"THE ROLE OF EXTRACELLULAR FORM OF VISFATIN (eNAMPT) IN MODULATING STRESS RESPONSES IN CULTURED MYOCYTES AS A MODEL OF SKELETAL MUSCLE AGEING"

BY

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"People in this world look at things mistakenly, and think that what they do not understand must be the void. This is not the true void. It is bewilderment."

Miyamoto Musashi, *A Book of Five Rings* **(1645)**

ABSTRACT

The understanding of the ageing process and of ageing-associated diseases represents a significant challenge for the scientific community, governments and society at large. I identified in skeletal muscle of murine models by microarray an increase in PPAR-β/δ expression during acute phase of hindlimb suspension (*accelerated ageing*), with a possible compensatory role, and an increase in expression levels of NR4A family of nuclear receptors in the skeletal muscle of caloric restricted rats (*decelerated ageing*). Adipose tissue has an endocrine role being actively involved in cross-talk with peripheral organs such as skeletal muscle. Visfatin is a recently discovered *adipokine* with pleiotropic functions. Unlike in other types of cells, in differentiated C2C12 myoblasts exogenous added visfatin (eNampt) did not act as an insulin-mimetic factor as shown by western blot and fluorescent assays. Visfatin treatment of differentiated C2C12 myotubes generated nevertheless an increase in the levels of reactive oxygen species as shown by fluorescent microscopy that was dependent on *de novo* transcription and translation of a new set of genes as revealed by RT-PCR. This increase in oxidative stress was independent of activation of the stress-activated protein kinases (MAPKs) such as ERK and p38, but dependent on NFkB activation as proved by western blot.

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As about the big meaning and the general relevance of the data that will be shown in the following chapters, nobody expressed it better than T.S. Elliot in the end of his masterpiece *Four Quartets*:

"We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time".

(from *Little Gidding,* 1942)

CONTENTS

ABBREVIATIONS

2-NBDG = 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose

2-DG = *2-Deoxyglucose*

2, 4-DNP = 2,4-dinitrophenylhydrozone

AP-1 = activator protein 1

ACOT1 = acyl-CoA thioesterase 1

ACLS3 = acyl-CoA synthetase long-chain family member 3

ACC = acetyl-CoA-carboxylase

ARNTL = aryl hydrocarbon receptor nuclear translocator-like

ABTS= 2, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate)

AMPK = AMP-activated protein kinase

PBEF = Pre-B-cell colony-enhancing factor

BCKDHA = branched chain ketoacid dehydrogenase E1

CuZnSOD = CuZn superoxide dismutase

CR/DR = caloric/dietary restriction

CNS = central nervous system

CPT-1b = carnitine palmitoyltransferase-1b

CPT-2 = carnitine palmitoyltransferase-2

CAV3 = caveolin 3

cGMP= cyclic GMP

CAT = catalase

COX2 = Cyclooxigenase 2

daf-16 = abnormal dauer formation-16

df/df= dwarf

DNPH = 2,4-dinitrophenylhydrazine

ERK1/2 = extracellular signal-regulated kinase 1

ER = endoplasmic reticulum

EHHADH = hydratase/3-hydroxyacyl Coenzyme A dehydrogenase

eNampt = extracellular Nampt

EAE = experimental encephalomyelitis

FADS3 = fatty acid desaturase 3

FABP3 = fatty acid binding protein 3

FASN = fatty acid synthase

FRTA = free radical theory of ageing

TPA = o-tetradecanoylphorbol-13-acetate

FBP2 = fructose-1, 6-biphosphatase

FDFT1 = farnesyl diphosphate farnesyl transferase 1

FGFs = fibroblast growth factors

GSK-3β = Glycogen synthase kinase 3 β

GPX-3 = glutathione peroxidase-3

GYS2 = glycogen synthase 2

SCD-2 = stearoyl-Coenzyme A desaturase 2

GPD1 = Glycerol-3-phosphate dehydrogenase 1

GLUT1 = glucose transporter 1

GPAM = glycerol-3-phosphate acyltransferase 1

GDP1 = glycerol-3-phosphate dehydrogenase 1

HEK293 = human embryonic kidney 293 cell line

HLS = hindlimb suspension method

H6PD = glucose 1-dehydrogenase

HK2 = hexokinase 2

HK1 = hexokinase 1

HUVECs = human umbilical vein endothelial cells

HIF1A = hypoxia-inducible factor 1A

IGF-IR = insulin-like growth factor receptor

iNampt = intracellular Nampt

IL-1β = interleukin-1β

IL-6 = interleukin-6

IL-2 = interleukin-2

IFN-γ = interferon-gamma

IPA = Ingenuity Pathway Analysis

IRS2 = insulin receptor substrate 2

IGFBP5 = insulin-like growth factor binding protein 5

LCT = long chain triglycerides

JNK = c-Jun terminal kinase

MCT = medium chain triglycerides

MTA = mitochondrial theory of ageing

MnSOD = Mn-superoxide dismutase

MPO = myeloperoxidase

MCP-1 = monocyte chemotactic protein-1

MAPK/SAPK = mitogen-/stress activated protein kinases

NR = Nuclear receptors

NAC = N-acetyl-L-cysteine

NMN = nicotinamide mononucleotide

NA = nicotinamide

Nam-PRTase/Nampt = Nicotinamide phosphoribosyltransferase

NAD = nicotinamide adenine dinucleotide

NSAID = Nonsteroidal anti-inflammatory drug

NFkB = nuclear factor kappa-light-chain-enhancer of activated B cells

NR1D1 = nuclear receptor subfamily 1 group D member 1

NR4A = nuclear receptor subfamily 4 group A

NRF1= nuclear respiratory factor 1

NRF2 = nuclear respiratory factor 2

NOX4 = NADPH oxidase 4

NMNAT1 = mononucleotide adenylyltransferase

NOS = nitric oxide synthase

NADH= **nicotinamide adenine dinucleotide**

PDGF = platelet-derived growth factor

PGC1α = PPAR-γ coactivator-1α

PGC1β = PPAR-γ coactivator-1 β

PKFL = phosphofructokinase

PGAM1 = phosphoglycerate mutase 1

PDHP1 = pyruvate dehydrogenase phosphatase 1

PDGF-B = platelet-derived growth factor-B

PDK4 = pyruvate dehydrogenase kinase isozyme 4

PI3K= phosphoinositide 3-kinase

PBF = pentafluorobenzenesulfonyl fluorescein

PTEN = Phosphatase and tensin homolog

PDK1 = Phosphoinositide-dependent protein kinase 1

ROS = reactive oxygen species

RXR = Retinoid x receptors

PPAR = peroxisome proliferator activated receptors

Ru (CO) 3Cl2]2 = tricarbonyldichlororuthenium

RT-PCR = real time PCR

RUNX1 = runt related transcription factor 1

SOD = superoxide dismutase

SOD1 = superoxide dismutase 1

SOD2 =superoxide dismutase 2

SREBP1c = sterol regulatory element binding protein-1c

Sir-2 = sirtuin-2

SCD-1 = stearoyl-Coenzyme A desaturase 1

SR = sarcoplasmic reticulum

TLR2 = Toll-like receptor 2

TLR4 = Toll-like receptor 4

TNF-α = tumor necrosis factor-α

TG = triglycerides

TRAF1 = TNF receptor-associated factor 1

TRAF6 = TNF receptor-associated factor 6

UCP-2 = uncoupling protein 2

UCP-3 = uncoupling protein 3

USD = US dollars

Visfatin = visceral fat-derived hormone

VEGFa = *vascular endothelial growth factor*

VLDLs *= very-low-density lipoproteins*

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PUBLISHED WORK ASSOCIATED WITH THIS THESIS

1. Mazzatti, D.J., Smith, M.A., **Oita**, **R.C**., Lim, F.L., White, A.J., and Reid, M.B. (2008). Muscle unloading-induced metabolic remodeling is associated with acute alterations in PPARdelta and UCP-3 expression. Physiol Genomics *34*, 149-161.

2. **Oita**, **R.C**., Mazzatti, D.J., Lim, F.L., Powell, J.R., and Merry, B.J. (2009). Whole-genome microarray analysis identifies up-regulation of Nr4a nuclear receptors in muscle and liver from diet-restricted rats. Mech Ageing Dev *130*, 240-247.

3. **Oita**, **R.C**., Ferdinando, D., Wilson, S., Bunce, C., and Mazzatti, D.J. (2010). Visfatin induces oxidative stress in differentiated C2C12 myotubes in an Akt- and MAPK-independent, NFkB-dependent manner. Pflugers Arch *459*, 619-630.

Chapter 1- INTRODUCTION

1.1 Ageing as a challenge for biomedical research in the 21st century

Based on World Health Organization predictions, by 2050 the aged segment of human population will grow from 650 million today to almost 2 billion and will have also an increased lifespan. Therefore, an in-depth theoretical understanding of the biological changes occurring during ageing is required for a better management of the pathological conditions associated (Benz and Yau, 2008; Franco et al., 2009; Vasto et al., 2009). As many as 300 distinct explanatory theories of ageing have been developed and the list is continuously growing (Medvedev, 1990). Ageing has been approached from different perspectives brought by different disciplines within biological research including evolutionism, developmental biology, genetics, genomics, cell biology, physiology and biochemistry. Ageing was initially conceptualized within the framework of evolutionary theories by A. Weismann who explained it as a normal consequence of *natural selection* (Gavrilov and Gavrilova, 2003). In 1952 P. Medawar developed the *"mutation accumulation"* theory by integrating within the evolutionary perspective the corpus of knowledge of classical genetics, conceptualizing ageing as the final state of a gradual accumulation of mutations with age, leading in the end to a reduced level of fitness culminating with death (Ljubuncic and Reznick, 2009). The *"antagonistic pleiotropy"* theory explained the ageing process as an accidental consequence of the action of genes that were selected as favourable during early life development, but were deleterious during later stages (Williams and Day, 2003). The evolutionary *"disposable soma"* theory (Drenos and Kirkwood, 2005) is the last theoretical construct in this line of thought explaining ageing as a consequence of the trading-offs required during development between reproduction, growth and repair. The scientific breakthroughs brought by biochemistry and cell and molecular biology fields in the last decades have also been incorporated into ageing research, the most notable paradigm proposed being the *free radical theory* of ageing discussed below.

1.1.1 The *free radical* theory of ageing

The idea that metabolism is the critical factor in determining the limits of lifespan was initially proposed in 1928 by Pearl's *"rate-of-living"* hypothesis that assumed a finite energy expenditure available to each living organism (Lints, 1989). Therefore, an accelerated metabolic rate and an increased rate of oxygen consumption should result in a reduced lifespan. Although not accepted anymore by scientific community due to the lack of a strict correlation between the rate of metabolism and lifespan or ageing phenotypes (Van Voorhies, 2004), the *"rate-of-living"* hypothesis nevertheless nurtured further research into the roles of oxygen and reactive oxygen species (ROS) in metabolism that culminated later in the *"oxygen poisoning"* hypothesis. The theory that oxygen can have deleterious effects on cellular structures and metabolism was proposed in 1954 by R. Gerschman when she noticed that the process of *"oxygen poisoning"* shares similar mechanisms of action with the phenomenon of x-irradiation through the generation of various species of free oxygen radicals (Gerschman et al., 1954). This idea was further employed by D. Harman two years later in his seminal paper (Harman, 1956). Harman based his theory on the assumption that the accumulation of ROS as *by-products* of normal cellular redox reactions will slowly, but certainly, lead to an increased number of genetic mutations, impaired cellular functions and eventually ageing. Harman predicted correctly that the main factors in this process are the proteins which directly use oxygen, especially those containing iron. The later identification of genes involved in oxygen metabolism like superoxide dismutase (SOD) gave Harman's theory solid empirical grounds (McCord and Fridovich, 1969). The basic components of initial Harman's theory were the following:

1. The *ageing* process correlates with *metabolic* rate;

2. The *metabolic* rate is proportional to the molecular *oxygen* used;

3. *Oxidation* process, catalyzed by multiple enzymatic reactions, generates *free radicals* that can have a deleterious impact on cell constituents.

4. A functional *antioxidant* defence system is critical for delaying or ameliorating the *ageing* process.

Recently, the *"free radical theory of ageing"* (FRTA) expanded to include other forms of activated oxygen such as peroxides and aldehides, and was renamed the *"oxidative stress theory of ageing"*(Sohal and Weindruch, 1996). The two versions are nevertheless considered to be identical for the communication purposes in the present thesis. After two decades of detailed research Harman further modified his initial hypothesis by focusing only on the specific roles of mitochondrial-derived free radicals, proposing the *"mitochondrial theory of ageing"* (MTA) (Harman, 1972). Recently, the mitochondrial theories of ageing (MTA) received additional support from independent studies exploring also the roles of mitochondrial genome stability and mutagenesis in cellular ageing (Miquel, 1992) and from the research on the roles of mitochondrial bioenergetics mechanisms in the production of ROS, such as electron transfer and oxidative phosphorylation (Lenaz et al., 2000). Besides their differences all FRTA and MTA theories can be classified broadly in two main categories: the *"accidental"* versions and the *"programmed"* versions of the *free radical* theory of ageing (Medvedev, 1990). The accidental version considers that the number and the amplitude of stochastic deleterious effects of oxygen metabolism shape the ageing process, while the programmed version states that ageing is the inevitable final stage of a predetermined developmental programme. Since both variants of the theory nevertheless assumed the core idea that the free radicals produced by living cells are regulating the rate and the extent of ageing process, it was possible to derive predictions based on theory that were tested experimentally. There are two main research directions in this aspect (Agarwal and Sohal, 1993). First of all, based on the theory, an *accelerated* rate of ageing should correlate with an *increased* production of free radicals resulting in a reduced lifespan. This led to a line of research that compared the levels of free radicals with the ageing phenotypes in different experimental animals or tissues (Adelman et al., 1988). Second, an *increase* in antioxidant defence should *decelerate* the

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ageing process, eventually increasing the lifespan. This line of research is based on the results of the studies of the effects of different mutations of antioxidant genes on the ageing process and on the effects of pharmacological/dietary interventions (Gems and Doonan, 2009). Both lines of research gathered significant arguments for and against the theory of free radicals of ageing. So far a comprehensive, widely accepted *unified theory* integrating all the relevant experimental data concerning the effects of the free radicals on ageing in a single explanatory system has not been achieved (Beckman and Ames, 1998). Nevertheless, it is generally accepted at least that the progression of ageing phenotype is dependent on the overall *balance* between the rate of free radicals produced and the action of the antioxidant defence system, and that a progressive *imbalance* between the two forces toward a reduced or impaired antioxidant status will result in an accelerated ageing process (Muller et al., 2007).

Simultaneously with the studies of the roles of free radicals on lifespan and ageing, other independent lines of research revealed their critical role in the development of many pathological conditions associated with ageing such as neurodegenerative diseases, cancer, cardiovascular diseases and impaired immunity (Ames et al., 1993). These studies were incorporated in the FRTA set of theories as arguments to justify the hypothesis that free radicals play a significant role in the development of the ageing process *itself*, not only in the modulation of its associated pathogenic aspects (Ku et al., 1993). The main postulate of this direction of research is that the differences in the generation of free radicals are the *main cause* of the differences in lifespan between individuals of the same species and also between species. Therefore, the scientific community dedicated to decipher ageing is currently divided between those defending the *"weak"* version of FRTA, that considers that free radicals play a major role *only* in the development of ageing-associated diseases which *indirectly* affects the lifespan, and those dedicated to defend a *"strong"*, supposedly the original interpretation of FRTA, that considers that the levels of the free radicals *directly* limit the lifespan and shape the ageing profile of any species (Beckman and Ames,

1998). While the *"weak"* concept has a broad support, the *"strong"* version is still highly debated and controversial. It was also shown that there are close relationships between antioxidant defence mechanisms and inflammatory status (Mulholland et al., 1999). For example, the death of endothelial cells occurring in models of atherogenesis generated by oxidative stress inducers or cytokines is reduced by the pre-incubation with antioxidants such as catalase (CAT) or N-acetyl-L-cysteine (NAC) (Dimmeler and Zeiher, 2000). In addition, antioxidants such as SOD and tocopherol are able to reduce the skin inflammation in mice (Lange et al., 1998). Since reactive oxygen species are by-products of usual cellular biochemical reactions, understanding the mechanisms that protect the integrity and the functionality of mammalian proteome and genome against their deleterious effects represents a central research goal of the study of ageing and regulation of lifespan. For example, it was shown that Drosophila models lacking copper/zinc-containing superoxide dismutase presented decreased lifespan (Phillips et al., 1989) and that homozygous null mice for Mn-superoxide dismutase (MnSOD) are not viable (Li et al., 1995). These reports proved the essential role of antioxidant protection not only for a proper ageing but also for a normal early development. In addition, they inspired a subsequent research based on the assumption that by improving the antioxidant capability of cells to resist the oxidative stress, the ageing process could be delayed or even reversed. The experimental attempts using *genetic, dietary/lifestyles* or *pharmacological* intervention strategies have had only partial or modest successes, sometimes leading even to contradictory results. For example, whilst the expression of the human mitochondrial catalase extended the lifespan in mice models (Schriner et al., 2005), the heterozygous mice lacking one allele of MnSOD did not present nevertheless any significant change (Van Remmen et al., 2003). Similarly, while null mice lacking CuZnSOD had reduced lifespan (Elchuri et al., 2005), its ubiquitous overexpression failed to increase it in the same model (Huang et al., 2000).

There are a few reports published that even seem to contradict the basic tenets of the

FRTA-derived predictions. For example, *C. elegans* mutants lacking mitochondrial SOD2 presented increased levels of ROS levels in mitochondria simultaneously with an *increased* lifespan, and incubation of these longevive mutants with antioxidants such as NAC *cancelled* the effects (Yang and Hekimi, 2010). Similarly, chronic conditioning of the same wild-type nematode models with a low concentration of an ROS-inducer such as paraquat not only generated higher global ROS levels, but increased also the lifespan values. In Drosophila, the simultaneous overexpression of catalase and MnSOD in mitochondria had surprisingly toxic effects reducing the lifespan, although improved the antioxidant defence and reduced the levels of free radicals produced (Bayne et al., 2005). The overexpression of the antioxidant genes in murine models had also toxic effects in some cases (Rando and Epstein, 1999). For example, the overexpression of the wild-type form of SOD1 in skeletal muscle led to significant myopathy and an accelerated aged phenotype due to an increased level of protein aggregation, and surprisingly higher levels of ROS (Wong and Martin, 2010). These results do not necessarily reject the FRTAs sets of predictions because they could be still explained through the concept of *hormesis* described below. Alternatively, such results might indicate that the quantification of oxidative stress levels *cannot* be reduced only to the measurement of the *free radicals* produced, a better understanding of the antioxidant defence status and of the mechanisms regulating it being crucial in this respect. For example, it was shown that while during physical exercise the production of the free radicals in skeletal muscle is greatly increased (Bejma and Ji, 1999), this event is nevertheless simultaneously activating the antioxidant defence mechanisms which in long term has a beneficial effect on myocytes physiology (Zainal et al., 2000). Ageing lowers and degrades the ability of skeletal muscle to activate during physical exercise the elements of antioxidant defence system such as heat-shock proteins (Liu and Steinacker, 2001), therefore the production of free radicals has a higher physiological impact with increased age. Better conceptual and experimental tools have to be developed in order to obtain a more accurate estimation of the levels of oxidative stress and of

the ability of antioxidant defence to protect the cellular constituents against the deleterious effects of the free radicals. In addition, less it is known about the roles of *repair* mechanisms that do not counteract the free radicals produced *per se,* but remove afterwards the oxidised products generated by such ROS such as carbonylated proteins, lipid peroxides or oxidized bases (Beckman and Ames, 1998). The reasons why some oxidative stress inducers induce a senescent phenotype and other do not could be explained through a differential ability of such repair mechanisms to remove the toxic by-products generated. Therefore, the main driving force behind the differential development of the senescent phenotype between different species or different individuals of the same species could lie in fact in the differences in the ability to *react* to the ROS experienced, not necessarily in the amount of free radicals produced.

The *pharmacological* interventions based on the administration of the known antioxidant supplements such as NAC (Miquel et al., 1995) or Ginko Biloba extracts (Sastre et al., 1998) successfully improved mitochondrial enzymatic parameters and antioxidant defence in mice models, but had no effect on lifespan. The only dietary intervention that proved so far to be effective in extending lifespan and delaying ageing of any experimental model employed is the *caloric* or *dietary* restriction (Fontana et al., 2010; Piper and Bartke, 2008; Zahn and Kim, 2007). The mechanisms through which caloric restriction improves the ageing phenotype are still unclear but it was shown at least to be partially dependent on reduced levels of inflammation and oxidative stress and a changed genomic expression profile leading to an improved antioxidant defence (Merry, 2002, 2004, 2005). Caloric restriction effects in an ageing context can be explained through the concept of *hormesis*, i.e. low level of damaging stimuli applied continuously which will have in the end global positive effects.

The future understanding of the relationships between *oxidative stress* and *ageing* will require the exploration of the distinct effects of ROS/antioxidant defence on individual tissues and organs, as well as an estimation of the contribution each tissue has on the development of the

overall ageing phenotype. Of great theoretical and practical importance will be the identification of tissue-specific ageing *markers* or of *mediators* that could have *pleiotropic* or even *opposite* effects in different cellular types or physiological contexts. For example, genes that have a pro-apoptotic role in one cell type but are anti-apoptotic in other cases, genes that have a prooxidant role in some of peripheral tissues and antioxidant in others, or genes that are associated with an insulin-resistant and pro-inflammatory state in some cases but with an insulin-sensitive and anti-inflammatory state in others. This will allow for differential *genetic* or *pharmacological* interventions at molecular, cellular or organism levels that will offer a good model to test the predictions made by the theories of ageing described above, by correlating the *state of ageing* in a particular tissue with the levels of *cellular stress*.

1.2   Adipose tissue- a critical regulator of the development of the inflammation and oxidative stress characterizing ageing-associated diseases

In the last decades many genes regulating the limits of lifespan and the development of ageing have been discovered in experimental models. For example, the transgenic C. elegans nematode models of *daf-16* gene that encodes for a transcriptional factor regulating insulin signalling presented not only an increased lifespan but also a healthier phenotype (Ogg et al., 1997). Similarly, Drosophila models overexpressing *methuselah* gene that encodes for a G-coupled receptor lived longer (Lin et al., 1998). The discovery that *S. cerevisae* yeasts overexpressing *sirtuin-2* (*sir-2*) gene that regulates epigenetic aspects of chromatin function showed an increased replicative lifespan (Kaeberlein et al., 1999) initiated an extensive stage of screening research of synthetic or natural compounds that can activate the human sirtuins to delay or prevent ageing (Alcain and Villalba, 2009). The first genetically-modified murine model

showing an increased lifespan was developed in 1996 when it was discovered that Ames dwarf mice (*df/df*) that were deficient in growth hormone, prolactin and thyroid-stimulating hormone lived longer (Brown-Borg et al., 1996). Recently it was found that the gene *klotho* that encodes for a type I membrane protein, possibly belonging to the beta-glucuronidase family, is also a hormone that has anti-ageing properties by inhibiting insulin signalling (Kuro-o, 2008). For example, while *klotho* null mice had a shorter lifespan with an accelerated ageing phenotype, *khlotho* transgenic mice had an increased lifespan (Kurosu et al., 2005). These reports indicate that regulation of lifespan, the rate of ageing and the development of ageing-associated diseases are affected by changes in gene expression of main regulators of insulin metabolism, changes in epigenetic status and changes in the signalling of the networks monitoring metabolism. The essential roles of inflammation, oxidative stress and of a dysfunctional metabolic regulation in the pathogenesis of ageing-associated diseases are already known. A persistent, chronic low level of inflammation is also a central element of the immunosenescent status (Krabbe et al., 2004). The ageing phenotype correlates with a change in the expression profile or in the activity pattern of the circulating cytokines and with an increased risk of mortality (Licastro et al., 2005).

In recent years adipose tissue has started to be understood not only as a fat depot, but also as an important *endocrine* organ (Jazet et al., 2003; Mohamed-Ali et al., 1998; Trayhurn and Wood, 2005). Adipokines are cytokines secreted by white or brown adipocytes that act in autocrine/paracrine (Karastergiou and Mohamed-Ali, 2010) or endocrine fashion (Kos and Wilding, 2009). Adipokines have complex roles such as the regulation of the adipose mass (Cawthorn et al., 2007; Cawthorn and Sethi, 2008), inflammation (Wang et al., 2005) or glucose (Rabe et al., 2008) and lipid metabolism (Lago et al., 2009). Since inflammation and oxidative stress characterize the ageing phenotype (Beauloye et al., 2007; Maury and Brichard, 2010; Maury et al., 2007; Rousseau et al., 1997) and since adipose tissue has an important endocrine impact on the physiology of other peripheral tissues, it is reasonable therefore to expect that there are close

relationships between the *ageing process* affecting peripheral tissues and *adipokines (*Picard and Guarente, 2005). In addition, the relationships between other closely related mediators of inflammation such as the cytokines and ageing are well established and deciphered. It is known for example that the levels of interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) change with age (Grubeck-Loebenstein and Wick, 2002), and it has also been shown that the ageing of immune system has a central role in the development of the *overall* ageing phenotype (Fulop et al., 2006). Similarly, it is reasonable to expect that changes in adipokine expression or activity could also have a major impact in the development of ageing-associated diseases, in whose progression inflammation and oxidative stress play a major role   (Picard and Guarente, 2005). The list of adipokines is continuously growing and includes besides already known cytokines (TNF-α, IL-6, IL-8, IL-10) other members with a less understood biology such as adiponectin, leptin, resistin, omentin, apelin, vaspin, chemerin, visfatin and monocyte chemotactic protein-1 (MCP-1) (Wozniak et al., 2009).

1.2.1 Visfatin- a new adipokine and modulator of oxidative stress and inflammation

Visfatin is a multifunctional protein belonging to adipokine family with a multistep history. The first historical stage started seventeen years ago when it was discovered that it had a synergistic effect on the maturation of human pre-B cell colonies in association with other interleukins, and it was named *PBEF* (Pre-B-cell colony-enhancing factor) (Samal et al., 1994). The precise nature of its cellular function remained obscured for another seven years until 2001 when it was discovered that PBEF gene sequence has a high level of homology with NadV gene from the prokaryote *Haemophilus ducreyi* (Martin et al., 2001). In the following year it was discovered that both human and bacterial genes were coding for a nicotinamide phosphoribybosiltransferase enzyme (Rongvaux et al., 2002) that synthesizes nicotinamide

mononucleotide (NMN) from nicotinamide (NA) and diphosphate, the human gene getting

consequently two more names, *Nam-PRTase* and *Nampt*. The fact that intracellular form of visfatin

synthesizes NMN, an intermediate of nicotinamide adenine dinucleotide (NAD), could indirectly

impact the ageing process and the development of ageing-associated diseases. For example, it was

discovered that sirtuin-2 gene that regulates the rate of lifespan and ageing phenotype encodes for a

histone deacetylase in yeasts which requires NAD as a substrate (Wenzel, 2006). Multiple

homologues of sirtuin-2 gene have been identified in human genome and all use NAD as a

substrate. The next stage of visfatin research was opened in 2005 when a controversial paper was

published (Fukuhara et al., 2005) that reported that PBEF/Nam-PRTase/Nampt is also an *adipokine*

being secreted into plasma by visceral fat adipocytes and acting as an insulin mimetic agent. As an

adipokine PBEF/Nampt is also known as *visfatin* (<u>vis</u>ceral <u>fat</u>-der<u>i</u>ved hormo<u>ne</u>) or extracellular

Nampt (**eNampt**), to distinguish it from the intracellular form of PBEF/Nampt (**iNampt**). The

identity of visfatin as an adipokine actively secreted by white and brown adipocytes from both

subcutaneous and visceral adipose depots is widely accepted today. Nevertheless, it is still unclear

if visfatin is a genuine insulin mimetic agent and the issue is still debated. Only a few independent

experiments were able to confirm the original hypothesis (Adya et al., 2008a, 2009; Lim et al.,

2008; Xie et al., 2007). Moreover, the original paper has been retracted by the publishing journal.

The present stage of research is focused on the exploration of the differential

roles of both intracellular (iNampt) and extracellular (eNampt) forms of visfatin in the modulation

of oxidative stress and inflammation. Independent experiments performed *in vivo*, *ex vivo* and *in

vitro* led to divergent conclusions. There are good arguments to consider that in some tissues or in

some physiological conditions visfatin has a *protective* role, defending cells from different types of

threatening stimuli like genotoxic stress (Rongvaux et al., 2008), ER-stress (Li et al., 2008) or

oxidative stress (Borradaile and Pickering, 2009). Such protection can have short-term anti-

apoptotic effects (Kendal-Wright et al., 2008) and in some cases even longer-term, anti-ageing

effects (van der Veer et al., 2007). However, there are solid arguments to assert that in other cells or tissues visfatin is in fact a powerful inducer of *oxidative stress* and *inflammation*, its overexpression or its increased function being involved in a large variety of pathological conditions associated with ageing ranging from leukaemia (Hasmann and Schemainda, 2003), lung injury (Ye et al., 2005), Alzheimer's (Adams, 2008) and arthritis (Nowell et al., 2006) to obesity (Berndt et al., 2005) and diabetes (Chen et al., 2006). So far the experiments measuring the synthesis of NMN by the intracellular form of visfatin (Busso et al., 2008), the levels of tissue-specific expression (Choi et al., 2007) or the levels of plasma circulating visfatin (Otero et al., 2006) showed that visfatin is associated with particular pathological conditions in some experimental models. Nevertheless, until now there is no global, unified theory about the underlying mechanisms and overall effects of either form of visfatin. Also, even when it is possible to make a reliable statistical correlation between a pathological biomarker and the level of visfatin expression or function, the precise physiological roles and relevance of such observed changes are still obscure. An increase in visfatin intracellular expression or extracellular secretion could be a *causative* factor in some of the diseases listed above, but in the same time it could have a *compensatory* or even a *preventive* function in other cases. The murine null knock-out models are unviable and the experiments that used PBEF-heterozygous mice did not clarify the most crucial questions about visfatin relationships with the regulation of oxidative stress and inflammation (Revollo et al., 2007). The usefulness of other types of models like lower invertebrates or prokaryotes to elucidate all functional properties of visfatin is also doubtful, although some progress has been made recently in this matter (Vrablik et al., 2009). Therefore, at the present stage of research it is not possible to definitively corroborate or dismiss any of the opposing hypotheses concerning the relationships between extracellular visfatin and cellular stress.

One important fact about visfatin is that it is highly expressed in skeletal muscle, which led to the suggestion that it might also act as a *myokine* (Krzysik-Walker et al., 2008). So far

there are just a few papers dedicated to explore the role of skeletal muscle intracellular visfatin in the generation or protection against oxidative stress and inflammation (Costford et al., 2010; Fulco et al., 2008), and until our published paper (Oita et al., 2010) there were no reports published about the effects of exogenous visfatin (eNampt) on skeletal muscle physiology.

Visfatin looks to have complex, even *antagonistic* effects in different tissues or in different pathological conditions, being associated with a prooxidant state/insulin resistance in certain tissues and with increased oxidative stress/insulin-sensitivity in others, with a pro-inflammatory state in some pathological cases and with an anti-inflammatory state in others. Therefore, a detailed research of its biology could offer a solid base for building a *theoretical model* to test the validity of the predictions made by different theories mentioned above about the relationships between the levels of oxidative stress and the extent or the intensity of ageing.

1.3   Nuclear receptors and ageing

Nuclear receptors (NRs) are a family of transcription factors that in the human genome are encoded by 48 different genes (Bensinger and Tontonoz, 2008). The general structure of NRs is composed of a ligand binding domain, a DNA binding domain and other modulator domains required for protein-protein interactions (Evans, 2005). Nuclear receptors can reside in the nucleus or in the cytoplasm. The majority of nuclear receptors are activated after the binding of ligand, while some are active in a constitutive fashion (Mangelsdorf et al., 1995). Based on structure and ligand, nuclear receptors can be classified in three main classes:

1. steroid subgroup that binds to DNA as heterodimers, comprising different subtypes such as glucocorticoid, androgen, estrogen and progesterone;

2. orphan subgroup that has no ligand identified yet, such as NR4As;

3. adopted orphan receptors subgroup that act as heterodimers with retinoid x receptors (RXRs) and need a ligand for activation, such as peroxisome proliferator activated receptors (PPARs) and liver X receptors (LXRs) (Chawla et al., 2001).

Nuclear receptors have important roles in regulating the transcription (by activation or repression) of genes that control the metabolism, inflammation and stress-responses (Giguere, 1999). Besides their role as transcriptional regulators nuclear receptors have also crucial non-genomic effects such as the cytosolic modulation of the activity of ion channels, protein kinases, protein phosphatases or phospholipases, therefore indirectly regulating the glucose and lipid metabolism or the antioxidant status (Ordonez-Moran and Munoz, 2009). The ability of nuclear receptors to bind hydrophobic, lipophilic ligands opened the possibility of screening new compounds (natural or synthetic) to be used in clinical practice to target the associated signalling networks regulating inflammation, metabolism or oxidative stress that are underlying the mechanisms of pathological conditions associated with ageing (Carlberg and Dunlop, 2006). It is already known the roles of some of nuclear receptors such as retinoid receptors (Saurat, 1995) and glucocorticoid receptors (Djordjevic-Markovic et al., 1999) in ageing, but less is known about relationships between PPARs and ageing.

Some of PPAR agonists were discovered to have PPAR-independent effects. For example, troglitazone could not increase PPRE activity in SZ95 cells without the previous overexpression of exogenous PPAR-γ and several types of antagonist treatment or ectopic expression of a dominant negative PPAR-γ form failed to change the inhibition of cyclin D1 expression and the regulation of the cell cycle (He et al., 2004). The safety of PPAR agonists came also into question recently after it was showed that TZDs administration in human patients with diabetes increased the risk for bone fracture (Kahn et al., 2008). Similarly, rosiglitazone increased the risk of cardiovascular diseases. The explanation

proposed was based on the fact that the above mentioned agonist blocked also the phosphorylation levels of PPAR-γ by Cdk5 at Ser273 residue, changing the expression of many genes involved in obesity and insulin-resistance.

1.3.1 PPARs and the ageing phenotype

Peroxisome proliferator activated receptors (PPARs) are a type of ligand-activated nuclear receptors that were considered as a distinct class because of a shared, typical domain structure. PPAR-α was the first member of the PPAR family to be identified when it was found to be activated by rodent hepatocarcinogenic agents that also caused proliferation of peroxisomes (Issemann and Green, 1990). The other two members, PPAR-γ and PPAR-β/δ, were cloned two years later due to structure similarity with PPAR-α (Dreyer et al., 1992). The PPARs large Y-shaped hydrophobic pocket of the ligand-binding domain can accommodate a large variety of lipophilic compounds with an acidic head group (Gampe et al., 2000). This distinct feature was exploited experimentally in order to screen for natural or synthetic compounds that could activate or block PPARs function. The first clue came from clinical trials of patients with type 2 diabetes that showed a reduced level of fasting glucose in plasma upon administration of clofibrate (Barnett et al., 1977), and later it was discovered that clofibrate is a ligand of PPAR-α (Mehendale, 2000). Recently it was also discovered that thiazolidinediones are potent PPAR-γ agonists that can be used in the treatment of insulin resistance (Ghazzi et al., 1997), and activation of PPAR-β/δ by its specific agonists such as L-165041 was shown to reduce the levels of circulating insulin and triglycerides in animal models of obesity (Leibowitz et al., 2000). These initial scientific breakthroughs stimulated an exploratory stage of screening new compounds targeting PPARs in order to treat many pathological conditions associated with ageing, although many of these

molecules were later shown to have significant clinical side effects (Cheatham, 2010).

The administration of PPARs agonists or changes in PPARs expression had so far no obvious effect on lifespan, but there are good reasons to consider that PPARs can influence indirectly the development of ageing phenotypes. For example, it was discovered that PPAR-γ agonists treatment increased in human embryonic kidney 293 cell line (HEK293) the expression of antiageing gene *klotho*, discussed above, through the binding to a noncanonical PPAR-response element (Zhang et al., 2008). Although PPAR-α agonists property of hepatocarcinogenicity in rodents (although not in humans) was shown to increase with age (Youssef and Badr, 2005), the activation of the receptor was able to reduce the inflammation in a model of T-cell ageing through the repression of T-bet (Jones et al., 2002). PPAR-β/δ signalling is also involved in ageing-associated diseases since it was shown that its activation induced myonuclear accretion and increase in the number of oxidative fibers in the skeletal muscle, delaying sarcopennia (Giordano et al., 2009). It was also shown that PPAR-β/δ is regulating the expression level of sirtuin1 gene that has anti-ageing properties (Okazaki et al., 2010). All PPARs have important antioxidant and anti-inflammatory effects in different tissues, with a few minor and specific differences not fully understood yet. Nevertheless, within PPAR family only **PPAR-β/δ** has the most consistent behaviour as an antioxidant and anti-inflammatory mediator across multiple tissues, especially in skeletal muscle (Luquet et al., 2003). In addition, PPAR-β/δ is expressed in skeletal muscle at higher values compared to the other two types (Muoio et al., 2002), which suggest a possible local preeminent role. Therefore, in the next chapters dedicated to PPARs a special attention will be paid to PPAR-β/δ.

I will present below the accumulated knowledge underlying the possible connections between PPAR-β/δ and the regulation of *cellular stress* and *inflammation* in the context of *ageing*-associated diseases. Although the focus of my thesis will be on skeletal muscle models of ageing, summarizing the current knowledge about the role of β/δ in regulating the

mechanisms of oxidative stress and inflammation in other tissues will be useful in interpreting the results presented in following chapters.

1.3.1.1 The role of PPAR-β/δ in regulating oxidative stress and inflammation

The first PPAR-β/δ knock-out model developed had no obvious **skin** defects, but the skin from mutant mice treated with the compound *o*-tetradecanoylphorbol-13-acetate (TPA) to induce epidermal differentiation showed a much bigger hyperplastic phenotype than the control group (Peters et al., 2000). In addition, the skin from PPAR-β/δ knock-out models showed an impaired or delayed reduction in TPA-induced inflammatory response compared to wild-type control group, when co-treated with the nonsteroidal anti-inflammatory (NSAID) anti-inflammatory drug *sulindac*. The increased degree of inflammatory cells infiltration in the epidermis of PPAR-β/δ knock-out models indicated that PPAR-β/δ has a significant function as an anti-inflammatory regulator. In addition, PPAR-β/δ expression was induced in primary keratinocytes by the treatment with cytokines such as TNF-α and interferon-gamma (IFN-γ) via activator protein 1 (AP-1) signalling, acting subsequently both as an anti-apoptotic factor and as a regulator of the keratinocyte differentiation and proliferation (Tan et al., 2001). The treatment of cultured keratinocytes with PPAR-β/δ agonists such as GW1514 significantly reduced the cytokine production upon TPA challenge and UV-B, and treatment with cytokines such as lipopolysaccharide (LPS) or TNF-α increased PPAR-β/δ expression in the same type of cells (Schmuth et al., 2004). Another relevant discovery was that PPAR-β/δ is upregulated in psoriasis by lipoxygenase products due to an aberrant interaction of PPAR-β/δ with nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) that could trigger an activation of a cellular

hyperproliferative programme in the skin (Westergaard et al., 2003). In another experiment the keratinocytes from PPAR-β/δ-null mice treated with TPA had increased myeloperoxidase (MPO) activity levels compared to the control keratinocytes, a marker of skin inflammation (Kim et al., 2006). Subsequent independent studies proved that PPAR-β/δ is more than a simple inhibitor of inflammation, having complex roles. For example, it was shown that PPAR-β/δ is involved in the regeneration process of wounded skin by modulating the temporal expression and spatial localization of growth factors such as fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) and of pro-inflammatory cytokines such as TNF-α and interleukin-1β (IL-1β) at certain sites around the injury (Michalik et al., 2001; Tan et al., 2004; Tan et al., 2002), events that are required for an efficient and fast regeneration. These discoveries proved that PPAR-β/δ could be an important target in the treatment of ageing-associated diseases affecting skin by modulating the expression of crucial genes regulating oxidative stress and inflammatory response.

PPAR-β/δ is the most promising member of PPARs family in treating **skeletal muscle** dysfunctionalities during ageing. It was shown for example that PPAR-β/δ has a major role in regulating oxidative stress responses and inflammation in skeletal muscle. An important clue came from a conditional transgenic model mice expressing PPAR-β/δ in skeletal muscle that showed a considerable increase in oxidative fibers in various fibers types like tibialis, plantaris and soleus based on the number of succinate dehydrogenase-positive cells (Luquet et al., 2003). PPAR-β/δ skeletal muscle-specific transgenic mice had also increased activities of antioxidant enzymes like citrate synthase and β-hydroxyacyl-CoA dehydrogenase, a leaner phenotype and a reduced amount of fat that it is similar with the phenotype of the exercise models of muscle activity in rat, mice and humans. Other studies showed that the administration of the PPAR-β/δ agonist GW-501516 improved the metabolic syndrome phenotype in skeletal muscle induced by a high-fed diet, enhancing metabolic rate and fatty acid β-oxidation, proliferation of mitochondria and reducing the levels of lipid droplets (Grimaldi, 2005; Luquet et al., 2005). The mechanisms regulated by the

PPAR-β/δ were deciphered *in vitro* by using rat L6 skeletal myotubes treated with GW-501516 agonist that showed an upregulation of genes involved in fatty acid transport, β-oxidation and mitochondrial respiration, switching therefore the fuelling choice from glucose to lipids (Tanaka et al., 2003). The transfection of mouse C2C12 skeletal myotubes with a PPAR-β/δ dominant negative construct reduced the anti-inflammatory effects induced by the compound tricarbonyldichlororuthenium ((([Ru(CO)3Cl2]2)) that inhibits NFkB activation by TNF-α (Woo et al., 2006). A significant proof that PPAR-β/δ is critical for antioxidant defence came from the discovery that in skeletal muscle PPAR-γ coactivator-1α (PGC1α) is also a target of PPAR-β/δ (Hondares et al., 2007). The role of PPAR-β/δ in regulating inflammatory response in muscle was suggested by the discovery that NFkB-signalling was activated *in vitro* in mouse C2C12 myotubes upon incubation with palmitate, event that was inhibited by the activation of PPAR-β/δ with the agonist L-165041 (Jove et al., 2005). Therefore PPAR-β/δ activation could effectively reduce the oxidative stress and inflammation underlying the development of ageing-associated diseases affecting skeletal muscle.

PPAR-β/δ is widely expressed in **brain**, especially in late embryonic stage (E18.5) (Woods et al., 2003). It is expressed mainly in the entorhinal cortex, cerebellum and hippocampus, with a higher level of expression in neurons and oligodendrocytes than in astrocytes (Heneka and Landreth, 2007). PPAR-β/δ null mice showed also a defective CNS myelination especially in *corpus callosum* (Peters et al., 2000). The modelling of focal cerebral ischemia in mice showed that infarct size was increased in PPAR-β/δ null animals that had bigger levels of lipid peroxidation and reduced levels of glutathione, uncoupling protein 2 (UCP-2) and superoxide dismutase (Arsenijevic et al., 2006), known antioxidants. The administration of PPAR-β/δ agonist GW0742 increased the overall protection in an animal model of experimental encephalomyelitis (EAE) by reducing the astroglial and microglial inflammatory activation (Polak et al., 2005). The administration of PPAR-β/δ agonists such as L-165041 and GW-501516 protected further against

damaging agents by reducing ROS levels in human dopaminergic neuroblastoma SH-SY-5Y cell line treated with thapsigargin to model Parkinson's effects (Iwashita et al., 2007). Furthermore, the activation of PPAR-β/δ in this SH-SY-5Y cellular model reduced the level of oxidative stress and the activation of caspase-3/7, a main executioner of apoptosis (Arsenijevic et al., 2006). The modelling of postnatal development of the brain *in vitro* by re-aggregating neuronal cultures showed that enzyme acyl coA synthetase 2 (ACS2) is a target for PPAR-β/δ (Basu-Modak et al., 1999), being involved in fatty acid metabolism, neurite outgrowth and inflammatory responses in spinal cord (Benani et al., 2003). PPAR-β/δ is therefore a potent antioxidant and anti-inflammatory mediator in brain.

PPAR-β/δ has an important **hepatic** function by decreasing glucose consumption and by increasing insulin sensitivity (Lee et al., 2006), by promoting fatty acid synthesis and by reducing the lipid droplets content (Nagasawa et al., 2006). In addition, PPAR-β/δ has immunomodulatory properties, interfering with IL6-induced acute phase reactions in liver and inhibiting the transcriptional activity of STAT3 (Kino et al., 2007). PPAR-β/δ agonist treatment also decreased the level of expression of cyclooxigenase-2 (COX-2) in human hepatocellular carcinoma line HepG2 (Glinghammar et al., 2003), which is a known regulator of inflammatory mechanisms and oxidative stress.

PPAR-β/δ is also an important regulator of lipid-induced inflammation and oxidative stress in the **cardiovascular system**. For example, the activation of PPAR-β/δ by its specific agonist GW0742X reduced the atherosclerotic markers in low density lipoprotein receptor (LDLR) null mice (Graham et al., 2005). Surprisingly, the deletion of PPAR-β/δ from macrophages further reduced the atherosclerotic lesions areas by 50% in LDLR null mice and lowered considerably the levels of inflammatory cytokines (Lee et al., 2003). The possible explanation could be the binding of PPAR-β/δ in unligated form to the transcriptional repressor Bcl-6, blocking its activation. Once activated by a specific ligand PPAR-β/δ releases Bcl-6 that will further block

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the expression of pro-inflammatory cytokines such as MCP-1. In knock-out models Bcl-6 is constitutively activated and therefore constantly represses inflammation, which explains the reduction in atherosclerotic lesions observed. In the current proposed model PPAR-β/δ acts a non-conventional *switch* by selectively repressing or activating the inflammatory pathways, depending on the status of the ligand binding to it. In the free form it sequesters inflammation repressors, but once interacting with the ligand it releases the regulators of inflammation. This proves that PPAR-β/δ has complex roles in regulating inflammatory mechanisms, and further research is still required.

