THE EFFECTS OF INFLAMMATION ON THE VASCULAR RESPONSES TO ACUTE MENTAL STRESS

By

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ABSTRACT

Evidence exists for the role of acute mental stress as a trigger for myocardial infarction. Despite the fact that the underlying mechanisms are not fully understood, inflammation and the vascular responses to stress are two mechanisms which