1.4 Skeletal muscle and ageing

During ageing skeletal muscle progressively develops an atrophic phenotype characterized by a reduced mass, fewer fibers and impaired contractility, a condition described also as *frailty* (Faulkner et al., 2007). The sarcopenic pathological condition affecting skeletal muscle is multifactorial (Fujita and Volpi, 2004). Sarcopenia can be managed by interventional approaches such as diet and exercise (Bautmans et al., 2009), strategies also known as *healthy ageing* (Franco et al., 2009). The roles of an improved antioxidant defence system and of a reduced level of inflammation in the development of a successful healthy ageing phenotype have been deciphered recently (McArdle et al., 2002). The progression of ageing and of ageing-associated diseases changes also the functionality of the signalling pathways regulated by different classes of nuclear receptors (Baltgalvis et al., 2010; Diep and Constantinou, 1999; Kannisto et al., 2006; Oudin et al., 1998; Steinbach and Chen, 1995; Torlinska et al., 2000). Particular attention was paid to PPARs family (Lopez-Soriano et al., 2006) since, as described in the previous subchapter, their agonists have been employed with moderate success in many clinical trials for the treatment of pathological conditions affecting skeletal muscle such as insulin resistance (Cha et al., 2001; Shibasaki et al.,

2003; Ye et al., 2001), metabolic inflexibility (Sugden et al., 2009), muscle waste (Moore-Carrasco et al., 2008), inflammation (Jove et al., 2005), sustained oxidative stress (Ceolotto et al., 2007) and hypoxia (O'Hagan et al., 2009). Therefore a deep understanding of the functional and structural changes in nuclear receptors-associated signalling pathways during myocyte ageing is required for a proper management of the diseases affecting skeletal muscle. The physiology of skeletal muscle is also modulated by the action of adipokines and cytokines (Dyck et al., 2006; Vu et al., 2007), especially in the case of antioxidant defence mechanisms and inflammatory mediators (Eckardt et al., 2008; Havekes and Sauerwein, 2010). The exploration of the effects of newly identified adipokines such as visfatin on skeletal muscle antioxidant defence mechanisms and inflammatory mediators will further improve our knowledge about relationships between adipokines and ageing, and hopefully it will make possible the development of effective and safe clinical interventions.

My thesis will explore the possible existence of any changes occurring during skeletal muscle ageing in the expression of nuclear receptors families and of their associated signalling pathways, especially PPARs, by using multiple *in vivo* and *in vitro* models. The thesis uses opposite *in vivo* models of ageing: a model of *accelerated* ageing affecting skeletal muscle (*hindlimb suspension* in mice), and a model of skeletal muscle *healthy ageing* strategy (*caloric restriction* in rats). The **hypothesis** is that during ageing there are *major* changes affecting nuclear receptors (NR) expression levels (especially PPARs) that could have an impact on the progression of ageing-associated diseases. To explore them I will use microarray assays to identify any changes in NR expression by the above mentioned modelling strategies followed by RT-PCR and western blot.

The second part of my thesis will be focused on the exploration of any possible effects of extracellular form of visfatin (eNampt) on skeletal muscle physiology, with a special emphasis on the oxidative stress production and antioxidant defence mechanisms, insulin sensitivity and inflammation. The hypothesis is that a possible *increase* in visfatin plasma concentration during

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ageing (yet to be determined) could have a significant impact on cellular processes affecting skeletal muscle relevant for the development of ageing-associated disease. For this I will employ an *in vitro* model (C2C12 mouse myotubes) and incubation with purified visfatin, followed by measurements of the levels of ROS, of the levels and pattern of oxidized products, estimation of the status of the antioxidant defence, of the activation/inhibition of signalling pathways relevant for insulin sensitivity and stress responses by using western blot and RT-PCR (PI3K/Akt, MAPKs and NFkB). The changes in NR expression such as PPARs will be monitored by RT-PCR and western blot. The study will hopefully offer a better theoretical understanding of the roles of nuclear receptors and visfatin in regulating oxidative stress and inflammation in skeletal muscle, information that could be used in the clinical practice focused on the treatment of ageing-associated diseases.

Chapter 2- MATERIALS AND METHODS

Cell culture

Murine-derived C2C12 cells were purchased from ECACC (European Collection of Cell Culture, 91031101) and proliferated as myoblasts in 4.5 g/l glucose Dulbecco's Modified Eagle's Medium (Sigma, Dorset UK D6546) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, P0791), 2mM glutamine (Sigma, Dorset UK G7513) and 15% FCS (Sigma, Dorset UK C5280). The medium was changed every two days and the semi-confluent cultures were seeded at a density of 2000/cm^2. To obtain the differentiated myotubes C2C12 myoblasts were grown until reaching 95% confluence and further incubated for 4-6 days in differentiation medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mM glutamine and 5% horse serum (Sigma, Dorset UK H1270). The differentiation medium was changed every day, and after reaching the level of optimal fusion and differentiation C2C12 myotubes were further cultured in antibiotic-free and serum-free medium for an additional 24 hrs before being subjected to any experimental procedures.

Visfatin and other pharmacological treatments

All visfatin and blocking treatments experiments were performed in antibiotic-free and serum-free differentiation medium. Murine visfatin produced in bacteria by using a His-tag (Biovision, Inc, Mountain View Ca, USA 4908-50; or MBL, Woburn, MA USA JM-4908-50) or in HEK 293 cells by using a FLAG-tag (Axxora, San Diego, CA USA ALX-201-364-C010) was resuspended initially in PBS at a concentration of 0.1 mg/ml and divided in small aliquots which were frozen at -20°C. Before each experiment one vial was de-frozen and further diluted to the final concentration of 100ng/ml in PBS. For the blocking experiments the following inhibitors diluted in DMEM were added to the C2C12-differentiated myotubes 1hr prior visfatin treatment: 10 µM U0126 to block MEK1/2, the upstream activator of ERK 1/2 (Cell Signalling, Danvers, MA

USA9903); 10 μg/ml of the *InSolution*™ p38 MAP Kinase Inhibitor III (Calbiochem, Darmstadt,

Germany 506148) to block p38 kinase; 0.1 μM of Cycloheximide Ready-Made Solution (Sigma,

Dorset UK C4859) to block translation; 1 μg/ml of Actinomycin D (Sigma, Dorset UK A9415) to

block transcription; 50 μg/ml IκB Kinase Inhibitor Peptide (Calbiochem, Darmstadt, Germany

401477) to inhibit IKK α/β, the upstream activator of NFkB; 1 mM N-acetyl-L-cysteine (NAC,

Sigma Dorset UK A9165) to scavenge ROS. Before incubation with visfatin cells were washed

three times with antibiotic-free and serum-free differentiation medium to remove any trace of

inhibitor.

To activate PPAR-β/δ signalling the agonist GW-501516 (Axxora, San Diego, CA

USA ALX-420-032) was resuspended initially in DMSO and diluted to the antibiotic-free and

serum-free differentiation medium to the working concentration of 10 nm before being added to

C2C12 cells for 24 hrs, DMSO being employed as a vehicle. To model the effects of accumulation

of lipids in cultured myotubes *Structolipid*^{TM} emulsion (Fresenius Kabi, Bad Homburg v.d.H.

Germany) was diluted 1/100 in the antibiotic-free and serum-free differentiation medium to a

concentration of 0.2 mg/ml of triglycerides and incubated for indicated times.

Measurement of oxygen free radical production

Cells were washed three times in PBS and incubated at 37°C with 25 μM carboxy-

H2DCFDA diluted initially in DMSO than in PBS at the working concentration (Invitrogen,

Paisley UK I36007) for 30 minutes, used for the detection of reactive oxygen species at 495/529

nm. In order to visualize the nuclei at 360/450 nm1 μM Hoechst 33342 from the same I36007 kit

was added in the last 5 minutes of incubation. For the detection of superoxide anions the kit

MitoSOX^{TM} (Invitrogen, Paisley UK M36008) was used by diluting the fluorescent compound in

initially in DMSO than in PBS at the working concentration of 5 μM for the same range of time,

followed by imaging at 510/580 nm. For the detection of hydrogen peroxide I used the compound pentafluorobenzenesulfonyl fluorescein (Cayman Chemical, Ann Arbor MI USA 10005983) diluted initially in DMSO than in PBS at the working concentration of 1 μg/ml for the same range of time, followed by imaging at 485/529. Cells were further washed three times in PBS, re-incubated in fresh PBS and subjected to the fluorescent-based assays that were performed in two ways. The first was based on fluorescent microscopy imaging by using a Leica DFC420 camera (Leica Microsystems, Wetzlar Germany) attached to a Leica DMIRBE microscope with the following filter cubes: A, I3 and N2.1. Image analysis was performed with the Image J 1.37c (Wayne Rasband, NIH, http://rsb.info.nih.gov/ij/plugins/imagej-updater.html).   The quantification of the fluorescent signal from 4-5 cells from each well was divided to the background/noise of the well and the statistical values (mean, standard deviation and significance) were computed by using Excel (Microsoft Office 2003, Microsoft Redmond, WA USA). The second choice was based on the Fluorometric Imaging Plate Reader FlexStation II (Molecular Devices, Sunnyvale CA USA) that performed automatically the detection of signal and the computation of values at the same wavelengths as specified above. In this case the nuclear staining step was avoided. The recording values were divided to the blank and the statistical values (mean, standard deviation and significance) were computed by using Excel (Microsoft Office 2003, Redmond, Washington, USA).

Measurement of the rate of glucose uptake

C2C12 cells were washed after treatment 3 times in warm PBS and incubated for 30 minutes at 37°C with 0.1 mg/ml of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Invitrogen, Paisley UK N13195), diluted in warm PBS. After the

incubation cells have been washed for 5 times with warm PBS, and the signal detection was performed by imaging at 495/529 nm.

Western blotting

Differentiated C2C12 myotubes grown in 12 wells plates were washed three times in ice-cold PBS and lysed by using ice-cold cell lysis buffer (Cell Signalling, Danvers, MA USA 9803) with 1mM PMSF. For the detection of phospho-proteins 5 mM NaF was also added (Sigma, Dorset UK). In the case of the experiment that tested the possibility of visfatin transport (Fig. 6.1) an additional step of cell washing for three times by using warm DMEM before the ice-cold PBS wash step was performed, in order to detach the visfatin that could have been bound to any receptor localized at the plasma membrane. Cells were scraped from the wells and transferred to 1.5 ml Eppendorf® Biopur™ tubes, which were incubated for an additional 40 minutes on ice with gentle inversion every 10 minutes, the degree of lysis being inspected visually. Cell lysates were centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant was transferred to a new tube from which five microliters of cell lysates were sampled for protein quantification by using the BCA kit (Perbio Science, North Nelson Industrial Estate Cramlington, Northumberland 23225).

For the detection of oxidized proteins the Oxyblot™ kit was used (Millipore, Billerica MA USA S7150). To detect the levels of oxidized proteins 50 mM DTT was added to the cell lysis buffer. From each sample 5 μl was mixed with 5 μl of 12% SDS to denature the proteins. To derivatize the proteins 10 μl of 1x DNPH was added and the tube was incubated for 15 minutes at RT. To end the reaction 7.5 μl of neutralization solution was added and the proteins were loaded on the gels without boiling. For the experiment described in chapter 3, skeletal muscle sample tissues taken from control and HLS-mice were homogenized initially in ice-cold cell lysis buffer by using a Precellys®24 system (Bertin Technologies, Enim group France) with CK28 ceramic beads, followed by the same procedure as above.

For the detection of phospho-proteins I used the following kits and antibodies: phospho-

Akt-pathway sample kit (Cell Signalling, Danvers, MA USA 9916); NFkB pathway sampler kit (Cell Signalling, Danvers, MA USA 9936); and phospho-MAPK family sampler kit (Cell Signalling, Danvers, MA USA catalogue number 9910). For the detection of visfatin I used a rabbit polyclonal antibody (Abcam, Cambridge UK catalogue number ab72128) or an antibody raised in mouse against the FLAG-tag (Abcam, Cambridge UK 18230). In chapter 3 I used the rabbit UCP-3 (Abcam, Cambridge UK ab3477) and rabbit PPAR-β/δ (Abcam, Cambridge UK 8937) antibodies. As loading controls, mouse monoclonal antibodies against GAPDH (Abcam, Cambridge UK 9484) and beta-actin (Abcam, Cambridge UK 8224) have been used. To detect the levels of nitro-tyrosines a mouse monoclonal antibody was used (Millipore, Billerica MA USA CC22.8C7.3).

Briefly, 60 μg of protein supplemented with 1x NuPage sample reduction agent (Invitrogen, Paisley UK NP0004) was boiled in NuPage LDS sample buffer (Invitrogen, Paisley UK NP0007) for 10 minutes at 70°C and then separated in 10 wells Novex Bis-Tris 4-12% gels (Invitrogen, Paisley UK NP0335BOX) or in 12 wells Novex Bis-Tris 10% gels (Invitrogen, NP0322BOX) by using NuPAGE SDS-MOPS (Invitrogen, Paisley UK NP0001) or NuPAGE SDS-MES running buffer (Invitrogen, Paisley UK NP0002). For electrophoresis I used XCell *SureLock™* Mini-Cell system (Invitrogen, Paisley UK EI0001) attached to PowerEase® 500 power supply (Invitrogen, Paisley UK EI86000). The proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel Germany) by using NuPAGE transfer buffer and Mini Trans-Blot Cell system (Bio-Rad, Hemel Hempstead UK 170-3930) connected to PowerEase® 500 power supply (Invitrogen, Paisley UK EI86000). After transfer the membrane was washed for 5 minutes in TBS before being blocked for 1 hr in TBS with 5% BSA and 0.1% Tween-20. All primary antibodies were diluted 1:1000 in 1x TBS with 5% BSA and 0.1% Tween-20 and incubated with the blot overnight at 4°C with gentle rocking (30 rpm). The membrane was washed with TBST three times for 15 minutes each, and then incubated for 1 hr with the goat anti-rabbit-HRP secondary antibodies (Cell Signalling, Danvers, MA USA 7074) provided by each kit 1:2000

in 1x TBS with 5% BSA and 0.1% Tween-2. For the detection of mouse-raised antibodies I used a secondary horse anti-mouse-HRP antibody (Cell Signalling, Danvers, MA USA 7076) or a donkey anti-mouse-HRP antibody (Jackson laboratories, 715-035-150) diluted 1:10,000 in 1x TBS with 5% BSA and 0.1% Tween-20. The membrane was further washed three times in TBST for 15 minutes each, followed by another wash for 5 minutes in TBS. The chemiluminescent signal was detected using SuperSignal West Pico substrate (Perbio Science, North Nelson Industrial Estate Cramlington, Northumberland 34077) and a ChemiDoc XRS system (BioRad, 170-870). The images were saved electronically by using Quantity One 1 (Bio-Rad, Hemel Hempstead UK 160-9601) and exported to Adobe Photoshop CS2 (Microsoft Office, Microsoft Redmond, WA USA). As a loading control, the HRP-bound GAPDH antibody was used (Abcam, Cambridge UK ab9482) diluted 1:5000 in 1x TBS with 5% BSA and 0.1% Tween-20.

RNA extraction and cDNA synthesis

To extract RNA from cells and tissues I used the TRIzol method (Invitrogen, Paisley UK 15596018) and Qiagen RNeasy kit (Qiagen, Düsseldorf Germany catalogue number 74104). I added 1ml of TRIzol directly on wells after a three-cycle washing step with warm PBS and transferred the cells to a tube to which I added 0.2 ml of chloroform. The tube was vortexed vigorously at RT for 10 seconds, and then incubated for another 2 minutes at RT. The tube was centrifuged at 4°C for 10 minutes at 14,000 rpm and after that 0.5 ml of the aqueous (upper phase) was taken from the tube. To this phase I added 0.5 ml volume of absolute ethanol cooled at -20C and mixed thoroughly, followed by filtering on a Qiagen RNeasy spin column and centrifugation for 1 minute at RT at 12,000 rpm. After 1 cycle of washing with 700 microliters of RW1 buffer and another two cycles of washing with 700 microliters and respectively 500 microliters of RPE buffer at RT for 30 seconds at 14,000 rpm, the column was dried by a further centrifugation for two

minutes at the same conditions. 50 microliters of DEPC-treated water was added to the column and incubated for 15 minutes at RT. The RNA was eluted by centrifugation at 12,000 rpm at RT for 1 minute. The quantification and quality control of RNA were performed by using Bioanalyzer 2100 system (Agilent, Santa Clara CA USA catalogue number G2938C) and RNA 600 Nano chips (Agilent, Santa Clara CA USA catalogue number G2938-90034). Before cDNA synthesis I digested the RNA with DNase I (Sigma, Dorset UK AMP-D1) in order to remove any trace of genomic DNA that might affect the RT-PCR assays. 0.5 micrograms of RNA was diluted in DEPC-treated water to a final concentration of 8 microliters and incubated with 1 microliter of 10x DNase reaction buffer and 1 microliter (1 unit) of Amplification Grade DNase I for 15 minutes at 37°C. After 15 minutes I added 1 microliter of STOP solution (50 mM EDTA) for the chelation of calcium and magnesium ions, and the tube was incubated at 70°C for 10 minutes followed by a 5 minutes step of cooling the tubes on ice. To obtain the cDNA I used the Superscript III First strand synthesis system (Invitrogen, Paisley UK catalogue number 18080-051). Briefly I mixed the RNA with 1 microliter of 10 mM dNTP mix and 1 microliter of 50 ng of random hexamers and incubated the mixture at 65°C for 5 minutes, then cooled it on ice for another five minutes. I further added 2 microliters of 10x first-strand buffer, 4 microliters of 25mM MgCl2, 1 microliter of 0.1M DTT, 1 microliter of RNaseOUT (40 units) and 1 microliter (200 unit) of SuperScript III RT to the tube. After gentle mixing the tube was incubated initially at 25°C for 10 minutes and later at 50°C for 60 minutes. The reaction was stopped at 70°C for 15 minutes, and then tube was chilled on ice. To remove any trace of RNA I added 1 microliter (2 units) of RNase H from E. coli for 20 minutes at 37°C.

The Bio-Rad I-Cycler (Bio-Rad, Hemel Hempstead UK) with FAM-490 system detection was employed for real time quantitative RT-PCR by using the Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Paisley UK catalogue number 11730-025). PCR thermocycler conditions were 50°C for two minutes, 90°C for two minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. All samples were run in triplicate with both primer sets and the control genes to normalize the differences in amount of starting material. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using Student's t-test. A p-value of less than 0.05 was considered significant. The following probes from Taqman (Applied Biosystems, Life technologies Carlsbad, California USA) have been used: **mouse samples** (**PPAR-β/δ,** Mm00803186_g1; **PPAR-α,** Mm00440939_m1; **PPAR-γ,** Mm00440945_m1; **NOX4**, Mm00479246_m1**; TNF-α**, Mm00443258_m1**; IFN-γ**, Mm99999071_m1; **IL-6**, Mm99999064_m1**; IL-15**, Mm00434210_m1; **UCP-3,** Mm01163394_m1**; GLUT-1,** Mm01192270_m1**; GLUT-3,** Mm00441483_m1**; GLUT-4,** Mm01245507_g1**; SOD2,** Mm01313000_m1**; GPX-3**, Mm00492427_m1**; SOD-1**, Mm01344233_g1**; HIF-1A**, Mm01283760_m1**; NRF-2** Mm00477786_m1**; VEGFa**, Mm01281449_m1**; TRAF1,** Mm00493827_m1**; nNOS,** Mm00435175_m1; **SOD1,** Mm01344233_g1; **CAT**, Mm00437992_m1; **NRF1**, Mm00447996_m1; **TRAF6,** Mm00493836_m1; **CPT-1b,** Mm00487200_m1; **CPT-2,** Mm00487205_m1; **UCP-3**, Mm00494074_m1; **ACCα,** Mm01304277_m1; **ACCβ**, Mm01204683_m1; RXRα, Mm00436264_m1; **AMPKα2,** Mm01264789_m1; **AMPKβ2,** Mm01257133_m1**; PGC-1α**, Mm00447183_m1; **PGC-1β**, Mm01258518_m1; **YKT6** homolog (Saccharomyces cerevisiae) snare protein: Mm00457727_m1; as control **GADPH** 4352932E and **ACTB** 4352933E); **rat samples** (**Arntl,** Rn00577590_m1; **Runx1,** Rn00569082_m1; **NR1D1**, Rn00595671_m1; **Tlr2**, Rn02133647_s1; **Mgmt,** Rn00563462_m1; **Uchl1**, Rn00568258_m1; **Epn3**, Rn01456494_g1; **Sln,** Rn02769377_s1; **Casq2**, Rn00567508_m1; **Apln,** Rn00581093_m1; **Psmb8**, Rn00589926_m1; **Psmb9**, Rn00562296_m1; **Casp3,** Rn00563902_m1; **Tmbim1,** Rn01400508_g1; **NR4A1**, Rn00666995_m1; **NR4A2,** Rn00570936_m1; **NR4A3**,

Rn00581189_m1; **SREBF1**, Rn01495772_g1; **FASN**, Rn00569117_m1; **SCD-1**, Rn00594894_g1; **GPAM**, Rn00568620_m1; **PPAR-γ,** Rn00440945_m1; **PPAR-β/δ,** Rn00565707_m1; **PPAR-α,** Rn00566193_m1; **PGC-1α**, Rn00580241_m1; **PGC-1β,** Rn00598552_m1; **AMPK γ3,** Rn01400861_g1; **UCP-3,** Rn00565874_m1; **FABP-4,** Rn00670361_m1; as controls **ACTB**, 4352931E, **GAPDH**, 4352338E).

Microarray assay

Labelling

600 ng RNA and 3 µl Agilent One-Colour RNA Spike-In RNA were labelled with the Agilent Low RNA Input Linear Amplification Kit PLUS, One-Colour according to instructions as follows: 1.2 µl T7 Promoter Primer was added to 600 ng RNA and 3 µl spike in control and denatured at 65 ºC. First Strand Buffer, DTT, dNTP MMLV and RNaseOut was added. The cDNA was synthesised during the following incubation step (2h at 40 ºC). After 10 min denaturation at 65 ºC and the addition of Cy-labelled CTP, Transcription Buffer, DTT, NTP, PEG, RNaseOUT, Inorganic Phosphatase, and T7 RNA Polymerase the synthesis of the fluorescent labelled cRNA was performed during the second incubation step (2 h at 40 ºC). The labelled cRNA was purified with the RNeasy Mini Kit (Qiagen Ltd, Düsseldorf Germany).

Hybridisation

The Agilent Hybridisation Kit (Agilent, Santa Clara CA USA catalogue number 5188-5242) was used in conjunction with Agilent Rat Oligo Arrays. 2µg of the labelled sample RNA were used for hybridisation according to the Agilent One-Colour Microarray-Based Gene Expression Analysis Protocol The hybridisation was performed for 17 h at 65 ºC at 10 rpm. Slides were them washed for

1 min at 22 ℃ in Wash Solution 1 (catalogue number 5188-5325) and 1 min at 22 ℃ in Wash Solution 2, pre-warmed to 37 ℃ (catalogue number 5188-5326). Slides were incubated for 30s in Agilent Stabilisation and Drying Solution (Agilent, Santa Clara CA USA catalogue number 5185-5979).

Scanning

The slides were scanned with the Agilent G2565BA Microarray Scanner System. For data extraction and quality control, the Agilent G2567AA Feature Extraction Software (v.9.1) was used.

Microarray bioinformatics Analysis

Extracted data were analysed using GeneSpring GX 7.3.1 (Silicon Genetics, CA USA). Agilent standard scenario normalisations for FE1-colour arrays were applied to all data sets. A subset of genes for data interrogation was generated that excluded controls, spots of poor quality, and gene probes that were present in less than 50% of samples. From these selected genes, relative expression in animals fed the *ad lib* diet was compared to animals on the caloric restriction diet. Genes differentially regulated by greater than 2.0-fold were selected. One-way, parametric, ANOVA tests were performed followed by Benjamini and Hochberg multiple test correction with a false discovery rate of 0.05. Microsoft Excel templates were prepared containing genes that were over- and under-expressed following CR.

Ingenuity^TM Pathway Analysis (IPA)

Ingenuity^TM Pathway Analysis (IPA) 3.0 (Ingenuity^TM Systems, CA USA) was utilised to assemble functional networks altered by CR. Ingenuity entry tool systematically encodes

findings presented in peer-reviewed scientific publications into ontologies, or groups of genes /proteins related by common function. Molecular networks of direct physical, transcriptional, and enzymatic interactions were computed from this knowledge base using the genes differentially regulated by CR. The resulting networks contain molecular relationships with a high degree of connectivity and every gene in the network is supported by published literature.

Measurement of the levels of oxidized lipids

To measure the levels of oxidized lipids I used a LPO kit (Cayman chemical, Ann Arbor MI USA 705002). C2C12 cells were incubated or not with 100 ng/ml of visfatin for 24 hrs. C2C12 cells were scrapped from wells and sonicated in HPLC-grade water (without any metals) followed by extraction of lipids by sonication. 500 microliters of sonicated extract from each sample was added to 500 microliters of Extract R provided by kit saturated with methanol, followed by adding of 1 ml of deoxygenated, ice cold chloroform to the tube. After centrifugation at 1,500xg for 10 minutes at 4°C, the bottom layer was collected which was further added to 450 microliters of a 1:1 mixture of deoxygenated, ice cold chloroform and deoxygenated methanol, followed by incubation with 50 microliters of chromogen provided by the kit (1:1 mixture of FTS reagent 1 consisting of 4.5 mM ferrous sulphate in 0.2 M HCl and FTS reagent 2 consisting of 3% of methanolic solution of ammonium thiocyanate). After 5 minutes of RT incubation, 300 microliters from each sample was taken and measured at 500 nm in a microplate reader by using a glass-bottom 96-well plate. The kit is based on the following reactions:

$$ROOH + Fe^{2+} \rightarrow RO^{\cdot} + Fe^{3+}$$
$$RO^{\cdot} + Fe^{2+} + H^{+} \rightarrow ROH + Fe^{3+}$$
$$Fe^{3+} + 5SCN^{-} \rightarrow Fe\,(SCN^{-})^{2-}_{5}$$

Measurement of antioxidant capability of the cells

To measure the antioxidant status I used a kit from Cayman chemical, Ann Arbor MI USA catalogue number 709001. C2C12 cells were incubated or not with 100 ng/ml of visfatin for 24 hrs, or with 100 μM of hydrogen peroxide for 30 minutes as a negative control. C2C12 cells were scrapped from the wells and sonicated in ice-cold buffer provided by the kit (5mM potassium phosphate, pH7.4 with 0.1% glucose and 0.9% sodium chloride) and centrifuged at 10,000xg at 4°C for 15 minutes. 10 microliters from the supernatant was mixed with 10 microliters of metmyoglobin resuspended in the assay buffer provided by the kit and with 150 microliters of **ABTS** chromogen (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate), resuspended in HPLC-grade water. 40 microliters of 441 μM hydrogen peroxide diluted in HPLC-grade water was added to each well to initiate the reaction and the plate was shaked at RT for 5 minutes in the dark. **Trolox** (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was used as a positive control instead of samples. The measurement was done at 405 nm in a microplate reader.

Measurement of cell viability

To measure the cell viability LDH-Cytotoxicity Assay Kit II was used (Biovision, Inc Mountain View, CA USA K313-500). If the plasma membrane is compromised than lactate is released in the culture medium and the lactate dehydrogenase from the kit will further oxidize lactate, generating nicotinamide adenine dinucleotide (NADH) that is recognized by the WST compound. The LDH reaction buffer is made of 200 microliters of WST substrate resuspended in HPLC-grade water with 10 ml of LDH assay buffer. Briefly, 10 microliters of culture medium were taken from cells incubated or not with 100 ng/ml of visfatin for 24 hrs or with 100 μM of hydrogen peroxide for 30 minutes as a positive control. The culture medium sampled was further added to
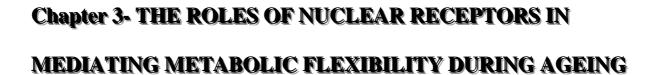
100 microliters of LDH reaction mix and incubated for 30 minutes at RT. After stopping the reaction with the STOP solution, the absorbance was measured at 450 nm.

ELISA

To detect the existence of visfatin (eNampt) in the culture medium of C2C12 cells in basal or stimulated an ELISA kit (Raybiotech, Norcross GA USA VIS-EIA-1) was used. 185 microliters of 1x Assay Diluent B containing biotinylated visfatin (BV) was mixed with 62.5 microliters of medium taken from wells with C2C12 cells or with the standards. The final concentration of the biotinylated visfatin in each tube was 10ng/ml. The 96 wells plate that had been already coated with goat anti-rabbit secondary antibody was further incubated with a rabbit anti-visfatin antibody. After 1.5 hrs the plate was washed 4 times and incubated in each well with either 100 microliters of mixed BV-standards provided by the kit, either with 100 microliters of mixed BV-sample medium from C2C12 cells prepared as above. After 2.5 hrs the plate was washed and further incubated in each well with 100 microliters of biotin-streptavidin solution for another 45 minutes at RT. After another washing step the TMB One-Step substrate was added to each well for 30 minutes with gentle shaking. 50 microliters of STOP solution was added and the reaction was measured at 450 nm. As positive a serial dilution of standards staring from 1000 ng until 0.1 ng was used and calibrated. The minimum visfatin concentration detectable was 379 pg/ml.

Statistical analysis

For computation of statistical values (average, mean, standard deviation, Student's t-Test, ANOVA) and for the graphical visualization I used the Excel 2003 software (Microsoft Office, Microsoft Redmond, WA USA). To perform ANOVA tests I used GraphPad Prism software (GraphPad, La Jolla, CA USA).

Chapter 3- THE ROLES OF NUCLEAR RECEPTORS IN MEDIATING METABOLIC FLEXIBILITY DURING AGEING

3A. Introduction

As it was recently predicted by a World Health Organization report (WHO, 2011), between 2000 and 2050 the aged population (over sixty years) will reach the landmark of two billion worldwide and 85% of them will live in the developing world. This aged population is expected to have an increased lifespan and a higher degree of an active lifestyle than at present. Medical problems encountered in current aged populations include cancer, osteoporosis, neurodegeneration and dementia, arthritis, cardiovascular diseases, stroke, sarcopennia and frailty, glaucoma and hearing loss, metabolic syndrome and obesity (Fontana et al., 2010). Therefore, a proper theoretical understanding of the cellular signalling pathways underlying the pathogenesis of ageing-associated diseases is required in order to design better therapies for the groups affected or better preventive strategies for the groups at risk. The role of the biomedical research is consequently to decipher the mechanisms leading to biological ageing and to imagine new ways to prevent (or at least delay) the detrimental symptoms associated with increased age.

During ageing the skeletal muscle is characterized by significant changes in myofibre number, mass, size and structure and by a defective excitation-contraction coupling system (Rossi et al., 2008). Such changes are associated with altered gene expression, increased cell death and reduced cell survival, increased oxidative and nitric stress, inflammation, ER-stress and hypoxia. These events result in a reduced muscle mass and strength, impaired endurance and contractility and reduced regenerative properties partially due to an increased cell death rate of satellite cells (Gopinath and Rando, 2008), being noticeable also a transition toward a slower-twitching muscle fibre type which exhibits a shift to a more aerobic-oxidative metabolism (Doran et al., 2009). In addition, the skeletal muscle from older human adults presents a significant accumulation of large intracellular lipid droplets and a changed interaction with a fewer number of mitochondria. This lipid accumulation during ageing is further associated with an impaired mitochondrial bioenergetic process that is increasing the levels of ROS (Crane et al., 2010).

This study used two approaches to model the effects of ageing on skeletal muscle. One was designed to study the effects of accelerated ageing process by using the *hindlimb suspension* method in mice (**HLS**), and one designed to explore the mechanisms of delayed or improved ageing phenotypes in rats subjected to *caloric* or *dietary restriction* (**CR/DR**). In order to investigate the changes induced by these two approaches a global genome profiling by using microarray was performed as described above, followed by data analysis. To confirm the changes identified by microarray the findings were further validated by using RT-PCR and western blot. The main *goal* of this chapter was to identify changes in the expression of families of nuclear receptors and other transcriptional regulators, since this could offer useful tools for targeting the ageing-associated diseases affecting skeletal muscle.

3B. Results
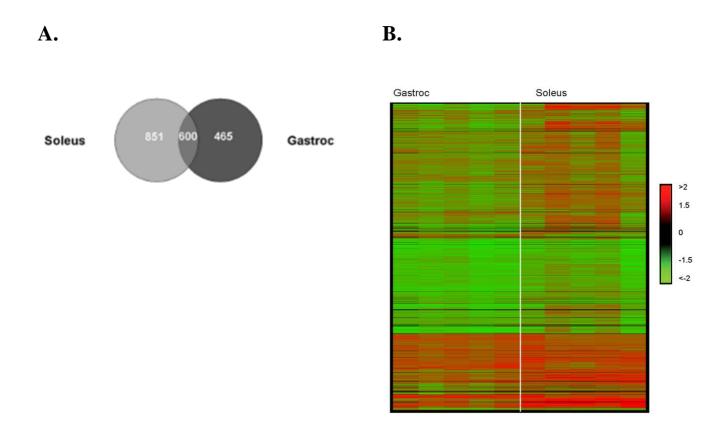
3.1 Identification of the early-responsive genes induced by hindlimb suspension in mice

The hindlimb suspension method (HLS) is widely employed in animal models to investigate the effects of inactivity on skeletal muscle physiology characterizing human skeletal muscle during ageing, bed rest or spaceflight (Cartee, 1995; Chapes and Ganta, 2005; Morey-Holton et al., 2005; Riley et al., 1995). Most studies have used this method to explore the long-term, *chronic* effects of inactivity (St-Amand et al., 2001), but less is known about the short-term, *acute* effects. Our collaborators from the University of Kentucky (Dr. Reid lab, Lexington, USA) performed the unloading experiments by keeping murine hindlimb suspended for short-term (1 day) or long-term (12 days), followed by the sampling of skeletal muscle (soleus and gastrocnemius) as described above (Matuszczak et al., 2004). In order to study the changes induced by acute HLS on global skeletal muscle gene expression profile, RNA was extracted in our laboratory from gastrocnemius and soleus samples from both control and 24 hrs HLS animals (n=5) and whole-genome microarray analysis was performed (Mazzatti et al., 2008). The raw data generated by the microarray experiment (GEO number GSE9802) were further analyzed by using GeneSpring GX 7.3.1 software. After the exclusion of probes that were absent or present in less than 50% of the samples, only the probes that showed at least 1.5-fold change between control and HLS animals and passed the ANOVA test (P< 0.05) were selected. The final list included 1,451 genes differentially expressed in soleus and 1,065 genes in gastrocnemius. In the case of soleus 417 genes were upregulated and 1,034 were downregulated, and in the case of gastrocnemius 318 genes were upregulated and 747 were downregulated. As seen from the Venn diagram depicted in Fig. 3.1A, only 600 genes were differentially expressed in both muscle types. This subset of commonly regulated genes was further subjected to gene cluster analysis by using a Pearson correlation. In

order to visualize the genomic differences between control and HLS mice a heat map was

constructed ordered by tree clustering and significance (Fig. 3.1B), in which each row represents a

probe and each column represents one sample. The upper part of the map show genes that did not

correlate between gastrocnemius and soleus, the middle part represents genes that were

downregulated in both muscle types and the lower part represents genes that were upregulated in

both muscle tissues. The red colour represents upregulation and the green colour represents

downregulation in gene expression, the intensity of the colour being correlated with the degree of

change normalized to the negative control values.

Fig. 3.1 Venn analysis and hierarchical clustering of hindlimb suspension (HLS)-altered gene probes.

A. Venn analysis of the gene expression profiling of control and unloaded muscle demonstrated that a total of 1,451 and 1,065 genes were differentially expressed after HLS in soleus and gastrocnemius muscle, respectively (>1.5-fold, $P < 0.05$); B. A gene cluster analysis was performed by using a Pearson correlation and heatmap generation of the 600 gene probes differentially regulated in both soleus and gastrocnemius muscles. Each row represents 1 of 600 probe sets and each column represents 1 sample. The intensity of colour in a cell represents the normalized expression of the probe, where green and red depict low and high expression, respectively (n=6).

A.

B.

3.2 Acute hindlimb suspension (HLS) changes the expression profile of genes involved in glucose and lipid metabolism

Gene Ontology (GO) analysis of genes that were significantly regulated in both soleus and gastrocnemius was used in order to investigate the effects of muscle unloading on individual biological processes. Table 3.1 shows the list of the ten most modulated biological processes changed by hindlimb suspension ordered by the P value, based on the computation of the number of the genes expected in the class normalized to the number of the genes observed in microarray. From a total of ten ontologies altered by HLS the first seven are associated with muscle development, skeletal muscle function and with protein synthesis. This discovery correlates with previous findings that deciphered such changes in skeletal muscle during ageing in both humans and rodent models (Jaspers et al., 1988; Tischler et al., 1985), further supporting the assertion that hindlimb suspension is an accurate model of investigation of the ageing phenotypes. Other ontologies significantly changed were cytoskeleton organization and the response to heat shock/stress. The skeletal muscle during ageing is characterized not only by structural changes but also by an impaired defence against infection and reduced resistance to stress, and these two processes are regulated partially by changes in the expression of genes involved in the response to heat stress and heat shock (Morton et al., 2009). My findings could therefore offer an explanation for the perceived changes in the aged skeletal muscle.

It has been previously shown that long-term unloading of skeletal muscle changed the expression profile of genes regulating lipid and glucose metabolism (Stein et al., 2005; Stein and Wade, 2005), leading to an increased reliance on glucose as a main fuel and a reduced utilization of lipids that will accumulate intracellularly. As many as 18 glucose-related ontologies were altered following the short term HLS in my experiment, especially gluconeogenesis and lipid metabolism (Table 3.2). In my experiment, while the expression level of key enzymes in gluconeogenesis and glycolysis such as fructose-1, 6-biphosphatase (FBP2), glycerol-3-phosphate

dehydrogenase 1 (GPD1) and glucose 1-dehydrogenase (H6PD) were *increased*, the expression

levels of two other critical enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase

were *not* modified (Table 3.3). It was also noticed a *reduced* expression of other crucial genes for

glucose metabolism such as hexokinase 2 (HK2), phosphoglycerate mutase 1 (PGAM1) and

glycogen synthase 2 (GYS2). In conclusion, some of genes involved in glucose metabolism

identified in the early stage of unloading fit to this paradigm identified in the chronic unloading, but

others did not. In addition, in contrast with the conclusions of independent long-term unloading

experiments I noticed that many genes involved in lipid metabolism presented in fact an *increased*

level of expression, such as acyl-CoA thioesterase 1 (ACOT1) which is critical for unsaturated fatty

acid biosynthesis, farnesyl diphosphate farnesyl transferase 1 (FDFT1) which is involved in

cholesterol biosynthesis, branched chain ketoacid dehydrogenase E1 (BCKDHA) which is involved

in branched fatty acid oxidation and acyl-CoA synthetase long-chain family member 3 (ACLS3)

which is involved in peroxisomal lipid metabolism. Stearoyl-Coenzyme A desaturase 2 (SCD-2),

which is involved in monounsaturated fatty acid synthesis, was nevertheless found to be

downregulated. The increased expression of many genes regulating lipid utilization during the

early stage of unloading as shown by my microarray assay contrasted with the results reported

independently about the chronic stage (Stein et al., 2005; Stein and Wade, 2005), which suggests

that the lipid metabolism could be in fact *upregulated* during this initial phase. Moreover, the

differential activation of genes involved in glucose metabolism as seen from my microarray results

suggests that in the early stage the utilization of glucose as a main energy substrate noticed by

independent studies in the chronic stage is in its *incipient* phase, but not yet fully established.

Therefore I can conclude that in the early stage is discernible an incipient process

of *adaptation* to metabolic dysfunction characterized by an *increased* lipid metabolism and a

perturbed glucose metabolism. Such putative adaptation occurs via changes in gene expression

profile that nevertheless disappear later, in the chronic stage. Such ontologies are not equivalent

nevertheless to molecular pathways and do not describe the dynamics of interactions between genes within the ontology. To address this issue Ingenuity Pathway Analysis (IPA) was used to identify molecular pathways affected by the short term hindlimb suspension in both soleus and gastrocnemius. This software searches a vast manually curated proprietary database of publicly available research data comprising all protein-protein interactions, protein-DNA interactions, signalling pathways, transcriptional networks, microRNAs and drug metabolism included in peer-reviewed scientific publications. The algorithms will identify any kind of direct and indirect relationships between genes in a certain given list, as the one generated by my microarray experiment, and the output will be represented as a network. The analysis of the 600 subset of genes by IPA showed that as many as 19 relevant networks were significantly changed after 24 hours of unloading in both types of muscle (Table 3.5). These networks are associated with cancer, organogenesis and development, regulation of cell cycle, drug metabolism, cell death and cell signalling, aspects known to be involved in progression of ageing. The network regulating specifically the glucose and lipid metabolism contains 23 focus genes and it is ranked the fifth with a total score of 33. The score represents the total number of relationships (*edges*) between the focus genes included in this particular network, a higher score meaning a higher statistical probability of physiologically relevant interactions. These relationships are depicted in Fig. 3.2 for soleus, the genes coloured in red being *upregulated* in the experiment, the genes coloured in green being *downregulated* and the genes that were *not changed* in my experiment being uncoloured. A central node in this network involved in lipid metabolism represented by PPAR-α was downregulated in both muscle types, simultaneously with its target genes PGC -1α and PGC-1β (Table 3.4 and Fig. 3.2). RXR, another central node in this network, was not changed.

Table 3.1 Top 10 most significantly regulated ontologies after acute unloading

Total number of genes included in each ontology (computed from entire mouse genome), expected % of genes reflected in each ontology (computed from entire mouse genome), the number of genes differentially regulated by hindlimb suspension (HLS) in each ontology (computed from the list of HLS-regulated genes) and % of genes in the ontology that were differentially regulated after HLS (computed from the list of HLS-regulated genes) are shown. P value reflects the significance of change in prevalence of genes in each ontology under the HLS condition relative to the expected prevalence of genes in each ontology.

Ontology	Total No. of Genes in Ontology	% of Genes in Ontology	No. of Genes changed by HLS	% of Genes changed by HLS in Ontology	p-value
GO:7517: muscle development	299	1.305	31	4.354	5.1E-09
GO:51258: protein polymerization	82	0.358	15	2.107	3.0E-08
GO:6936: muscle contraction	131	0.572	18	2.528	1.3E-07
GO:7519: striated muscle development	212	0.925	23	3.23	2.1E-07
GO:48741: fiber development	124	0.541	17	2.388	3.0E-07
GO:48747: muscle fiber development	124	0.541	17	2.388	3.0E-07
GO:48637: skeletal muscle development	139	0.607	18	2.528	3.2E-07
GO:7010: cytoskeleton organization and biogenesis	917	4.238	57	8.006	3.4E-06
GO:9408: response to heat	44	0.192	9	1.264	6.9E-06
GO:48513: organ development	1937	8.462	92	12.92	2.9E-05

Table 3.2 Ontologies associated with glucose and lipid metabolism significantly regulated by HLS in soleus muscle

P value reflects the significance of change in prevalence of genes in each ontology under the HLS condition relative to the expected prevalence of genes in each ontology.

ontologies	p-value
GO:19216 regulation of lipid metabolism	0.000178
GO:6094 gluconeogenesis	0.000675
GO:44255 cellular lipid metabolism	0.00109
GO:45444 fat cell differentiation	0.00141
GO:43255 regulation of carbohydrate biosynthesis	0.00242
GO:6638 neutral lipid metabolism	0.00285
GO:6629 lipid metabolism	0.00947
GO:8610 lipid biosynthesis	0.0103
GO:19217 regulation of fatty acid metabolism	0.0161
GO:45923 positive regulation of fatty acid metabolism	0.0169
GO:1676 long-chain fatty acid metabolism	0.018
GO:7005 mitochondrial organization and biogenesis	0.0194
GO:6839 mitochondrial transport	0.0247
GO:6006 glucose metabolism	0.0258
GO:1678 cellular glucose homeostasis	0.029
GO:19915 sequestration of lipid	0.029
GO:44242 cellular lipid metabolism	0.03
GO:45834 positive regulation of lipid metabolism	0.0433
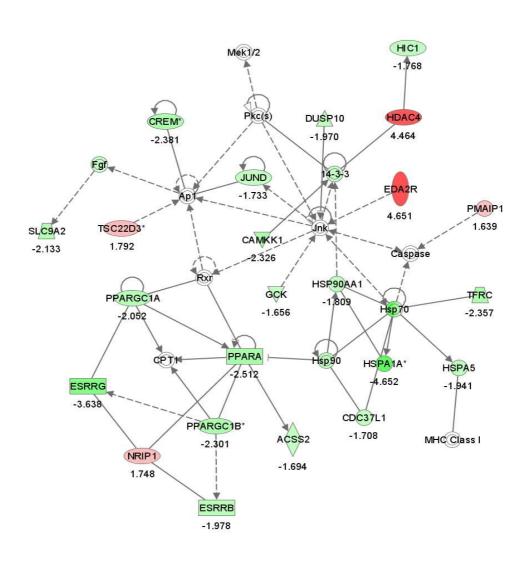
Table 3.3 List of the relevant genes involved in lipid and glucose metabolism,
significantly regulated by acute HLS in both soleus and gastrocnemius

Gene identifier, symbol, common name and microarray fold-change observed in CR-rats compared to *ad lib*

gene identifier	symbol	common name	fold-change
NM_007994	FBP2	fructose bisphosphatase 2	1.89
NM_010271	GPD1	glycerol-3-phosphate dehydrogenase 1	1.94
NM_173371	H6PD	glucose 1-dehydrogenase	1.66
NM_013820	HK2	hexokinase 2	- 1.59
NM_023418	PGAM1	phosphoglycerate mutase 1	- 1.9
NM_145572	GYS2	glycogen synthase 2	- 2.1
NM_012006	ACOT1	acyl-CoA thioesterase 1	3.34
NM_010191	FDFT1	farnesyl diphosphate farnesyl transferase 1	2.37
NM_007533	BCKDHA	branched chain ketoacid dehydrogenase E1	1.52
NM_028817	ACSL3	acyl-CoA synthetase long-chain member 3	2.02
NM_009128	SCD-2	stearoyl-Coenzyme A desaturase 2	- 1.56

Fig. 3.2 Analysis of lipid and glucose metabolism molecular networks regulated by HLS in soleus muscle.

Differentially expressed genes from analysis of soleus muscle after HLS were analyzed by the Ingenuity Pathway Analysis (IPA) tool. The most significant network involved in glucose and lipid metabolism is shown as regulated by HLS in soleus muscle. Differentially regulated genes are shown in red and green, depicting upregulation and downregulation after HLS (compared to ambulatory control), respectively. Bold colour indicates a high degree of regulation (2.5-fold), while pale colour indicates 1.5- to 2.5-fold differential regulation. Noncolored genes were not found to be differentially expressed after treatment. Positive regulatory interactions are marked by solid arrows (direct interactions) or dashed arrows (indirect interactions). Negative interactions are shown by inhibitory arrows.

3.3 Acute hindlimb suspension modifies PPAR-dependent signalling pathways

In order to have a better picture of the **PPAR-α** signalling the Ingenuity Pathway Analysis (IPA) was used to identify possible changes in the networks that it regulates. The visualization of canonical PPAR-α signalling pathway which is depicted in the Fig. 3.3 shows that acute HLS altered in soleus the expression of some of its components such as PPAR-α, HSP90, STAT5B, c-FOS, IKBKG, NGFR and NCOA (Table 3.4). The fact that some of the genes modified by unloading are nodal points within the PPAR-α signalling pathway investigated indicates that the change in its expression was physiologically relevant. In order to validate the changes observed by microarray, I further performed RT-PCR for all three members of PPAR family and discovered that while PPAR-α and PPAR-γ were downregulated by HLS in both types of skeletal muscle, the expression level of PPAR-β/δ was upregulated in soleus. Subsequent RT-PCR assays showed that while the levels of PPAR-α target genes such as PPGC-1α and PGC-1β were downregulated in both soleus and gastrocnemius, the expression levels of other PPAR target genes such as carnitine palmitoyltransferase-1b (CPT-1b), carnitine palmitoyltransferase-2 (CPT-2) and uncoupling protein 3 (UCP-3) were significantly upregulated in soleus (Fig. 3.4).

In gastrocnemius the expression level of CPT-2 was downregulated while the other two target genes (CPT-1b and UCP-3) showed only a trend toward upregulation. RT-PCR was again used to further assess the possible change in expression of several genes that are critical for lipid and glucose homeostasis such as AMPK and ACC. While the expression level of β2 subunit of AMP-activated protein kinase (AMPK) in soleus was slightly downregulated by HLS, the expression level of α2 subunit was upregulated. Similarly, while the expression level of α subunit of acetyl-CoA-carboxylase (ACC) was not changed, the expression of β subunit was upregulated in both muscle types by HLS. These results demonstrate the differential regulation of PPAR-dependent signalling pathways and of genes involved in lipid and glucose metabolism in different types of muscle such as soleus and gastrocnemius. Western blotting was used to certify the changes

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identified at transcriptomic level. As seen from Fig. 3.5, the acute hindlimb suspension increased the protein levels of uncoupling protein 3 (UCP-3) and PPAR-β/δ in soleus compared to ambulatory animals, confirming the microarray and RT-PCR results. To investigate whether the changes observed in the acute phase were present also in the chronic stage, an RT-PCR assay for UCP-3 and PPAR-β/δ probes was performed by using samples taken from soleus muscle of mice subjected to HLS for 12 days. As seen from Fig. 3.6, UCP-3 expression was upregulated but PPAR-β/δ expression reverted to control levels. This could indicate that UCP-3 expression is regulated in the chronic phase by other signalling pathway besides PPAR signalling. PPAR-β/δ expression is increased only in the acute phase of the unloading, suggesting that the compensatory effect is overwhelmed after a long-term suspension. Alternatively, the initial increase in PPAR-β/δ expression during the acute phase could be functionally sufficient for the long-term increase in UCP-3 expression identified even after 12 days of HLS, when PPAR-β/δ expression is back to normal.

Table 3.4 Genes belonging to PPAR-α canonical pathway significantly regulated by acute HLS in soleus

Gene identifier, symbol, common name and fold-change in microarray experiment observed in CR-rats compared to *ad lib*

NM_011144	**PPAR-α**	peroxisome proliferator activated receptor alpha	- 2.512
NM_008904	**PGC -1α**	peroxisome proliferative activated receptor γ coactivator 1 alpha	- 2.05
NM_133249	**PGC-1β**	peroxisome proliferative activated receptor γ coactivator 1 beta	- 2.213
NM_010480	**HSP90**	heat shock protein 90kDa alpha	- 1.8
NM_011489	**STAT5b**	signal transducer and activator of transcription 5B	1.56
NM_010234	**c-FOS**	FBJ osteosarcoma oncogene	- 1.9
NM_010547	**IKBKG**	inhibitor of kappaB kinase gamma	- 1.6
NM_033217	**NGFR**	TNFR superfamily, member 16	1.9

Table 3.5 Networks significantly regulated in soleus and gastrocnemius muscle after HLS as determined by   Ingenuity Pathway Analysis (IPA)

Network score, number of HLS-regulated genes (focus genes), and main cellular functions or conditions (diseases) in which these genes play a role are displayed for each of these networks.

Score	Focus Genes	Top Functions or Conditions/Diseases
44	28	Cancer, Reproductive System Disease, Cell Cycle
42	27	Auditory and Vestibular System Development and Function, Connective Tissue Development and Function, Digestive System Development
37	25	Cellular Movement, Cancer, Neurological Disease
35	24	Cancer, Cell Cycle, Reproductive System Disease
33	23	Carbohydrate Metabolism, Small Molecule Biochemistry, Lipid Metabolism
29	21	Carbohydrate Metabolism, Small Molecule Biochemistry, Cellular Assembly and Organization
25	19	Cancer, Cellular Growth and Proliferation, Nervous System Development and Function
21	17	Cell Death, Haematological System Development and Function, Cellular Development
19	16	Connective Tissue Development and Function, Immunological Disease, Lipid Metabolism
18	15	Drug Metabolism, Gene Expression, Lipid Metabolism
18	15	Gene Expression, Small Molecule Biochemistry, Cardiovascular Disease
18	15	Genetic Disorder, Skeletal and Muscular Disorders, Dermatological Diseases and Conditions
18	15	Cancer, Cell Death, Gene Expression
16	14	DNA Replication, Recombination, and Repair, Cell Cycle, Cancer
16	14	Organism Development, Haematological System Development and Function, Cellular Function and Maintenance

Fig. 3.3 PPAR- canonical pathway in soleus muscle after HLS.

Genes that were differentially expressed in soleus muscle after HLS were clustered into canonical pathways with the Ingenuity Canonical Pathway tool. The PPAR signaling pathway was significantly regulated by HLS in both soleus muscle ($P < 0.001$). Coloured genes were identified by microarray analysis as differentially upregulated or downregulated in HLS samples compared with control (red and green, respectively). Other uncoloured nodal genes are directly or indirectly associated with the differentially expressed genes but were not found to be significantly regulated by HLS. Genes are linked by their subcellular location (extracellular, cytoplasmic, nuclear; from top to bottom, respectively). Main processes modulated by transcriptional regulation are shown. The legend of the figure is included in Supplementary Figure 1.
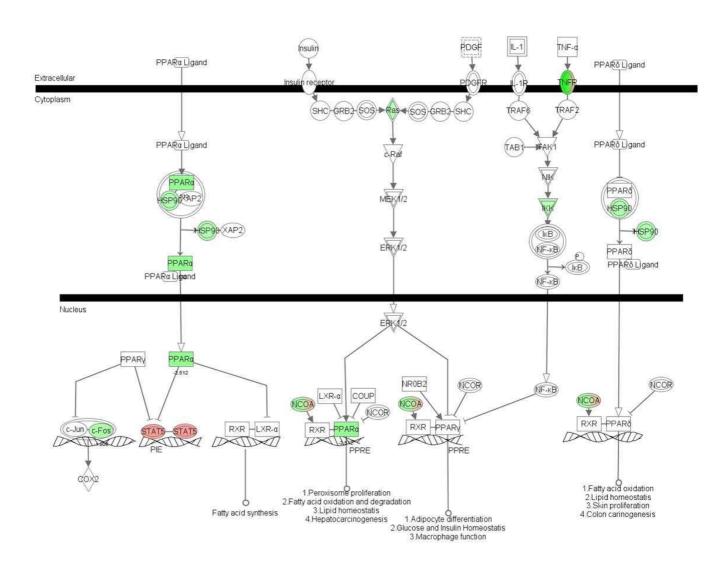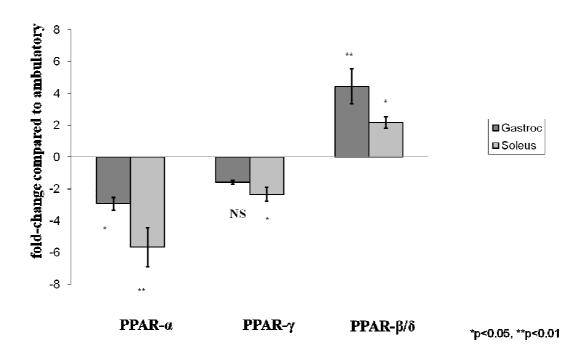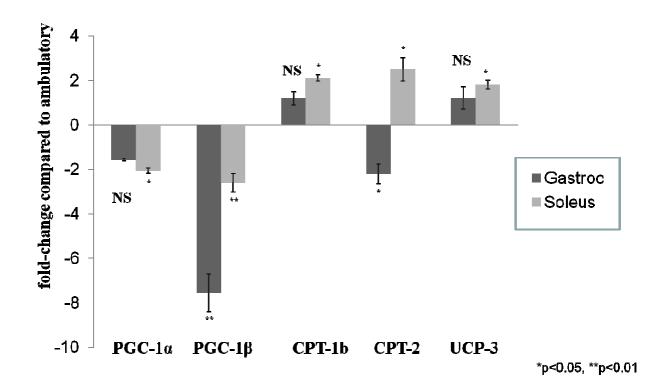
Fig. 3.4 Acute HLS changes the expression profile of PPAR family and of their associated signaling networks in skeletal muscle samples

       Soleus and gastrocnemius tissue samples were isolated from control animals and animals after 1-day HLS. RNA was extracted from ambulatory ($n = 4$) and 1-day HLS ($n = 4$) conditions, followed by RT-PCR for PPAR-α, -γ, -β/δ probes **(A)**; for PGC-1α, PGC-1β, CPT-1b, CPT-2 and UCP-3 **(B);** and for ACC (α and β isoforms) and AMPK (α2, β2 subunits) **(C)**. All samples were run in triplicate with test probes and the control gene mouse beta-actin to control for differences in amount of starting material. Fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the beta-actin amplified control and then comparing to gene expression in ambulatory control animals. n = 5/per group; +/- SEM; *$P < 0.05$ by ANOVA; **$P < 0.01$ by ANOVA; NS = not significant.

A.

B.

*p<0.05, **p<0.01

C.

'p<0.05, ''p<0.01
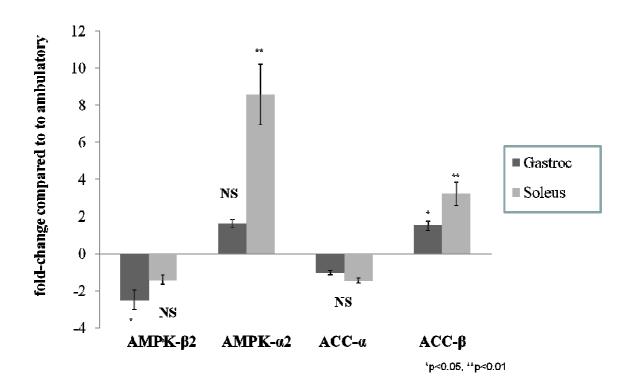
Fig. 3.5   PPAR-β/δ and UCP-3 protein expression levels are increased in soleus muscle of acute-HLS mice

A. Tissue extracts from soleus muscle of ambulatory (n=3) and 24-hrs HLS mice (n=5) were used to detect by ECL western blot the levels of PPAR-β/δ and UCP-3 protein expression; *B.* Quantization was performed by densitometric analysis followed by normalization of values to β-actin expression in each sample. *A*verage PPAR-β/δ and UCP-3 expression in soleus muscle of ambulatory and HLS soleus depicted graphically relative to ambulatory control. +/- SEM; *$P < 0.05$ by ANOVA; **$P < 0.01$ by ANOVA; NS = not significant.

A.

B.
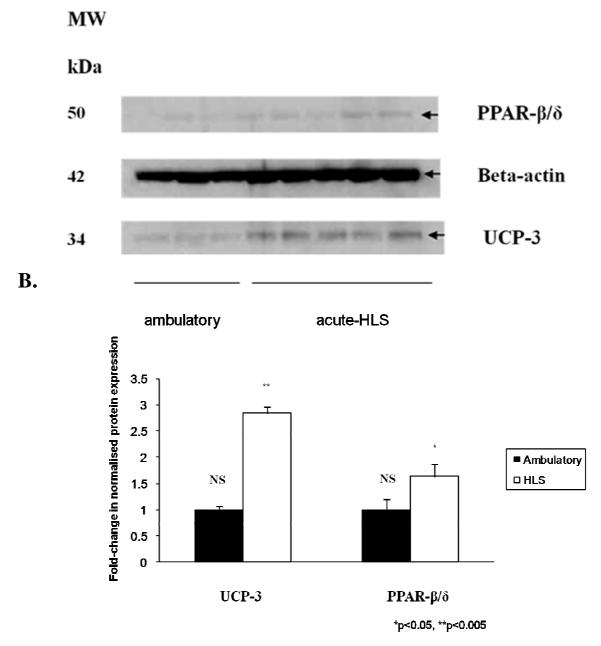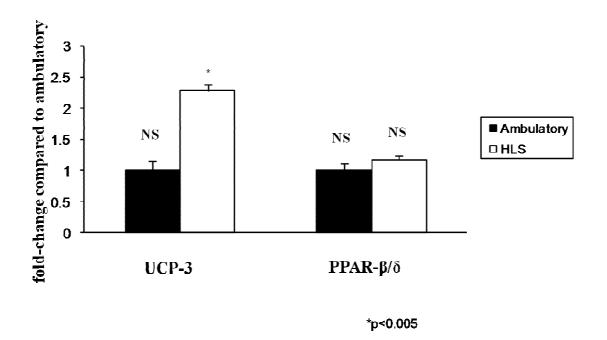
Fig.3.6   UCP-3 expression is increased during chronic stage but PPAR-β/δ expression reverts to normal in HLS-soleus muscle.

Soleus tissue was isolated from control animals and animals after 12-days HLS. RNA was extracted from ambulatory ($n = 4$) and 12-day HLS ($n = 4$) conditions, followed by RT-PCR for UCP-3 and PPAR-β/δ probes. All samples were run in triplicate with test probes and the control gene mouse beta-actin to control for differences in amount of starting material. Fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the beta-actin amplified control and then comparing to gene expression in ambulatory control animals. +/- SEM; *$P < 0.05$ by ANOVA; NS = not significant.

3.4   Caloric or dietary restriction (CR/DR) has a significant impact on the global genomic profile of rodent skeletal muscle

Caloric or dietary restriction (CR/DR) is the only successful dietary intervention which extends the lifespan of all experimental animal models tested so far (Bergamini et al., 2003; Masoro, 2005). In addition, CR has anti-ageing properties by delaying or reducing the impact of ageing-associated diseases such as cancer (Longo and Fontana), sarcopenia (Colman et al., 2008), neurodegeneration (Contestabile, 2009), metabolic syndrome (Opie, 2009), insulin resistance and obesity (Palou et al. 2010). The physiological and metabolic consequences of caloric restriction have been extensively investigated and detailed in the literature (Colman et al., 2009; Cruzen and Colman, 2009). Nevertheless, the underlying regulatory mechanisms have not been explored in detail so far. A main goal of the present chapter was deciphering the possible roles of nuclear receptors in modulating the anti-ageing effects induced by caloric restriction. A theoretical breakthrough in this respect could offer effective intervention tools for the groups affected or at risk for the diseases listed above. To explore the effects of caloric restriction on skeletal muscle gene expression profile an whole-genome microarray (Agilent platform) was performed in our laboratory by using the rat gastrocnemius samples kindly offered by our collaborators from Liverpool University (Dr. BJ Marry laboratory).

Briefly, the rats have been separated by our collaborators in two groups immediately after weaning: the control group that was fed *ad libitum* and the experimental group that was subjected to caloric or dietary restricted conditions for the rest of their life. This rodent model has been already validated and used for extensive research on mitochondrial metabolism and oxidative stress (Lambert et al., 2004; Merry, 2004, 2005). The rats that were subjected to caloric restriction lived longer, weighted slightly less and presented improved metabolic parameters (Merry, 2005). After 28 months of differential diet the rats have been sacrificed and the skeletal muscle samples taken from both control *(ad libitum)* and test (CR) animals were used for a whole

genome DNA microarray experiment. After extraction of the raw values I used GeneSpring software to further analyze the data. After exclusion of absent calls and probes that were expressed (present or marginal) in less than half of experimental samples, 20,023 out of an initial 41,105 probes (50%) were considered for analysis. Only probes with a differential expression of at least 2.0-fold and which passed ANOVA testing at $p < 0.05$ were considered to be truly differentially expressed by CR compared to *ad libitum*, the final list containing a total of 478 probes. Of these probes differentially expressed, 52 were upregulated and 426 were downregulated by CR (Supplemental Table 1). To better analyze them a gene cluster analysis was done by using a Pearson correlation, generating a heatmap where each row represents one of the 478 probes and each column represents one skeletal muscle sample from one different animal. Expression of the 478 probes in skeletal muscle of each animal is shown in Fig. 3.7 where the *intensity* of colour in a cell represents the normalized expression of the probe, and *green* and *red* colouring depict low and high expression, respectively. Expression of each probe in the samples from the six animals fed the CR diet and six animals fed *ad libitum* are shown from left to right. Biological processes were further investigated by using Gene Ontology (GO) analysis of the genes significantly regulated in CR- compared to *ad libitum*-fed animals. The list from Table 3.6 shows the 18 most significantly regulated GO biological processes in dietary restricted muscle with associated p-value. Although functions known to be affected by CR (including glucose and lipid metabolism) are not among the top 18 most-regulated ontologies, several other metabolic ontologies were found to be significantly regulated ($p < 0.05$) such as *glycogen metabolism*, *aspartate metabolism*, *protein metabolism*, *nucleoside metabolism*, *adipose tissue differentiation* and *mitochondrial biogenesis*.

Additionally, I observed a significant regulation of cell cycle, growth and apoptosis-related ontologies in CR samples. I noticed for example that caloric restriction reduced the expression of many genes associated with cell death such as cell death effectors (caspase-1,-3,-4 and -9), apoptosis mediators (BAX, PDCD4, DIDO1, TRAF2, TRAF3, DAP, TMBIM1, RELT),

DNA fragmentation effectors (DFFA, CIDEA) and signalling regulators (RIPK2, DAPK2). Other known negative mediators of apoptosis such as BCL6 and tumor suppressors such as BCoR-L1 were upregulated. This reduction in expression of pro-apoptotic genes could explain the reduced the atrophy and sarcopennia seen in the muscle of animals and human volunteers placed upon caloric restricted conditions (Colman et al., 2008).

Fig. 3.7 Hierarchical clustering of CR-sensitive gene probes.

The 478 gene probes significantly differentially regulated in skeletal muscle samples from CR compared to *ad libitum*-fed animals were subjected to gene cluster analysis using a Pearson correlation. A heatmap was generated where each row represents one of 478 probe sets and each column represents one skeletal muscle sample from an individual animal. The intensity of colour in a cell represents the normalized expression of the probe, where green and red depicts low and high expression, respectively, compared to the average expression of each probe across all samples. n = 6 animals in each group.
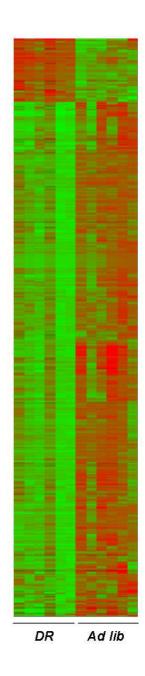
Table 3.6   Top 18 significantly CR-regulated ontologies and associated p-values.

Ontology	p-value
GO:48589: developmental growth	3.11E-06
GO:6952: defence response	9.57E-06
GO:9607: response to biotic stimulus	2.56E-05
GO:6955: immune response	2.95E-05
GO:6878: copper ion homeostasis	3.05E-05
GO:7517: muscle development	8.58E-05
GO:6936: muscle contraction	0.000104
GO:19882: antigen presentation	0.000131
GO:42535: positive regulation of tumour necrosis factor-alpha biosynthesis	0.000142
GO:42534: regulation of tumour necrosis factor-alpha biosynthesis	0.000142
GO:42533: tumour necrosis factor-alpha biosynthesis	0.000142
GO:48668: collateral sprouting	0.000264
GO:48669: collateral sprouting in the absence of injury	0.000264
GO:48738: cardiac muscle development	0.000264
GO:48739: cardiac muscle fibre development	0.000264
GO:45736: negative regulation of cyclin-dependent protein kinase activity	0.000333
GO:30333: antigen processing	0.000397
GO:9611: response to wounding	0.00044

3.5   Caloric restriction changes the genomic profile of networks regulating glucose and lipid metabolism in skeletal muscle and liver

As seen from Table 3.7, some of the crucial genes involved in glycolysis such as phosphofructokinase (PFKL), ketohexokinase (KHK), phosphoglycerate mutase 1 (PGAM1) and hexokinase 1 (HK1) were downregulated (-1.69, -2.04, -1.51 and - 1.77 times, respectively) and this fits with the already accepted and established paradigm that caloric restriction inhibits the process of glycolysis and consequently reduces the toxic metabolic by-products such as methylglyoxal and ROS (Hipkiss, 2006, 2007). I noticed also that the expression of insulin receptor substrate 2 (IRS2) was increased 1.52 times in the skeletal muscle of caloric restricted animals. This change in expression is similar to the results of other independent experiments where laboratory animals that were subjected to exercise showed an improved glucose metabolism and reduction in the production of free radicals, process that was partially mediated by the signalling pathway acting downstream of IRS2 (Park et al., 2007). Glycerol-3-phosphate dehydrogenase 1 (GPD1), which is a key NAD-dependent enzyme involved in glycerol biosynthesis, was also increased 1.76 times and this correlates with the discovery that deletion of the gene in mutant yeasts showing increased lifespan cancelled the anti-ageing effect (Du et al., 2005), suggesting that it is positively associated with delayed ageing. Previously it was shown that caloric restriction improved insulin sensitivity and I also discovered that many mediators that are associated with insulin resistance and diabetes such as insulin-like growth factor binding protein 4 (IGFBP4) and insulin-like growth factor binding protein 5 (IGFBP5) were reduced too (-1.6 and -2.2 times). It is known already that caloric restriction increases mitochondrial biogenesis, subsequently reducing the oxygen consumption and the free radicals produced (Lambert and Merry, 2004), but less is known about peroxisomal biogenesis and the role of peroxisomal enzymes in reducing ROS levels upon caloric restriction in ageing models. I discovered for example that hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (EHHADH) which is a critical enzyme for peroxisomal fatty acid

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beta-oxidation was increased 1.624 times in the skeletal muscle of caloric restricted animals. I detected also increased expression of many transporters localized at both mitochondria and plasma membrane such as mitochondrial transporters of ADP: ATP (SLC25A30 + 2.6 times, SLC25A25 + 3.14 times and similar to mitochondrial carrier protein MGC4399 +1.52 times) or aminoacids transporters (SLC38A3, SLC35C2 and SLC43A1 + 1.5 times). I further noticed a reduced expression of genes critical for fatty acid biosynthesis and transport such as fatty acid desaturase 3 (FADS3, -1.6 times), fatty acid binding protein 3, (FABP3, -1.7 times) and fatty acid binding protein 5 (FABP5, -1.9 times). These changes could explain the reduced accumulation of lipid droplets in the skeletal muscle of obese human patients undergoing fasting or losing weight (Goodpaster et al., 2000). Therefore the above mentioned genomic changes of networks regulating glucose and lipid metabolism correlate with previous reports detailing the improved metabolic flexibility and reduced oxidative stress in CR models.

Table 3.7 Expression of critical genes regulating lipid and glucose metabolism altered by caloric restriction as revealed by microarray analysis

GeneBank	Symbol	Common name	array fold-change
L25387	PFK-C	phosphofructokinase	-1.69
NM_031855	KHK	ketohexokinase	-2.04
NM_053290	PGAM1	phosphoglycerate mutase 1	-1.51
NM_012734	HK1	hexokinase 1	-1.77
XM_001076309	IRS2	insulin receptor substrate 2	1.53
NM_022215	GPD1	glycerol-3-phosphate dehydrogenase 1	1.77
NM_001004274	IGFBP4	insulin-like growth factor binding protein 4	-1.6
AW917764	IGFBP5	insulin-like growth factor binding protein 5	-2.2
NM_133606	EHHADH	enoyl-Coenzyme A, hydratase	1.62
NM_173137	FASD3	fatty acid desaturase 3	-1.6
NM_024162	FABP3	fatty acid binding protein 3	-1.7
NM_145878	FABP5	fatty acid binding protein 5	-1.9

Table 3.8 The expression of previously known NR4A2 regulated genes and interacting partners in CR-samples as revealed by microarray

GeneBank	Symbol	Common name	array fold-change
NM_012740	TH	tyrosine hydroxylase	- 1.53
NM_022548	WIG1	wild-type p53-induced gene 1	- 1.5
XM_215812	TP53BP1	transformation related protein 53 binding protein 1	- 2.3
NM_030989	p53	tumor protein p53	- 1.5
NM_001008349	NFkB2	NFkB light polypeptide gene enhancer 2	- 1.6
NM_171992	CCND1	cyclin D1	- 2.3

3.6 Validation of arrays results by using RT-PCR

In order to validate the results obtained by using microarrays I arbitrarily selected 17 genes and assessed mRNA expression by RT-PCR. Table 3.9 compares CR-regulated gene expression in skeletal muscle by gene array and RT-PCR. All 17 genes selected for validation demonstrated similar expression patterns for the microarray and real-time RT-PCR measurements, yet with different magnitudes, validating array results. I detected in our microarray experiments a reduced expression of many nuclear receptors and transcription factors associated with cancer such as ARNTL (aryl hydrocarbon receptor nuclear translocator-like, -6.5 times), RUNX1 (runt related transcription factor 1, -5.34 times) and NR1D1 (nuclear receptor subfamily 1 group D member 1, -2.88 times). In addition, the expression levels of many receptors involved in inflammation such as Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) were reduced, which could explain the reduced inflammatory process noticed independently in caloric restricted models (Lee et al., 2010).   Also, genes involved in the development of sarcopenic phenotype such as caspase-3, ubiquitin carboxy-terminal hydrolase L1, calsequestrin 2 and proteasomal subunits 8 and 9 were downregulated by CR, suggesting a possible involvement.   Another gene involved in insulin sensitivity regulation such as apelin was downregulated too, with an unknown role in this case.

Table 3.9 Comparison of CR-regulated genes in skeletal muscle by microarray and RT-PCR.

Gene identifier, symbol, common name, fold-change in microarray experiment observed in CR-rats compared to *ad lib*, fold-change in RT-PCR experiment observed in CR-rats compared to *ad lib* (p < 0.05)
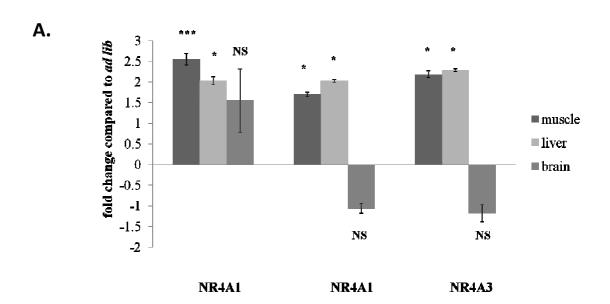
GeneBank	Symbol	Common name	microarray	RT-PCR
NM_024362	ARNTL	aryl hydrocarbon receptor nuclear translocator-like	-6.58	-6.13
NM_017325	RUNX1	runt related transcription factor 1	-5.35	-9.20
NM_145775	NR1D1	nuclear receptor subfamily 1, group D, member 1	-2.88	-3.05
NM_198769	TLR2	Toll-like receptor 2	-2.86	-1.20
NM_012861	MGMT	O-6-methylguanine-DNA methyltransferase	-2.76	-2.42
NM_017237	UCHL1	ubiquitin carboxy-terminal hydrolase L1	-3.70	-2.30
NM_001024791	EPN3	epsin 3	-3.22	-8.83
NM_001013247	SLN	sarcolipin	-6.54	-3.03
NM_017131	CASQ2	calsequestrin 2	-3.13	-1.50
NM_031612	APLN	apelin, AGTRL1 ligand	-2.54	-3.15
NM_080767	PSMB8	proteosome (prosome, macropain) subunit, beta type 8	-2.25	-3.00
NM_012708	PSMB9	proteosome (prosome, macropain) subunit, beta type 9	-2.15	-3.25
NM_012922	CASP3	caspase 3, apoptosis related cysteine protease	-2.42	-3.80
NM_001007713	TMBIM1	transmembrane BAX inhibitor motif containing 1	-3.90	-1.33
NM_024388	NR4A1	nuclear receptor subfamily 4, group A, member 1	2.2	2.6
NM_019328	NR4A2	nuclear receptor subfamily 4, group A, member 2	3.02	1.8
NM_017352	NR4A3	nuclear receptor subfamily 4, group A, member 3	3.7	2.1
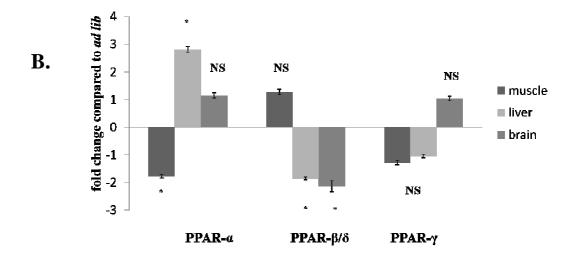
3.7 Altered expression of NR4A family in skeletal muscle, liver and brain of CR animals

Included in the transcripts found to be differentially regulated in skeletal muscle by CR were three functionally related genes: NR4A1, NR4A2 and NR4A3. The nuclear receptor subfamily 4 group A (NR4A) family of nuclear receptors was initially included in the class of the early responsive genes for growth factors (Milbrandt, 1988) and has since been implicated in regulating the response to insulin (Fu et al., 2007). In order to determine if these receptors were differentially expressed in a tissue-specific manner following CR, I further assessed their gene expression by RT-PCR in skeletal muscle, liver and whole brain of CR- and *ad libitum*-fed animals. In muscle and liver the upregulation of all three NR4A receptors (NR4A1, NR4A2 and NR4A3) was observed in dietary restricted animals compared to the control (Fig. 3.8A). In contrast, in brain the dietary restriction did not significantly alter expression of any NR4A receptor. These data highlight the complex regulation of these receptors and suggest that each NR4A receptor may perform diverse functions in different tissues in different developmental stages or physiological conditions (Pei et al. 2006).

Fig. 3.8 RT-PCR assay of gene expression of NR4As and PPARs nuclear receptor families in multiple tissues from CR-animals compared to control.

Animals were grouped into CR and *ad libitum* conditions and relative mRNA expression in each group was determined by RT-PCR; 8A: Relative expression of NR4As in muscle, liver and brain of CR animals compared to *ad lib*; 8B: Relative expression of PPARs in muscle, liver and brain of CR animals compared to *ad lib*. All samples were run in triplicate with test probes and the control rat genes β-actin to control for differences in amount of starting material (n = 6 animals per group; +/- SEM; *P < 0.05 by ANOVA; *** P < 0.001 by ANOVA; NS = not significant.).

3.8   Altered expression of genes belonging to NR4A-associated signaling pathways
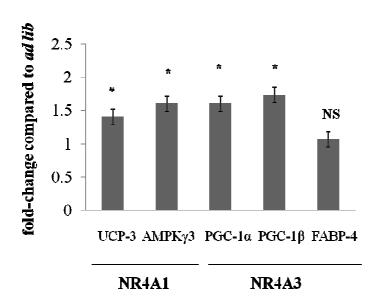
Similar to other steroid receptors each NR4A receptor contains a DNA-binding domain, a transactivation domain and a putative ligand-binding domain. Initially classified as orphan receptors due to the lack of a known ligand, it now seems probable that they do not require ligand binding for their physiological function. It was already shown that NR4A members interact with RXRs (Calgaro et al., 2007) and that RXR ligands increase NR4As activity (Wallen-Mackenzie et al., 2003). In addition, it was shown that caloric restriction decreased the expression values of RXRs in skeletal muscle of mice models. Therefore, I hypothesized that RXR expression might be upregulated by caloric restriction, thereby allowing the possibility of an increased transcriptional activation of target genes. However, microarray analysis identified no significant effects of CR on any RXR isoform (-α, -β, -γ). I next investigated by RT-PCR (Fig. 3.8B) whether CR affected expression of an important class of RXR-interacting proteins that have previously been shown to be altered as a consequence of CR, namely the peroxisome proliferator-activated receptors (PPARs) (Masternak and Bartke, 2007). CR resulted in downregulation of PPAR-α in muscle and up-regulation in liver compared to control. In contrast, PPAR-β/δ was downregulated in both liver and brain but not in muscle. No significant change in PPAR-γ expression was observed in any of the three tissues investigated. In previous studies it was also shown that caloric restriction changed PPARs expression but had no effect on RXRs (Masternak et al., 2005a; Masternak et al., 2005b). Additionally, in liver and heart PPARs expression was found to be both increased or decreased, depending on the isoform and tissue investigated (Masternak et al., 2004), suggesting that regulation of these nuclear hormone receptors by CR is complex and occurs in a tissue-specific and isoform-specific manner. In order to better understand the significance of altered NR4A expression in muscle of CR rats, I further investigated known transcriptional targets of NR4A receptors (Fig. 3.9). To date, very few NR4A transcriptional targets are known. Attenuation of

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NR4A1 expression in muscle results in decreased expression of genes important for glucose and lipid metabolism such as including uncoupling protein-3 (UCP-3), glucose transporter 4 (GLUT4), CD36, caveolin 3 (CAV3) and AMP-activated protein kinase $\gamma3$ subunit (AMPK-$\gamma3$) (Maxwell et al., 2005). Two recent reports have also described NR4A3 transcriptional targets in muscle. These include fatty acid binding protein-4 (FABP-4), PPAR gamma-coactivators alpha and beta (PGC-1α and β), lipin1a and pyruvate dehydrogenase phosphatase 1 (PDHP1) (Pearen, Ryall et al. 2006). I next investigated muscle-specific expression of these targets by RT-PCR. Fig. 3.9 shows relative gene expression of two NR4A1 targets (UCP-3 and AMPK-$\gamma3$) and three NR4A3 targets (PGC-1α, -1β and FABP-4) in muscle from animals fed *ad libitum* or with the CR diet. I observed significant upregulation of UCP-3, AMPK-$\gamma3$, PGC-1α and PGC1-β, but no change in expression of FABP-4 following CR. These data are consistent with increased transcriptional activity of NR4A1 and NR4A3. Since PPAR-α was downregulated and PPAR-β/δ not changed upon CR in muscle, it is likely that the increase in UCP-3, AMPK-$\gamma3$, PGC-1α and PGC1-β expression values was caused by NR4As, not by PPARs. Previously it was also shown that NR4A1 overexpression is able to repress the expression levels of many genes involved in mitochondrial metabolism of lipids belonging to sterol regulatory element binding protein-1c (SREBP1C)-pathway such as glycerol-3-phosphate acyltransferase 1 (GPAM), SCD-1 and fatty acid synthase (FASN) (Pols et al., 2008). I performed RT-PCR for these target genes and noticed that GPAM and SCD-1 expression levels were significantly downregulated in the skeletal muscle of caloric restricted rats (Fig. 3.10), suggesting a possible NR4A1 involvement. Consequently, I used Ingenuity Pathway Analysis[TM] (IPA) to investigate whether additional NR4A-interacting proteins were regulated by CR. IPA identified one network in which all three NR4A receptors are depicted together with interacting proteins (Fig. 3.11). Coloured genes were identified by microarray analysis as differentially upregulated or downregulated by CR (red and green colouring, respectively). IPA analysis of the genes affected by CR identified one homodimeric protein, platelet-derived growth factor-B (PDGF-

B) that directly stimulates expression of all three NR4A receptors. PDGF-B is a potent mitogen for vascular smooth muscle cells and was found to inhibit the induction of nitric oxide synthase activity in vascular smooth muscle cells (Schini et al., 1992). Interestingly, NR4A3 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation (Nomiyama et al., 2006), a role for NR4As in modulating the protection against vascular diseases being recently been hypothesized (Pols et al., 2007). Microarray analysis revealed a trend towards decreased expression of PDGF-B in the CR condition (1.2-fold compared to *ad libitum*), but this was not statistically significant (p = 0.20), suggesting that the up-regulation of NR4A receptors following long-term CR is independent of PDGF-B activity. Taken together, pathway analysis identified multiple potential mechanisms of regulation of NR4A expression which may contribute to the pleiotropic effects of CR.

Fig. 3.9 CR changes the expression of genes regulated by NR4A1 and NR4A3 in skeletal muscle of CR rats

Animals were grouped into CR and *ad libitum* conditions and relative mRNA expression in each group was determined by RT-PCR. Relative expression of the listed genes in muscle of DR animals compared to *ad lib* is shown. All samples were run in triplicate with test probes and the control rat genes β-actin to control for differences in amount of starting material. A. NR4A1 and NR4A3 positively regulated genes; B. NR4A1 negatively regulated genes; +/- SEM; *P < 0.05 by ANOVA; *** P < 0.001 by ANOVA; NS = not significant.

A.

B.
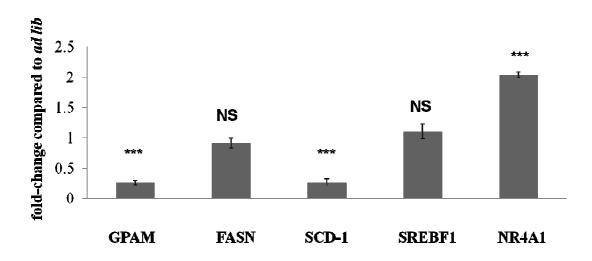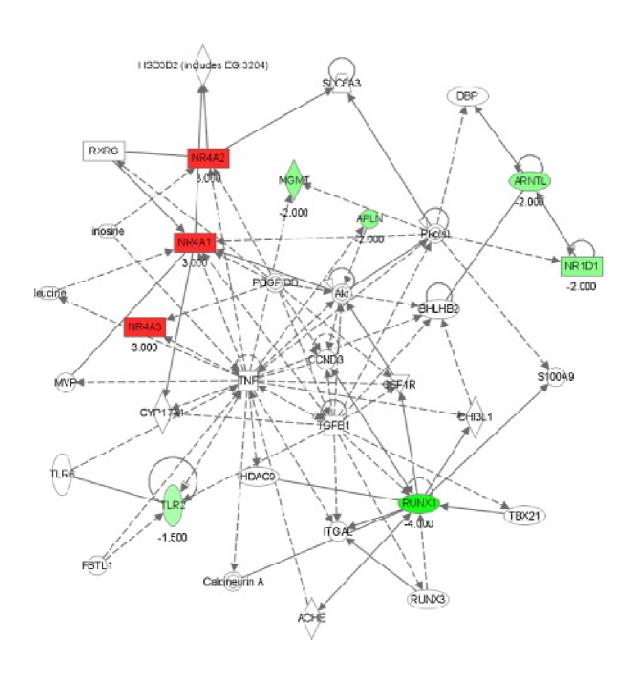
Fig.3. 10 Network of genes differentially expressed in skeletal muscle of CR animals compared to ad libitum-fed animals.

NR4A-interacting genes were analyzed by using IPA. The network shown was significantly associated with immune and lymphatic system development and function, tissue morphology, and cell cycle (P < 0.001). Coloured genes listed in Table 3.9 were identified by microarray analysis as differentially upregulated (red colouring) or downregulated (green colouring) in the CR condition compared to control. Fold-change values were rounded to the nearest half-integer. Other nodal genes in the network are directly or indirectly associated with the differentially expressed genes.

3C. Discussion

1. Hindlimb suspension (HLS) as a model of accelerated ageing

During ageing human skeletal muscle is characterized by reduced mass, impaired functionality and altered metabolic flexibility. The phenotype of the aged muscle is partially similar, but not completely identical, to the phenotype acquired by skeletal muscle after short-term or long-term inactivity (bed rest), or after an impaired level of activity (spaceflight) (Dorrens and Rennie, 2003; Harridge, 2003; Hebuterne et al., 2001). Hindlimb suspension (HLS) is a useful method to model in rodents the effects of such inactivity on human skeletal muscle metabolism and in recent years several groups have used this approach to study especially the changes in gene expression induced by long-term, chronic suspension (Knox et al., 2004; Matsushima et al., 2006). The results of these studies conclusively proved that muscle unloading is characterized by a reduced rate of fatty acid oxidation and decreased transcription of genes involved in lipid metabolism, simultaneously with increased levels of expression of genes involved in glycolysis and glycogen synthesis. Such perturbation will lead to an increased reliance on glucose as a preferred energy source, signifying in fact a loss of metabolic flexibility (Grichko et al., 2000; Henriksen and Tischler, 1988; Langfort et al., 1997). The effects of acute, short-term HLS on global genome expression have not been previously explored in detail. Therefore, in the present chapter I presented the conclusions of the microarray assay which identified the changes induced by such a short-term suspension on skeletal muscle genomic expression profile, followed by RT-PCR and western blot for confirmation.

In my study I focused on identifying the early-responding signaling and regulatory genes that might explain the loss of metabolic flexibility in muscle. A changed profile of genes regulating lipid and glucose metabolism was observed which indicates that such metabolic inflexibility develops in the acute phase of the unloading (Fig. 3.2). The chronic exposure to unloading was shown already to increase the expression of genes involved in glycolytic pathway

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such as hexokinase, phosphofructokinase, and pyruvate kinase (Stein et al., 2002). As shown in Table 3.3, I detected in the acute HLS-samples (24 hours) from soleus an increased expression of genes regulating glycolysis and gluconeogenesis (FBP2, GPD1 and H6PD), an unexpected reduced expression of other crucial genes (HK2, PGAM1 and GYS2) and no change for other important genes such as phosphofructokinase and pyruvate kinase. I also noticed that many other genes involved in lipid metabolism showed surprisingly increased levels of expression such as ACOT1, FDFT1, BCKDHA, ACSL3, only SCD-2 having a reduced expression. These seemingly contradictory data show first of all that during the acute phase it is discernable a *perturbed* glucose metabolism as suggested by the changed genomic profile of some of the rate-limiting genes. Nevertheless, since not every gene involved in glucose metabolism had an increased expression upon acute HLS and since some crucial genes had even a decreased expression, I can conclude that the process of adaptation is still in an *incipient* form during the first 24 hours.

Moreover, since some of the genes crucial for lipid metabolism are increased it is possible that the lipid metabolism process is in fact *accelerated* during the early phase. This could indicate that unlike in the chronic phase when the genes relevant for lipid oxidation are downregulated, the acute phase might develop a very early type of *compensatory adaptation* to acute unloading-induced metabolic deregulation, via an *increased* rate of lipid metabolism. Such possible adaptation will be nevertheless overwhelmed during the chronic phase characterized by an enhanced reliance on glucose utilization concomitant with increased lipid accumulation, leading to the loss of metabolic flexibility.

2. The role of PPARs-dependent signalling during acute HLS

Microarray and RT-PCR assays showed a significant reduction in PPAR-α and PPAR-γ and an increase in PPAR-β/δ expression levels (Fig. 3.4A), simultaneously with a decrease in PGC-1α and PGC-1β expression (Fig. 3.4B). The increase in UCP-3 expression in soleus suggests that the acute upregulation of PPAR-β/δ following short-term HLS may represent an active response of skeletal muscle in order to reduce the deleterious impact on its physiology. It is therefore possible that the loss of PPAR-β/δ expression after 12 days may explain the noticed inflexibility during chronic stage of immobility. This means that activation of PPAR-β/δ could have clinical beneficial effects in the case of muscle diseases during ageing.   Gene arrays demonstrated no significant change in pyruvate dehydrogenase kinase isozyme 4 (PDK4) or FABP3 expression in soleus or gastrocnemius muscle after 24-h HLS. However, I did observe a significant increase in expression of CPT-1b and CPT-2 in soleus muscle after unloading, in contrast to the observations made in the models of long-term exposure to HLS (Fig. 3.4B and 3.4C).

In the present study I observed in the soleus muscle after acute HLS a significant increase in the expression of the known PPAR-β/δ target gene **UCP-3**, but not in the gastrocnemius. On the basis of its homology with other members of uncoupling proteins family UCP-3 has been implicated mainly in the regulation of energy expenditure (Jia et al., 2009). So far UCP-3 was shown to have at least three main roles: facilitating the fatty acid oxidation; removing LCFA and translocating lipid peroxide anions; and activating a proton leak (MacLellan et al., 2005). As a consequence UCP-3 reduces the levels of oxidative stress and lowers the mitochondrial membrane potential, thereby maximizing the oxidation state of mitochondrial respiratory chain carriers. This results in decreased ROS production and protection of the mitochondria from damage. UCP-3 transgenic mice tended to have lower body weight, increased oxygen consumption and decreased mitochondrial proton motive force than the control mice (Clapham et al., 2000). In

addition, they have lower fasting plasma glucose level and insulin levels and increased glucose

clearance rate. Several reports have reported that fatty acids induce UCP-3 gene expression in

skeletal muscle (Boss et al., 1998). UCP-3 expression is correlated with the levels of free fatty

acids and with lipid peroxidation markers, suggesting a switch toward fat oxidation. UCP-3 was

also shown to protect against lipotoxicity in a cachexia model (Minnaard et al., 2006). The

cachectic state is often accompanied by increased rates of adipose tissue lipolysis, reduced

mitochondrial volume and changes in mitochondrial protein synthesis rate. Therefore it was

proposed that increased expression of UCP-3 protects mitochondria against the oxidative damage

induced by nonesterified fatty acids (Schrauwen et al., 2001). In addition, recently it was

demonstrated the role for UCPs in the adaptive response to long-term overfeeding by regulating

substrate oxidation (Ukkola et al., 2001).

Several investigations linked upregulated PPAR-β/δ with high levels of UCP-3

expression (de Lange et al., 2007; Fritz et al., 2006; Son et al., 2001). The UCP-3 gene promoter

contains a PPAR response element (Riquet et al., 2003) and specific PPAR-β/δ agonist treatment

resulted in upregulation of UCP-3. The same study reported that modulation of UCP-3 by targeting

PPARs appeared specific to PPAR-β/δ because neither PPAR-α nor PPAR-γ were able to

transcriptionally regulate UCP-3. Moreover, it was also shown that low-intensity exercise increased

skeletal muscle protein expression of PPAR-β/δ and UCP-3 in type 2 diabetic patients, suggesting

that regulation of these two serve to improve clinical outcome (Fritz, Kramer et al. 2006). I

hypothesized that UCP-3 functions may be polyvalent and differ in the context of acute and chronic

activation of the protein. Although the loss of UCP-3 function appears not to be involved in the

pathogenesis of obesity-related conditions, it is possible that upregulation of UCP-3 may improve

metabolic flexibility.

It was also recently demonstrated that the effects of caloric restriction on

metabolic responses including regulation of glucose and lipid utilization may involve **AMPK** or

ACC and it is important to stress that PPAR-β/δ directly interacts with AMPK (Kramer et al., 2007). In the present study both ACC-β and AMPK-α1 subunits were upregulated in the soleus muscle by HLS. AMPK is a major player in the regulation of energy homeostasis, being mainly, but not exclusively, activated by changes in AMP: ATP ratio due either to a reduction in ATP synthesis or to an increase in ATP consumption. Described initially as a *"fuel gauge"* or as a "*guardian of energy status*" by Hardie, AMPK acts as a metabolic master *switch*, being a main regulator of oxidative capacity and mitochondrial biogenesis by activating the catabolic ATP-producing pathways and by inhibiting the anabolic ATP-consuming processes (Hardie and Carling, 1997). The AMPK-α subunit is coded by two different genes: α1, expressed mainly in the oxidative/glycolytic type IIa and oxidative type I fibers; and α2 type, expressed in all types. In my experiments I detected an increased expression of AMPK- α2 expression only. AMPK-α2 but not AMPK-α1 KO mice are resistant to aminoimidazole carboxamide ribonucleotide (AICAR)-induced hypoglycaemic effects and whole body α2 deletion results in a mild insulin-resistant phenotype and impaired glucose tolerance test (Towler and Hardie, 2007). The differential role of the two genes is further supported by other transgenic experiments that showed that the mice expressing an inactive AMPK-α2 subunit have a lower endurance exercise (Koh et al., 2008; Park et al., 2008). AMPK is activated by many stimuli like nutrient depletion, oxidative stress, hypoxia, heat shock, ischemia or prolonged exercise by the action of upstream kinases such as LKB1, CamKKIIβ and possibly Tak1. Once activated AMPK will phosphorylate many of its targets such as ACC-2, mTOR, e2FK and HMG-CoA reductase modifying in short-term their function or changing the genomic expression profile of its target genes in long-term. AMPK activation was also correlated with increased glucose metabolism mediated not only by translocation of GLUT4 through the plasma membrane in an AMPK-dependent and phosphoinositide 3-kinase (PI3K)-independent fashion, but also by its increased expression as a consequence of AMPK-mediated effects on gene expression (Song et al., 2010). I did not find nevertheless a change in expression of GLUT4 in the microarray experiment.

Moreover, since PGC-1α is required for this global AMPK-dependent effect on gene expression (Thomson and Winder, 2009), and since in my case I noticed a significant reduction in PGC-1α expression level, I could speculate that AMPK has no significant effect on transcription in hindlimb suspended mice. AMPK activation will lead to an increased rate of fatty acid oxidation, glucose uptake and mitochondrial biogenesis. Switching between glucose and lipids as fuel of choice is a constant property of normal healthy muscle, but this ability is reduced during ageing and after chronic inactivity. The hindlimb suspension reduces in long-term the metabolic flexibility characterizing the normal muscle by preferentially using glucose as a fuel of choice and not lipids. I did not measure the levels of fatty acid oxidation in my model of suspended mice, therefore I can only speculate about a possible change in its rate. Nevertheless, a possible increased activation of AMPK in the acute stage could reflect a genuine increased rate of fatty acid oxidation and this is contrasting with the long-term phenotype of unloading.

Another important question is whether AMPK acts upstream or downstream of UCP-3. An increase in UCP-3 expression will also lead to an increase in ATP: AMP ratio that regulate AMPK activity, which could occur via mitochondrial uncoupling (Dokladda et al., 2005), suggesting that AMPK could act downstream of UCP-3. Since PPAR-β/δ agonist treatment also activated AMPK signalling through an unknown still mechanism, it is possible that AMPK acts downstream of UCP-3 and PPAR-β/δ (Kramer et al., 2007). Nevertheless, AMPK activation was also shown to lead to increased expression of UCP-3 as proved by AICAR stimulation (Putman et al., 2003). This issue is a matter of future studies, my data being insufficient to draw a clear and final conclusion in this respect at this point.

3. The role of NR4A family in mediating the multiple effects of caloric restriction on skeletal muscle physiology in rats

Caloric restriction is the only dietary intervention that increases the lifespan of any experimental model used so far and prevents or delays the development of ageing–associated diseases (Masoro, 2005). During ageing skeletal muscle is characterized by loss of muscle mass and inervation, reduced muscle strength and impaired metabolic flexibility (Bautmans et al., 2009; Hebuterne et al., 2001; Narici et al., 2008; Ryall et al., 2008). These changes are associated with increased oxidative stress (Droge, 2005) and insulin resistance (Serrano et al., 2009; Ye et al., 2006), accumulation of intramuscular ectopic lipids (Slawik and Vidal-Puig, 2006) and reduced regenerative capabilities of satellite cells (Renault et al., 2002), partially due to an increased occurrence of inflammation or cell death (Kayo et al., 2001). Caloric restriction was shown to improve the ageing phenotype by reducing the levels of oxidative and nitric stress, by increasing insulin sensitivity and mitochondrial biogenesis, by inhibiting pro-inflammatory and pro-apoptotic signals and by improving metabolic flexibility (Carmeli et al., 2002; Lal et al., 2001; Leeuwenburgh et al., 1997; Marzetti et al., 2009b).

By using microarray and RT-PCR assays I noticed an increase in expression of the family of NR4A receptors upon caloric restriction in rats. It is likely that NR4A subgroup has no natural ligand because there is no defined ligand pocket due to the presence of bulky hydrophobic amino acids in the ligand binding domain. This conclusion is supported further by the fact that DHR38, the only known orthologue of NR4A3 in Drosophila, presents similar characteristics. The lack of a ligand-dependent mechanism of regulation of activation suggests that NR4As are *constitutively* active and that there are other mechanisms regulating their activity such as post-translational modifications, binding of other mediators to AF-1 transactivation domain, different subcellular localizations and changes in expression (Maxwell and Muscat, 2006). The most important post-translational modification proved to regulate NR4A subgroup functions is

phosphorylation, and so far it was shown that NR4A receptors are phosphorylated at multiple sites by many protein kinases such as RSK, ERK, Akt and MSK1. In addition, even if there are no known ligands it was discovered nevertheless that NR4A subgroup activity is increased by diverse stimuli such as 6-mercaptopurine that binds to AF-1 domain of NR4A3, PGA2 that binds to the ligand-binding domain of NR4A3 and the compound DIM-C-pPhCl (1,1-bis(3¶-indolyl)-1-(p-chlorophenyl)-methane) that activates NR4A1 activity through the binding to the C-terminus. Another mechanism of control of NR4A function is differential localization. For example NR4A1 can be localized not only in the nucleus but also in cytosol and mitochondria, and such extranuclear localization is involved in the induction of apoptosis. It is now well established that NR4A subgroup expression levels are modified by the action of diverse stimuli such as free fatty acids, oxidative stress, prostaglandins, growth factors, cytokines, peptide hormones, phorbol esters and neurotransmitters through the phosphorylation of many transcriptional factors such as AP-1, CREB, NFkB and Sp1 (Maxwell and Muscat, 2006). Therefore, the increased expression noticed can be directly correlated with an increased level of activity.

The nuclear hormone receptor 4A (NR4A) subgroup comprises three different receptors: neuron-derived clone 77 (NR4A1, Nurr 77, NGFI-B), nuclear receptor related 1 (NR4A2, Nurr1) and neuron-derived orphan receptor 1 (NR4A3, NOR-1). The typical NR4As DNA binding motif sequence is known as NGFI-B response element (NBRE) and is defined by two adenines followed by the classical NR sequence AGGTCA. NR4A can bind to the specific DNA sequences either as monomers, either as dimers. Interestingly enough, NR4A1 and NR4A3 can heterodimerize with RXR and this interaction confers the ability to bind not only to NBRE but also to DR5 elements. The initial studies demonstrated that NR4A receptors are important in cell survival and apoptosis and recent reports have linked them to the regulation of DNA repair (Pols and de Vries, 2008).

Since the roles of NR4A family members in regulating metabolism and energy expenditure are relatively a recent discovery and since systematic reports about their specific function in aged skeletal muscle are scarce, I will present below a short summary of the current knowledge of their functional profiles in order to better decipher and integrate the potential relevance of my data in the previous corpus of information.

3.1 NR4A1

There are multiple independent reports indicating that NR4A1 has a critical role in regulating glucose and lipid metabolism in skeletal muscle (Kanzleiter et al., 2009). In addition, the experimental mice lacking NR4A1 develop insulin resistance and obesity upon a high-fat diet showing reduced oxygen consumption, increased accumulation of intracellular lipids in myocytes and impaired insulin receptor phosphorylation compared to the wild-type mice fed on the same type of diet (Chao et al., 2009). The silencing of NR4A1 in C2C12 myogenic cell line resulted in the subsequent decrease in expression of many genes involved in lipid transport (CD36, caveolin 3), glucose uptake (GLUT4), fatty acid oxidation (UCP-2, UCP-3, AMPK-γ3) and signalling (adiponectin receptor 2) (Maxwell et al., 2005). To confirm the changes identified by microarray an RT-PCR assay was performed for some of the NR4A1 known targets, noticing that the expression levels of UCP-3 and AMPK-γ3 were upregulated in the skeletal muscle of the caloric restricted rats (Fig. 3.9). This result is in agreement with the silencing experiment performed in C2C12 cells mentioned above, suggesting a possible involvement of NR4A1 in my model's phenotype. In addition, it was reported that in liver NR4A1 blocks SREBP1 activity (Pols et al., 2008) and reduces the expression of SREBP1 target genes such as SCD-1, GPAM and FASN. I also noticed by RT-PCR that while the expression of SREBP1 was not significantly decreased, the expression values of SCD-1 and GPAM were reduced in skeletal muscle of CR models (Fig. 3.10). In another independent report the overexpression of NR4A1 in C2C12 cells

increased also the expression of genes relevant for glucose transport (GLUT4), glycolysis (BPGM, PGAM2, ENO3, PFKM) glycerophosphate shuttle (GPD1 and SLC37A2), glycogenolysis (PYGM, PHKA1 and PHKG1) and glycogen storage (GYS3 and PPP1R3C) (Chao et al., 2007), but it did not affect the genes involved in lipid metabolism. In my microarray experiment an increased expression it was detected only for GPD1 and PGAM1, the rest of the genes from the list being unchanged (Table 3.8). Furthermore, in another report it was shown that whilst the overexpression of the gene in L6 *in vitro* had no effect on lipid oxidation, it had nevertheless a significant effect on the rate of glucose uptake in both basal and insulin-stimulated conditions due to an increased expression of GLUT4 and glycogenin (Kanzleiter et al.). Nevertheless, in my microarray experiment I did not see a significant change in the skeletal muscle of the caloric restricted rats in the expression of the above mentioned target genes.

NR4A1 was proved also to have a powerful effect on cell death and oncogenesis, events affecting the skeletal muscle during ageing. For example, in pancreatic beta-cell lines its expression was induced by free fatty acids and this correlates with the degree of apoptosis *in vivo* (Kuang et al., 1999). Moreover, in stimulated T-cells the silencing of the gene prevented apoptosis (Liu et al., 1994). Another possibility is that NR4A1 is involved in blocking the inflammatory signals in aged caloric restricted animals since it was shown that it is an inhibitor of NFkB-dependent signalling (Evans, 2009) through the interaction with IkBa, the inhibitor of p65 subunit of NFkB (You et al., 2009). Therefore it can be concluded that NR4A1 might be a crucial mediator in modulation of the changes induced by caloric restriction in skeletal muscle in the expression of many genes that regulate glucose and lipid metabolism. Nevertheless, since there are no published reports about the effects of acute caloric restriction on NR4A1 expression, I can not speculate if such an effect is an early or late-stage event.

3.2 NR4A2

The data analysis of the microarray experiment followed by RT-PCR showed an increased expression of NR4A2 in the skeletal muscle samples taken from caloric restricted rats. In different tissues NR4A2 has different functions and changes in its expression have been correlated with diverse pathological conditions characterized by oxidative stress and inflammation. Reduced expression of NR4A2 was reported in the neuronal samples from patients with Parkinson disease (Le et al., 2008) and mutations affecting the NR4A2 gene were further associated with familial forms of this neurodegenerative disease (Federoff, 2009; Sleiman et al., 2009; Sutherland et al., 2009). One possible explanation was suggested by the discovery that the silencing of NR4A2 resulted in a significant increase in α-synuclein gene expression due to a reduced inhibitory binding of the nuclear receptor to the gene promoter (Yang and Latchman, 2008). Since α-synuclein is also important in the pathogenesis of many protein aggregation disorders affecting skeletal muscle during ageing (Askanas et al., 2000), I verified its expression in my microarray experiment but noticed that it was nevertheless unchanged.

Less is known about the role of NR4A2 in skeletal muscle physiology besides the fact that its expression is moderately increased in skeletal muscle after exercise (Mahoney et al., 2005) and beta-adrenergic treatment (Myers et al., 2009). I verified in my microarray experiment data set any change in expression values of previously reported NR4A2 target genes in other tissues. I detected a reduced expression of genes involved in lipid metabolism such as FABP3 and FABP5 previously reported to be positively regulated by NR4A2 (Volakakis et al., 2009). Another gene that has been reported to be increased by NR4A2 activation (Li and Tai, 2009) such as cyclin D1 was also decreased. It was noticed in the same set of data a decreased expression of many growth factors associated with skeletal muscle mass, atrophy and differentiation such as FGF2, FGF9 and FGF7, but not FGF20 and FGF8, reported previously to be regulated by NR4A2 (Grothe et al., 2004; Kim et al., 2003).

NR4A2 was shown also to have complex effects on cellular stress and inflammation in different tissues. In the case of cell death affecting the dopaminergic neurons NR4A2 proved to act as a neuroprotective agent by blocking the gene expression of many inflammatory mediators in astrocytes and microglia (Bensinger and Tontonoz, 2009). The overexpression of the receptor in neural stem cells improved also the resistance against oxidative stress (Sousa et al., 2007). The precise mechanisms are still unclear but it was already discovered that NR4A2 is interacting with astrocytal CoREST complex, blocking the expression of many inflammatory mediators that once released by astrocytes could have neurotoxic effects (Saijo et al., 2009). The overexpression of NR4A2 in macrophage cell line THP-1 reduced further the levels of oxidized lipids and inflammatory mediators possibly through the transrepression of NFkB signalling pathway (Pei et al., 2005). A similar conclusion was also suggested by recent experiments using neuronal models (Sousa et al., 2007). This protective effect in the case of astrocytes, neurons and macrophages is nevertheless contradictory to the effect shown by the overexpression of the receptor in a model of a immortalized synoviocyte cell line that presented increased expression of many pro-inflammatory mediators such as IL-8, amphiregulin and kit ligand (Davies et al., 2005; O'Kane et al., 2006), genes that were nevertheless unchanged in my microarray experiment. A similar conclusion was derived from independent studies of NR4A2 overexpression in cartilage (Mix et al., 2007). In addition, in the T-cells from the CNS of the mouse models of multiple sclerosis (EAE) NR4A2 had a higher level of expression compared to the wild-type animals and the overexpression of NR4A2 *in vitro* resulted in an increased expression of pro-inflammatory cytokines such as IL-17 and IFN-γ. In the skin of psoriatic patients NR4A2 expression level is increased significantly in both cytosol and nucleus, and blocking of TNF-α signalling by treatment with the drug *infliximab* was able to reduce NR4A2 expression level to the normal values and restore the predominant cytosolic localization seen in the skin of healthy patients (O'Kane et al., 2008). I did not detect nevertheless any change in expression levels of the above

mentioned target genes in my experiment with caloric restricted rats, therefore the possible role of NR4A2 in reducing inflammation in my experimental caloric restricted model is a matter to be explored further in the future. In NR4A2 heterozygous mice the striatal levels of nNOS were increased and the neuropathy seen in the dopaminergic neurons was dependent on nitric oxide production (Imam et al., 2005). I did not detect nevertheless a reduced expression of nNOS in the caloric restricted mice.

NR4A2 was also reported to be regulated in some tissues by NFkB (McEvoy et al., 2002). The interplay between NR4A2 and the regulation of cell cycle was also proved by the fact that in the same type of neurons it is interacting with p53 and has a negative effect by repressing its transcriptional activity with anti-apoptotic effects (Zhang et al., 2009). In my microarray experiment p53 was downregulated simultaneously with some of its binding proteins such as TP53BP1 and some of its target genes such as WIG1. The discovery that glucocorticoid receptor is interacting with then N-terminus of NR4A2 increasing its transcriptional activity (Carpentier et al., 2008) is contrasting nevertheless with the fact that caloric restriction is reported to downregulate the GR expression in some tissues (Gursoy et al., 2001).

Therefore the downstream gene targets for NR4A2-dependent signalling in the case of skeletal muscle are still undeciphered, but it is probable that an increase in its expression might be involved in the repression of pro-inflammatory mediators upon caloric restriction conditions.

3.3   NR4A3

In my experiment it was noticed also an increased NR4A3 expression in the skeletal muscle of caloric restricted rats, receptor that was cloned in 1994 (Ohkura et al., 1994) and has different splice variants with different functional properties (Ohkura et al., 1998). The specific role of NR4A3 in skeletal muscle was recently explored and it was proved for example that in

C2C12 murine cultured myotubes its expression was increased by beta-adrenergic treatment in PKA-dependent manner (Kanzleiter et al., 2009; Pearen et al., 2006). The silencing of NR4A3 gene reduced the expression level of UCP-2 and increased the expression level of UCP-3. Since UCP-3 was found to be upregulated in caloric restricted samples it is likely that this is due to NR4A3-independent regulatory mechanisms. Silencing of NR4A3 expression in myogenic C2C12 cell line reduced palmitate oxidation, increased lactate concentration and shifted the metabolism toward a more anaerobic process due to a lower expression of genes involved in lipid oxidation and pyruvate use (Pearen et al., 2008) such as PGC1-α and PGC-1β. This correlates with my discoveries that in the skeletal muscle of the caloric restricted rats the expression levels of PGC1-α and PGC-1β genes were increased while lactate dehydrogenase was decreased (Fig. 3.9). Nevertheless, other target genes regulating lipid oxidation reported to be reduced in the NR4A3-silenced C2C12 cells such as PDK4, FOXO1 and lipin1a were not changed in my experiment.

An increase in expression of NR4A3 could also affect glucose metabolism, since some of NR4A3 polymorphisms are associated with a better insulin secretion in non-diabetic patients (Weyrich et al., 2009). The overexpression of NR4A3 in 3T3-L1 adipocytes increased the rate of glucose transport (Fu et al., 2007) upon insulin treatment though an increased GLUT4 translocation to the plasma membrane in a manner dependent on PI3K/Akt-associated signalling, and the silencing of the gene generated the opposite phenotype. NR4A3 has also clear effects on the hormonal regulation of food intake since it was shown before to be expressed at lower values in the hypothalamus of leptin-deficient obese animal models compared to controls, independently of 5-HT2C signalling. The injection of NR4A3 siRNA in animal models suppressed the daily food intake (Nonogaki et al., 2009) which might suggest a compensatory role in obese animals.

NR4A3 has also an important but still not fully understood role in the regulation of cell death and oncogenesis. For example, it was reported that overexpression of NR4A3 prevented endothelial cell death under hypoxia conditions and that silencing of the gene increased

the number of apoptotic cells (Martorell et al., 2009). In T-cells the overexpression of NR4A3 induced nevertheless apoptosis through the upregulation of CD25. In addition, the induction of apoptosis upon TCR activation is accompanied by upregulation of NR4A3 and NR4A1 (Cheng et al., 1997). The cell death of MCF7 cancer cell line induced by treatment with calcium ionophore A23187 (Ohkubo et al., 2000) increased the expression levels of NR4A3, and a similar event was also noticed in the early stage of rat liver resection-reperfusion injury (Ohkubo et al., 2002). When spleen cells from NR4A3 transgenic mice were treated with prostaglandin A2 (PGA2), the number of the apoptotic cells tripled compared to control (Kagaya et al., 2005). By using cell-free systems the same authors showed that PGA2 is able to transactivate NR4A3 though binding to LBD. NR4A3 has therefore much more complex and intriguing relationships with the regulation of cell death and oncogenesis than the other two members of the family. For example, as a result of the t(9;22) translocation NR4A3 was reported to fuse with EWS (EWS/NOR1) and the new product was proved to have a role in the development of extraskeletal myxoid chondrosarcoma (EMC) (Antonescu et al., 1998). The mechanisms underlying this process of oncogenesis are still debated but it was shown so far that the fusion protein is able to modulate transcription by affecting pre-mRNA splicing (Ohkura et al., 2002). Another reports showed that PARP-1 was able to bind to NR4A3 but not to EWS/NOR1, consequently inhibiting the NR4A3 transactivation properties and its interactions with the global transcriptional machinery (Ohkura et al., 2008).

NR4A3 has also important effects on inflammatory mediators since it was shown that the administration of purine anti-metabolite 6-mercaptopurine, an antineoplastic and antiproliferative agent with additional anti-inflammatory properties, was able to transactivate NR4A3 in AF-1 dependent manner (Wansa et al., 2003). In addition, IL-1B treatment of human synovial and gingival fibroblasts increased both NR4A3 expression levels and its DNA binding activity (Borghaei et al., 1998). The list of upstream regulators and signalling pathways of NR4A3 expression include so far CAM-KK cascade and the subsequent CREB signalling in neurons

(Inuzuka et al., 2002) and smooth muscle cells (Martinez-Gonzalez et al., 2003), and PKA-signalling in cultured osteoblasts upon parathyroid hormone treatment (Pirih et al., 2003). In endothelial cells NR4A3 expression is increased by vascular endothelial growth factor (VEGF) in a CREB-dependent manner regulating further cell cycle, DNA synthesis and cell growth (Rius et al., 2006). The role of NR4A3 in mediating vascular smooth muscle cells proliferation was shown by the fact that its increased expression upon statin treatment was inhibited by simvastatin by interfering with CREB phosphorylation through inhibition of RhoA/ROCK pathway (Crespo et al., 2005).

Therefore, based on these particular experiments performed already on the cultured myotubes I can speculate that the increase in NR4A3 expression in the skeletal muscle of caloric restricted rats might mediate an increase in the rates of lipid oxidation, mitochondrial biogenesis and reduced oxidative stress, which could explain the improvements seen in skeletal muscle during CR (Nadeau et al., 2006) .

Chapter 4-THE EFFECTS OF VISFATIN (eNAMPT) ON OXIDATIVE STRESS LEVELS AND INSULIN RECEPTOR-DEPENDENT SIGNALLING IN CULTURED MYOTUBES

4A. Introduction

Adipokines are cytokines secreted by the adipose tissue acting in autocrine (Karastergiou and Mohamed-Ali, 2009), paracrine (Vona-Davis and Rose, 2007) or endocrine fashion (Wozniak et al., 2009). Since adipokines can influence the metabolism of other peripheral tissues such as skeletal muscle (Kim et al., 2009), myocardium (Schram and Sweeney, 2008), liver (Marra and Bertolani, 2009), pancreas (Stadler et al., 2009) or components of immune system (Curat et al., 2004), their endocrine effect has overall a major physiological impact. Any changes in adipokines expression, activity or localization play a significant role in the development of many pathological conditions such as obesity (Trayhurn and Wood, 2005), metabolic syndrome (Kobayashi, 2005), inflammation (Aldhahi and Hamdy, 2003) or lipodystrophy (Sweeney et al., 2007). As discussed in the previous chapter, during ageing skeletal muscle is characterized by progressive structural changes and decaying functionality (Rossi et al., 2008). These phenotypes are partially underlined by changes in expression profile of the networks regulating glucose and lipid metabolism, insulin sensitivity (Serrano et al., 2009; Ye et al., 2006) and antioxidant defence (Droge, 2005), the same type of processes that are affected in the pathological conditions associated with adipokines mentioned above. In addition, a significant part of the published literature revealed the critical role of adipokines in modulating the signalling pathways regulating insulin sensitivity and antioxidant defence in peripheral tissues during ageing (Di Gregorio et al., 2004; Esposito et al., 2006; Fulop et al., 2006; Miles et al., 2008; Picard and Guarente, 2005; Schutte et al., 2007; Terlain et al., 2006).

Visfatin is a newly discovered member of adipokine family with the unique particularity within the group of having two variant forms, identical in sequence and coded by the same gene, but with different localizations: the first is intracellular (iNampt), and the second extracellular (eNampt). The intracellular form of visfatin is ubiquitously expressed and it was proved so far to synthesize NMN, an intermediate of NAD, through its phosphoribybosiltransferase activity (Wang T. et al., 2006). The extracellular form was found to be actively secreted through a non-

classical and still obscure regulated pathway (Revollo et al., 2007) by both white adipocytes (Fukuhara et al., 2005) and brown adipocytes (Wang et al., 2009), justifying its inclusion in the adipokine group. Recently, a more complex picture emerged since it was reported that visfatin is secreted also by many other types of cells such as macrophages (Curat et al., 2006) and hepatocytes (Garten et al., 2010). The discovery that intracellular visfatin (iNampt) is highly expressed in skeletal muscle inspired initially the speculation that it might act also as a myokine after the secretion from myocytes, similarly to interlukin-6 (IL-6) or inrerleukin-15 (IL-15) (Krzysik-Walker et al., 2008), and recently it was reported also that visfatin was detected in the culture medium of rat L6 differentiated myotubes (Wang et al., 2010).

There are no definitive conclusions about the relationships between extracellular visfatin (eNampt) and the ageing process or the ageing phenotype, but there are multiple arguments to implicate the extracellular form of visfatin in the development of the pathological conditions associated with ageing. Measurements of the levels of plasma visfatin revealed significant differences between the healthy human subjects (control group) and diverse cohorts of patients suffering from a large variety of pathological conditions such as chronic kidney disease (Mu et al., 2010), acute pyelonephritis (Mazaki-Tovi et al., 2010), diabetic nephropathy (Kang et al., 2010), inflammation (Chang et al., 2010b), thyroid dysfunctions (Ozkaya et al., 2009), ischemic stroke (Lu et al., 2009a), polycystic ovary syndrome (Panidis et al., 2008), coronary artery disease (Liu et al., 2009a), arthritis (Jurcovicova et al., 2010), markers of increased oxidative stress (Bo et al., 2009), endothelial dysfunction (Takebayashi et al., 2007) and insulin sensitivity (Li et al., 2006). In almost all these pathological cases the level of plasma visfatin was found to be *higher* in the disease group compared to the healthy one, correlating moreover with the *degree* of the symptoms. Nevertheless, it is not clear yet whether such increased concentration of visfatin represents a *cause* of the disease, an accidental *side effect* or an indication of a *protective/compensatory* role (as discussed in the case of HLS and PPAR-β/δ before). For example, whilst overweight patients showing weight loss under a

hypocaloric diet had lower visfatin plasma levels (de Luis et al., 2008), in a different model of hemorrhagic shock/resuscitation the plasma concentration of visfatin correlated positively with the protective therapeutic hypothermia intervention (Beiser et al., 2010). Also, it was shown that visfatin expression is upregulated in skeletal muscle upon exercise in human patients (Costford et al., 2010).

It is known already that adipokines or cytokines such as TNF-α (Lo et al., 2007), IL-6 (Lihn et al., 2008), resistin (Junkin et al., 2009), omentin (Winkler and Cseh, 2009), vaspin (Rabe et al., 2008), leptin (Solinas et al., 2004) and adiponectin (Vu et al., 2007) have a major impact on skeletal myotubes survival and lifespan, stress responses, inflammation and insulin sensitivity. As previously reported (Adya et al., 2008a,b; Xie et al., 2007; Lim et al., 2008), once secreted in the plasma by adipocytes, hepatocytes or macrophages the extracellular form of visfatin (eNampt) is able to bind to the insulin receptor expressed by endothelial cells, osteocytes and cardiomocytes and trigger the activation of PI3K/Akt signalling cascade, leading to an increase in the rate of glucose uptake. The possibility that visfatin could be used as an insulin-mimetic agent opened a new direction in the research dedicated to find new solutions for the treatment of insulin resistance, obesity and metabolic syndrome (Fukuhara et al., 2005). Previous *in vitro* or *ex vivo* research showed that the incubation of primary or immortalized cell lines originating from different tissues with different concentrations of visfatin (eNampt), changes in its intracellular expression levels (iNampt) by genetic interventions (transgenic or knock-out), or pharmacological-based inhibition of its phosphoribosyltransferase function can have different, even opposite effects on cellular viability, stress responses and inflammatory signals.

For example, it was reported that incubation of macrophages (Li et al., 2008), cardiomyocytes (Lim et al., 2008) or amnion cells (Kendal-Wright et al., 2008) with visfatin (eNampt) improved the cellular viability and prevented apoptosis induced by various toxic agents. In addition, the blocking visfatin phosphoribosyltransferase function induced apoptosis of tumour cells (Khan et al., 2007; Khan et al., 2006). Nevertheless, it was also shown that silencing of the gene

reduced the levels of oxidative stress induced by IL-1β in lung endothelial cells (Zhang et al., 2008) and that incubation of human umbilical vein endothelial cells (HUVECs) with visfatin activated the NFkB-associated pathway in an ROS-dependent manner (Kim et al., 2008). Although there are no reports about changes in visfatin plasma levels during ageing, it was reported nevertheless that overexpression of visfatin in endothelial cells increased their replicative lifespan and improved their resistance against cellular stress in a high glucose environment (Borradaille, N.M. et al. 2009). Skeletal muscle is the main system responsible for the removal of the circulating glucose and its physiology is modified by changes in the levels of plasma circulating adipokines, especially in the case of insulin sensitivity, inflammatory status and antioxidant defence (Dyck et al., 2006). Since there were no previous reports about the effects of plasma visfatin on skeletal muscle stress responses and insulin sensitivity, I explored these questions in the present chapter whose main findings were published in the attached paper (Oita et al., 2010). In my research I used C2C12 cell line that it is an established, robust and stable cellular model (Kubo, 1991), being an attractive choice to study *in vitro* the effects of different adipokines on skeletal muscle physiology.

The *first* goal of the present chapter was to test by using fluorescent-based assays whether the incubation of C2C12 myotubes with exogenous visfatin induced a change in oxidative stress levels. The *second* was to test if exogenous visfatin activated insulin receptor-dependent signalling.

4B. Results

4.1 Visfatin (eNampt) incubation increases the levels of oxidative stress in cultured and differentiated murine C2C12 skeletal myotubes

In order to study the effects of free, plasma circulatory, extracellular form of visfatin (eNampt) on skeletal muscle stress-responses a commercially available mouse visfatin recombinant protein (BioVision, Inc) has been used. To confirm the results and to remove any reasonable doubt about the validity of the protein chosen for my experiments, the main experimental conclusions have been further verified by using two other versions available on the market, reaching the same conclusions. One was purchased from MBL International and it was produced in bacteria, and the other was purchased from Axxora Ltd and it was produced in mammalian HEK 293 cells, version that was already validated in a previously published paper (Li et al., 2008).

To explore the possibility that extracellular visfatin (eNampt) could induce oxidative stress in a cultured model of skeletal muscle, I incubated fused and differentiated murine C2C12 myotubes with different concentrations of visfatin (0-200 ng/ml) for a variable range of time (0-24 hours). The levels of ROS generated intracellularly were measured by using the fluorogenic specific probe carboxy-H2DCFDA. This probe is easily transported across the plasma membrane into the cytosol where it is deacetylated by nonspecific cellular esterases to the compound carboxy-DCFH. The free radicals generated intracellularly reduce the fluorescein further to the compound carboxy-DCF that shows an increased bright green fluorescent emission upon UV excitation. This signal was detected by fluorescent microscopy and images were further processed by software-based analysis. In order to double-check the results, I also verified the imaging results by using an automatic microplate reader attached to a PC, reaching the same conclusions.
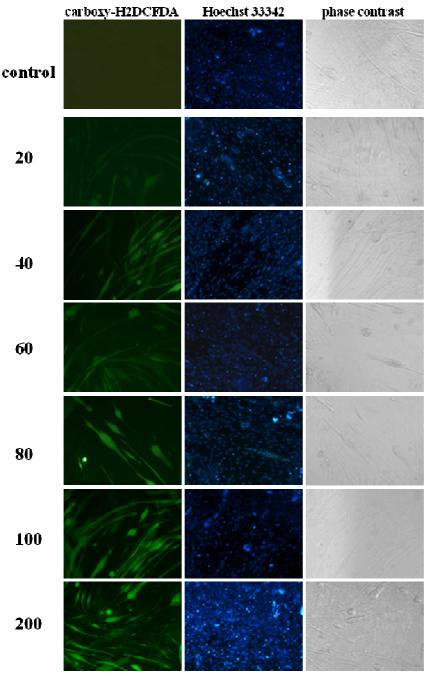
As seen from figure 4.1, the incubation of cells with different concentrations of visfatin for 24 hrs induced a significant increase in the level of free radicals from a starting value of 20 ng/ml, and since the maximum signal detected was reached at 100 ng/ml of visfatin I decided to

use this value as the working concentration. This concentration is consistent also with previous published papers exploring the effects of visfatin on stress responses in other types of cells (Lovren et al., 2009; Moschen et al., 2007; Xie et al., 2007).   To identify the earliest time point of a significant ROS detection I incubated cultured myotubes with 100 ng/ml of visfatin for a different range of time (1- 24 hours), and as seen from figure 4.2 by using the fluorescent microscopy imaging I discovered a significant increase in ROS production between 4 and 6 hours of incubation, with a maximum signal value reached after 18 hours. Longer incubation time failed to further increase the production of the free radicals. In conclusion, for the rest of experiments performed in this chapter I used the concentration of 100 ng/ml of visfatin and an incubation period of 18 hours as optimal conditions for inducing and measuring oxidative stress levels.

To certify that the signal is genuine, I pre-incubated the C2C12 cells with 1mM NAC (N-acetyl-l-cysteine, a gluthatione precursor that is widely used as a scavenger for free radicals) for 1 hour before visfatin treatment and repeated the carboxy-H2DCFDA fluorescent-based assays. As seen from figure 4.3, the signal from cells pre-treated with NAC before visfatin challenge was reduced with cca. 40% compared to signal detected from cells incubated only with visfatin. Since carboxy-H2DCFDA-based microscopy is not discriminating efficiently between different species of oxygen free radicals, I further explored the detection of individual species like superoxide and hydrogen peroxide by using type-specific probes. To detect the presence of hydrogen peroxide I used the probe pentafluorobenzenesulfonyl fluorescein (Fig. 4.4A) that becomes fluorescent upon perhydrolysis of the sulfonyl linkage and to detect the generation of superoxide I used the mitochondrial-specific probe MitoSOX^{TM} Red (Fig. 4.4B). The fluorescent-based assays experiments using the probes listed above proved that after 18 hours of incubation of the cells with 100 ng/ml of visfatin both types of free radical species were detected.

Fig. 4.1 Chronic incubation of differentiated myocytes with various concentrations of visfatin induces oxidative stress

Differentiated C2C12 myotubes were incubated for 24 hrs with different concentrations of recombinant visfatin (0-200 ng/ml). After 24 hrs cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect the levels of intracellular ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to negative control data is shown. Data shown are the average of quadruplicate samples per experiment, from three independent experiments +/− SEM. *Denotes $p<0.05$ compared to control. **Denotes $p<0.01$ compared to control.
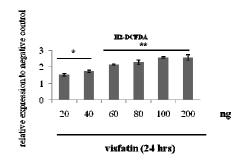
Fig. 4.2 Incubation of differentiated myocytes with a high concentration of visfatin induces oxidative stress after 6 hrs

Differentiated C2C12 myotubes were incubated for a different range of time (0-24 hrs) with 100 ng/ml recombinant visfatin. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control data is shown. Data shown are the average of quadruplicate samples per experiment, from three independent experiments +/− SEM. *Denotes $p < 0.05$ compared to control. **Denotes $p < 0.01$ compared to control. NS denotes no difference between treatment and control.
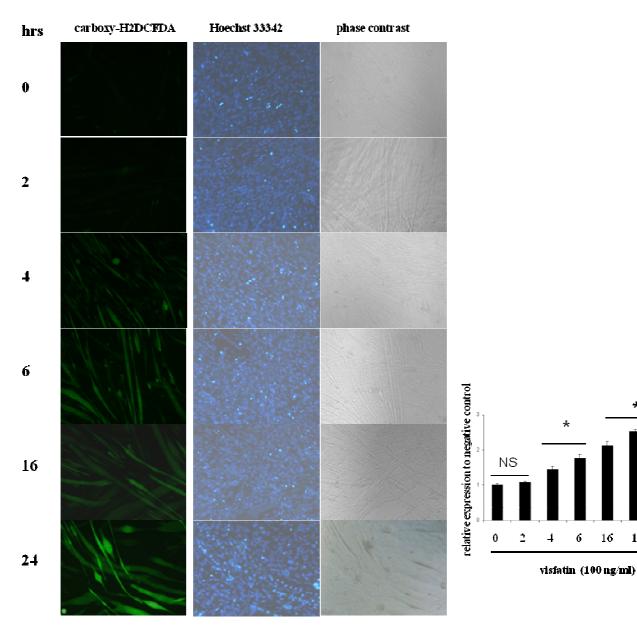
Fig. 4.3 The oxidative stress induced by the treatment of cultured myocytes with visfatin is reduced by the pre-treatment with antioxidants

Differentiated C2C12 myotubes were incubated for 18 hrs with 100 ng/ml recombinant visfatin after the pre-treatment in the presence or absence of 1 mM NAC for 1 hr to scavenge the free radicals. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control data is shown. Data shown are the average of quadruplicate samples per experiment, from three independent experiments +/− SEM. **Denotes p<0.01 compared to control and between visfatin treatment plus NAC compared to visfatin alone.
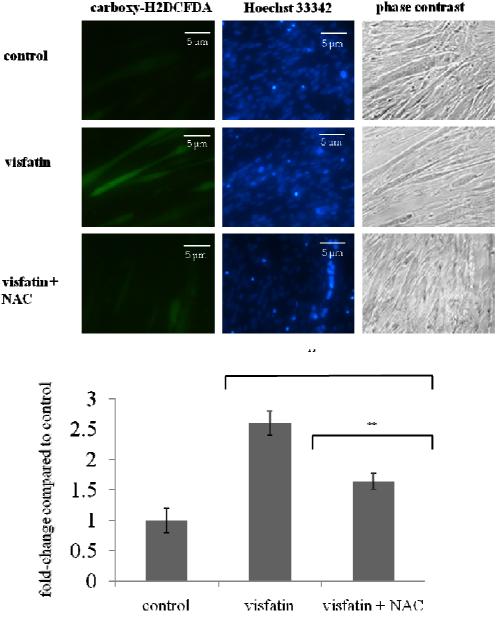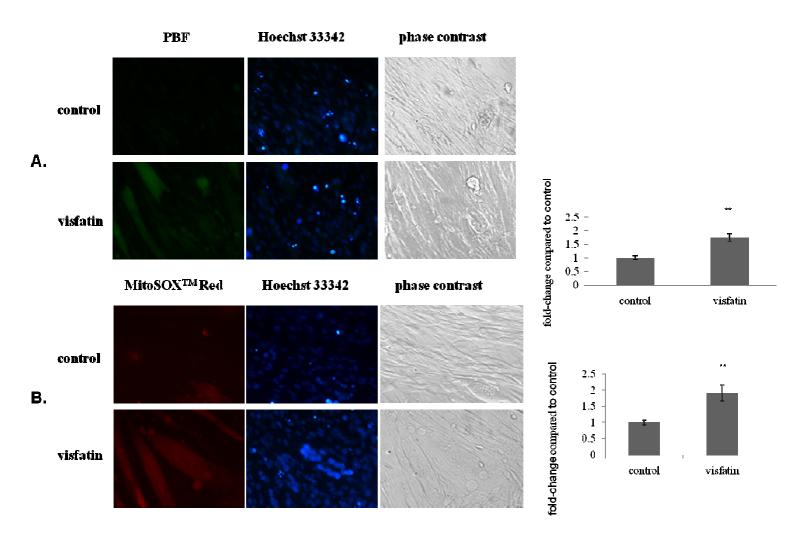
Fig. 4.4 Visfatin treatment of cultured myocytes generates both superoxide and hydrogen peroxide reactive oxygen species.

C2C12 differentiated myotubes were incubated for 18 hrs with 100 ng/ml of visfatin as shown before. A. Production of hydrogen peroxide was detected by incubating myoblasts with 1 µg/ml of pentafluorobenzenesulfonyl fluorescein for 25 min followed by 5 min incubation with 1 µM Hoechst 33342 (to stain the nuclei); B. Superoxide generation was detected by incubating differentiated cells with 5 µM MitoSOX™ Red for 15 min followed by 5 min incubation with 1 µM Hoechst 33342 (to stain the nuclei). Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments. +/− SEM. **Denotes p<0.01 compared to control.

4.2 Visfatin (eNampt) treatment is not increasing the levels of oxidized proteins and lipids and it is not changing the myocytes global antioxidant capability

Oxidation can change the structure and the function of proteins (le-Donne et al., 2006), DNA/RNA (Radak et al., 1999) or lipids (Rosenfeld, 1998) and an increased level of ROS can affect the proper metabolism of skeletal muscle (Singh et al., 2008). Therefore I further verified if I could detect a change in the levels of the oxidized forms of lipids and proteins upon incubation with visfatin. To detect and quantify the levels of the oxidized forms of the proteins I used the Oxyblot[TM] assay method that detects the levels of carbonylated residues introduced in the side-chains of the proteins by the action of oxygen free radicals. Briefly, the denatured and reduced cell lysates extracted were treated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize the carbonyl groups to 2,4-dinitrophenylhydrozone (2,4-DNP) residues. Such derivatized 2,4-DNP residues were recognized by a specific antibody through a typical western blot assay and the levels of oxidation were further quantified by densitometric analysis. As seen from figure 4.5, there were no significant differences between the levels of oxidized proteins from cell lysates extracted from the wells incubated with visfatin for 16, 24 or 48 hours compared to cell lysates extracted from control, untreated cells. The specificity of the assay was proved by the lack of chemiluminescent signal in the samples treated only with the derivatization-control solution (buffer) instead of DNPH.

I tested also the possibility that the free radicals induced by visfatin could increase the levels of oxidized lipids. To test this hypothesis I used an LPO assay kit that is based on the capability of thiocyanate ions (the chromogen) to recognize the ferric ions produced by the reaction of lipid hydroperoxides with the ferrous ions from the assay solution. As seen in figure 4.6, I did not detect any significant increase in the levels of oxidized lipids in the samples taken from the cells treated with visfatin for 16, 24 or 48 hours compared with to control.

Because these two sets of results concerning the levels of oxidized products were apparently contradictory with the results presented in the subchapter 4.1, I hypothesized that visfatin

could increase simultaneously not only the production of ROS but also the global cellular antioxidant defence capacity that might prevent such free radicals to have any significant oxidizing effect on proteins and lipids. To test this possibility I measured colorimetrically the global capability of the cell lysates to prevent the oxidation of the compound ABTS$^{®}$ by metmyoglobin, and as seen from figure 4.7 visfatin treatment for 24-48 hours failed to change the global antioxidant capacity compared to control, unlike hydrogen peroxide that reduced it.

These experiments proved that the free radicals produced by incubation with visfatin did not increase significantly the levels of oxidized lipids and proteins. Additionally, the lack of a general simultaneous increase in global cellular antioxidant defence upon incubation with visfatin might suggest the possibility of *a partial* or *localized* increase in such antioxidant defence. This subject will be explored in detail in the following chapters.

Fig. 4.5 Visfatin treatment does not increase the levels of oxidized proteins

C2C12 differentiated myotubes were incubated for 16, 24 and 48 hrs with 100 ng/ml of visfatin. The denatured and reduced cell lysates extracted were treated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize the carbonyl groups to 2,4-dinitrophenylhydrozone (2,4-DNP) residues, or only with buffer as a negative control. The cell lysates were resolved by using a standard western blot procedure by using an antibody against the derivatized 2,4-DNP residues. Quantization of the expression was performed by densitometric analysis and the values were further to the untreated cells. Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between visfatin treatment and control.

Fig. 4.6   Visfatin treatment does not increase the levels of oxidized lipids

C2C12 differentiated myotubes were incubated for 16, 24 and 48 hrs with 100 ng/ml of visfatin as shown before. The lipids were extracted and the levels of lipid hydroperoxides were quantified based on the capability of thiocyanate ions (the chromogen) to recognize the ferric ions produced by the reaction of lipid hydroperoxides with the ferrous ions from the assay solution. Quantization of the expression was performed by colorimetric analysis and the values were further normalized to the negative control. Each data point is the average of duplicates samples per experiment from three independent experiments +/− SEM. NS denotes no significant difference between visfatin and control.
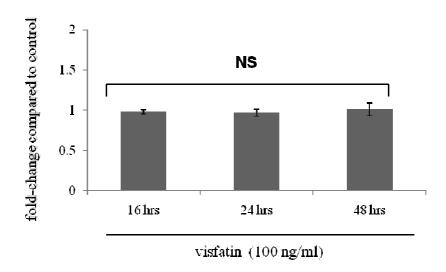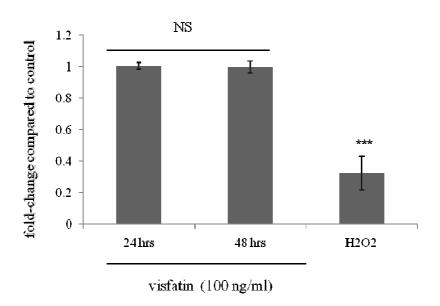
Fig. 4.7   Visfatin treatment does not increase the myocytes global cellular antioxidant capability

C2C12 differentiated myotubes were incubated for 24 and 48 hrs with 100 ng/ml of visfatin as shown before or with hydrogen peroxide as a positive control for 30 minutes. Cells were scraped and the cell lysates extracted were used to block the oxidation of ABTS compound by metmyoglobin. The levels of oxidation were assayed by colorimetry, relative to control. Each data point is the average of duplicates samples per experiment from three independent experiments +/− SEM. ***Denotes $p < 0.001$ compared to control. NS denotes no significant difference between visfatin treatment and control.

4.3 Visfatin (eNampt) treatment is not affecting the cellular viability and is not increasing the levels of nitrosylated proteins

A sustained oxidative stress in cultured skeletal myocytes can also have cytotoxic effects that could lead to a reduced cellular viability (Liao et al., 2006). In order to measure the cellular viability after visfatin treatment I used a commercially available assay kit (BioVision, Inc) that is measuring the activity of the enzyme lactate dehydrogenase which is released from the cells in the supernatant if the integrity of the plasma membrane is compromised, generating NADH following lactate oxidation. The kit quantifies the levels of NADH generated by using the specific compound WST. Visfatin treatment for 24-48 hours did not change significantly the levels of NADH in the supernatant unlike hydrogen peroxide, proving that the oxidative stress generated by visfatin had no significant effect on cellular viability (Fig. 4.8). This fact correlated with the previous finding that there was no significant increase in the levels of oxidized products, offering a limited ground to the hypothesis that a *partial* and *localized* increase in the mechanisms regulating antioxidant defence might explain the lack of effects of the detected free radicals on cellular integrity and physiology.

An increase in the levels of ROS is accompanied usually by a higher production of nitric oxide that can modify cell physiology through a further generation of oxygen free radicals mediated by increased production of peroxynitrate (Supinski and Callahan, 2007), through an increase in cyclic GMP (cGMP) levels (Drenning et al., 2009), or through the post-translational modifications of proteins like nitrosylation of specific residues (Nogueira et al., 2009;Sharov et al., 2006). The process of protein nitrosylation represents a significant challenge to the proper functionality of the cells (Riederer et al., 2009) and it was reported already to be involved in the development of the ageing phenotypes (Butterfield et al., 2007; Murdaugh et al., 2010; Musci et al., 2006). Therefore I looked by western blot at the levels of nitrotyrosine residues, which unlike S-nitrosylation modifications are irreversible changes induced by nitric oxide (Stamler et al., 2008),

targeting for example proteins associated with calcium signalling in skeletal muscle (Viner et al., 1996). As seen from figure 4.9, there was no significant increase in the levels of nitrotyrosines residues (YT) by the incubation with visfatin for 1-48 hours.

This paragraph proved that the oxygen free radicals induced by visfatin did not affect the cellular viability and that the nitric oxide potentially produced by such ROS did not increase the levels of nitrosylated proteins.

Fig. 4.8   Visfatin treatment does not change the myocytes cellular viability

C2C12 differentiated myotubes were incubated for 24 and 48 hrs with 100 ng/ml of visfatin or with hydrogen peroxide as a positive control for 1hr. Quantization of NADH levels from supernatant was performed by colorimetric analysis. NADH is produced by the oxidation of lactate by the enzyme lactate dehydrogenase that is released in the supernatant if the plasma membrane is damaged. To quantify NADH levels I used WST compound and the values were further normalized to the negative control. Each data point is the average of duplicates samples per experiment from three independent experiments +/− SEM. ***Denotes p<0.001 compared to control. NS denotes no significant difference between visfatin challenge and control.
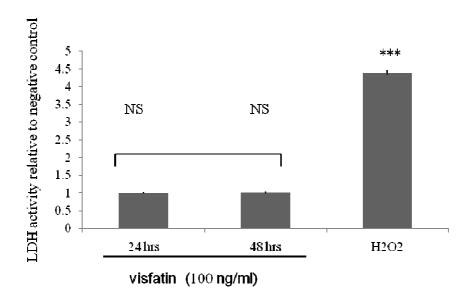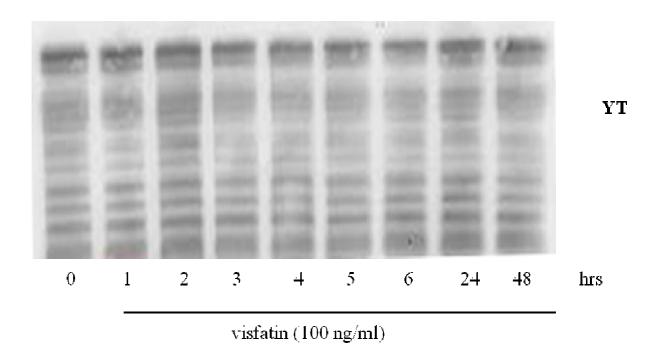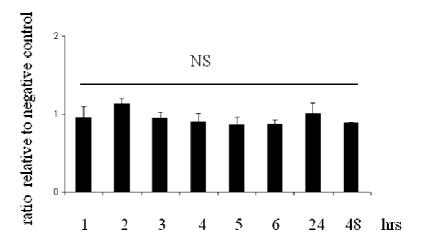
Fig. 4.9   Visfatin treatment does not increase the levels of irreversible nitrosylated proteins

C2C12 differentiated myotubes were incubated between 1-48 hrs with 100 ng/ml of visfatin as shown before. Protein lysates were resolved by a typical western blot to detect the presence of nitrotyrosines (YT). Quantization of the expression was performed by densitometric analysis and the values were further normalized to the negative control. Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between visfatin challenges and control.
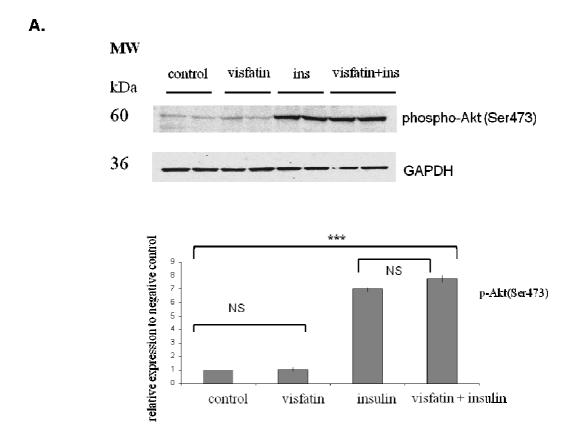
4.4 Acute or chronic visfatin (eNampt) treatment is not activating the insulin receptor–dependent PI3K/Akt signalling
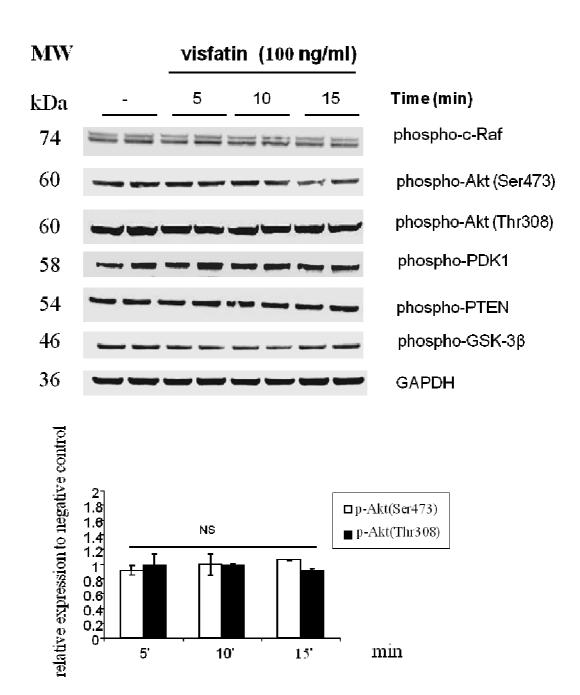
As previously reported, exogenous visfatin (eNampt) binds to insulin receptor expressed by many types of cells like HUVECs (Adya et al., 2008a), osteoblasts (Xie et al., 2007), mesangial cells (Song et al., 2008) and cardiomyocytes (Lim et al., 2008) and increases the glucose uptake rate through the activation of phosphoinositide 3-kinase (PI3K/Akt) signalling, acting therefore as an insulin mimetic agent. It appears nevertheless that visfatin does not compete with insulin since it binds to a different site of the insulin receptor (Fukuhara et al., 2005). To test if the same effect can be identified in the case of the cultured skeletal myocytes I incubated differentiated C2C12 cells with 100 ng/ml of visfatin for 5, 10, and 15 minutes and verified the possible activation of the Akt pathway by western blot followed by densitometric analysis. I tested in this respect the levels of the phosphorylated forms of Akt (at Ser^{473} and Thr^{308} residues), the phosphorylated forms of Akt-upstream positive and negative regulators (PTEN and PDK1) and the phosphorylated forms of Akt-downstream targets (GSK-3β and c-Raf). While 100 nM insulin was able to induce a significant increase in phospho-Akt^{Ser473} levels after 5 minutes of treatment (figure 4.10A), visfatin failed to change in the first 15 minutes the levels of phospho-Akt^{Ser473} and phospho-Akt^{Thr08}, the levels of phosphorylated forms of Akt upstream activator (PDK1) and inhibitor (PTEN), as well as the levels of phosphorylated forms of Akt downstream targets like GSK-3β and c-Raf (figure 4.10B). In addition, as seen from figure 4.10A, the chronic incubation with visfatin for 18 hrs did not change the levels of phospho-Akt at Ser^{473} residue and neither the ability of insulin to activate PI3K/Akt pathway after 5 minutes. These experiments proved that the incubation of C2C12 cells with extracellular visfatin did not activate the PI3K/Akt pathway and did not interfere with the activation of the same pathway by insulin. This proves that in the case of cultured myocytes visfatin is *not* acting as an insulin-mimetic agent.

Fig. 4.10   Visfatin treatment does not activate the insulin receptor-associated PI3K/Akt signalling

A. Phosphorylation of Akt at Ser473 residue was detected at baseline, in the cells with chronic visfatin exposure (100 ng/ml) for 18 hrs, following 100 nM insulin stimulation for 5 min and in the cells showing treatment with visfatin for 18 hrs followed by insulin stimulation for 5 min. Quantization of Akt phosphorylation at Ser473 or Thr308 was performed by densitometric analysis compared to the negative control; B. Western blot analysis was performed by using antibodies raised against the phosphorylated forms of Akt at Ser473 or Thr308 residues, against the phosphorylated forms of Akt upstream regulators (PDK1 and PTEN) and of its downstream targets (c-Raf and GSK-3β) following visfatin treatment at 100 ng/ml (no treatment, 5, 10 and 15 min). Quantization of Akt phosphorylation at Ser473 or Thr308 was performed by densitometric analysis compared to the negative control. Each data point is the average from three independent experiments +/− SEM. ***Denotes $p<0.001$ compared to control. NS denotes no significant difference between visfatin and insulin treatment compared to insulin alone in chronic exposure, or between control and visfatin in both chronic and acute exposures.

B.
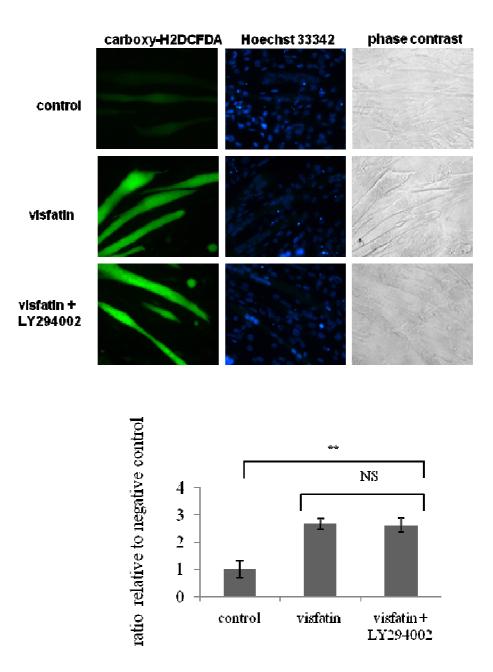
4.5. Blocking PI3K is not reducing the oxidative stress levels increased by visfatin (eNampt)

As shown above in the present chapter, visfatin treatment did not activate PI3K/Akt pathway in the acute stage of incubation (1- 15 minutes), nor in the chronic stage (18 hours). Nevertheless, PI3K/Akt pathway could be activated by visfatin treatment in an interval between these two time points and such activation could be missed by my western blot experiment. It was already shown that the chronic activation of Akt signalling characterizing early oncogenesis increased the aerobic glycolysis rate (the *Warburg effect*) (Robey and Hay, 2009) leading to an increase in the levels of free radicals (van Gorp et al., 2006). Therefore, to test the possibility that an eventual Akt activation upon visfatin challenge could mediate the increase in ROS levels detected previously, I blocked PI3K by its specific inhibitor LY294002 (50 μM) for 1 hour before the incubation with 100 ng/ml of visfatin for another 18 hours. As seen from figure 4.11 there was no change in the signal detected by fluorescent assays in the cells with blocked PI3K/Akt signalling before visfatin challenge compared to the signal detected from cells incubated only with visfatin. This experiment proved that the ROS production is independent of PI3K/Akt signalling pathway.

Fig. 4.11   The oxidative stress induced by visfatin is not mediated by PI3K/Akt signalling pathway
Differentiated C2C12 cells were pre-treated for 1 hr with 50 μM LY294002, an inhibitor of PI3K, before being subjected to another 18 hrs of challenge with 100 ng/ml of visfatin. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/− SEM. **Denotes $p < 0.01$ compared to control. NS denotes no significant difference between the treatment of visfatin plus PI3K inhibitor compared to visfatin alone.

4C. Discussion

Visfatin is a new member of adipokine family with one form but with two different localizations, one being intracellular (iNampt) and another extracellular (eNampt) (Garten et al., 2010). The intracellular form was proved so far to be involved in the NAD synthesis pathway through its phosphoribosyltransferase activity (Wang T. et al., 2006), and the extracellular form was discovered to be actively secreted by diverse types of cells such as macrophages, adipocytes and hepatocytes (Fukuhara et al., 2005;Wang et al., 2009;Curat et al., 2006). Initially considered to be an insulin minetic agent, visfatin proved to be also an inducer of oxidative stress and inflammation.

Because there were no previous reports about the effects of an increased levels of plasma visfatin on the stress responses of skeletal muscle *in vivo* (both animal models or human patients), and since skeletal muscle is a target for the rest of adipokines with a major influence on insulin sensitivity, stress markers and inflammatory status, I explored such possible effects of an increased concentration of plasma visfatin on skeletal muscle by using an *in vitro* approach.

1. **The relationships between exogenous visfatin (eNampt) and the levels of oxidative stress**

I showed in the present chapter that incubating murine C2C12 fused and differentiated myotubes with different concentrations of exogenous murine visfatin (eNampt) (<200 ng/ml) induced oxidative stress as monitored by fluorescent-based assays (Figs.4.1-4.3). This event occurred in the chronic stage, after a minimum of 5 hours, not in the acute stage as shown to happen in the case of stimulation of endothelial cells (Kim et al., 2008). While visfatin treatment increased free radicals, it did not affect the cellular viability that indicates that such a stress is not impairing significantly the myotubes survival (Fig. 4.8). Visfatin did not induce an increase in the levels of

oxidized lipids or oxidized proteins either. The detection of an increase in ROS levels without an increase in the levels of oxidized products or impaired viability after visfatin challenge suggested an existence of a simultaneous improvement of the antioxidant capacity of the cells to resist such a stress. The measurement of this global cellular ability to protect against oxidative stress was performed by using the ABTS assay that did not show any global change in the antioxidant potential of C2C12 cells after the treatment with visfatin compared to control (Fig. 4.7).

The additional metabolic consequences and the upstream mediators of the increased levels of free radicals are still unknown, therefore the main questions still to be answered in the following chapters are:

1.   *What pathways mediate the increased levels of ROS induced by visfatin?*

2.   *What pathways could mediate the protection against such oxidative stress that might explain the lack of oxidized products or loss of viability?*

3.   *What consequences such increased ROS levels could have on the signalling pathways relevant for glucose metabolism?*

2. Visfatin (eNampt) and insulin-dependent signalling pathway

Previously it was shown that visfatin can act as an insulin mimetic agent, but the issue is still controversial and debated (Fukuhara et al., 2005). Since there were no previous reports about the effects of exogenous visfatin on skeletal muscle, which is a main target for adipokines and the main organ responsible for the removal of the glucose from the plasma, I tested whether visfatin can activate the PI3K/Akt signalling pathway that mediates the effects of insulin on the rate of glucose uptake. The stimulation of C2C12 cells with 100 ng/ml visfatin did not induce the phosphorylation of Akt at Ser^{473} or at Thr^{308} residues in the first 15 minutes (Fig. 4.10B), unlike the insulin challenge that triggered a robust response after 5 minutes at the Akt^{Ser473} (Fig. 4.10A). Visfatin did not change either the phosphorylated levels of Akt upstream activator (PDK1), upstream

inhibitor (PTEN) or of Akt downstream targets like GSK-3β and c-Raf (Fig.4.10B). The pre-incubation of C2C12 cells with visfatin for 18 hrs did not impair or augment the ability of insulin to activate PI3K/Akt after 5 minutes and the incubation of cells with visfatin alone for 18 hrs did not change either the levels of phosphorylated Akt at Ser[473] residues (Fig. 4.10A). This showed that in C2C12 cells visfatin did not activate PI3K/Akt signalling pathway, indicating that in the case of cultured skeletal myocytes it is ***not*** an insulin-mimetic agent.

The possibility of an increase in the rate of glucose uptake by visfatin, independently of PI3K/Akt signalling, will be explored in the future chapters. Nevertheless, such activation of Akt pathway could occur later through mechanisms independent of insulin receptor phosphorylation status and such eventual activation could mediate a subsequent oxidative stress response. For example, chronic activation of Akt observed in the early stages of oncogenesis can increase the levels of aerobic glycolysis (the *Warburg effect*) and an increased glycolysis rate is associated with a higher production rate of free radicals (van Gorp et al., 2006). Therefore I blocked PI3K activity by its specific inhibitor before visfatin treatment and repeated the fluorescent-based measurement of ROS levels that did not show any changed values comparing to visfatin-only treated cells (Fig. 4.11). This proved that the observed ROS production induced by visfatin treatment in C2C12 cells is independent of Akt signalling.

Because the long-term incubation of cultured myotubes with visfatin (>24 hours) might still modify the rate of glucose uptake or glucose metabolism independently of Akt signalling, possibly through the modulation of other physiological processes like a robust NAD metabolism as shown before (Revollo et al., 2007), the next question to be raised and answered in the following chapter is the following:

What is the effect of exogenous visfatin on glucose uptake rate? And if I will see such an effect, what are the pathways mediating it?

Chapter 5- THE DIFFERENTIAL EFFECTS OF VISFATIN (eNAMPT) AND TRIGLYCERIDES ON THE EXPRESSION OF ROS-REGULATORY GENES, GLUCOSE UPTAKE AND INSULIN SENSITIVITY

5A. Introduction

In the previous chapter it was showed that exposure of C2C12 cells to exogenous visfatin (eNampt) increased intracellular ROS levels after 18 hours. Nevertheless, the cellular viability was not impaired. Furthermore, no significant increase in the levels of oxidized proteins or oxidized lipids was detected, usually generated by a sustained, chronic production of reactive oxygen species (Nader-Djalal et al., 1998). To explain this initial apparent discrepancy a provisional hypothesis was proposed, based on the theoretical possibility that visfatin could increase simultaneously not only the levels of *intracellular ROS* but also the cellular *antioxidant* defence status. It was shown for example that physical exercise increased in skeletal muscle not only the levels of ROS but also the antioxidant defence status (Bejma and Ji, 1999). This hypothesised improved antioxidant defence could block any significant oxidizing effects on cellular constituents such as proteins and lipids by the simultaneously produced ROS. Since such an antioxidant response was not observed at the pancellular level by using the ABTS assay, the first goal of the present chapter was to inquire whether visfatin might induce a transient or localized antioxidant response. This potential localized increase in antioxidant capability by visfatin, undetectable at the global, pancellular level, could explain the lack of oxidized products. The identity and the mechanisms of such transient or localized responses have been already explored in macrophages (Flesch et al., 1994), placenta (Ejima et al., 1999) and plant-pathogen interactions (Piterkova et al., 2009). To explore the possibility of such localized responses, an RT-PCR assay was performed to quantify the expression levels of certain selected genes already known to be involved in ROS production or antioxidant defence.

To establish a reliable control in order to compare possible expression changes induced by visfatin within an ageing context, a lipid-based challenge was used. Intramyocellular triglycerides are an important source of energy for normal, healthy skeletal muscle especially in the case of chronic, low level exercise (Gorski, 1992). Nevertheless, during ageing the human skeletal

muscle is characterized by an accelerated accumulation of intramyocellular lipids depots that are associated with a progressive increase in the level of oxidative stress, generalized insulin resistance and chronic inflammation (Franklin and Kanaley, 2009; Crane et al., 2010; Machann et al., 2005). These intracellular events are also involved in the development of ageing-associated diseases involving skeletal muscle such as metabolic syndrome (Georg and Ludvik, 2001), sarcopennia (Borst, 2004; Fulle et al., 2004) and frailty (Lang et al., 2009; Faulkner et al., 2007). Interestingly, it appears that skeletal muscle contains lipid droplets in the extracellular, interstitial space (Russell et al., 1998), with unknown origin and unclear function. Skeletal muscle can use different types of lipids from circulation for energy purposes, such as the free fatty acids released by adipocytes in plasma (Martin et al., 1993) or triglycerides. The plasma triglycerides are structured as chylomicrons or as very-low-density lipoproteins (VLDLs) (Voshol et al., 2009). The established method previously used to model *in vitro* the lipid-related toxicity seen in ageing and ageing-associated diseases was the incubation of primary or immortalized muscle cells with BSA-bound free fatty acids such as palmitate or oleate (Garcia-Martinez et al., 2005; Lu et al., 2000). This method presents nevertheless multiple technical pitfalls, increasing the dangers of non-specific results (Wang X. et al., 2010) or artefacts (Bickerton et al., 2007). In addition, less it is known about the effects on skeletal muscle of lipids stored as triglycerides (VLDL or chylomicrons), and not as free fatty acids (Kraegen et al., 2001). Consequently, in order to avoid the problems seen in the experiments using free fatty acids bound to BSA and to further explore the effects of lipids stored as triglycerides, I used a new alternative method, namely the incubation of cultured myocytes with the commercially available fat emulsion *Structolipid^{TM}*. This 20% fat emulsion is composed of equimolar amounts of long chain triglycerides (LCT) from refined soy oil and medium chain triglycerides (MCT) from coconut oil in the ratio of 64% (w/w) and 36% (w/w) respectively, the fatty acids being randomly distributed within the same triglyceride molecule. Structolipid^{TM} is currently used in clinical practice as a parenteral nutrition regimen choice by intravenous

administration in order to supply energy and essential fatty acids (1-1.5 g/kg body weight/day) (Ellborg et al., 2010), but it was also successfully used to model *in vitro* the effects of intramyocellular accumulation of lipid depots seen in aged or dysfunctional skeletal muscle. For example, it was shown already that incubation with Structolipid[TM] at high concentrations (0.2 mg/ml) induced oxidative stress in cultured cells (Delaigle AM, 2006; Delaigle et al., 2004). Therefore I used this fat emulsion to induce a lipid-based challenge in C2C12 cells.

The *first* goal of the present chapter was to confirm that this new type of lipid challenge (Structolipid[TM]) is a genuine model of lipid overload of C2C12 cells by testing its transport and accumulation in cytosol, followed by measurements of oxidative stress markers as performed in previous chapter. After these preliminary experiments the existence of any possible changes in the expression of some of the genes relevant for oxidative stress and antioxidant defence induced by visfatin or fat emulsion are to be explored by using RT-PCR.

It was shown before that the treatment with exogenous visfatin increased the rate of glucose uptake in many types of cells such as pancreatic β cells (Brown et al., 2010) and osteoblasts (Xie et al., 2007), similarly to insulin. This effect was dependent on activation of Akt signalling that was not detected in the experiments presented in previous chapter. The chronic treatment with exogenous visfatin could nevertheless modify the glucose uptake rate indirectly, through the regulation of other signalling pathways. For example, it is known that glucose metabolism and uptake can be modified by changes in NAD metabolism that are known to be regulated by the activity of the intracellular form of visfatin, iNampt (Revollo et al., 2007). It was also shown that incubation of smooth muscle cells with exogenous visfatin increased the intracellular NAD levels and subsequently activated the NFkB signalling pathway, but the upstream mechanisms are still unclear (Romacho et al., 2009). I tested therefore the possibility that chronic exogenous visfatin (eNampt) treatment could change the rate of glucose uptake in cultured myotubes by using a new fluorescent method based on measurements of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-

deoxyglucose (2-NBDG). This method was developed recently (O'Neil et al., 2005; Zou et al., 2005) and it was already proved to be an efficient, reliable and safer alternative to the classical radioactive (2-DG) method both *in vitro* (Zou et al., 2005; Ball et al., 2002) and *in vivo* (Ye et al., 2008). The *second* goal of the present chapter was therefore testing whether the incubation of C2C12 cells with exogenous visfatin or triglycerides can change the glucose uptake rate in both basal and insulin-stimulated conditions by using 2-NBDG.

Previously it was shown that the physiological effects induced by lipids on the rate of glucose uptake are partially mediated by an impaired activation of PI3K/Akt pathway (Nogueira et al., 2008) and by changes in expression of transporters such as glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) (Bloch-Damti and Bashan, 2005). The *third* goal of the present chapter was therefore to test whether the triglyceride challenge can interfere with the PI3K/Akt signalling pathway by using the western blot method to quantify the levels of phosphorylated forms of Akt, as performed in the previous chapter in the case of visfatin treatment. In addition, RT-PCR was used to quantify the possible change induced by visfatin or triglycerides in the expression of GLUT1, which is involved in the basal glucose uptake, and GLUT4, that is mediating the insulin-stimulated glucose uptake process.
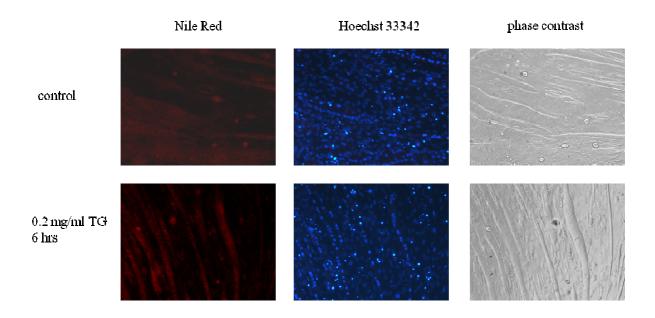
5B. Results

5.1 Triglyceride treatment is an accurate and efficient method to model the effects of lipids in skeletal muscle

Structolipid[TM] was previously proven to induce oxidative stress in cultured skeletal myocytes (Delaigle et al., 2006; Delaigle et al., 2004), and I used it as an alternative to the free fatty acids method to model the effects induced by lipids on skeletal muscle physiology characterizing the ageing-associated diseases such as obesity and metabolic syndrome (Samuel et al., 2010). C2C12 cultured and differentiated myotubes were incubated with the fat emulsion that was diluted 1/100 in serum-free DMEM to a final concentration of 0.2 mg/ml of triglycerides (TG). To confirm the transport and accumulation of the triglycerides in C2C12 cells I performed fluorescent-based assays by using the specific dye Nile Red (1 µg/ml). A marked increase in lipid depots was detected by Nile Red staining after 6 hours of treatment with 0.2 mg/ml of triglycerides (Fig. 5.1). This shows that triglycerides are transported into the cytosol of cultured myotubes.

Fig. 5.1 StructolipidTM triglycerides are transported into the cytosol of C2C12 differentiated myotubes

C2C12 cells were incubated for 6 hrs with 0.2 mg/ml of *Structolipid^{TM}* fat emulsion that was diluted 1/100 in serum-free DMEM to a final concentration of 0.2 mg/ml of triglycerides (TG). Cells were further incubated with 1 µg/ml Nile Red diluted in PBS for 15 minutes, followed by 5 min incubation with 1 µM Hoechst 33342 to stain the nuclei. Fluorescent microscopy was performed at 560 nm for Nile Red and at 460 nm for Hoechst 33342.

5.2 The incubation with triglycerides induces oxidative stress in cultured C2C12 cells after 24 hours

To test whether 0.2 mg/ml of triglycerides treatment can generate oxidative stress in C2C12 cells after 24 hours of incubation I used the carboxy-H2DCFDA fluorescent-based assay as described in previous chapter and noticed a significant increase in oxidative stress levels, as seen from figure 5.2. This showed that triglyceride challenge induced oxidative stress. In order to investigate in detail the effects of incubation with triglycerides on the skeletal myocytes physiology, I explored also the possible detection of oxidized forms of proteins by using the Oxyblot[TM] system followed by densitometric analysis, as performed in the previous chapter for visfatin challenge. Figure 5.3 shows that the incubation of C2C12 cells with 0.2 mg/ml triglycerides increased with almost 80% the global levels of carbonylated proteins after a minimum incubation time of 24 hours, with a significant increase of a band around 30 kDa. This proved that the cellular stress pattern induced by triglyceride treatment is different from the one generated by exogenous visfatin (eNampt). The pattern of carbonylated proteins triggered by triglyceride challenge in C2C12 cells is different also from the pattern seen in the skeletal muscle from diabetic animal models (Oh-Ishi et al., 2003) and from the pattern shown by aged skeletal muscle (Griffin et al., 2008). This shows that triglyceride treatment is an acceptable model of the effects of ageing on skeletal muscle when an accumulation of intramyocellular lipids occurs.

Fig. 5.2 Incubation of differentiated myocytes with triglycerides induces oxidative stress after 24 hours

C2C12 cultured and differentiated myotubes were incubated for 24 hrs with *Structolipid*^{TM} fat emulsion that was diluted 1/100 in serum-free DMEM to a final concentration of 0.2 mg/ml of triglycerides. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Fluorescent microscopy was performed at 520 nm for carboxy-H2DCFDA and at 460 nm for Hoechst 33342, and quantification was further done by using software-based analysis. Relative expression compared to negative control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/– SEM. ***Denotes p<0.001 compared to control.
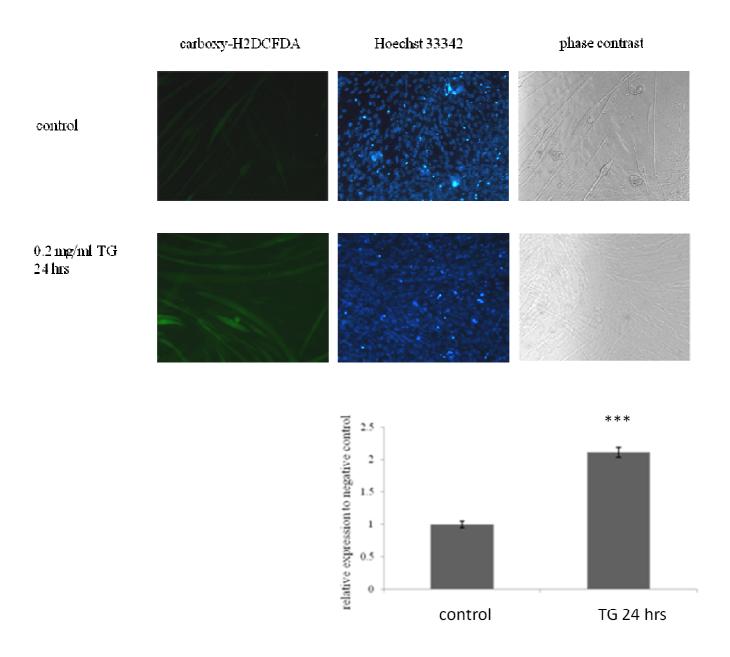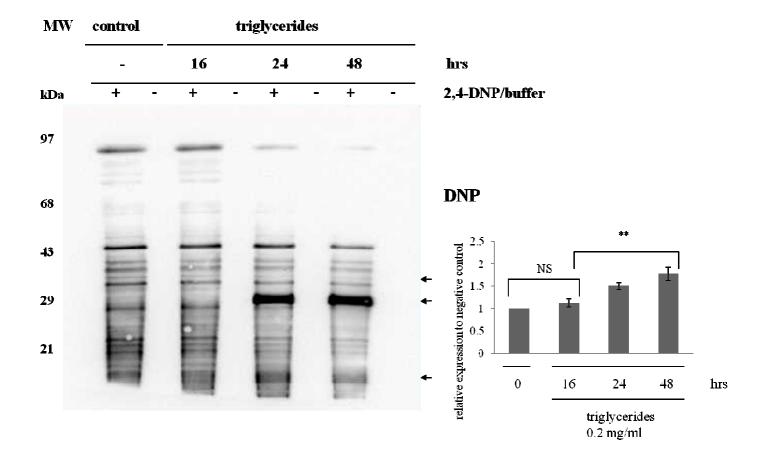
Fig. 5.3 Incubation of differentiated myocytes with triglycerides increases the levels of oxidized proteins after 24 hours

C2C12 differentiated myotubes were incubated for 16, 24 and 48 hrs with 0.2 mg/ml of triglycerides in serum-free DMEM. The denatured and reduced cell lysates extracted were treated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize the carbonyl groups to 2,4-dinitrophenylhydrozone (2,4-DNP) residues. The cell lysates were resolved by using a standard ECL western blot procedure by using an antibody against the derivatized 2,4-DNP residues. Quantization of the expression was performed by densitometric analysis and the values were further normalized to negative control. Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between treatment and control. **Denotes $p < 0.01$ compared to control.

5.3   The incubation of cultured C2C12 cells with triglycerides changes the expression of genes involved in oxidative stress and antioxidant defence in a different manner compared to visfatin challenge

In order to decipher the mechanisms involved in ROS production and antioxidant defence changed by visfatin or triglycerides in cultured myocytes I performed RT-PCR for a number of genes already known to be involved in the process. As seen from figure 5.4, the incubation of C2C12 cells for 24 hours (but not for 6 hours) with 0.2 mg/ml of triglycerides was able to increase significantly the expression levels of many genes involved in antioxidant defence such as catalase (CAT), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and of transcriptional factors required for antioxidant defence or ischemic response such as hypoxia-inducible factor 1A (HIF1A), nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2). Triglyceride treatment did not change the expression levels of other genes responsible for free radical production such as NADPH oxidase 4 (NOX4), a subunit of NADPH oxidase complex. In comparison, as seen from figure 5.5, incubation of C2C12 cells with 100 ng/ml of visfatin for 24 hours increased the expression level of SOD1 and NOX4 genes only, leaving the rest of the genes mentioned above unchanged. The incubation of skeletal myocytes with triglycerides, but not with visfatin, was also able to reduce after 24 hours the expression of glutathione peroxidase-3 (GPX3), a powerful antioxidant factor (Fig. 5.6). This could indicate a possible reduction in the general antioxidant defence ability by triglycerides as proved already for skeletal muscle from diabetic patients or from the animal models of metabolic syndrome (Chung et al., 2009).

In conclusion, the pattern induced by visfatin treatment was different compared to the pattern shown by lipid emulsion. The fact that the levels of expression of transcriptional factors such as NRF1, NRF2 and HIF1A and antioxidant proteins such as GPX3 were not changed after visfatin treatment corroborates with the finding from previous chapter that visfatin did not oxidize proteins nor lipids.

Fig. 5.4 Incubation of differentiated myocytes with triglycerides increases the expression levels of some of the ROS-regulatory genes

C2C12 differentiated myotubes were incubated for 6 or 24 hrs with 0.2 mg/ml of triglycerides in serum-free DMEM. After RNA extraction and cDNA synthesis RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin) and then comparing the values from lipid-challenged samples to the values from untreated cells. ***Denotes $p<0.001$ compared to control. NS denotes no significant difference between treatment and control. Each data point is the average from three independent experiments +/− SEM.
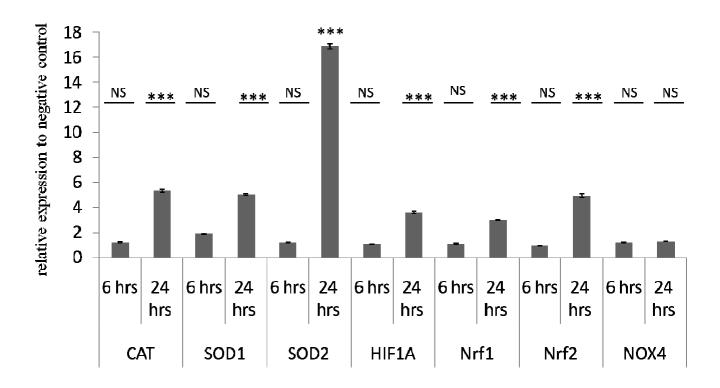
Fig. 5.5 Incubation of differentiated myocytes with visfatin increases the expression levels of some of the ROS-related genes differently compared to triglyceride challenge

C2C12 differentiated myotubes were incubated for 24 hrs with 100 ng/ml of visfatin in serum-free DMEM. After RNA extraction and cDNA synthesis RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin), and then comparing the values from visfatin-challenged samples to the values from untreated cells. **Denotes $p<0.01$ compared to control. Each data point is the average from three independent experiments +/− SEM.
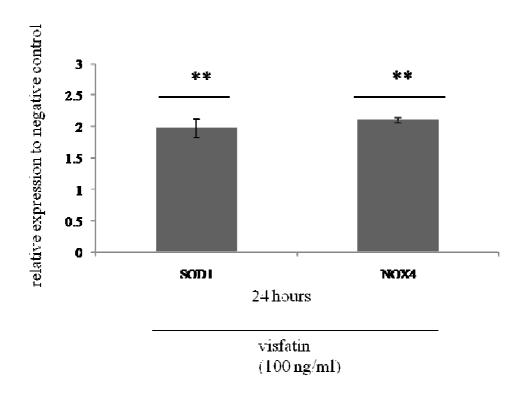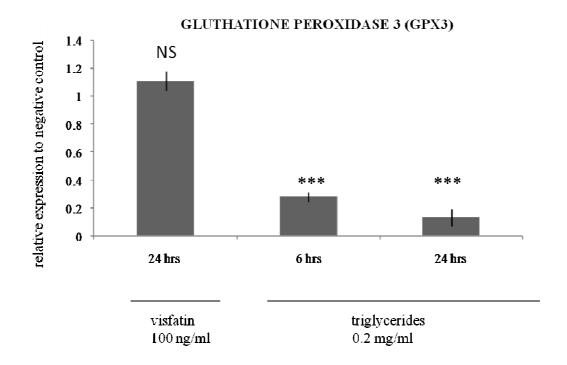
Fig. 5.6   Incubation of differentiated myocytes with triglycerides, but not visfatin, decreases the expression levels of GPX3, a powerful antioxidant gene

C2C12 differentiated myotubes were incubated for 24 hrs with 100 ng/ml of visfatin or with 0.2 mg/ml triglycerides in serum-free DMEM for 6 or 24 hrs. After RNA extraction and cDNA synthesis RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin) and then comparing the values from treated samples to the values from untreated cells. *** Denotes $p<0.001$ compared to control. NS denotes no significant difference between treatment and control. Each data point is the average from three independent experiments +/− SEM.

GLUTHATIONE PEROXIDASE 3 (GPX3)

5.4 Incubation of cultured myotubes with triglycerides, but not with visfatin, changes the rate of glucose uptake in cultured myocytes

Previously it was shown that long term changes in the expression level or in the enzymatic activity of intracellular form of visfatin (iNampt) could affect the levels of glucose uptake and glucose metabolism through the modulation of NAD levels (Revollo, 2008), independently of PI3K/Akt signalling. I explored this possibility of indirect effects of glucose uptake rate also in the case of chronic exogenous visfatin (eNampt) challenge that failed to activate PI3K/Akt pathway, as shown in previous chapter. In order to test this hypothesis I used a new available method, recently developed, that is based on fluorescent microscopy measurements of the compound 2-NBDG (Ye et al., 2008; Fukushima et al., 2007; Zou et al., 2005; Ball et al., 2002; Yamada et al., 2000). C2C12 cultured cells, treated or not with visfatin for 24 hours, were incubated with the compound 2-NBDG (0.1 mg/ml) diluted in PBS for 30 minutes followed by f1uorescent microscopy imaging (Fig.5.7B). As seen from figure 5.7C, there were no differences in the recorded values between visfatin–treated and control cells, indicating that visfatin has no effect on the rate of glucose uptake in the case of cultured myotubes. This correlates with the fact that only in the cellular types that presented an activation of PI3K/Akt signalling pathway by visfatin such as osteocytes and endothelial cells it was also noticeable an increased rate of glucose uptake (Xie et al., 2007). In contrast, the incubation of C2C12 cells with triglycerides (0.2 mg/ml) for 24 hours increased the levels of basal transport of glucose by approximately 40%. This discovery correlates with previous independent experiments elucidating the mechanisms of insulin resistance in skeletal muscle. For example, it was already shown by 2-DG method that palmitate treatment of cultured myotubes is increasing the rate of glucose uptake in the basal state (Montell et al., 2001), confirming the usefulness of Structolipid™ in obesity research.
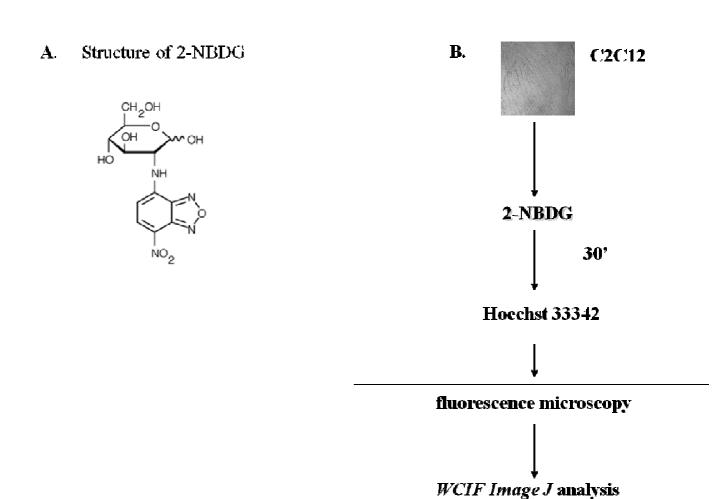
To study the effects of triglycerides or visfatin on the insulin-stimulated glucose uptake I incubated C2C12 cells with 100 ng/ml of visfatin or with 0.2 mg/ml of triglycerides for 24

hours, followed by the incubation with 100 nM insulin diluted in PBS for 30 minutes (Fig. 5.8A). To quantify the rate of glucose uptake I repeated the 2-NBDG assay as described above. The fluorescent microscopy measurements of 2-NBDG signal showed that insulin treatment alone increased the levels of glucose uptake in C2C12 cells more than 2.5 times compared with non-treated cells (Fig.5.8B), confirming the usefulness of the 2-NBDG assay for glucose measurements in C2C12 cells (del Aguila et al., 1999). While the pre-incubation with visfatin for 24 hours did not affect the insulin-dependent glucose uptake, triglyceride treatment nevertheless was able to reduce it by a third. This shows that extracellular form of visfatin did not change the rate of glucose uptake in both basal and insulin-stimulated states, unlike the modulation of its intracellular form (iNampt) that increased the rate of NAD synthesis and indirectly the rates of glucose uptake and glucose metabolism (Revollo, 2008). The decrease in glucose uptake by triglycerides in insulin-stimulated condition correlates also with the fact that palmitate treatment of cultured myotubes reduced the glucose uptake rate in the insulin-stimulated state as shown by 2-DG method (Ragheb et al., 2009).
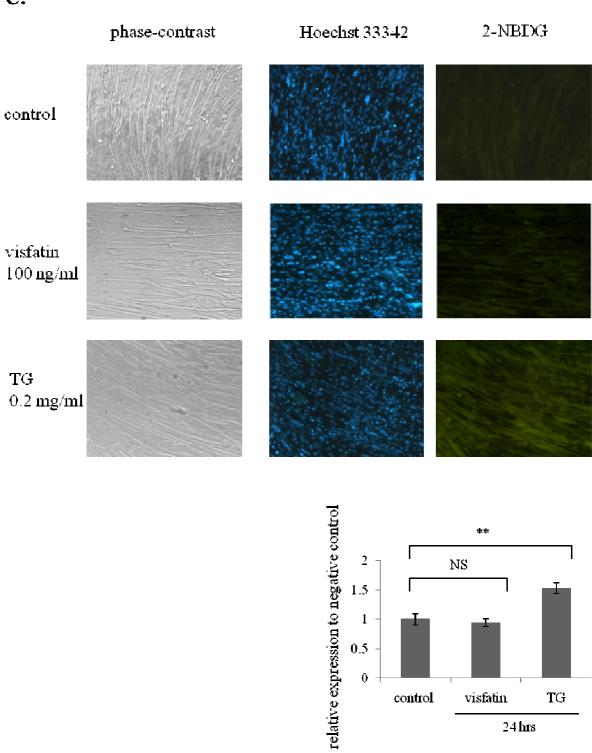
The increase in the glucose uptake in basal state and reduction in the insulin-stimulated state upon triglyceride challenge can be explained by changes in PI3K/Akt signalling that regulates the translocation of glucose transporters at the plasma membrane (Nakamura et al., 2009; Nogueira et al., 2008; Kelly et al., 2008), and by changes in the expression levels of glucose transporters such as GLUT1 (Dimopoulos et al., 2006; MacLaren et al., 2008) and GLUT4 (Armoni et al., 2007), aspects that were approached next.

Fig. 5.7 Incubation of differentiated myocytes with triglycerides, but not visfatin, increases the rate of the basal glucose uptake

C2C12 differentiated myotubes were incubated for 24 hrs with 100 ng/ml of visfatin or with 0.2 mg/ml of triglycerides in serum-free DMEM for 6 or 24 hrs. To measure the rate of glucose uptake cells were incubated with 0.1 mg/ml of 2-NBDG (A) in PBS for 30 min followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei (B). Fluorescent microscopy was performed at 520 nm for 2-NBDG and at 460 nm for Hoechst 33342, and quantification was further done by using software-based analysis (C). Relative expression compared to control data is shown. Data shown are the average of quadruplicate samples per experiment, from three independent experiments +/− SEM. **Denotes p<0.01 compared to control. NS denotes no significant difference between treatment and control.

A.   Structure of 2-NBDG

B.   C2C12

2-NBDG

30'

Hoechst 33342

fluorescence microscopy

WCIF Image J analysis

C.

	phase-contrast	Hoechst 33342	2-NBDG
control			
visfatin 100 ng/ml			
TG 0.2 mg/ml			
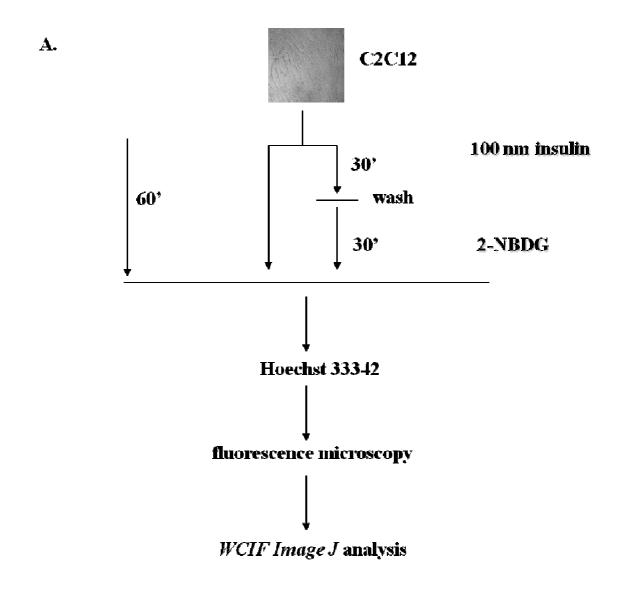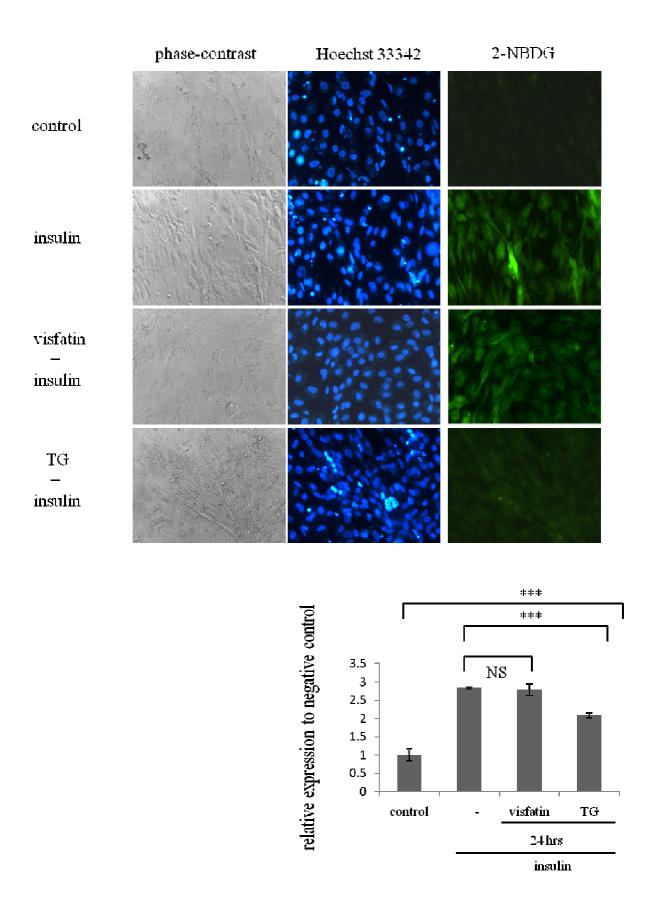
Fig. 5.8 Incubation of differentiated myocytes with triglycerides, but not visfatin,
decreases the rate of the insulin-stimulated glucose uptake

C2C12 differentiated myotubes were incubated for 24 hrs with 100 ng/ml of visfatin or with 0.2 mg/ml of triglycerides. Cells were washed and pre-treated with 100 nM of insulin in PBS for 30 minutes before the incubation with 0.1 mg/ml of 2-NBDG in PBS for an additional interval of 30 min, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei (A). Fluorescent microscopy was performed at 520 nm for 2-NBDG and at 460 nm for Hoechst 33342, and quantification was further done by using software-based analysis. Relative expression compared to control data is shown (B). Data shown are the average of quadruplicate samples per experiment, from three independent experiments +/− SEM. ***Denotes p<0.001 compared to control. NS denotes no significant difference between treatment and control.

B.
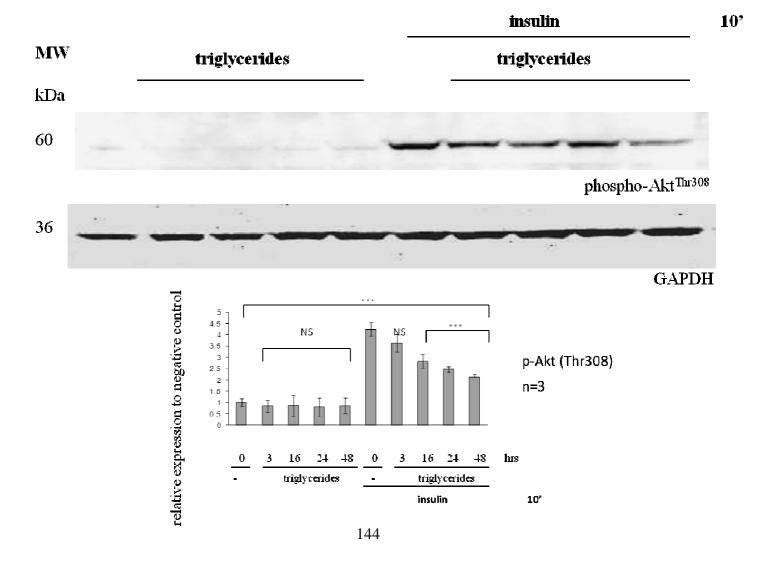
5.5. Triglyceride treatment impairs the insulin-stimulated activation of PI3K/Akt signalling
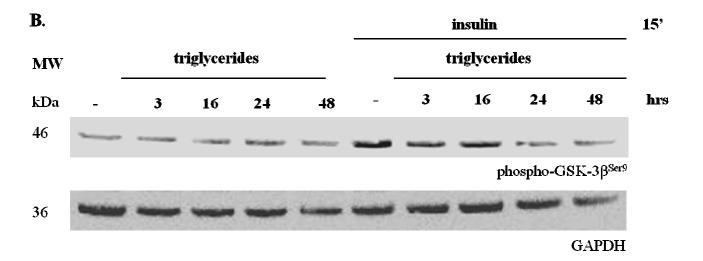
As shown in the third chapter exogenous visfatin treatment did not change the levels of phosphorylated Akt and this correlates with the unchanged rate of glucose uptake shown above. It was reported before that the incubation of cultured cells with free fatty acids can induce insulin resistance partially by interfering with insulin-dependent PI3K/Akt pathway activation (Nakamura et al., 2009; Nogueira et al., 2008; Kelly et al., 2008) that regulates GLUT4 translocation at the plasma membrane (Storz et al., 1999). I further verified by western blot the phosphorylated levels of Akt at Thr308 and of GSK-3β, which once phosphorylated by Akt at Ser9 residue upon insulin challenge has reduced activity (Hajduch et al., 2001). As seen in figure 5.9A, the incubation of cells with 0.2 mg/ml triglycerides for a minimum of 24 hours reduced the levels of phospho-Akt^{Thr308} in the insulin-treated cells, but did not change the levels of phospho-Akt^{Thr308} in the basal state. Also, as seen from figure 5.9B, while triglycerides treatment for a minimum of 24 hours did not affect the basal level of phospho-GSK-3β^{Ser9}, it reduced nevertheless the increase in its levels induced by the stimulation with insulin. This correlates also with previous data about the effects of free fatty acids such as palmitate on Akt activation (Dimopoulos et al., 2006). The fact that triglycerides treatment impaired the activation of Akt pathway by insulin could explain the decrease in the rate of glucose uptake in the insulin-stimulated conditions shown above since this might impair the translocation of GLUT4 transporter to the plasma membrane. The lack of any significant change in phospho-Akt^{Thr308} or phospho-GSK-3β^{Ser9} levels in the basal condition after triglyceride challenge shows that the increase in glucose uptake noticed before is not mediated by the PI3K/Akt signalling pathway.

Fig. 5.9 Incubation of differentiated myocytes with triglycerides impairs the activation of PI3K/Akt pathway by the insulin

C2C12 differentiated myotubes were incubated for 3, 16, 24 and 48 hrs with 0.2 mg/ml of triglycerides in serum-free DMEM. Cells were further treated or not with 100 nM insulin for 10 minutes (A) or for 15 minutes (B). Cells extracts were used to detect by ECL western blot the levels of phospho-Akt^{Thr308} (A) and phospho-GSK-3β^{Ser9} (B). As loading control an antibody against GAPDH was used. Quantization of phosphorylation was performed by densitometric analysis compared to the loading control, followed by normalization of values to the negative control. Each data point is the average from three independent experiments +/− SEM. ***Denotes p<0.001 compared to control. NS denotes no significant difference between treatment and control.

A.

B.

		triglycerides				insulin 15' triglycerides				
MW kDa	-	3	16	24	48	-	3	16	24	48	hrs
46											phospho-GSK-3β^{Ser9}
36											GAPDH
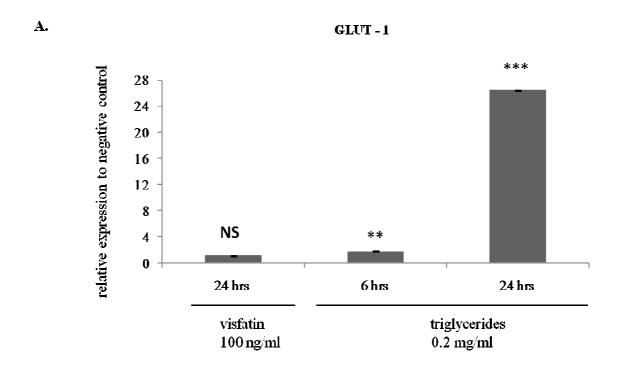
5.6 Incubation with triglycerides, but not visfatin, changes the expression levels of glucose transporters

Because in the experiments presented above triglyceride treatment increased the level of basal glucose uptake but decreased the level of insulin-stimulated glucose uptake, I verified by RT-PCR the expression levels of GLUT1 and GLUT4 transporters that are responsible for the above mentioned processes. Figure 5.10A-B shows that while visfatin treatment for 24 hours had no effect on the expression levels of both transporters, the treatment of cells with 0.2 mg/ml of triglycerides significantly increased the gene expression levels of GLUT1 after a minimum incubation time of 6 hours and reduced the expression levels of GLUT4 after 24 hours. The fact that GLUT1 gene expression is upregulated might explain the increase in the rate of glucose uptake by triglycerides in the basal state as detected by 2-NBDG assay. Also, this suggests that the increase in GLUT1 expression is not regulated by PI3K/Akt-dependent downstream events since there was no change in phospho-Akt^{Thr308} level in the basal state. The fact that GLUT4 is decreased upon triglyceride challenge correlates also with my previous findings of the effects of triglycerides on the rate of glucose uptake in the insulin-stimulated state and with the effects of triglycerides on phospho-Akt^{Thr308} or phospho-GSK-3β^{Ser9} levels in the insulin-stimulated state. The lack of effect of exogenous visfatin on the expression levels of glucose transporters correlates also with the lack of effect on the PI3K/Akt signalling pathway and the unchanged rate of glucose uptake, proving that visfatin is **NOT** an insulin mimetic agent in the case of cultured myocytes.

Fig. 5.10 Incubation of differentiated myocytes with triglycerides, but not visfatin, changes the expression levels of glucose transporters

C2C12 differentiated myotubes were incubated for 24 hrs with 100 ng/ml of visfatin or with 0.2 mg/ml triglycerides in serum-free DMEM for 6 or 24 hrs. After RNA extraction and cDNA synthesis, an RT-PCR assay was performed for GLUT1 (A) and GLUT4 (B). All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin) and then comparing to gene expression in untreated cells. **Denotes $p<0.01$ compared to control. ***Denotes $p<0.001$ compared to control. NS denotes no significant difference between treatment and control.

A.

GLUT - 1

147

B.

GLUT - 4
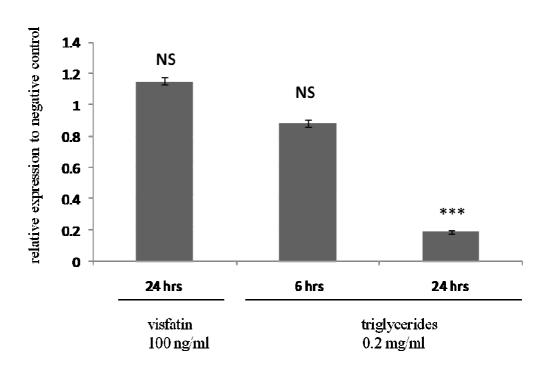
Table 5. 1 Composition of Structolipid^{TM}

Oil source (% by wt)	Coconut (36%), soybean (64%)
Ratio of n–6 to n–3 PUFAs	7:1
Fat content (g/L)	200
Molecular weight	683
Phospholipids (g/L)	12
Glycerol (g/L)	22.5
pH	8.0
Osmolarity (mOsmol/L)	350
Energy (kcal/L)	1960

5C. Discussion

1. Triglycerides treatment induces oxidative stress in a different manner compared with visfatin

Structolipid^{TM} is a commercially available fat emulsion extensively used in the parenteral medicine (Weigt et al., 2002). It was also employed in molecular biology experiments as an alternative to the established BSA-bound free fatty acids method in order to model the lipid-induced oxidative stress in cultured cells (Delaigle et al., 2004; Delaigle et al., 2006). By diluting the fat emulsion to a final concentration of 0.2 mg/ml of triglycerides in serum-free medium I was able to model in an accurate way the physiology of skeletal muscle seen during lipid-induced stress in obesity and ageing. This emulsion is an accelerated and robust method for delivery and transport of triglycerides in C2C12 cells (Fig. 5.1) that avoids the technical difficulties or the artefacts characterizing the free fatty acids treatment previously used (Bickerton et al., 2007). In addition, this lipid form models the effects of lipids stored in the blood as triglycerides and not as free fatty acids, aspect that has been not extensively studied previously.

The treatment of C2C12 cells with 0.2 mg/ml triglycerides for 24 hours induced oxidative stress as measured by fluorescent microscopy (Fig. 5.2). I noticed an increase in the level of oxidized proteins by 80% compared with the negative control after a minimum incubation time of 24 hours (Fig. 5.3), a band localized around 30 kDa showing a significant increase in the carbonylated form. The identity of such a band is still unexplored and this question will not be covered in the present thesis. So far I did not identify another independent report to confirm or support my particular finding. The pattern is also different from the one noticed in the skeletal muscle of diabetic animal models presenting increased depots of intracellular lipids in skeletal muscle (Oh-Ishi et al., 2003), the one seen in the skeletal muscle of mice fed with a high-fat diet (Bonnard et al., 2008) or in the aged skeletal muscle (Feng et al., 2008). Some of the bands from the lanes with control and with 16 hrs of triglycerides treatment samples are *decreasing* after 24 hrs

and 48 hrs of TG treatment. This could signify that the big band seen around 30 kDa after a minimum of 24 hrs of treatment is an *aggregation* of carbonylated proteins undigested by the proteasome. Nevertheless, it could be argued with equal strength that since triglycerides treatment will likely *decrease* the expression values of some of proteins expressed in C2C12 cells, than their basal levels of oxidation upon the derivatization step will also be decreased (assuming that they are not oxidized by triglycerides-induced oxidative stress), therefore the bands present a decreased signal. For example, the triglycerides treatment reduced the expression of GPX3 and GLUT4 at transcription level as seen in chapter 5. Whether this is a single band or an aggregation of undigested proteins is still to be determined.

By using an RT-PCR assay I also showed that visfatin had a different effect on genes regulating ROS production or antioxidant defence compared to lipid-based challenge. For example, triglycerides increased expression values of crucial genes such as SOD1, CAT and SOD2 and of transcription factors such as NRF1, NRF2 and HIF1A (Fig. 5.5), reducing simultaneously expression of GPX3, an important antioxidant factor. Visfatin increased nevertheless only expression of NOX4, a member of NOX family, and of SOD1. NOX4 is a member of NADPH oxidase family with 7 members that present a core structure of 6 transmembrane domains and a long cytosolic domain containing FAD and NADPH binding domains (Bedard and Krause, 2007). The oxidation mechanism is based on the transfer of one electron from the NADPH to the molecular oxygen generating superoxide. The first described member of the family (NOX2) was identified more than 20 years ago (Harper et al., 1984) as a member of an enzymatic complex involved in the generation of ROS in the human neutrophils during phagocytosis. The phagocytic NADPH complex is formed of a membrane-bound cytochrome b_{558}, three cytosolic factors named p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase Rac2 that has a regulatory role. During the activation of the phagocyte NADPH oxidase complex the cytosolic proteins translocate to the plasma membrane initiating the production of superoxide (Geiszt, 2006). In the recent years it was shown that NADPH family members are

expressed also in non-immune cells and fulfil multiple functions. NOX4 was identified initially in the kidney (Cheng et al., 2001) and is the most elusive member of the family, its functions being only partially understood. Besides kidney, NOX4 is widely expressed in a large variety of tissues such as foetal liver, smooth muscle cells, adipocytes, vascular endothelial cells, osteoclasts, hematopoietic stem cells and skeletal muscle. The fact that its overexpression alone increased to the production of superoxide indicated that it is *constitutively* activated (Ambasta et al., 2004), unlike other NOX members. The subcellular localization of NOX4 is also diverse. In HUVECs cells NOX4 was localized in the nucleus (Kuroda et al., 2005), in smooth muscle cells in nucleus and ER (Hilenski et al., 2004) and in the skeletal myocytes it was localized in cytosol and close to plasma membrane. Nevertheless, in an independent report the production of superoxide by NADPH oxidases in cultured myocytes was identified only in the sarcoplasmic reticulum (SR) fraction (Xia et al., 2003). Dysregulations in the NOX4 activity or changes in its expression levels have been reported in the development of diabetic nephropathy (Gorin et al., 2005) and cardiovascular diseases (Sorescu et al., 2002). Of interest, recently it was also reported that NOX4 mediates the production of ROS in cultured 3T3-L1 adipocytes after insulin challenge (Mahadev et al., 2004), partially by inhibiting the action of tyrosine phosphatases (PTPases). In addition, it was shown that exogenous visfatin treatment of vascular endothelial cells stimulated the accumulation and aggregation of gp91 and p47 phox subunits of NADPH oxidase complex in the membrane rafts domains, leading to an increase in the production of superoxide (Xia et al., 2010). The specific role of NOX4 in skeletal muscle is still unexplored, besides the fact that it is an important regulator of proliferation and differentiation (Mofarrahi et al., 2008). Therefore, the increased expression of NOX4 by visfatin treatment could partially explain the increased levels of ROS detected by the H2DCFDA fluorescent method. Nevertheless, since MitoSOX^{TM} probe used in the previous chapter is specific only for the superoxide produced by mitochondria, and since NOX4 was reported so far to be present only in cytosol, around the plasma membrane, in the SR but not in mitochondria, it is likely that the

increased superoxide production upon visfatin challenge did not reflect a direct involvement of NOX4, suggesting independent mechanisms. Nevertheless, the measurement of NOX4 activity is not covered in the present thesis and whether the increase in NOX4 expression by visfatin indicates that the production of ROS by visfatin occurs though its activation is a subject for future studies. Interestingly, NOX4 was not increased by the triglyceride treatment, although it was shown independently that high-fat diet was able to induce in mice models an increase in its expression and that palmitate-treated endothelial cells silenced with a NOX4-specific construct had a reduced level of activation of NFkB signalling (Maloney et al., 2009). The upstream regulators for such increase in NOX4 levels upon visfatin challenge are unknown so far. The relevance of this discovery was further reinforced by other recent independent reports showing that exogenous visfatin challenge induced also changes in localization and/or activity of other members of NADPH oxidases family increasing the levels of ROS produced, with a potential relevance for the development of many pathological conditions affecting ageing such as metabolic syndrome, inflammation and arthritis (Boini et al., 2010; Malam et al., 2011; Xia et al., 2011).

Therefore, the next question to be approached in the coming chapter is the following:

What signalling pathways are regulating the ROS production and the underlying changed genomic profile upon visfatin treatment in C2C12 cells?

2. Triglyceride incubation induced insulin resistance in C2C12 cells by affecting PI3K/Akt signalling

As already known, once activated by insulin challenge the PI3K-dependent signalling induces the translocation of GLUT4 transporter to the plasma membrane within the first hour of incubation (Valverde et al., 2005). I reported in this chapter an increased rate of glucose uptake in skeletal myocytes after 30 minutes of incubation with insulin (Fig. 5.7), likely a

consequence of the increased translocation of GLUT4 to the plasma membrane. As shown in the previous chapter, short- or long-term treatment of cultured myocytes with exogenous visfatin alone did not change the phosphorylated levels of PI3K/Akt-associated signalling pathway. Furthermore, the pre-incubation of C2C12 cells with visfatin for 24 hours did not influence the subsequent activation of Akt by the acute insulin stimulation as shown in the previous chapter. In the present chapter I showed also that the incubation of C2C12 cells with visfatin for 24 hours did not change gene expression levels of glucose transporters such as GLUT1 and GLUT4 (Fig. 5.10A,B). In addition, visfatin had no effect on the rate of glucose uptake in both basal (Fig. 5.7C) and insulin-stimulated conditions (Fig. 5.8B), as measured by 2-NBDG assay. This proved that in the case of cultured skeletal myocytes visfatin is **not** an insulin mimetic agent, which is also a strong argument to dismiss the possibility of an insulin mimetic effect on skeletal muscle *in vivo* too. 2-NBDG is a fluorescent derivative of glucose that is taken up from the extracellular space into the cytosol of cultured myotubes by glucose transporters and consequently can be visualized by fluorescent microscopy. This alternative method is also applicable for automatic microplate readings. In addition, unlike the radioactive assay, 2-NBDG approach is also useful for the identification of localization patterns and it is applicable for live, real-time imaging (Gaudreault et al., 2008). Previously, by using 2-DG method it was shown that skeletal muscle from obese rats had an impaired glucose uptake response compared to the skeletal muscle from wild-type animals (Jacob et al., 1996), and the same effect was shown also in the case of L6 rat cultured skeletal myotubes treated with palmitate (Kelly et al., 2008). My findings correlate with previous data that indicated that the increase in the rate of glucose uptake upon the challenge with the exogenous visfatin (eNampt) was noticed only in those cells that also presented an activation of PI3K/Akt signalling pathway (Lim et al., 2008), event missing in my experiments.

I also showed that the chronic triglyceride treatment reduced the levels of phosphorylated forms of Akt and GSK-3β (one of Akt substrates) only in the insulin-stimulated

state, but not in the basal state (Fig. 5.9A,B). In the same time, as shown by 2-NBDG assay, the triglyceride challenge increased the rate of glucose uptake in basal state (Fig. 5.7C), but reduced it in the insulin stimulated state (Fig. 5.8B). These findings correlate with the reduced insulin-stimulated activation of Akt signalling by triglycerides. Triglycerides reduced also the expression level of GLUT4 after 24 hours (Fig. 5.10B), possibly contributing to the reduced glucose uptake seen in the insulin-stimulated state. The fluorescent-based assays showed also that triglycerides treatment increased the glucose uptake in basal state and this correlates with the increased expression of GLUT1 transporter (Fig. 5.10A). The lack of reduction in phospho-Akt levels after triglycerides treatment in the basal state showed that PI3K/Akt signalling pathway is not involved in the increased GLUT1 expression.

The relevance of the increase in GLUT1 expression upon triglyceride treatment is still debated. It was shown for example that GLUT1 translocation at the plasma membrane is higher in the human patients with diabetes and obesity and that the metabolic inflexibility seen during diet-induced obesity is partially dependent on an *increased,* unregulated level of glucose uptake in basal state (Miele et al., 1997). Alternatively, the increase in GLUT1 expression and in the rate of basal glucose uptake in C2C12 cells upon TG treatment might act as a *compensatory* mechanism to counteract the reduced expression of GLUT4 and the impairment of PI3K/Akt-signalling pathway that mediates the translocation of GLUT4 at the plasma membrane upon insulin challenge in order to assure a steady supply level of glucose. For example, an increase in GLUT1 expression was shown already to have a positive, compensatory effect on cardiomyocytes upon the *in vivo* pressure-load-induced hypertrophy (Morissette et al., 2003).

Therefore, the next question to be asked is this:

What are the effects of visfatin on inflammatory signalling pathways?

Chapter 6- THE EFFECTS OF VISFATIN (eNAMPT) ON THE INTRACELLULAR SIGNALLING PATHWAYS IN CULTURED SKELETAL MYOCYTES

6A. Introduction

In the fourth chapter it was shown that exogenous visfatin (eNampt) treatment increased ROS levels in C2C12 cells (Fig. 4.1), but independently of insulin receptor-dependent signalling and of PI3K/Akt-associated pathway (Fig. 4.10-11). One possible explanation for this ROS increase could be the binding of extracellular visfatin (eNampt) to another as yet unidentified receptor. The hypothetical existence of such a membrane receptor was already proposed in the literature (Li et al., 2008), but there are no published reports of attempts to identify it. The screening of such putative visfatin-specific membrane receptor was also beyond the scope of the present thesis, being a subject for future studies. Another hypothesis to explain the increase in ROS levels is based on the possibility of exogenous visfatin (eNampt) transport from extracellular space across plasma membrane and the accumulation in the intracellular compartments of C2C12 cells. An active transport across membrane structures was reported previously for other cytokines like leptin (Bouret, 2008), leukaemia inhibitory factor (Pan et al., 2008) and TNF-α (Pan et al., 2002). It is reasonable to assume that an active transport of exogenous visfatin (eNampt) from extracellular space across plasma membrane will result in an intracellular increase of visfatin expression (as iNampt) in C2C12 cells, assuming the absence of a simultaneous process of proteasomal degradation or additional re-secretion. Nevertheless, since the precise subcellular localization of intracellular form of visfatin is still debated (Kitani et al., 2003), the possible destination of such a transport is also unclear. In the testes of prepubertal chickens the intracellular form of visfatin (iNampt) was localized mainly in the nucleus of Sertoli and Leydig cells, but in the testes of adult chickens the pattern was cytosolic in both cell types (Ocon-Grove et al., 2010). Similarly, visfatin was localized mainly in the cytosol of proliferating PC-12 and 3T3-L1 cells, but in the differentiated state of both cell lines the pattern was nuclear (Kitani et al., 2003). An increase in the expression of intracellular form of visfatin (iNampt) could lead to higher levels of ROS as a consequence of the modulation of cellular processes such as to nicotinamide adenine dinucleotide

(NAD) synthesis (Pilz et al., 2007), independently of its putative binding to a membrane receptor. Nicotinamide mononucleotide (NMN) is synthesized by intracellular form of visfatin (iNampt) from nicotinamide through its phosphoribybosiltransferase activity (Wang T. et al., 2006), and NAD will be synthesized from NMN by the activity of another enzyme, mononucleotide adenylyltransferase (NMNAT1) (Garavaglia et al., 2002). Increased expression of iNampt correlates with higher levels of NAD in cultured fibroblasts (Imai, 2009a) and visfatin heterozygous$^{+/-}$ females mice further presented reduced levels of NAD in pancreatic islets (Revollo et al., 2007). The fact that NMNAT1 is localized also in nucleus (Berger et al., 2005) further suggests the possible existence of different functions for intracellular form of visfatin (iNampt) if localized in cytosol or in nucleus, unknown yet. Nevertheless, an increase in ROS levels as a direct, immediate effect of higher levels of NAD is improbable. A reduction in NAD levels increased the levels of oxidative stress in *C. elegans* (Vrablik et al., 2009) and nicotinamide supplementation prevented mitochondrial dysfunctionalities reducing ROS levels (Jia et al., 2008). Such effect could be nevertheless indirect, such as an inflammatory response. For example it was shown that incubation of smooth muscle cells with exogenous visfatin (eNampt) activated NFkB-signalling through its phosphoribybosiltransferase activity, independently of PI3K/Akt-signalling pathway (Romacho et al., 2009).

It is also likely that intracellular form of visfatin (iNampt) might fulfil other functions besides NMN synthesis which could induce a sustained ROS production, as suggested already (Li et al., 2008). For example, it was shown that overexpression of visfatin gene in primary human endothelial cells increased ROS levels, the process being blocked by the inhibition of mitochondrial NADH oxidase complex I function (Zhang et al., 2008). Furthermore, the silencing of the visfatin/PBEF gene by siRNA was able to reduce ROS levels induced by the incubation of the same type of cells with the cytokine IL-1β. A possible explanation proposed by the authors for this increase in ROS levels was the fact that visfatin was shown to directly interact with other

proteins already proved to be involved in oxidative stress production and inflammation like NADH dehydrogenase subunit 1 (ND1) and ferritin light chain and interferon induced transmembrane 3 (IFITM3). Therefore, the *first* goal of the present chapter was testing whether incubation of C2C12 cells with exogenous visfatin between 1-24 hours was followed by its active transport from extracellular space into the cytosol by using western blot method.

The *second* aspect to be investigated in the present chapter was the possible activation of intracellular signalling pathways (other than PI3K/Akt) that could explain the increase in ROS levels. For example, it was reported that acute exogenous visfatin treatment (<30 min) activated mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1 (ERK1/2) in ventricular cardiomyocytes (Lim et al., 2008) and p38 in HUVECs cells (Liu et al., 2009b). It is already known that MAPKs, also known as stress-activated kinases (SAPKs), are important mediators of oxidative and nitric stress responses (Cao et al., 2004). Interestingly, so far there are no reports about the possible activation of c-Jun terminal kinase (JNK) by visfatin treatment, another member of MAPK family which is critically involved in ROS production (Shen and Liu, 2006), and this possibility will not be tested in the present thesis either. It was previously shown that exogenous visfatin challenge (eNampt) induced the activation of NFkB pathway in the first hour of treatment in endothelial cells (Lee et al., 2009) and after 4 hours in smooth muscle cells (Romacho et al., 2009). It is also well known that an activation of NFkB signalling could induce an increase in ROS production by activating the transcription of some of its pro-inflammatory target genes that subsequently can mediate a chronic and sustained stress response like TNF-α (Munhoz et al., 2004), COX-2 (Munhoz et al., 2008), IL-6 (Paule et al., 2007), IFN-γ (Briscoe et al., 2006) and iNOS (Pinlaor et al., 2008). Recently it was also discovered that MAPKs-induced effects can be independent of transcriptional regulation through the direct phosphorylation of cellular targets, other than regulators of gene expression. For example, it was reported that JNK, another member of MAPK family, can phosphorylate proteins regulating axodendritic growth in

neurons such as microtubule-associated protein 2 (MAP2) (Bjorkblom et al., 2005) and superior cervical ganglion-10 protein (SCG10) (Tararuk et al., 2006), affecting neurogenesis. Therefore, it would be possible also for p38 and ERK to directly phosphorylate proteins involved in antioxidant defence and oxygen metabolism and modify their enzymatic properties, eventually leading to increased levels of ROS. There are also multiple reports about the relationships between visfatin expression or function and the levels of circulating NFkB-regulated cytokines associated with oxidative stress. For example, it was shown that cultured human monocytes treated with exogenous visfatin expressed higher levels of TNF-α (Moschen et al., 2007). Similarly, human monocytes or synovial fibroblasts transfected or stimulated with exogenous visfatin showed increased TNF-α expression values via NFkB and AP-1 signalling (Brentano et al., 2007). In other report, the experimental mice of collagen-induced arthritis presented increased visfatin and TNF-α plasma levels, and the pharmacological inhibition of phosphoribybosiltransferase activity of visfatin reduced circulating TNF-α levels (Busso et al., 2008). Furthermore, decreased visfatin expression values correlated with decreased levels of plasma circulating interferon-γ (IFN-γ) upon caloric restriction in human volunteers (Lee et al., 2010). Also, exogenous visfatin (eNampt) treatment of cultured macrophages increased not only the gene expression but also the secretion values of an important cytokine such as IL-6 in endothelial cells (Kim et al., 2009). To test the possible activation of MAPKs (ERK and p38) and NFkB signalling pathways in C2C12 cells by exogenous visfatin (eNampt), western blot was performed for the phosphorylated forms of MAPKs (p38, ERK ½) and NFkB pathway (p65, IKKα/β and IkBa). Such pathways could be nevertheless activated during chronic stages of incubation with visfatin (eNampt), events that might not be detected by my western blot experiment. Therefore, ERK, p38 and NFkB activations were blocked by using their specific inhibitors prior visfatin challenge, and intracellular ROS levels were measured by fluorescent-based assays, as performed in previous chapters.

6B. Results

6.1 Visfatin (eNampt) was not transported from the extracellular space across plasma membrane into the cytosol of C2C12 cells
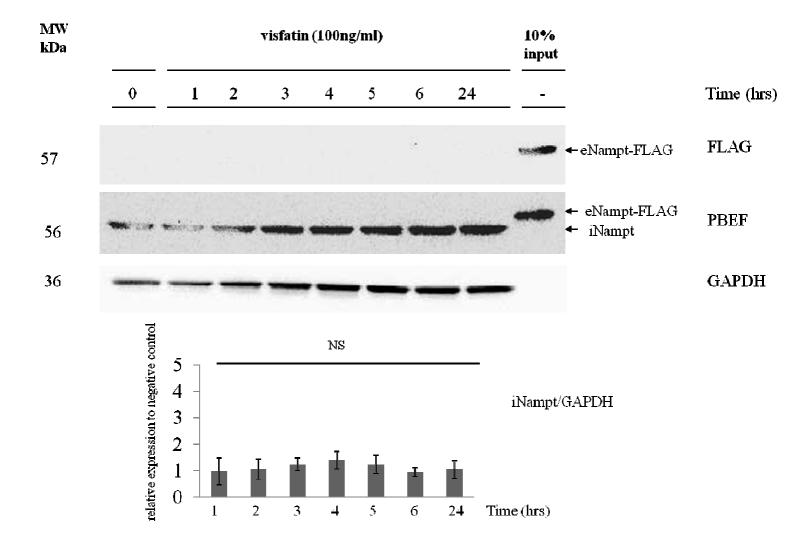
To test the possibility that visfatin was transported across the plasma membrane, C2C12 cells were incubated with 100 ng/ml visfatin for an interval between 1-24 hours followed by western blot assay by using cell lysates. Since the commercially available visfatin chosen for this particular experiment had a FLAG-tag at the N-terminus, two different antibodies have been used to perform western blot. The first antibody was raised against the tag, and the second was raised against the visfatin, recognizing both intracellular (iNampt) and extracellular (eNampt) forms. As positive control 10 ng of tagged exogenous visfatin diluted in PBS was used (10% of the input). This value reflects approximately the same concentration which should have been detected in the amount of protein lysates loaded in each well (60 ng) if the membrane transport efficiency for exogenous visfatin had been at maximum levels (100%), assuming the absence of a simultaneous proteasomal degradation process.

As seen from figure 6.1, when the antibody specific for FLAG-tag has been used no band was detected in cell lysates from the samples treated with visfatin for 1-24 hrs. Moreover, the western blot using the antibody specific for visfatin did not detect either any extra band of the size predicted for the exogenously added extracellular form (eNampt) which, due to the attached tag, is approximately 1 kDa bigger than the intracellular form (iNampt) endogenously expressed by C2C12 myotubes and also recognized by the antibody. Had exogenous visfatin (eNampt) been transported from the culture medium into the cytosol of C2C12 cells two distinct bands should have been detected by using the second antibody: one for the intracellular form of visfatin expressed endogenously by C2C12 cells (iNampt) of 56 kDa, and another corresponding to the extracellular version (eNampt-FLAG) transported across plasma membrane of cca. 57 kDa. Therefore, this blotting experiment shows that visfatin is not transported from the extracellular space into the cytosol of C2C12 cells. This allows

the speculation that the effects of visfatin on myocytes cellular stress responses are likely the consequences of its binding to a membrane-bound receptor, other than insulin receptor. Once bound to this putative receptor visfatin (eNampt) could activate/repress different intracellular signalling pathways other than PI3K/Akt, leading to increased levels of ROS through still unknown mechanisms. The identity of this putative receptor is still unexplored in the literature, its existence only theoretically proposed (Li et al., 2008).

Fig. 6.1 Visfatin is not transported from the extracellular space into the cytosol of C2C12 myocytes

C2C12 differentiated myocytes were incubated with visfatin (100ng/ml) diluted in serum-free medium for 1-24 hrs and 10% of the initial input has been used (10ng) as positive control. Protein lysates were resolved by a typical western blot and probed by using the antibody raised against the FLAG-tag (above) or visfatin (middle). As a control an antibody against GAPDH was used (below). Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between treatment and control.

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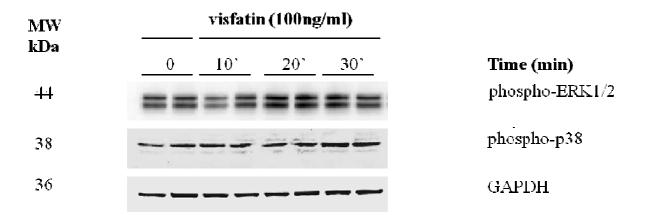
6.2 Acute visfatin (eNampt) treatment did not activate MAPKs such as p38 and ERK

Previously it was reported that exogenous visfatin challenge can modulate intracellular signalling pathways by increasing the phosphorylated levels of mitogen-activated kinases (MAPKs) like ERK and p38 in the first 30 minutes of treatment, in an Akt-dependent manner (Adya et al., 2008a; Kim et al., 2008; Lim et al., 2008). It is already known that activation of MAPKs such as p38 (Cao et al., 2004) and ERK (Ding et al., 2007) can further mediate an increased production of oxygen free radicals. As seen in figure 6.2, western blot experiments showed that visfatin treatment did not induce any increase in the phosphorylated forms of p38 and ERK in the first 30 minutes of incubation. Since there were no previous reports of JNK activation by exogenous visfatin (eNampt), this possibility was not explored in the present thesis either.

Nevertheless, MAPKs could be activated in the later, chronic stages of visfatin treatment independently of Akt-signalling pathway, leading potentially to higher levels of ROS (Perry et al., 1999). Therefore, C2C12 cells were pre-incubated for 1 hour with the inhibitor U0126 that blocks MEK 1/2 activation (ERK 1/2 upstream activators) or with *InSolution^{TM}* p38 MAP kinase inhibitor III that blocks p38α activity, followed by exogenous visfatin treatment (100 ng/ml) for another 18 hours. The measurement of free radicals by using carboxy-H2DCFDA-based assays showed no differences in ROS levels between the samples pre-treated with MAPKs inhibitors followed by visfatin challenge compared to the signal detected from visfatin-only treated cells (Fig. 6.3). This proved that the oxidative stress induced by exogenous visfatin is independent of p38 and ERK signalling pathways.

Fig. 6.2   Visfatin is not activating the MAPKs (p38 and ERK 1/2) in the first 30 minutes of incubation

C2C12 differentiated myocytes were incubated with visfatin (100ng/ml) diluted in serum-free medium for the indicated times (0, 10, 20 and 30 minutes). Protein lysates were resolved by a typical western blot and probed by using the antibodies raised against the phosphorylated forms of p38 (above) or ERK ½ (middle). As a control an antibody against GAPDH was used (below). Quantization of phospho-p38 and phopsho-ERK1/2 levels, compared to the negative control was done by densitometric analysis. Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between visfatin treatment and control.
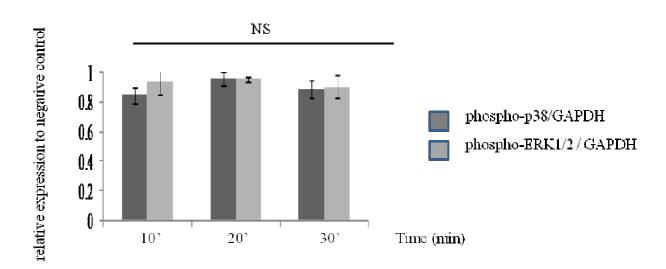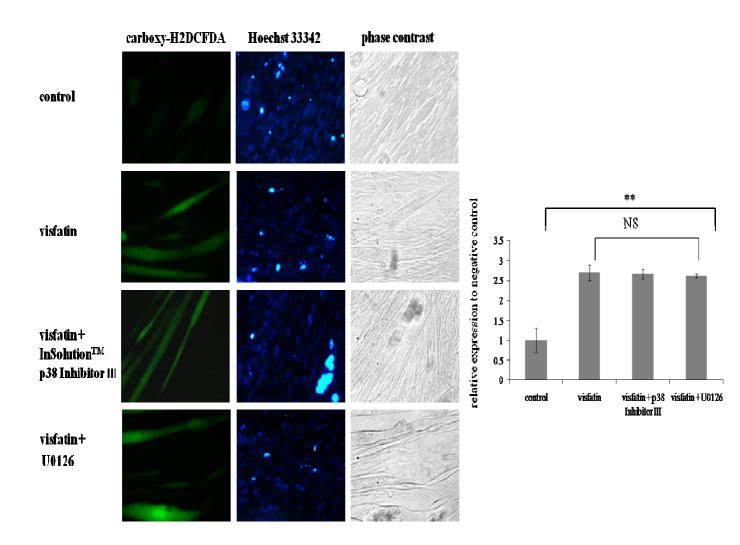
Fig. 6.3   *The oxidative stress induced by visfatin is independent of MAPKs signalling pathway*

Differentiated C2C12 cells were pre-treated for 1 hr with 10 μM U0126, an inhibitor of ERK activation, or with 10 μg/ml *InSolution^{TM}* p38 MAP kinase inhibitor III that blocks specifically p38 activation, before being subjected to another 18 hrs of challenge with 100 ng/ml of visfatin. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/− SEM. **Denotes $p<0.01$ compared to control. NS denotes no significant difference between inhibitors followed by visfatin treatment compared to visfatin alone.

6.3   Chronic visfatin (eNampt) treatment activated NFkB signalling

Previously it was reported that exogenous visfatin treatment activated NFkB-signalling after 1 hour of incubation of cultured synovial fibroblasts (Brentano et al., 2007) and after 4 hours in smooth muscle cells (Romacho et al., 2009). Such an activation of NFkB-signalling could induce an increase in the expression of many of its targets that could act as pro-inflammatory mediators, increasing further the production of ROS. For example, visfatin challenge increased in non-skeletal muscle cells the expression levels of pro-inflammatory mediators such as iNOS (Romacho et al., 2009), eNOS (Lovren et al., 2009) and TNF-α (Luk et al., 2008; Moschen et al., 2007), genes that are associated with sustained nitric and oxidative stress responses. The quantification by western blot and densitometric analysis of phosphorylated levels of NFkB (p65) showed a significant increase in its activation after 4 hours of incubation of C2C12 cells with extracellular visfatin (100 ng/ml) (Fig. 6.4). The activation of NFkB pathway was also confirmed by the increased phosphorylation level of the upstream activator of p65 subunit (IKK α/β) and of IkBα, the p65 inhibitor that once phosphorylated by IKK α/β is degraded by proteasome. The activation of NFkB did not occur in the acute stage of incubation with visfatin (within the first hour) as previously reported in synovial fibroblasts, macrophages or endothelial cells, but in the chronic stage (cca. 4-5 hours), approximately at the same time point when increased ROS production was detected. Because it was previously reported that NFkB signalling could increase the expression levels of many cytokines that can further mediate an additional oxidative stress response (Kanitkar et al., 2008), C2C12 cells were pre-incubated for 1 hour with 50 μg/ml IκB Kinase Inhibitor Peptide, a specific blocker of IKK activation, followed by the treatment with exogenous visfatin for an additional 18 hours. As seen from figure 6.5, a significant reduction in the levels of ROS produced (cca. 30%) was noticed by a fluorescent-based assay in C2C12 cells pre-treated with the IKK inhibitor followed by visfatin challenge compared to cells treated only with visfatin. This showed that the oxidative stress induced by visfatin is partially mediated by NFkB-dependent signalling.

Fig. 6.4 Visfatin is activating the NFkB signalling pathway after 4 hrs of incubation

C2C12 differentiated myocytes were incubated with visfatin (100ng/ml) diluted in serum-free medium for the indicated times (0-6 hrs). Protein lysates were resolved by a typical western blot and probed by using the antibody raised against the phosphorylated form of p65 subunit of NFkB (above), the phosphorylated forms of IKKα/β (middle) and IkBa (below). As a control an antibody against GAPDH was used (bottom). Quantization of p65 phosphorylation levels compared to negative control was done by densitometric analysis. Each data point is the average from three independent experiments +/− SEM. NS denotes no significant difference between visfatin and control. **Denotes p<0.01 compared to control.
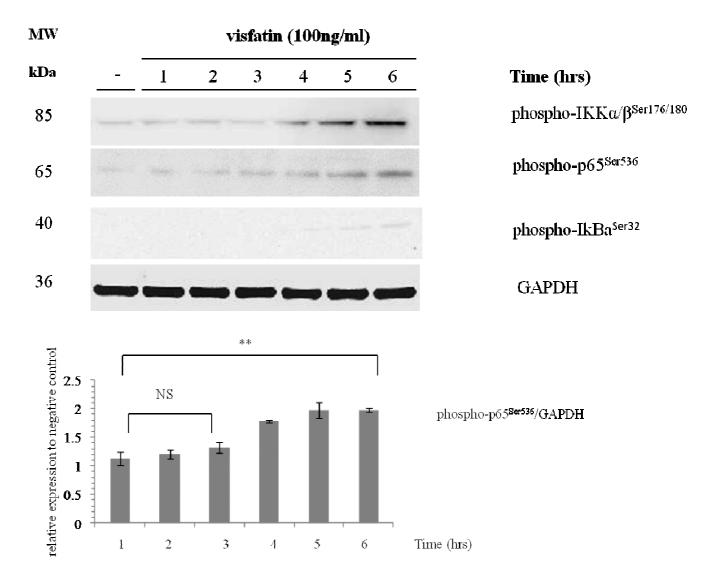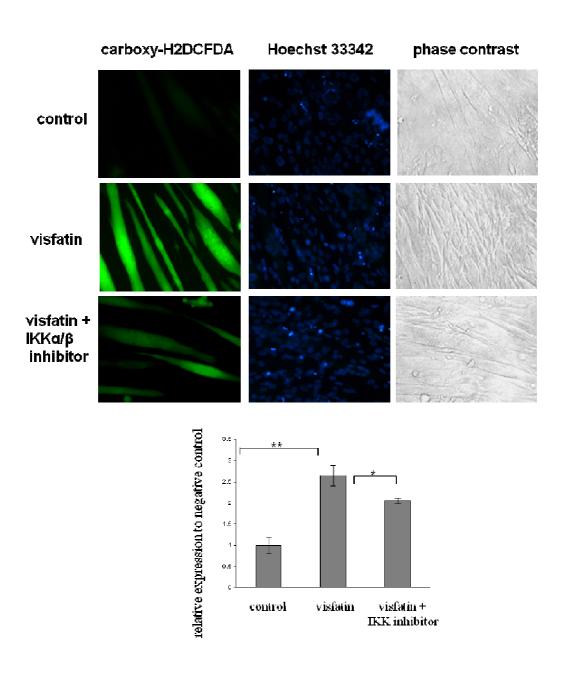
Fig. 6.5   Blocking the activation of the NFkB signalling pathway is reducing the oxidative stress levels induced by visfatin with cca. 40%

Differentiated C2C12 cells were pre-treated for 1 hr with 50 μg/ml IκB Kinase Inhibitor Peptide, an inhibitor of NFkB activation, before being subjected to another 18 hrs of challenge with 100 ng/ml of visfatin. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/− SEM. **Denotes p<0.01 compared to visfatin. **Denotes p<0.01 compared to control.
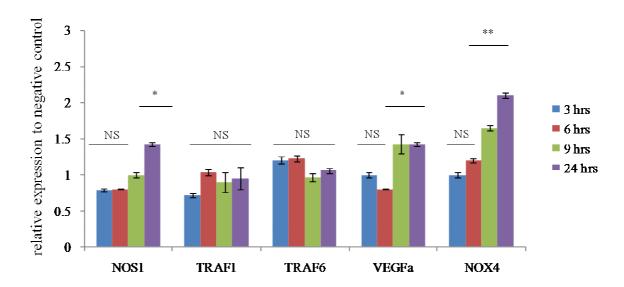
6.4 Activation of NFkB by visfatin (eNampt) treatment changed the expression level of many of its regulated genes involved in oxidative stress production or antioxidant defence

NFkB signalling regulates the transcription levels of many genes that were already reported to be positively or negatively associated with oxidative stress like TNF-α, IFN-γ, IL-6, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and VEGFa (Bowie and O'Neill, 2000). To test the possibility that visfatin incubation of C2C12 cells could change their expression levels an RT-PCR assay was performed by using different time points (0-24 hours). As seen in figure 6.6A, NFkB target genes known to be involved in the regulation of inflammatory signals such as TNF receptor-associated factor 1 (TRAF1) (Lee and Choi, 2007) and TNF receptor-associated factor 6 (TRAF6) (Wu and Arron, 2003) were not changed by visfatin treatment. VEGFa, a NFkB target gene proved to be upregulated upon visfatin treatment in non-skeletal muscle cells (Adya et al., 2008), showed only a moderate increased expression. Visfatin treatment further moderately increased the expression of nitric oxide synthase 1 (NOS1), named also the neuronal nitric oxide synthase (nNOS), enzyme involved in nitric oxide synthesis (Keser et al., 2011). Visfatin challenge increased also the level of expression of IL-6 and IL-15 (Fig. 6.6B) after 24 hours of treatment, myokines regulated by NFkB that have a major role in skeletal muscle energy metabolism and stress responses (Chan et al., 2004). Exogenous visfatin challenge of C2C12 cells surprisingly decreased expression levels of other cytokines such as TNF-α and IFN-γ, previously reported to mediate a stress response (Kim et al., 2005). In the previous chapter it was shown a significant increase in NOX4 expression levels after 24 hours of visfatin treatment (Fig. 5.6). Previously it was discovered that NOX4 gene expression is regulated by NFkB pathway (Manea et al., 2010). Therefore, the activation of IKK pathway was blocked by using the same inhibitor as before prior visfatin treatment for 24 hrs and the expression level of NOX4 was verified by RT-PCR. As seen from figure 6.7, the inhibition of NFkB activation reduced the expression level of NOX4 induced by visfatin by almost 50%. This shows that NOX4 expression is only partially regulated by NFkB.

Fig. 6.6 Visfatin treatment changes the expression levels of some of NFkB-regulated target genes

C2C12 differentiated myocytes were incubated with visfatin (100ng/ml) diluted in serum-free medium for 0-24 hrs. After RNA extraction and cDNA synthesis RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin) and then comparing to gene expression in untreated cells. ***Denotes $p<0.001$ compared to control. **Denotes $p<0.01$ compared to control. *Denotes $p<0.05$ compared to control. NS denotes no significant difference between visfatin and control.

A.

B.
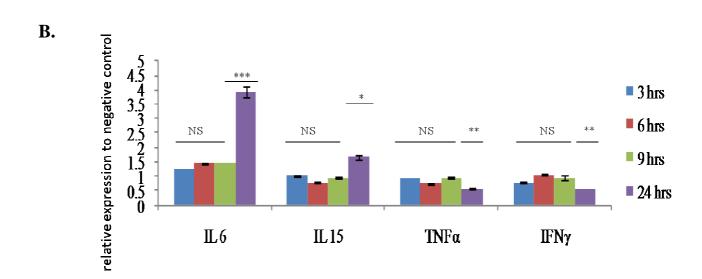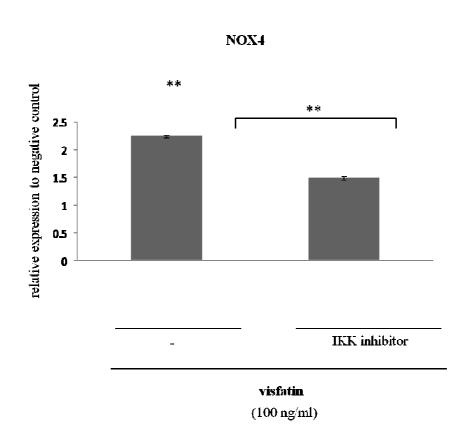
Fig. 6.7 Blocking NFkB-signalling pathway reduces in half the expression level of NOX4 upregulated by visfatin challenge

Differentiated C2C12 cells were pre-treated for 1 hr with 50 μg/ml IκB Kinase Inhibitor Peptide (an inhibitor of NFkB activation), before being subjected to another 24 hrs challenge with 100 ng/ml of visfatin. After RNA extraction and cDNA synthesis RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin) and then comparing to gene expression in untreated cells. **Denotes $p<0.01$ compared to control. **Denotes $p<0.01$ compared to visfatin.
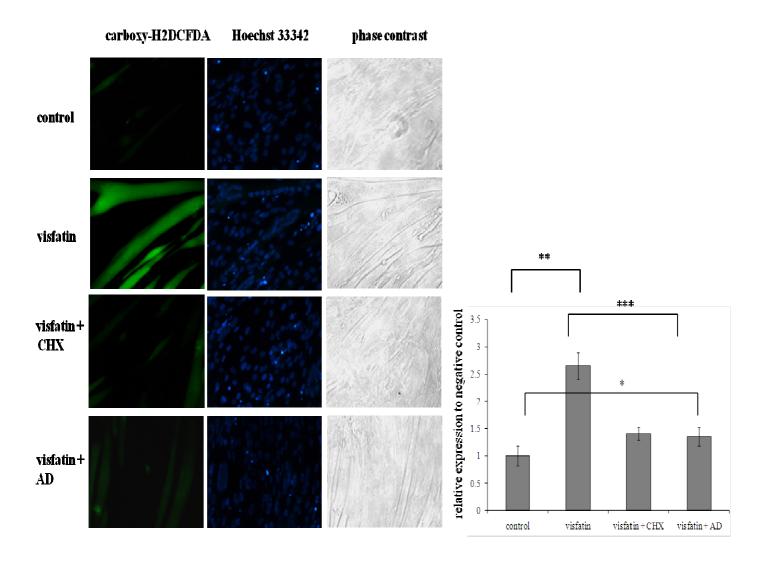
6.5 Blocking transcription and translation reduced the ROS levels increased by visfatin (eNampt)

Because the inhibition of NFkB activation reduced ROS levels increased by visfatin the logical conclusion was that ROS production is dependent on *de novo* synthesis of a new genomic profile. To investigate this possibility transcription or translation were blocked by using their specific inhibitors (Actinomycin D and Cycloheximide, respectively) for 1 hour prior the treatment with exogenous visfatin for another 18 hours, and fluorescent-based assays to measure ROS levels were repeated as described before. As seen from figure 6.8, a reduction of almost 70% in the levels of ROS was noticed in cells pre-treated with inhibitors followed by visfatin challenge compared to the cells treated only with visfatin. This suggests that there are other as yet unidentified pathways besides NFkB signalling that are mediating the stress response. The identity of these signalling pathways is a question unanswered yet, being a subject for future studies that will not be covered by the present thesis.

Fig. 6.8   ROS induced by visfatin is dependent on the de novo transcription of a new set of genes

Differentiated C2C12 cells were pre-treated for 1 hr with 1 µg/ml of Actinomycin D (AD), an inhibitor of transcription, or with 0.1 µM of Cycloheximide Ready-Made Solution (CHX), an inhibitor of translation, before being subjected to another 18 hrs of challenge with 100 ng/ml of visfatin. Cells were incubated with 25 µM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 µM Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/− SEM. ***Denotes $p<0.001$ compared to visfatin. **Denotes $p<0.01$ compared to control. *Denotes $p<0.05$ compared to control.

6C. Discussion

1. Visfatin (eNampt) *transport* hypothesis vs. visfatin (eNampt) *receptor-binding* hypothesis

The fact that circulating pro-inflammatory cytokines such as leptin, TNF-α, IL-1α or LIF are actively transported across membrane structures such as blood-brain barrier is already known (Bouret, 2008;Pan et al., 2008;Pan et al., 2002), but the transporters and the regulatory mechanisms involved are still elusive. An active transport of exogenous visfatin (eNampt) from extracellular space into the cytosol of C2C12 cells could result in an intracellular accumulation as iNampt. Nevertheless, the possible destination compartment for such a transport is not clear because the subcellular localization of intracellular form of visfatin is diverse, as detailed in introduction. A higher expression of visfatin as iNampt in the intracellular compartments of C2C12 cells could increase ROS levels, as it was shown already in endothelial cells (Zhang et al., 2008). There were no previous reports about the possible transport of visfatin (eNampt) from extracellular space across the plasma membrane into the cytosol of any mammalian cells. Therefore, this hypothesis was tested in C2C12 cells by using the western blot method (Fig.6.1). In conclusion, the experiments showed that there was no proof of a transport of exogenous visfatin (eNampt) into the cytosol of C2C12 cells in the first 24 hours of incubation. The western blot experiment using the antibody raised against FLAG-tag did not detect any band in the cell lysates taken from the samples treated with exogenous visfatin (eNampt). Furthermore, the western blot experiment using the antibody raised against visfatin did not detect any extra band corresponding to the size of the exogenous form that is almost 1kDa bigger in size than the intracellular form of visfatin (56 kDa), due to the attached tag. The incubation of C2C12 cells with exogenous visfatin (eNampt) did not change further the protein levels of endogenous, intracellular form of visfatin (iNampt), suggesting further no change in intracellular NAD levels. Since in the fourth chapter it was shown that visfatin treatment did not activate the insulin receptor-dependent PI3K/Akt signalling (Fig.4.10), it is likely that the stress responses induced by visfatin are generated

by the binding to an unknown membrane receptor. This putative receptor could induce an increase in the intracellular ROS levels only in the tissues or cells where it is expressed or functional by activating/repressing yet to be defined downstream intracellular signalling pathways. The same speculative conclusion about the possibility of a visfatin-specific receptor, other than insulin receptor, was reached by another independent report that also failed to prove an insulin mimetic property for extracellular form of visfatin (Li et al., 2008). One possible candidate for such a putative receptor for visfatin could be for example the insulin-like growth factor receptor (IGF-IR) which once activated can increase the levels of glucose uptake (Shefi-Friedman et al., 2001). Previously it was shown that in HUVECs cells exogenous visfatin challenge induced the activation of Akt/PI3K-signalling pathway, similarly to insulin. Since it was also proved that in HUVECs cells IGF-IR is expressed at higher values than insulin receptor (Nitert et al., 2005), it is possible therefore that visfatin insulin-mimetic effects on HUVECs are mediated in fact by IGF-IR, not by insulin receptor. This issue is nevertheless a subject for future studies.

2. Visfatin (eNampt) treatment and signalling pathways activation

Visfatin did not activate MAPKs such as ERK and p38 in the acute stage of treatment, as shown before in non-muscle cells (Lim et al., 2008; Adya et al., 2008; Kim et al., 2007). The lack of activation of MAPKs like p38 and ERK in C2C12 cells in the acute phase of treatment correlates with previous findings that this activation by visfatin is dependent on the upstream Akt-signalling (Lovren et al., 2009) event undetected in my case as shown in chapter 4 (Fig. 4.10). It is still possible nevertheless that such MAPKs activations might occur later, but since blocking of MAPKs had no effect on ROS levels induced by visfatin this issue was not pursued further. Also, it is possible that visfatin could activate JNK, the other member of MAPK family, but this is a subject for future studies.

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Visfatin activation of NFkB in the first 6 hours of treatment opened the possibility that it could mediate the ROS production induced. The blocking of p65 activation showed a significant reduction in ROS levels upon subsequent visfatin challenge. The IC50 of NFkB inhibitor is 10 μM for the LPS-induced IkB degradation by IkB kinases (IKK) in RAW 264.7 cells. NFkB is an important mediator of cellular responses to oxidative stress and inflammation (Schmid et al., 2006). Nevertheless, I did not notice by RT-PCR an increase in NFkB-target genes such as TNF-α and IFN-γ, proved before to mediate a chronic inflammation and oxidative stress. The blocking of transcription and translation reduced the ROS levels by almost 70%, showing that there other pathways besides NFkB mediating such increase. The potential role of NOX4 in visfatin-induced effects deserves additional studies since blocking of NFkB reduced the increase in NOX4 expression triggered by visfatin challenge for 24 hrs.

Chapter 7 – THE RELATIONSHIPS BETWEEN EXOGENOUS VISFATIN (eNAMPT) AND PPARs IN C2C12 CELLS

7A. Introduction

As discussed in the third chapter, the experimental animal models of skeletal muscle accelerated ageing (*hindlimb suspension*) and of skeletal muscle delayed ageing (*caloric restriction*) presented significant changes in expression profiles of nuclear receptors families, such as PPARs and NR4As. During ageing it was noticed a progressive modification of adipose tissue mass and physiology accompanied by different expression and secretion profiles of adipokines (Esposito and Giugliano, 2004; Scrivo et al., 2010). Adipokines have a major *endocrine* impact on other peripheral tissues by modulating gene networks regulating insulin sensitivity, antioxidant defence and inflammation (Esposito and Giugliano, 2004). The known experimental models of ageing mentioned above are characterized by changes in expression or function of adipokines (Crisostomo et al., 2010). For example, caloric restriction reduced not only the body weight and adipose tissue mass but also plasma levels of circulating visfatin in obese women subjects (Lee et al., 2010). In addition, it is established that caloric restriction has anti-ageing effects (Niemann et al., 2010). These effects were shown to be partially dependent on the activation of sirtuin family which use as a substrate NAD previously synthesized from NMN generated by intracellular form of visfatin (iNampt) (Picard and Guarente, 2005), as discussed in detail in previous chapters. This conclusion was further reinforced by the discovery that transgenic mice models of *sirtuin1* gene presented a phenotype similar to the experimental animals subjected to caloric restriction with a delayed ageing process (Bordone et al., 2007).

The complex connections between expression levels of adipokines and nuclear receptor activation in an ageing context have been unravelled recently. For example, incubation of primary human trophoblast cells with adiponectin increased the expression of PPAR-α (Jones et al., 2010), and the same phenotype was shown by the treatment of C2C12 cells with leptin (Bendinelli et al., 2005). Therefore, any changes in adipokines expression profiles, already certified to occur during progression of ageing and ageing-associated diseases (Esposito and Giugliano, 2004), could

have a significant impact on expression and function of skeletal muscle specific-PPARs. As such, a change in plasma visfatin levels during ageing could indirectly influence the outcome of a clinical therapy employing PPARs agonists/antagonists to manage the ageing-associated diseases affecting skeletal muscle. There are no reports published so far about the activation or repression of skeletal muscle-specific PPARs-associated signalling pathways or changes in expression of skeletal muscle-specific PPARs by exogenous form of visfatin (eNampt). It is known nevertheless that visfatin treatment (eNampt) increased the expression levels of PPAR-γ in cultured preadipocytes, affecting adipogenesis (Yang et al., 2010). The *first* goal of the present chapter was to test whether the exposure of differentiated C2C12 myotubes to exogenous visfatin (eNampt) could change the expression levels of PPARs by using an RT-PCR assay.

As discussed in the third chapter, PPAR-β/δ could be an important target in treating the metabolic dysfunctions in aged skeletal muscle. I further showed in the third chapter that expression of PPAR-β/δ was increased in acute phase of mice hindlimb suspension, possibly mediating a transient *metabolic adaptation* to the stress incurred. Significant progress has been recorded recently also in the development of specific and effective PPAR-β/δ agonists and antagonists. **GW-501516** is a selective agonist for PPAR-β/δ that is currently employed in clinical trials at different stages to treat different pathological conditions such as dyslipidaemia (clinical trial NCT00158899), heart failure (clinical trial NCT00318617), obesity (clinical trial NCT00388180) and cardiovascular diseases (clinical trial NCT00841217) (Clinical Trials-NIH, 2011). GW-501516 was used also successfully in reducing the phenotype of experimental animal models of brain inflammation and neurodegeneration (Defaux et al., 2009). At the cellular level, the treatment of myocytes with GW-501516 was shown to increase the rates of fatty acid oxidation through the upregulation of relevant genes (Sprecher, 2007) and changed the expression profile of genes involved in energy production and antioxidant defence (Dressel et al., 2003). As a consequence, GW-501516 treatment changed in skeletal muscle the fuel preference from glucose to

lipids (Brunmair et al., 2006) and improved overall the metabolic syndrome phenotype (Tanaka et al., 2003), although it did not change directly the rate of glucose uptake (Dimopoulos et al., 2007). This PPAR-β/δ agonist could be also used to target the underlying inflammatory mechanisms in skeletal muscle because it was already shown that it blocks the activation of NFkB pathway by palmitate in cultured myocytes (Coll et al., 2010). In conclusion, the *second* goal of the present chapter was to test whether the activation of PPAR-β/δ-dependent signalling by using the agonist GW-501516 can change oxidative stress levels and inflammatory signalling pathways induced by exogenous form of visfatin (eNampt). To test this possibility multiple fluorescent-based assays, western blot and RT-PCR methods were used.

The usefulness of PPAR agonists in modulating the expression, activity or secretion levels of adipokines in multiple tissues of possible clinical interest for ageing management has already been proven. For example, activation of PPAR-γ by the agonist BRL 49653 reduced leptin levels in cultured adipocytes (De Vos et al., 1996). In addition, it was shown that PPARs-associate signalling pathways mediate the regulation of adiponectin expression levels induced by diet-derived fatty acids (Swarbrick and Havel, 2008). There are also multiple reports detailing the effects of PPARs activation or repression on visfatin expression or activity. For example, it was shown that administration of fenofibrate (a PPAR-α agonist) or rosiglitazone (a PPAR-γ agonist) increased visfatin gene expression in adipose depots of obese diabetic rats (Choi et al., 2005). In addition, PPAR-γ agonists such as GW1929 increased expression of visfatin in human, although not in murine macrophages (Mayi et al., 2010). Pioglitazone, another PPAR-γ agonist, decreased nevertheless the expression of visfatin in visceral adipose tissue of obese rats (Lv et al., 2009). The murine hepatic expression of visfatin was also decreased by administration of WY-14643, a PPAR-α agonist (Dahl et al., 2010). Furthemore, the effect was cancelled in a PPAR-α null mice model, decrease which was proved to be important in the context of nonalcoholic fatty liver disease. The administration of L-165041, a PPAR-β/δ agonist, increased further visfatin

expression in adipose tissue from obese rats fed with a high-fat diet and in cultured 3T3-L1 adipocytes (Choi et al., 2005). These reports suggest that there are tissue-specific mechanisms regulating visfatin expression by PPARs, unknown yet. Nevertheless, there are no reports published so far about the effects of PPARs agonists on skeletal muscle expression or function of intracellular form of visfatin (iNampt).

PPAR agonists can change not only the intracellular expression levels but also secretion levels of circulating visfatin from different cellular types such as adipocytes and macrophages. For example, *in vitro* studies showed that incubation of human adipocytes or HUVEC cells with PPAR-γ agonists such as rosiglitazone, telmisartan or valsartan increased the extracellular secretion of visfatin as eNampt (Storka et al., 2008). The administration of GW1929, another PPAR-γ agonist, was also able to increase the expression and secretion of visfatin in murine macrophages (Mayi et al., 2010). In another study the administration of PPAR-γ agonist pioglitazone nevertheless reduced the circulating levels of visfatin in obese rats, not only the intracellular expression in adipocytes (Lv et al., 2009), and the administration of fenofibrate did not change the circulating levels of visfatin in human subjects with type 2 diabetes (Pfutzner et al., 2008). Furthermore, it appears that in some tissues or physiological conditions PPARs and visfatin belong to similar functional signalling networks since the analysis of their expression values shows similar statistical patterns upon genetic-/pharmacological-based interventions. For example, treatment of bone marrow stromal cells (BMSCs) with tigogenin, a compound that inhibits adipocytic differentiation, reduced simultaneously the expression levels of PPAR-γ and the secretion level of visfatin (Zhou et al., 2007). The same conclusion was reached by the treatment of adipocyte 3T3-L1 cell line with LPS that impaired adipogenesis by reducing expression levels of both PPAR-γ and visfatin (Poulain-Godefroy and Froguel, 2007). Whether visfatin is also a genuine *myokine*, as it was theoretically proposed based on its high level of expression in skeletal muscle (Krzysik-Walker et al., 2008), is an issue still highly debated. The recent discovery that

culture medium of rat L6 myocytes contains significant levels of visfatin is a pioneer discovery (Wang et al., 2010), but the result was not reproduced independently. Since PPARs agonists are effective in the treatment of insulin resistance and metabolic syndrome affecting skeletal muscle, and given the fact that exogenous visfatin (eNampt) induces oxidative stress and inflammation in cultured myotubes, it is therefore important to establish first of all whether visfatin is a genuine myokine in the *basal*, unstimulated conditions. Also, it is important to test whether such a possible secretion event of visfatin from skeletal muscle is changed by the activation of PPAR-β/δ signalling upon *agonist* treatment. Therefore, the *third* goal of the present chapter was to explore whether visfatin can be detected in the culture medium of differentiated and fused C2C12 myotubes and whether the incubation with GW-501516 can induce a change in such possible secreted values. To do this an ELISA assay was employed. Since the role of intracellular form of visfatin (iNampt) in regulating oxidative stress is not a particular focus issue of the present thesis, the possibility that GW-501516 can change only the intracellular expression of visfatin (iNampt) in C2C12 cells without a subsequent secretion was not tested, which is a subject for future studies.

NR4A family of nuclear receptors is also an attractive target for the treatment of insulin resistance in skeletal muscle (Fu et al., 2007) as discussed in the third chapter, but so far there are no reports about any connection between NR4As expression and visfatin. In addition, there are no specific reliable agonists or antagonists developed yet targeting NR4As activity (Myers et al., 2009). Therefore, in the absence of a specific NR4As agonist or antagonist, the issue of possible connections between NR4As and visfatin was not explored in the present study, being a subject for future projects.
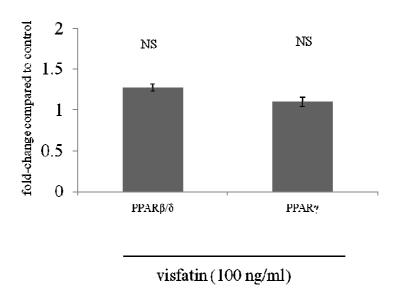
7B. Results

7.1 Visfatin **did not change significantly the expression levels of PPARs in C2C12 cells**

To test whether the incubation of C2C12 cells with 100 ng/ml of exogenous visfatin (eNampt) for 24 hours changed the expression levels of PPAR family, an RT-PCR assay was performed. As seen from figure 7.1, no significant change was noticed in expression levels of PPAR-β/δ and PPAR-γ upon visfatin challenge for 24 hours. PPAR-α expression level in differentiated and fused C2C12 myotubes was too low to be detected by RT-PCR in the cDNA range used for the detection of the other two PPARs, and the identification of PPAR-α level was not further approached through western blot. This showed that unlike other adipokines that can modify PPARs expression levels in skeletal muscle (Jones et al., 2010), exogenous visfatin had no significant effect in this aspect.

Fig. 7.1   Visfatin did not change significantly the gene expression levels of PPARs in C2C12 cells

C2C12 differentiated myocytes were incubated with visfatin (100ng/ml) diluted in serum-free DMEM medium for 24 hrs. After RNA extraction and cDNA synthesis, RT-PCR was performed. All samples were run in triplicate and fold change in gene expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin), and then comparing the values to the gene expression in untreated cells. NS denotes no significant difference between visfatin and control. Each data point is the average of triplicate samples per experiment from three independent experiments +/− SEM.
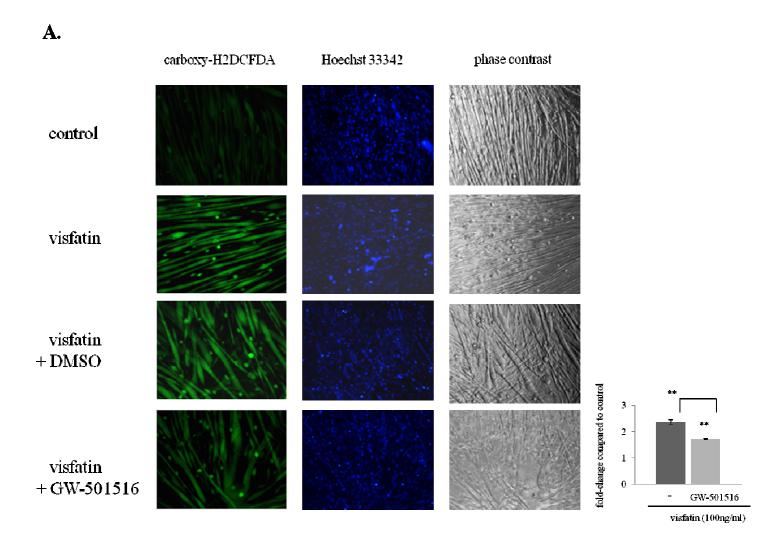
7.2. Administration of GW-501516, a PPAR-β/δ agonist, reduced the oxidative stress induced by visfatin in C2C12 cells

To explore the possibility that activation of PPAR-β/δ-dependent signalling can modify ROS levels induced by exogenous visfatin treatment, C2C12 cells have been incubated for 24 hours with its specific agonist GW-501516 (10nM) followed by the treatment with 100 ng/ml of exogenous visfatin (eNampt) for another 18 hours. The fluorescent microscopy experiment showed that the oxidative stress induced by exogenous visfatin was reduced almost in half in C2C12 cells pre-treated with GW-501516, compared to the cells treated only with visfatin (Fig. 7.2A). This showed that PPAR-β/δ activation reduces the oxidative stress levels induced by visfatin. Nevertheless, as seen in Fig. 7.2B, although the treatment of C2C12 cells with GW-501516 for 24 hours had no significant effect on NOX4 expression levels, it did nevertheless reduce with cca. 30% the increase in NOX4 expression induced by visfatin challenge after 24 hours.
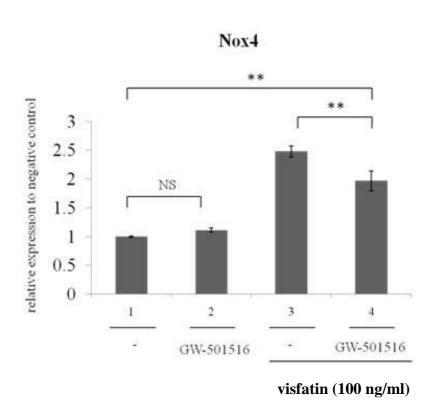
Fig. 7. 2   Administration of GW-501516, a PPAR-β/δ agonist, reduced the oxidative stress induced by visfatin in C2C12 cells

A. Differentiated C2C12 cells were pre-treated or not for 24 hrs with GW-501516 (10nm), being later subjected to another 18 hrs of challenge with 100 ng/ml of visfatin diluted in serum-free DMEM medium. Cells were incubated with 25 μM carboxy-H2DCFDA for 25 min to detect ROS, followed by 5 min incubation with 1 μM Hoechst 33342 to stain the nuclei. Relative expression compared to control is shown. Each data point is the average of quadruplicate samples per experiment from three independent experiments +/− SEM. **Denotes p<0.01 compared to visfatin. **Denotes p<0.01 compared to control.

A.

B. C2C12 differentiated myocytes were pre-treated or not for 24 hrs with GW-501516 (10nm), followed by the incubation with visfatin (100ng/ml) diluted in serum-free DMEM medium for another 24 hrs. After RNA extraction and cDNA synthesis, RT-PCR was performed. All samples were run in triplicate and fold change in expression was calculated by normalizing the test gene crossing threshold (C_t) with the control (beta-actin), and then comparing to the gene expression in untreated cells. NS denotes no significant difference between control and agonist alone. **Denotes $p<0.01$ agonist plus visfatin compared to visfatin alone. **Denotes $p<0.01$ visfatin plus or minus agonist compared to negative control. Each data point is the average of triplicate samples per experiment from three independent experiments $+/-$ SEM.

B.
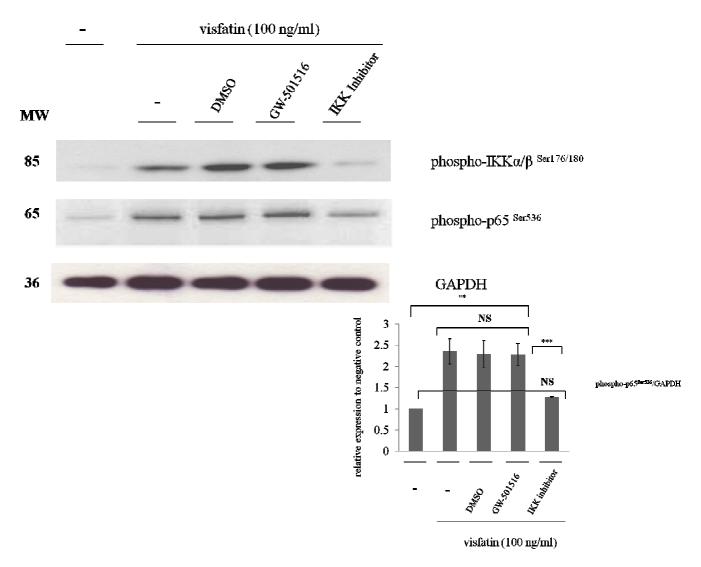
visfatin (100 ng/ml)

7.3 Administration of PPAR-β/δ agonist failed to reduce the activation of NFkB activation by visfatin

As shown in a previous chapter, visfatin treatment increased the phosphorylated levels of NFkB (p65 subunit) after a minimum incubation time of 4 hours. To test whether the PPAR-β/δ agonist can reduce this activation, C2C12 cells have been incubated for 24 hours with its specific agonist GW-501516 (10nM) followed by the treatment with 100 ng/ml of exogenous visfatin (eNampt) for another 6 hours. The EC_{50} of this agonist is 1.1nM and has a specificity of almost 1000 times compared to the other two PPAR isoforms. To quantify activation of NFkB-pathway a western blot for the phosphorylated form was performed. As shown by the Fig. 7.3, C2C12 cells pre-treated with the agonist GW-501516 before exogenous visfatin challenge for 6 hours showed no difference in phospho-p65^{Ser536} and phospho-IKKα/β$^{Ser176/180}$ levels compared to cells treated only with visfatin. This showed that the reduction in ROS levels by GW-501516 is not mediated by an inhibition of NFkB-dependent signalling pathway and that the increase in antioxidant defence by agonist treatment did not affect the activation of NFkB. In addition, the blocking of NFkB activation by its specific inhibitor reduced almost completely the increase in phosphorylated p65 values induced by visfatin treatment, proving that the partial reduction in ROS levels shown in previous chapters was not due to a reduced efficiency of NFkB blocking. This further suggests that activation of NFkB is not mediated by an initial increase in ROS levels, as shown in other cases (Ho et al., 1999).

Fig. 7. 3   Administration of PPAR-β/δ agonist failed to reduce the activation of NFkB activation by visfatin

Differentiated C2C12 cells were pre-treated or not for 24 hrs with DMSO as vehicle, the agonist GW-501516 diluted in DMSO (10nm) or with IκB Kinase Inhibitor Peptide (50 μg/ml) for 1 hr, before being subjected to another 6 hrs of challenge with 100 ng/ml of visfatin. Protein lysates were resolved by a typical western blot and probed by using the antibody raised against the phosphorylated form of p65 subunit of NFkB (above), phosphorylated form of IKKα/β (middle) or an antibody against GAPDH as control (bottom). Quantization of p65 levels of phosphorylation, compared to the negative control was done by densitometric analysis. Each data point is the average from three independent experiments +/− SEM. **Denotes p<0.01 visfatin compared to control. NS denotes no significant difference between visfatin and agonist/vehicle compared to visfatin alone. **Denotes p<0.01 inhibitor plus visfatin compared to visfatin alone.
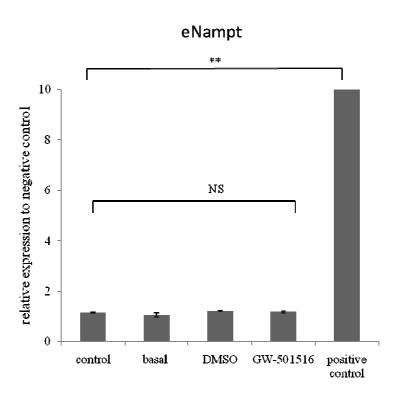
7.4   Visfatin is not a myokine in the basal condition and the administration of PPAR-β/δ agonist did not induce a secretion of visfatin from C2C12 cells

Previously it was discovered that visfatin can be detected in the supernatant of cultured L6 rat skeletal myotubes as a secreted myokine (Wang et al., 2010). It was shown also that visfatin secretion levels from diverse types of cells such as adipocytes or macrophages can be modified by PPAR agonists (Choi et al., 2005). Therefore, to test whether visfatin is a genuine myokine in my cellular model the serum-free DMEM supernatant of basal un-stimulated C2C12 myotubes was taken after 24 hours of culture and the levels of circulating visfatin were determined by an ELISA assay. As seen from figure 7.4, visfatin was not detected in the basal state, proving that is not a myokine secreted under normal conditions in my murine model. To test whether PPAR-β/δ-dependent signalling can induce a secretion of visfatin as a myokine, C2C12 cells were treated with GW-501516 (10nm) for 24 hours or with DMSO as a vehicle, followed by collection of the supernatant that was subjected to the same ELISA assay. As seen from figure 7.4, visfatin was not detected either in the supernatant of C2C12 cells treated with PPAR-β/δ agonist. This experiment shows that in cultured C2C12 cells visfatin is **not** a genuine **myokine** and that any therapy targeting PPAR-β/δ will not induce a secretion of visfatin from skeletal muscle.

Fig. 7.4   Visfatin is not a myokine in the basal condition and the administration of PPAR-β/δ did not induce a secretion of visfatin from C2C12 cells

Differentiated C2C12 cells were treated or not for 24 hrs with GW-501516 (10nm), DMSO being also used as a vehicle. The medium was taken from both control and agonist-stimulated cells and subjected to a competitive ELISA assay by using a RayBio® mouse visfatin enzyme Immunoassay kit (RayBiotech Inc, VIS-EIA-1). The 96 wells plate that was already coated with goat anti-rabbit secondary antibody has been incubated with rabbit anti-visfatin antibody followed by the incubation with the supernatant taken from cells or with the visfatin-standard mixed with biotinylated visfatin. The plate was finally incubated with biotin-streptavidin solution and TMB substrate. The measurement of the signal was performed at 450 nm. Each data point is the average from three independent experiments +/− SEM. **Denotes $p < 0.01$ positive control compared to negative control. NS denotes no significant difference between treatments and control.

eNampt

7C. Discussion

Multiple human clinical trials and animal experiments using PPAR agonists had so far promising results in the treatment of insulin resistance and obesity (Erol, 2007). It was also shown previously that exogenous visfatin challenge changed the expression levels of PPARs in non-muscle cells (Zhou et al., 2007). This implies that a possible change of visfatin plasma levels during ageing could theoretically affect a clinical therapy targeting PPARs in order to treat skeletal muscle ageing-associated pathologies by changing their expression levels, such as a therapy for HLS. Therefore, an in-depth and detailed understanding of any possible relationships between plasma levels of extracellular form of visfatin (eNampt), nuclear receptors expression or activity in skeletal muscle and regulatory mechanisms of oxidative stress and inflammation in the context of ageing and ageing-associated diseases is a requirement for any clinical intervention strategy targeting PPARs or circulating visfatin (Sinclair, 2005; Yang et al., 2006). To explore the roles of any possible changes of visfatin plasma level on PPARs-associated signalling pathways in skeletal muscle, the same experimental conditions with exogenous visfatin (eNampt) treatment of cultured C2C12 myotubes have been used, as described in previous chapters.

The first goal of the present chapter was to test whether incubation of cultured C2C12 cells with exogenous visfatin changed the expression levels of PPAR family by using RT-PCR. As shown in figure 7.1, exogenous visfatin treatment (100 ng/ml) for 24 hours did not change significantly the expression of PPAR-β/δ and PPAR-γ. PPAR-α expression was too low to be detected in differentiated and fused C2C12 myotubes by RT-PCR, although it was detected previously in proliferating C2C12 myoblasts (Suzuki et al., 2007). This experiment indicated that a possible visfatin plasma increase during ageing or ageing-associated diseases did not affect at transcriptional level the targeting of PPAR-β/δ signalling by using specific agonists/antagonists to treat pathologic conditions affecting skeletal muscle (Kopelovich et al., 2002). Nevertheless, a possible visfatin plasma increase could affect the activity of PPARs independently of transcription,

but this is a subject for future studies that are not covered in the present thesis.

Also, it is important to know whether PPAR agonists are effective in reducing the oxidative stress and inflammatory responses induced by exogenous form of visfatin in cultured C2C12 cells. Additionally, it is also important to decipher through what mechanisms such putative process might occur. In the present chapter it was further showed that administration of a PPAR-β/δ agonist reduced the levels of oxidative stress induced by exogenous form of visfatin (Fig. 7.2A). It is unclear still if this reduction is due to a reduced expression of the genes that are involved in ROS production upon visfatin challenge, or due to a predictable increase in global antioxidant defence status after PPAR-β/δ activation as shown before (Dressel et al., 2003).The activation of PPAR-β/δ alone with the agonist GW-501516 did not change the gene expression level of NOX4 in C2C12 cells after 24 hours (Fig.7.2B). Nevertheless, pre-treatment of C2C12 cells with the agonist GW-501516 for 24 hours reduced with 30% the increase in NOX4 levels induced by the subsequent challenge of C2C12 cells with exogenous visfatin (eNampt) for another 24 hours. This proves that PPAR-β/δ interferes with visfatin action, but since GW-501516 did not change NOX4 gene expression levels in the basal state than it is likely that this effect is only *indirect*.

Whether visfatin is a genuine myokine as previously proposed (Krzysik-Walker et al., 2008) is an issue still debated, and it was shown already that it was detected in the supernatant of cultured L6 rat myotubes (Wang et al., 2010). A possible secretion of visfatin from skeletal muscle will add a new level of *complexity* to the issue discussed in this chapter. For example, interleukins such as IL-6, IL-8 and IL-15 are secreted by skeletal muscle in regulated fashion as genuine myokines too, having a crucial role in modulation of oxidative stress and inflammation levels in skeletal muscle through an autocrine and paracrine effect (Pedersen et al., 2007). Moreover, while as a cytokine IL-6 is a *pro-inflammatory* mediator, as a myokine IL-6 is secreted by skeletal muscle during contraction or physical exercise, having essentially *protective* and immunomodulatory properties. For example, as a myokine IL-6 was shown to suppress the

production of TNF-α in skeletal muscle upon endotoxin challenge (Starkie et al., 2003). This suggests that as a *myokine* visfatin could fulfil different roles than visfatin secreted as an *adipokine*, possibly by acting in autocrine or paracrine fashion. Furthermore, visfatin secretion from skeletal muscle could (positively or negatively) interfere with an exogenous challenge of visfatin, as the one employed in my experiments. To test if visfatin is secreted by fused and differentiated C2C12 myotubes in the supernatant an enzyme-linked immunosorbent assay (ELISA) assay has been used. As seen from figure 7.4, there were no differences between the negative control and the culture medium which was taken from C2C12 cells after 24 hours of culture. This shows that in this interval of time the exogenous form of visfatin (eNampt) is not secreted in the basal state from skeletal muscle differentiated myotubes.

Furthermore, it was previously shown that the secreted values of visfatin can be modified by PPAR agonists in different cellular types (Choi et al., 2007; Choi et al., 2005; Pfutzner et al., 2008). Since PPAR-β/δ agonist treatment reduced the level of oxidative stress in cultured myocytes upon stimulation with exogenous visfatin, it was important to establish whether the treatment of C2C12 cells with GW-501516 can also modify the levels of visfatin secretion in the culture medium. Such event could interfere with exogenous visfatin (eNampt) challenge, positively or negatively. To test this possibility C2C12 cells have been treated for 24 hours with GW-501516 and the levels of visfatin in supernatant were measured subsequently by using the same ELISA assay as above. As shown by the same figure, activation of PPAR-β/δ by GW-501516 did not induce a secretion of visfatin from C2C12 cells after 24 hours. This indicates that in the case of cultured C2C12 mouse cells visfatin is not a myokine upon the activation of PPAR-β/δ-signalling.

Chapter 8 - DISCUSSION

The healthy ageing strategy can be managed through diet, exercise and pharmacological-based interventions. As the 2009 Pharma R&D Annual Review report shows (Pharmaprojects, 2009), the global overall pipeline for all the drugs in active development grew by 5% in 2009 compared to 2008 (>9600) due to a significant increase of products in the preclinical stage, but the number of *finally* approved drugs reaching the market was still modest. Even more intriguing, while the last decade experienced a sustained biomedical revolution (in both theoretical and technological aspects), the number of applications submitted for approval to FDA in US *decreased* in the same period (Wood, 2006). This gaps occurs because the rate of R&D success in phases II and III of drug development is still below 50% (Mervis, 2005) which makes the overall pre-approval average cost of any drug to 800 million USD (DiMasi et al., 2003). This makes the biomedical research for *ageing* management to be financially both risky and costly. Therefore, a solid in-depth understanding of the complex connections between the progression of *ageing*, the effects of *adipokines* (such as visfatin) and the functional dynamics of *nuclear receptors* families (such as NR4As and PPARs) is a critical factor for the faster development of *safer*, more *efficient* and more *affordable* drugs for the treatment of ageing-associated *diseases*

In the present thesis I investigated the effects of exogenous added visfatin (eNampt) on *in vitro* cultured murine myotubes, with a special focus on oxidative stress, inflammation and insulin resistance parameters. The other major goal of the thesis was the identification of any significant changes in gene expression profiles of different families of nuclear receptors in two animals models of skeletal muscle ageing, since improving metabolic flexibility through the specific targeting of nuclear receptors-associated signalling may represent a novel intervention strategy to prevent the adverse physiological effects characterizing skeletal muscle during ageing.

8.1   The roles of nuclear receptors in ageing

To model the effects of altered pace of ageing on skeletal muscle physiology in rodents two complimentary approaches have been used: one designed to accelerate it (hindlimb suspension, *HLS*) and one designed to decelerate it (caloric restriction, *CR*).

8.1.1 The role of PPARs in the metabolic adaptation to HLS

Acute HLS (24 hrs) induced a significant reduction in mouse PPAR-α and PPAR-γ and an increase in PPAR-β/δ expression levels (Fig. 3.4A), as summarized in our published paper (Mazzatti et al., 2008). The decrease in PPAR-α expression was simultaneously accompanied by a decrease in expression of its known targets PGC-1α and PGC-1β (Fig. 3.4B). Nevertheless, in the acute phase no significant decrease in expression of genes involved in fatty acid transport and oxidation was detected by microarray, suggesting that there are alternative pathways that compensate for the loss of PPAR-α and PPAR-γ. One possible candidate is PPAR-β/δ, and it has already been shown that in some cases its increased expression or activation can compensate for the loss in PPAR-α expression (Muoio et al., 2002). In contrast to the other PPARs that were downregulated by HLS, PPAR-β/δ expression level was upregulated in both muscle types concomitantly with an increased UCP-3 expression in soleus as proved by both RT-PCR (Fig. 3.4B) and western blot assays (Fig. 3.5). Uncoupling proteins like UCP-3 are known to be critical for the maintenance of ATP: AMP ratio and AMPK signalling, having a robust effect on the regulation of energy expenditure (Klip et al., 2005). Because of the proven role of PPAR-β/δ in regulating oxidative capacity and fuel switching it is possible that its increased expression following short-term HLS represents an *adaptive*, compensatory response to regulate the fuel utilization and to maintain the metabolic flexibility under stress. This hypothesis is further

supported by the finding that PPAR-β/δ expression is restored to the basal levels after 12 days of HLS (Fig. 3.6), approximately at the same time when muscle atrophy and loss of lipid oxidative capacity were observed. From these data it can be hypothesized that the loss of PPAR-β/δ expression may contribute to the metabolic inflexibility and adverse physiological alterations observed after long-term unloading. Improving metabolic flexibility through PPAR-β/δ regulation may represent a novel intervention strategy to prevent the adverse metabolic and physiological effects of muscle unloading.
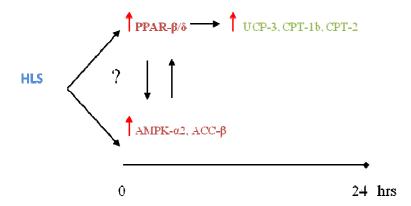
Several pieces of independent evidence suggested already that PPAR-β/δ may respond to such unloading events with a possible *compensatory* effect for the global metabolic deregulation. For example, PPAR-β/δ was shown to be involved in the remodelling of skeletal muscle by increasing type I oxidative fibers number which also reduced the body fat content (Wang et al., 2004). Increased PPAR-β/δ expression and activity has been previously implicated in the metabolic and structural adaptations to long-term fasting and endurance exercise, and increased expression was also an indicator of improvement in diabetic patients after training (Luquet et al., 2004). In addition, previous investigations have demonstrated that transgenic mice expressing an activated form of PPAR-β/δ have enhanced fatty acid utilization and are protected against the high-fat diet-induced obesity (Wang et al., 2003). Furthermore, PPAR-β/δ agonist treatment induced changes in muscle fuel metabolism as shown by increased lipid oxidation and decreased carbohydrate utilization (Constantin et al., 2007). PPAR-β/δ could therefore be a part of an adaptive, compensatory regulatory complex designed to protect against further metabolic complications. For example, it is already known that PPAR-β/δ can change the expression levels of genes involved in fatty acid oxidation and lipid homeostasis such as fatty acid binding protein 3 (FABP3) which is involved in uptake and transport of fatty acids (Zimmerman and Veerkamp, 2001), CPT-1 that is a rate-limiting enzyme in mitochondrial fatty acid oxidation and PDK4. Microarrays analysis demonstrated no significant change in PDK4 or FABP3 expression in soleus

or gastrocnemius muscle after acute HLS, but it was observed a significant increase in expression of CPT-1b and CPT-2 in soleus muscle after the acute stage of unloading. In the models of long-term exposure to HLS (12 days) the expression of these two genes were nevertheless decreased, further indicating that the adaptation during the acute stage is only *transient*. Hindlimb suspension has a major physiological impact by leading to a preferential use of glucose as a fuel of choice instead of lipids, therefore reducing in long-term the metabolic flexibility characterizing the healthy normal muscle. Such metabolic inflexibility is also a characteristic of aged skeletal muscle. The increase in PPAR-β/δ expression during the acute phase and the subsequent reversal to the normal state in the chronic stage could signify that PPAR-β/δ is an important member of such metabolic *switch*. The return to the basal state in PPAR-β/δ expression during the chronic phase of unloading could be partially responsible for the loss in metabolic flexibility shown in later stages. This interpretation of PPAR-β/δ as a *switch* is also supported by its role in regulating Bcl-6 activation, as discussed in the introduction. Therefore, the changed expression level of several proteins such as PPAR-β/δ, UCP-3, AMPK and CPT-1/2 noticed in acute HLS might have a substantial role in the maintenance of metabolic flexibility and represent potential targets. Because of its interactions with the other putative targets PPAR-β/δ appears particularly promising, and since enhanced UCP-3 expression is associated with improvements in glycemic regulation, it can be hypothesized that inducing PPAR-β/δ-dependent upregulation of UCP-3 and other genes could prevent the metabolic and physiological alterations associated with muscle unloading (Fig. 8.1). This model of PPAR-β/δ as a switch could be also used to test the predictions made by different theories of ageing about the relationships between senescence and oxidative stress.

Fig. 8.1   The proposed role of PPAR-β/δ in regulating the adaptation to metabolic inflexibility during the acute phase of skeletal muscle immobilization

Acute HLS increased in the suspended hindlimb the expression of PPAR-β/δ and of its target genes (UCP-3, CPT-1b, CPT-2), as well as of AMPK and ACC. Such changes could mediate a process of metabolic adaptation to the stress induced by unloading. Whether PPAR-β/δ-signalling acts upstream, downstream or independently of AMPK-signalling is still to be determined.

METABOLIC ADAPTATION?

↑PPAR-β/δ ⟶ ↑ UCP-3, CPT-1b, CPT-2

HLS

?

↑AMPK-α2, ACC-β

0   24 hrs

Legend

↑   increased expression/activity

↓   decreased expression/activity

⟶ ↓↑   causal relationships/interactions

⟶   time

8.1.2 The role of NR4A family in mediating the multiple effects of caloric restriction on skeletal muscle physiology in rats

In the CR model it was demonstrated for the first time an increased expression of all three members of NR4A family in rodent skeletal muscle (Fig. 3.8A). No significant changes were observed in brain, demonstrating tissue-specificity in expression patterns following CR (Oita et al., 2009). Upregulation of several known NR4A transcriptional targets such as UCP-3, AMPK-γ3, PGC-1α and PGC-1β was also observed (Fig. 3.9). NR4A receptors respond to changes in the cellular environment, including neuroendocrine stimuli, regulating the expression of various genes in the hypothalamus–pituitary–adrenal (HPA) axis including POMC, the precursor to adrenocorticotropic hormone which is the main regulator of adrenal glucocorticoid synthesis (Philips et al., 1997). Alterations to neuroendocrine status are one of the many proposed mechanisms by which CR increases lifespan and retards ageing, and it was shown that food-restricted rats exhibit daily periods of hyperadrenocorticism (Nelson et al., 1995) which may be a major contributor to CR-induced delay of ageing processes and lifespan extension.

The knock-out or transgenic models of NR4A subgroup did not show any obvious changes in lifespan or in the ageing phenotypes, although they have not been especially investigated in this respect (Mullican et al., 2007; Pan T. et al., 2008; Zheng et al., 1998). The fact that NR4A are expressed mainly in energy-dependent tissues such as skeletal muscle, brain, adipose tissue, heart and liver suggests that they have a critical role in regulating energy and metabolism. NR4A1 is regulated by glucose levels (Susini et al., 1998) and a recent report showed that while insulin or thiazolidinediones challenges increased NR4A1 and NR4A3 expression (Fu et al., 2007), their expression was nevertheless reduced in animal models of insulin resistance. These findings provide evidence for a link between NR4A receptors and the complex *metabolic shift* observed in animals following CR. Interestingly, increased expression of all three NR4A receptors is also associated with skeletal muscle recovery following endurance exercise (Mahoney et al.,

2005; Mahoney and Tarnopolsky, 2005). In addition to their function in muscle metabolism NR4A receptors are also thought to be involved in adipose tissue and in liver metabolic regulation. In 3T3-L1 cultured adipocytes all three members of NR4A subgroup are rapidly and transiently induced by the PPAR-γ agonist rosiglitazone (Fu et al., 2005). Since adipose, liver and muscle are major sites of lipid and glucose metabolism and are important targets for metabolic therapies, it is possible that the modulation of NR4A receptors may represent a novel strategy for treatment of metabolic disorders. This concept has already been explored (Hsu et al., 2004) and has generated a great deal of interest in recent years, but further investigation is necessary to determine whether any of the NR4A receptors represent suitable targets for pharmaceutical intervention.

A recent report (Pei et al., 2006) demonstrated that NR4A receptors act as downstream mediators of cAMP action *in vitro* and in response to glucagon or fasting *in vivo*. These data support our observed upregulation of NR4A receptors in muscle and liver following CR and suggest that these receptors respond to nutritional cues such as insulin levels through yet unknown mechanism. This hypothesis is consistent with the previously described role of mammalian target of rapamycin (mTOR)-dependent nutrient signaling in mediating the effects of caloric restriction (Linford et al., 2007). Although there is no direct link between mTOR and NR4As, there are indications of a possible indirect crosstalk between AMPK and mTOR has been established (Hardie, 2008a, b). Since NR4A receptors are regulated by cAMP it is possible that they respond to changes in AMP: ATP ratio similarly to both AMPK and uncoupling proteins. These data suggest that multiple nutrient-sensing and signaling mechanisms may contribute to the complex metabolic adaptations that occur following CR and that NR4A receptors might a role in this process.
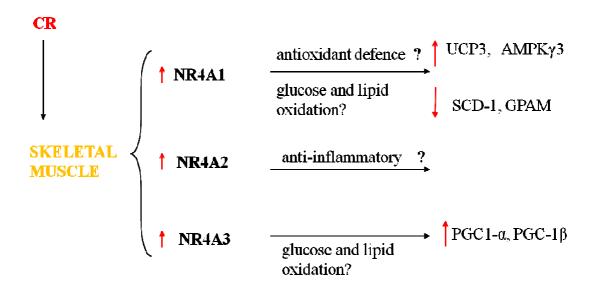
In conclusion, I identified an increased expression of the members of NR4A subgroup in the skeletal muscle of the caloric restricted animals simultaneously with a change in expression of some of the previously known NR4A target genes regulating lipid and glucose
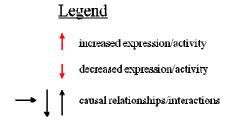
metabolism, oxidative stress and inflammation. Based on previous filtered knowledge, detailed in the discussion from chapter 3, it can be speculated that in the case of caloric restricted skeletal muscle NR4A1 acts mainly on the glucose and lipid metabolism, NR4A2 has an important impact on inflammatory response and NR4A3 mainly regulates genes critical for lipid metabolisms (Fig. 8. 2). The prospect of screening of selective activators/inducers of NR4As (unavailable yet) to reduce the metabolic inflexibility in aged skeletal muscle justifies further research in this area.

Furthemore, since the change in NR4A family expression was restricted only to some of the tissues investigated so far, than they could be used as *markers* to estimate the tissue-differential aspects of ageing, as explained in the introduction.

Fig.8. 2 The proposed role of NR4A subgroup of nuclear receptors in modulating the effects of caloric restriction on lipid and glucose metabolism, oxidative stress and inflammation

CR increased in skeletal muscle the expression levels of NR4As family of nuclear receptors. The simultaneous increased expression of some of NR4A1 positively-regulated genes (UCP-3, AMPKγ3), of NR4A1 negatively-regulated genes (SCD-1, GPAM) and of NR4A3 positively-regulated genes (PGC1-α and PGC1-β) suggests that such increase in NR4As expression is physiologically relevant, but the particular significance in this context remains to be determined.

8.2 The putative effects of visfatin (eNampt) on skeletal muscle ageing- related processes

8.2.1 Visfatin (eNampt) and insulin-dependent signalling pathway

The most contentious academic issue concerning the extracellular, circulating version of visfatin is the hypothesis that it is able to bind to the insulin receptor expressed by peripheral tissues and activate PI3K/Akt signalling pathway, increasing therefore the rate of glucose uptake (Fukuhara et al., 2005). While the identity of visfatin as a genuine secreted adipokine is firmly established and widely accepted (Antuna-Puente et al., 2008; Beltowski, 2006; Hammarstedt et al., 2006), the insulin-mimetic property of such a secreted form is still highly contentious and controversial (Moschen et al., 2007). The initial paper proposing this insulin mimetic hypothesis (Fukuhara et al., 2005) was retracted by the publishing journal for scientific improprieties and subsequent independent experiments done *in vivo*, *ex vivo* or *in vitro* discovered that such activation occurs only in a few limited cases of cellular systems and experimental conditions (Adya et al., 2008a; Xie et al., 2007; Lim et al., 2008). Numerous independent experiments in other cellular systems failed to prove any insulin mimetic properties upon incubation with visfatin (Li et al., 2008; Revollo et al., 2007; Wanecq et al., 2009; Wang et al., 2009).

Since there were no previous reports about the effects of exogenous visfatin on skeletal muscle which is a main target for adipokines action and the main organ responsible for the removal of the glucose from the plasma, I tested whether visfatin can activate PI3K/Akt signalling pathway that mediates the effects of insulin on the rate of glucose uptake through the translocation of GLUT4 transporter to the plasma membrane (Valverde et al., 2005). The stimulation of C2C12 cells with 100 ng/ml visfatin did not induce an increase in the phosphorylation of Akt at Ser^{472} or

at Thr^{308} residues in the first 15 minutes as seen by western blot (Fig. 4.10B), unlike insulin challenge that triggered a robust response after 5 minutes at the Akt^{Ser472} residue (Fig. 4.10A). Since the culture medium for C2C12 differentiation lacked insulin there is no reason to assume that C2C12 cells were insulin-resistant/-insensitive. Visfatin did not change either the phosphorylated levels of Akt upstream activator (PDK1) and upstream inhibitor (PTEN), or of the downstream targets like GSK-3β and c-Raf (Fig. 4.10B). Furthermore, pre-incubation of C2C12 cells with visfatin for 18 hours did not impair nor augment the ability of insulin to activate PI3K/Akt after 5 minutes (Fig. 4.10A). This showed that in C2C12 cells visfatin did not activate the PI3K/Akt signalling pathway in acute or chronic stages, suggesting that in the case of skeletal muscle visfatin is **not** an insulin-mimetic agent.

The concentration used (100ng/ml) is consistent also with previous reports (Lovren et al., 2009; Moschen et al., 2007; Xie et al., 2007) and higher concentration values (up to 500 ng/ml) had no effect on PI3K/Akt-signalling pathway either (data not shown). The concentration chosen in this thesis is within the limits identified as physiological relevant by measurement of human visfatin plasma levels in both normal and pathological samples (Dogru et al., 2007), and an eventual activation of PI3K/Akt by using higher than physiological concentrations of visfatin will have no medical applicability nor theoretical relevance. Also, since three different versions of commercially available visfatin have been employed, produced in bacteria or in mammalian cells, and since all these three versions showed the same consistent results it can be concluded therefore that the experimental conclusions of the thesis presented were both accurate and representative. It is important to stress nevertheless that my experiments did not explore whether visfatin can bind to insulin receptor in cultured and differentiated myotubes, only if such incubation can activate the insulin receptor-dependent PI3K/Akt signalling pathway because this is a better proof of a possible effect on the rate of glucose uptake. Multiple explanations could be proposed for the discrepancies between the conclusions derived from independent experiments

published so far. For example, different cell types could be characterized by significant differences in the number of insulin receptors expressed or localized at the cellular membrane, or they could differ in the sensitivity of activation of PI3K/Akt signalling pathway. The cellular models showing PI3K/Akt activation upon visfatin treatment like endothelial cells, osteoblasts and ventricular cardiomyocytes could alternatively present a higher expression of other membrane receptors such as IGF-IR that was proved already to mediate an additional PI3K/Akt activation upon insulin treatment. Interestingly, it was proved that in HUVEC cells that showed a visfatin-dependent phosphorylation of PI3K/Akt pathway after 5 minutes of incubation (Adya et al., 2008b) the IGF-IR gene is expressed at significantly higher levels than insulin receptor gene (Nitert et al., 2005). This could indicate that the membrane receptor which mediates the visfatin action on PI3K/Akt-signalling pathway was in fact IGF-IR, not the insulin receptor. Nevertheless, since I did not quantify IGF-IR levels of expression in C2C12 cells, at the present stage this remains only a hypothesis yet to be explored. Another theoretical possibility independently proposed already is the existence of another, still unidentified, membrane receptor specific only for visfatin that could activate the PI3K/Akt pathway in the tissues or cells where it is expressed or functional (Li et al., 2008). Nevertheless, until now there are no published reports detailing any attempts to identify such a putative receptor, and this subject was not covered in the present thesis.

The long-term incubation of cultured myotubes with visfatin (<24 hours) might still modify the rate of glucose uptake or glucose metabolism independently of Akt signalling, possibly through the modulation of other physiological processes like a robust NAD metabolism as shown before (Revollo et al., 2007). Therefore the next question to be answered was whether the chronic incubation of C2C12 cells with visfatin could modify the levels of glucose uptake. The measurement of the rate of glucose uptake in both basal (Fig. 5.7C) and insulin-stimulated conditions by 2-NBDG assay (Fig. 5.8B) showed no difference between control and 24 hours visfatin-treated cells. In addition, the incubation of C2C12 cells with visfatin for 24 hours did not

change the gene expression levels of known glucose transporters such as GLUT1 and GLUT4 (Fig. 5.10A, B). This proved that in the case of cultured skeletal myocytes visfatin is *not* an insulin mimetic agent, which is also a strong argument to dismiss the possibility of any effects on skeletal muscle *in vivo* too. My findings correlate with previous data indicating that the increase in the rate of glucose uptake upon the challenge with the exogenous visfatin (eNampt) was noticed only in those cells that presented simultaneously an activation of PI3K/Akt signalling pathway (Lim et al., 2008), event missing in my experiments.

Unlike visfatin treatment, chronic triglyceride challenge reduced the levels of phosphorylated forms of Akt and GSK-3β (one of Akt substrates) in the insulin-stimulated state, but not in the basal state (Fig. 5.9A, B). In the same time, as shown by 2-NBDG assay, triglyceride challenge increased the rate of glucose uptake in basal state (Fig. 5.7C), reducing it nevertheless in the insulin stimulated state (Fig. 5.8B). The decrease in the rate of glucose uptake upon stimulation with insulin after the pre-treatment with triglycerides correlates with the reduced insulin-stimulated activation of Akt signalling. In addition, both findings correlate with the research done previously *in vitro* by using palmitate (Ragheb et al., 2009) and *in vivo* by using animal models fed with a high fat diet (Vinayagamoorthi et al., 2008). In addition of blocking PI3K/Akt signalling, the treatment of cultured myotubes with triglycerides reduced also the expression level of GLUT4 after 24 hours (Fig. 5.10B). Such a reduction in GLUT4 gene expression could also explain the impaired glucose uptake in the insulin-stimulated state upon triglyceride challenge as shown by the 2-NBDG assay. The fluorescent-based assays showed also that triglycerides treatment increased the glucose uptake in basal state and this correlated with previous findings that used palmitate challenge and with other studies on the mechanisms of insulin resistance (Hardy et al., 1991; Usui et al., 1999). This phenomenon can be explained by the increased expression of GLUT1 transporter (Fig. 5.10A) after triglyceride challenge that likely increased the rate of glucose uptake. The lack of reduction in phospho-Akt levels after triglycerides treatment in the basal state indicates that PI3K/Akt signalling

pathway is not mediating the increased GLUT1 expression noticed.

These experimental conclusions about the triglyceride treatment correlate with the independent data reported previously about the effects of ectopic accumulation of lipids in skeletal muscle *in vivo* during advanced stages of obesity and with the results of *in vitro* studies that used free fatty acids such as palmitate. This certifies also that the fat emulsion Structolipid[TM] can be used to model *in vitro* the effects of the lipid-dependent stress as an alternative to the free fatty acids strategy.

8.2.2   Visfatin and the regulation of oxidative stress levels

In the fourth chapter it was shown that incubating murine fused and differentiated C2C12 myotubes with different concentrations of exogenous murine visfatin (eNampt) (<200 ng/ml) induced oxidative stress as monitored by fluorescent-based assays (Fig. 4.1- 4.4). Unlike previous reports that identified the generation of free radicals in the first hour during the early stage of the incubation of endothelial cells with visfatin (Kim et al., 2008), the experiments shown in chapter 4 indicated that the earliest time point for a significant increase was in the chronic stage of treatment between 4-6 hours, with a maximum peak reached after 18 hours (Fig. 4.2). Additional incubation time did not further increase the levels of free radicals detected. Therefore, visfatin showed a different ROS-inducing pattern in cultured myotubes compared to cytokines such as TNF-α (Li et al., 1999;Li et al., 1998) or endotoxins like LPS (Maitra et al., 2009) that can generate reactive oxygen species within the first hour of treatment, being more similar in this respect with other adipokines such as leptin (Xu et al., 2004) and adiponectin (Ikegami et al., 2009). Nevertheless, this increase in free radicals by visfatin did not change the total levels of oxidized proteins (Fig. 4.5) or the levels of oxidized lipids (Fig. 4.6). This is not unusual since in many other cases there were recorded differences in measured values for different markers of oxidative stress. For example, there were recorded differences in the quantified values

of ROS and of the oxidized products by measuring the levels of carbonylated proteins and other biochemical methods in cultured myocytes (Astruc et al., 2007), between the levels of carbonylated proteins and fluorescent microscopy-derived values in the case of the nematode *C. elegans* (Yasuda et al., 2006), or between the levels of carbonylated proteins and fluorescent microscopy-derived values in the rat liver after endurance exercise (Sun et al., 2009). Carbonylation of proteins is an irreversible change that occurs after a prolonged and sustained level of oxidative stress, and such carbonylated proteins are usually digested by the proteasomal complex (Nystrom, 2005) . The existence of an increased level of free radicals in the absence of increased oxidized products suggests that such event is not affecting the integrity of cellular processes or the cell structure.

The increased levels of intracellular ROS by visfatin could be explained by an increased production of ROS, by a reduced/impaired antioxidant capability of the cells (Lyakhovich et al., 2006), or by a combination of both factors. Such events could appear as a consequence of the reactions occurring at the enzymatic complexes localized not only at mitochondria and peroxisomes but also as a consequence of the activation of NADPH oxidases (NOX) that were discovered recently to be localized in the cytosol and sarcoplasmic reticulum of skeletal myocytes (Xia et al., 2003), with still unclear roles.

A more theoretically revealing and critical question than whether a certain agent induces oxidative stress as measured by fluorescent-assays or by quantifying the levels of oxidized products is the question if such a stress has any effect on the physiological *status-quo* or on the structural integrity of the cell. As shown by NADH assay, visfatin treatment did not affect the cellular viability which indicated that such a stress is not impairing significantly myotubes survival (Fig. 4.8). This is not an unique phenomenon since many oxidative stress inducers do not generate apoptosis or cellular damage, and in certain circumstances such increase in free radicals has even an anti-inflammatory, protective role (Tang et al., 2009).

Whether the increased level of intracellular ROS detected is caused by an increased

production of free radicals or by an impaired antioxidant defence is still unclear. Because it was

detected an increase in ROS levels without an increase in the levels of oxidized products or

impaired viability after visfatin challenge, I further hypothesized that this might be explained by a

simultaneous improvement of the antioxidant capacity of the cells to resist such a stress. It was

already shown that incubation of other types of cells like macrophages (Li et al., 2008) and

cardiomyocytes (Lim et al., 2008) with visfatin improved the resistance against toxic agents and

this process might occur through an improved antioxidant protection. Such a hypothetical scenario

would correlate therefore with a case of increased levels of free radicals as a consequence of an

accelerated production of ROS, not an impaired antioxidant defence. The measurement of this

global cellular ability to protect against oxidative stress was performed by using the ABTS assay

that did not show any global change in the antioxidant potential of C2C12 cells after the treatment

with visfatin, compared to the control (Fig. 4.7). This suggests that such a potential increase in

antioxidant ability of cells by visfatin could be *localized*, not global, targeting the organelles and

cellular compartments where free radicals are produced such as peroxisomes, mitochondria and SR.

Such a putative limited and localized improved antioxidant defence might not be detected therefore

by ABTS method. Furthermore, since it was not detected by ABTS assay a reduction in the

antioxidant capability upon visfatin challenge it is likely that the higher ROS level noticed is

caused by an *increased* production of free radicals, not by an impaired defence status.

The treatment of C2C12 cells with 0.2 mg/ml triglycerides for 24 hours induced

similar oxidative stress as measured by fluorescent microscopy (Fig. 5.2). Unlike in visfatin

treatment case it was noticed an increase in the level of oxidized proteins by 80% after a minimum

incubation time of 24 hours (Fig. 5.3). A band localized around 30 kDa showed a significant

increase in the carbonylated form, but the identity of such a band is still unexplored and this

question was not covered in the present thesis. So far I did not identify another independent report

to confirm or support my particular finding. The pattern is also different from the one noticed in the

skeletal muscle of diabetic animal models presenting increased depots of intracellular lipids in

skeletal muscle (Oh-Ishi et al., 2003), in the skeletal muscle of mice fed with a high-fat diet

(Bonnard et al., 2008) or from the pattern shown by the aged skeletal muscle (Feng et al., 2008).

8.2.3 Identification of the gene networks possibly involved in ROS production by visfatin

Triglycerides treatment for 24 hours increased the expression of genes that have an

antioxidant role such as SOD1, CAT and SOD2 and of transcription factors involved in the

antioxidant defence such as NRF1, NRF2 and HIF1A (Fig. 5.4). This increase indicates that the

stress induced by triglycerides was significant and this correlates with previous reports about the

effects of free fatty acids or high-fat diet on skeletal muscle. For example, it was shown that

palmitate treatment increased the expression of SOD2 in cultured human myocytes, similarly to the

pattern shown by obese or insulin-resistant skeletal muscle (Reyna et al., 2008). In addition, it was

shown that palmitate treatment increased the expression of NRF2 in cultured hepatoma cells (Das

et al., 2010). The increase in the expression of the above mentioned antioxidant defence genes by

triglycerides suggests that the oxidative stress induced is critical, subsequently triggering the

activation of defensive, antioxidant mechanisms. This interpretation of my results is further

supported by the previous discovery that skeletal muscle of obese children with metabolic

syndrome had a reduced antioxidant defence status (Molnar et al., 2004) and by the fact that

overexpression of antioxidant genes such as SOD1, CAT and SOD2 reduced the activation of

caspase-3 and the apoptotic process triggered by palmitate challenge of cultured cells (Cacicedo et

al., 2005). Furthermore, it is also known that mice heterozygous for SOD2 presented increased

oxidative stress levels in liver (Kokoszka et al., 2001) and that cardiomyocytes derived from

NRF2-null mice had increased ROS levels when cultured in high glucose levels conditions

compared to the control cells (He et al., 2009). In addition, the fact that triglyceride treatment

decreased the expression of GPX3 gene (Fig. 5.6), another important antioxidant defence member, could indicate a significant reduction in the antioxidant defence ability (Chung et al., 2009). It was shown for example that a reduction in GPX3 expression or function is a marker of obesity and this correlated with an increased level of oxidative stress (Lee et al., 2008). This decrease in GPX3 levels was not observed nevertheless in the cells challenged with visfatin, suggesting a non-critical level of oxidative stress induced in this case.

On the other hand, the only genes from the list that were changed by visfatin treatment were SOD1 and NOX4 (Fig. 5.5). The fact that NOX4 was increased opened the possibility of a new pathway of ROS production by visfatin, possibly independent of mitochondria and peroxisomes, but this is a subject for future studies. Cu/ZnSOD (SOD1) is another important member of the antioxidant defence network changed by visfatin challenge. It was shown before to be localized mainly in the cytosol, nucleus and mitochondria (Okado-Matsumoto and Fridovich, 2001). Mutations in the motor neuronal-specific Sod1 gene were shown to be important in the development of ALS (Miao and St Clair, 2009). The role of SOD1 in the skeletal muscle physiology is less explored and it was studied so far mainly in the relationship with the pathogenesis of ALS. The SOD1 -/- mutant mice had decreased muscle mass, increased levels of ROS and accelerated signs of ageing resembling sarcopennia (Muller et al., 2006). Nevertheless, although in the cells with high levels of superoxide production as cardiomyocytes an increased expression or activity of SOD1 has a protective role (Tanaka et al., 2004), in the cells with low levels of superoxide an overexpression of SOD1 alone has surprisingly deleterious effects. For example, the expression of wild-type human SOD1 in the skeletal muscle of transgenic mice led to significant myopathy and motor neurological degenerative phenotypes through aggregation and increased levels of nitric and oxidative stress (Wong and Martin, 2010). In another report, the expression of *G93A* mutant allele of the gene in the wild-type healthy skeletal muscle led to increased muscle atrophy due to higher levels of oxidative stress, mitochondrial dysfunctions and

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sarcolemmal damage (Dobrowolny et al., 2008). The increased expression of SOD1 upon visfatin challenge could signify the presence of a *localized* antioxidant defence response against the oxidative stress generated simultaneously that can explain the lack of the oxidized proteins or lipids, but this issue requires future studies. The fact that visfatin treatment did not increase the expression levels of many other antioxidant factors as the triglycerides challenge indicate a different pattern of ROS production and possibly a non-critical oxidative stress level, which could explain the lack of oxidized products noticed. The conclusion is reinforced by the fact that GPX3 expression level was not changed by exogenous visfatin.

8.2.4 Exploration of possible signalling pathways regulating the increase in ROS by visfatin

Another way to elucidate the mechanisms beyond ROS production by visfatin was the identification of any signalling pathways already known to modulate it.

8.2.4.1 Visfatin (eNampt) treatment and PI3K/Akt signalling pathway

I showed in the forth chapter that acute or chronic treatment visfatin did not activate the PI3K/Akt pathway. To further dismiss the possibility that PI3K/Akt pathway is involved in the increase in ROS noticed, PI3K activity has been blocked by its specific inhibitor before visfatin treatment. Additional fluorescent-based measurement of ROS levels did not show any changed values in the cells pre-treated with inhibitor prior adipokine challenge comparing to visfatin-only treated cells (Fig. 4.11). This proved that the observed ROS production induced by visfatin treatment in C2C12 cells is independent of Akt signalling.

8.2.4.2 Visfatin (eNampt) treatment and MAPKs-dependent signalling

Previously it was reported that in non-skeletal myocytes exogenous visfatin treatment can activate MAPKs such as p38 and ERK1/2 in the first 30 minutes of incubation (Lim et al., 2008; Adya et al., 2008a; Kim et al., 2007). It is also well known that MAPKs activation can lead to an increase in intracellular ROS levels (Cao et al., 2004). MAPKs/SAPKs are mitogen- or stress-activated kinases that can modify cellular physiology by changing the global genomic profile through the phosphorylation of numerous transcriptional factors like ATF-2 (Raingeaud et al., 1995), Max (Zervos et al., 1995), Elk-1 (Marais et al., 1993), c-Jun (Leppa et al., 2001) and MEF2 (Kyriakis and Avruch, 2001). Recently it was also discovered that MAPKs-induced effects can be independent of transcriptional regulation through the direct phosphorylation of cellular targets, other than regulators of gene expression. For example it was reported that JNK, another member of MAPK family, can phosphorylate cytosolic proteins regulating axodendritic growth in neurons such as microtubule-associated protein 2 (MAP2) (Bjorkblom et al., 2005) and superior cervical ganglion-10 protein (SCG10) (Tararuk et al., 2006), affecting neurogenesis. Therefore, it is also possible for p38 and ERK to directly phosphorylate proteins involved in antioxidant defence and oxygen metabolism and modify their enzymatic properties, eventually leading to increased levels of ROS. The western blots experiments for the phosphorylated forms of p38 and ERK1/2 showed nevertheless no significant increase in their levels in the first 30 minutes of incubation of C2C12 cells with exogenous visfatin compared to control cells (Fig.6.2). This correlates with previous published research data showing that MAPKs activations in non-muscle cells by exogenous visfatin were dependent on Akt (Adya et al., 2008a,b), event absent in my experiments. Since there were no previous reports of JNK activation upon visfatin challenge this possibility was not tested either, being a subject for future studies.

Nevertheless, it is still possible that MAPKs activations can occur later in the chronic stages of treatment of C2C12 cells with visfatin, independently of PI3K/Akt signalling, and

such activations could mediate a ROS increase. To test this possibility the activities of p38 and ERK have been blocked by using their specific inhibitors before incubation with exogenous visfatin. The fluorescent-based detection of the signal from the cells pre-treated with MAPKs inhibitors followed by visfatin challenge failed to show any reduction in oxidative stress levels generated, compared to visfatin-only treated cells (Fig.6.3). This proved that the ROS increase noticed upon visfatin treatment is independent of p38 and ERK signalling pathways.

8.2.4.3 Visfatin (eNampt) treatment and NFkB-regulated signalling

The activation of NFkB by visfatin was previously reported to occur in the acute phase of incubation (<60 minutes) of endothelial cells (Lee et al., 2009; Kim et al., 2008), or in the chronic phase of incubation of the smooth muscle cells (Romacho et al., 2009). Therefore, the possible activation of NFkB-associated signalling pathway was tested by performing western blot for the phosphorylated form of p65 subunit of NFkB. The western blot results showed an increase in phosphorylated levels of p65 subunit at Ser536 residue after 4 hours of incubation with visfatin, with a maximum value around 6 hours of incubation (Fig.6.4). The activation of NFkB signalling pathway was proved also by the increased phosphorylated levels of p65 upstream activator (IKK α/β at Ser176/180) and of p65 inhibitor (IkBα at Ser32) in the same time interval. After the phosphorylation by IKK at Ser32 the inhibitor IkBα is degraded through proteasome, releasing a fully functional p65 subunit (Magnani et al., 2000). The fact that NFkB signalling pathway was activated by visfatin treatment opened the possibility that it might be involved in the increased levels of oxidative stress noticed. Therefore, the activation of IKK (p65 upstream activator) was blocked for one hour before visfatin treatment for another 18 hours by using a specific inhibitor. Fluorescent microscopy assays of the signal from the cells pre-treated with NFkB inhibitor followed by visfatin challenge showed a reduction in ROS levels with cca. 40% compared to cells challenged only with visfatin (Fig.6.5). This indicates that visfatin-induced oxidative stress is

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dependent on *de novo* synthesis of a new set of genes. To explore the possibility that other transcriptional pathways besides NFkB might be involved in the ROS increase, transcription and translation were blocked by using their specific inhibitors before visfatin treatment. This showed that the levels of ROS produced after 18 hours were reduced with almost 70% compared to the cells treated only with visfatin (Fig.6.8). Therefore, it can be concluded that there are other pathways mediating the stress responses induced by visfatin besides NFkB, unknown yet.

8.2.4.4 Visfatin (eNampt) treatment and inflammatory signals

NFkB-dependent signalling can increase the expression levels of many genes that could further induce a positive loop in ROS production such as nitric oxide synthases (Taylor et al., 1998), TNF-α (Wullaert et al., 2006) and IFN-γ (Briscoe et al., 2006; Malmberg et al., 2001). Nitric oxide is an important mediator of stress responses in skeletal muscle, being synthesized by nitric oxide synthases (Mungrue et al., 2003). The dominant isoform in skeletal muscle is the neuronal type (nNOS) that has higher levels of expression in myocytes than the inducible (iNOS) and endothelial (eNOS) forms, and is localized mainly beneath the sarcolemma (Grozdanovic, 2001). An increased expression of nitric oxide synthases can lead therefore to higher levels of nitric oxide that could be further converted to peroxynitrate by the reaction with superoxide anion, generating additional ROS (Yang et al., 1998). RT-PCR assay showed that the expression level of nitric oxide synthase 1 (NOS1) was only moderately increased. The fact that I did not detect a significant increase in the levels of nitrotyrosine residues after 24 hours of incubation of C2C12 cells with visfatin suggested nevertheless that there was no significant nitric stress produced (Fig. 4.9). There are also previous reports indicating a positive association of higher visfatin plasma levels with increased TNF-α expression and inflammatory phenotypes (Erten et al., 2008; Samara et al., 2008). TNF-α treatment was also proved to lead to a further increase in ROS levels in gastric cells (O'Hara et al., 2009), in the arteries of diabetic mice models (Gao et al., 2008) and in the

intestinal epithelial cells (Jin et al., 2008). Interestingly, TNF-α is expressed by skeletal muscle and although blocking TNF-α by using monoclonal antibody-based therapy reduces oxidative stress and necrosis levels in muscular dystrophy animal models (Grounds and Torrisi, 2004), the specific deletion of TNF-α in skeletal muscle impaired nevertheless the regeneration ability of skeletal myocytes (Collins and Grounds, 2001), possibly through a reduced expression of myogenic differentiation protein 1 (MyoD) (Warren et al., 2002). A similar pattern was identified also in the case of IFN-γ (Yoo et al., 2008; Higuchi et al., 1997). For example, while IFN-γ treatment induced ROS production in cultured keratinocytes (Qi et al., 2011) and microglial cells (Jung et al., 2010), the reduction of IFN-γ expression in skeletal muscle was nevertheless associated with an impaired regeneration capability of cultured skeletal myocytes (Cheng et al., 2008). RT-PCR assays showed that expression levels of these two known mediators of oxidative stress production such as TNF-α and IFN-γ were in fact reduced. This shows that ROS production is not mediated by an increased expression of TNF-α or IFN-γ. The relevance of such a decrease for the lack of oxidized products upon visfatin challenge is unknown yet.

Since NOX4 is also a NFkB target (Moe et al., 2006; Basuroy et al., 2009; Park et al., 2006) and, as shown in the fifth chapter, its gene expression is increased by visfatin treatment (Fig.5.5), the activation of NFkB was blocked for one hour prior visfatin challenge for another 24 hours. RT-PCR assay showed that NOX4 gene expression was reduced in cells pre-treated with IKK inhibitor followed by visfatin challenge with cca. 50% compared to visfatin-only treated cells (Fig.6.7). This indicates that there are other pathways mediating NOX4 gene transcription besides NFkB, unknown yet. The precise role of NOX4 and NOX complexes in the generation of oxidative stress in the case of skeletal muscle represents a recent discovery and its elucidation will require further studies. Whether this reduction in NOX4 expression levels upon NFkB inhibition is responsible for the reduction in oxidative stress noticed is still unknown. It was noticed also an increased expression of VEGFa gene upon exogenous visfatin stimulation, another target gene of

NFkB. Increased expression of VEGFa was previously associated with decreased antioxidant defence in vitreous fluid in the case of diabetic patients (Yokoi et al., 2005). It was also shown that VEGFa expression was increased in the serum and skeletal muscle samples of patients with inflammatory diseases affecting skeletal muscle such as polymyositis and dermatomyositis (Grundtman et al., 2008). The precise mechanisms possibly affected by increased expression of VEGFa in skeletal muscle are not clear still.

Other known cytokines such as IL-6 and IL-15 were also increased upon visfatin challenge of C2C12 cells. Increased IL-6 expression was previously associated with a higher level of oxidative stress in brain (Penkowa and Hidalgo, 2000), alveolar epithelia (Haddad et al., 2001), macrophages (Chang et al., 2010a) and vascular cells (Szeto et al., 2008). IL-6 was reported also to be secreted by skeletal myocytes as a genuine myokine (Pedersen and Febbraio, 2008). Nevertheless, the relevance of such muscle-specific secretion of IL-6 for the skeletal muscle stress responses and inflammation is still contentious and highly debated. It is established at least that skeletal muscle-specific IL-6 has a significant role in regulation of fatty acid oxidation and glucose metabolism, especially during endurance exercise (Nielsen and Pedersen, 2007). Furthermore, changes in IL-6 expression have a major impact on the regulation of skeletal mass (Frost et al., 2004). It appears therefore that while plasma level of IL-6 is associated with a pro-oxidant state, as a myokine IL-6 is correlated nevertheless with an increased level of antioxidant defence in skeletal muscle (Sacheck et al., 2006). IL-15 is also a myokine with a clear role in the regulation of adipose tissue mass and inflammation (Almendro et al., 2008; Nielsen and Pedersen, 2007) which was increased by exogenous visfatin challenge. It was shown so far that IL-15 has a lower expression during ageing and ageing-associated diseases (Marzetti et al., 2009a) and that reduced plasma level of IL-15 is associated with reduced expression of glutathione and increased oxidative stress in obese patients (Di Renzo et al., 2010). The relevance of IL-15 in the context of skeletal muscle is currently unknown, besides the fact of being broadly regulated by inflammatory signals and stress

inducers (Perera, 2000). Whether such an increase in IL-6 and IL-15 cytokines is involved in ROS production or antioxidant defence upon visfatin challenge in C2C12 cells is still unclear.

Interestingly, a previous report (Adya et al., 2008a) showed that while visfatin treatment increased NFkB activity in human endothelial cells, it was able nevertheless to reduce an additional NFkB activation upon TNF-α challenge. These data suggest that the relationships between visfatin and NFkB activation are not linear and only partially understood. The complex effects of visfatin on NFkB signalling indicate that in different tissues or pathologic conditions it has diverse and versatile immunomodulatory properties, only partially mapped and explored. As in the case of PPAR-β/δ during acute HLS discussed above, visfatin could act also as a complex *switch* between pro/antioxidative stress forces or between pro- and anti-inflammatory signalling pathways affecting skeletal muscle. This is nevertheless a hypothetical scenario that will require further studies.

Another hypothesis to explain the effects of extracellular form of visfatin on cellular stress is based on the putative synthesis of NMN from nicotinamide and 5-phosphoribosyl 1-pyrophosphate in the plasma or in the extracellular space, not only in the intracellular space. Once synthesized by eNampt in extracellular space, NMN could be transported across plasma membrane through specialized transporters (Revollo et al., 2007) and generate ROS as a consequence of an increase in NAD synthesis as proposed (Romacho et al., 2009). Since nicotinamide is present in the DMEM culture medium used for C2C12 differentiation, the possibility attracted considerable attention. Nevertheless, the hypothesis of visfatin as a systemic enzyme was rejected recently (Hara et al., 2011), based on the fact that the reaction requires the presence of ATP at miliomolar levels, and culture medium used lacks ATP.

8.2.5 Visfatin (eNampt) treatment and PPARs

In the seventh chapter I showed that the activation of PPAR-β/δ-dependent signalling reduced the ROS levels induced by visfatin. Diverse studies performed *in vitro*, *ex vivo* and *in vivo* (animal models or human subjects) already proved that the activation of PPAR-β/δ signalling pathway has antioxidant properties in multiple tissues. Treatment of cultured cardiomyoblasts with GW-501516 conferred protection against oxidative stress-induced apoptosis through the upregulation of protective genes such as catalase (Pesant et al., 2006). The role of PPAR-β/δ as a sensor of oxidative stress levels was further proved by the fact that the activity of the receptor was increased in the hepatocytes treated with the compound 4-hydroxynonenal (4-HNE), a lipid species that induces oxidative stress (Coleman et al., 2007). In addition, in the same paper it was reported that activation of PPAR-β/δ in hepatocytes by the agonist GW-9662 conferred protection and resistance against the lipid-dependent toxicity. The repetitive low-level oxidative stress challenge by using hydrogen peroxide activated PPAR-β/δ activity in cultured endothelial cells, protecting against a critical subsequent oxidative stress-mediated apoptotic event (Jiang et al., 2009). In vascular endothelium the activation of PPAR-β/δ by GW-501516 increased the expression levels of hem oxygenase-1 (HO-1), reducing further the levels of oxidative stress induced by an additional treatment with hydrogen peroxide and overall the atherogenic phenotype (Ali et al., 2010). Some of the genes relevant for antioxidant defence such as carnitine palmitoyltransferase-1 (CPT-1b) and pyruvate dehydrogenase kinase 4 (PDK4) were shown to be upregulated by GW-501516 (Coll et al., 2010). An increase in the expression of these two genes and of PPAR-β/δ was also identified in the acute phase of hindlimb suspension, as discussed in the third chapter. In endothelial cells it was shown that the activation of PPAR-β/δ by GW-501516 increased the expression of many crucial antioxidant genes such as catalase, superoxide dismutase 1 and thioredoxin (Fan et al., 2008). Furthermore, in human primary skeletal cells GW-501516 treatment increased expression level of UCP-3 gene that has antioxidant effects (Terada et al.,

2006). These reports suggest that activation of PPAR-β/δ in C2C12 cells reduced the oxidative stress levels induced by exogenous visfatin likely by improving the pre-existent antioxidant defence ability to resist the stress, rather than directly interfering with visfatin-induced effects.

The nexus role of PPAR-β/δ as anti-inflammatory mediator was shown by the fact that while the administration of GW-501516 was able to reduce the inflammation in a T-cell mediated autoimmune disease model of multiple sclerosis (Kanakasabai et al., 2010), it was nevertheless ineffective in a case of an antibody-induced demyelination that acts independently of inflammation (Defaux et al., 2009). Furthermore, GW-501516 ameliorated the insulin resistance induced by the treatment of cultured skeletal myotubes with palmitate by modifying the expression levels of genes involved in the fatty acid oxidation, AMPK phosphorylation and antioxidant defence (Coll et al., 2010). Activation of PPAR-β/δ had also anti-inflammatory effects in cultured keratinocytes upon UV-B exposure and in skin psoriasis models (Rivier et al., 1998). Nevertheless, as shown by Fig. 7.3, administration of the agonist GW-501516 failed to reduce in C2C12 cells the activation of NFkB signalling pathway by exogenous visfatin treatment. This is not surprising since it was already shown that the pre-treatment with GW-501516 agonist reduced the activation of NFkB by LPS in cultured adipocytes by blocking the upstream activation of ERK ½ kinase (Rodriguez-Calvo et al., 2008), activation that was missing in my case. This shows that the reduction in ROS levels by GW-501516 is occurring independently of the inhibition of NFkB-signalling. This suggests also that NFkB activation occurs independently of pre-existing reactive oxygen species since PPAR-β/δ treatment did not reduce its activation. This also correlates with the fact that inhibition of NFkB reduced only partially NOX4 levels induced by visfatin (Fig. 6.7).

It appears also that visfatin expression and secretion levels can be modulated by PPARs agonists in tissues of interests for ageing research such as skeletal muscle, adipose tissue and immune cells (Choi et al., 2005). The roles of exogenous visfatin in the modulation of skeletal muscle physiology have not been directly addressed until our published paper (Oita et al., 2010).

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As discussed in detail in the fourth chapter, there are likely differences between the intracellular form (iNampt) and the extracellular form of visfatin (eNampt) in this respect, especially in the case of oxidative stress and inflammatory regulatory mechanisms relevant for ageing. For example, it was shown that free, circulating visfatin plasma levels were reduced in overweight women subjected to caloric restriction, simultaneously with a reduction in body weight and adipose tissue mass (Lee et al., 2010). The intracellular form of visfatin is also crucial for the anti-ageing effects triggered by activation of sirtuins in adipocytes or in skeletal muscle upon caloric restriction challenge through the modulation of NAD metabolism (Hsu et al., 2008; Imai, 2009b; Lu et al., 2009b).

By using an ELISA assay I showed that visfatin is not secreted in the culture medium of C2C12 cells in neither basal nor in GW-501516-stimulated conditions. Why visfatin was detected in the supernatant of cultured L6 rat myocytes (Wang et al., 2010) and not in the supernatant of C2C12 mouse myocytes (Fig. 7.4) is a question unanswered yet, being a subject for future studies. A possible explanation could be the fact that in the previously reported experiment with rat L6 cells the culture medium was taken after 3 days of incubation, an interval that partially reflected the process of L6 differentiation from proliferating myoblasts to differentiated and fused myotubes in low-level serum culture medium. In my experiments C2C12 cells have been used only after reaching full differentiation status in serum-free medium. Therefore, it is possible that visfatin could be involved in the process of differentiation and be secreted only at the myoblast stage, or during the transition process from myoblasts to myotubes. Nevertheless, since the focus of the present thesis was the effect of visfatin on differentiated skeletal muscle as a model of ageing, the possibility that visfatin could be detected in culture medium of proliferating C2C12 myoblasts instead of differentiated C2C12 myotubes was not explored.

Therefore, there are no indications so far that exogenous visfatin challenge could have an effect on PPARs expression in adult skeletal muscle, and no indication that the activation

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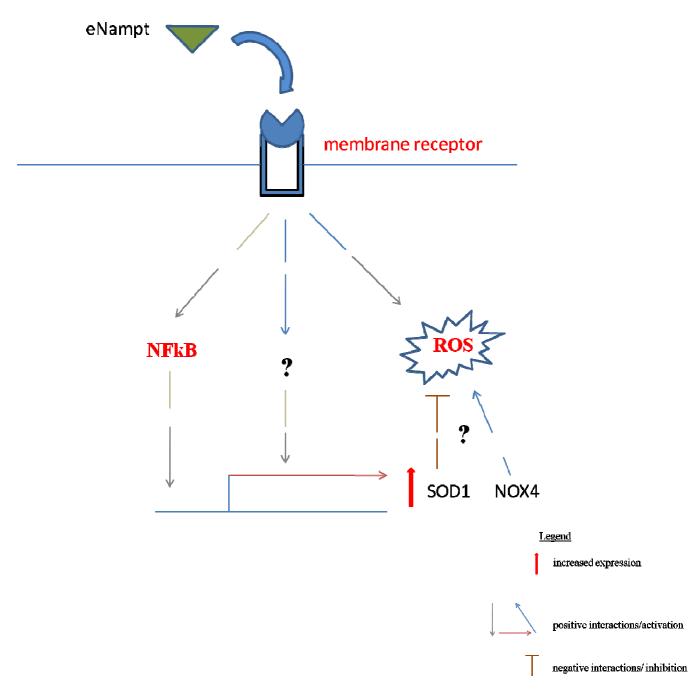
of PPAR-β/δ induced a secretion of visfatin from skeletal muscle as a myokine. Whether visfatin is a genuine myokine is still debated, and in my case it was not discovered in the conditioned medium from C2C12 differentiated and fused myotubes. Activation of PPAR-β/δ reduced the oxidative stress produced by visfatin, likely through the upregulation of its known target genes that increased the antioxidant defence status in cultured myocytes, but it did not affect directly the activation of NFkB.

8.3 Visfatin- a promising and intriguing adipokine relevant for skeletal muscle research

In conclusion, although exogenous visfatin treatment did not induce an insulin-mimetic effect in cultured myotubes, it triggered nevertheless an increase in oxidative stress levels and inflammation after a chronic stimulation. The fact that the oxidative stress induced was different from the pattern generated by ectopic lipid accumulation suggests that it harbours still interesting unknown aspects. As seen from the model depicted in Fig. 8.3, visfatin could increase simultaneously both the levels of free radicals through the action of prooxidant enzymes such as NOX4 and the antioxidant ability to resist such as stress through the increase in expression of enzymes such as SOD1. As such, visfatin research could be used to construct a useful model for ***testing*** the theoretical predictions of the ***free radical theory of ageing*** described in the introduction since it has versatile effects, being in different tissues pro- or antioxidant, pro- or anti-inflammatory, or being associated with insulin resistance or insulin sensitivity. The **next** crucial step in elucidating visfatin function will be the identification of its putative membrane receptor(s) that mediates its effects, as well as the downstream signalling pathways. Whether the insulin-mimetic effects in non-muscle cells are mediated by the *same* membrane receptor that induces ROS and inflammation is another important question to be approached.

Fig. 8.3 Schematic diagram of the proposed mechanism of action of visfatin (eNampt) on skeletal muscle myocytes

Visfatin binds to a membrane receptor expressed by skeletal muscle (unidentified yet) and triggers and increase in ROS levels, process partially dependent on NFkB-signalling pathway. Simultaneously, visfatin increases the expression levels of genes involved in antioxidant defence such as SOD1 and of genes that could mediate the increase in ROS levels such as NOX4.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1- The legend of the IPA network images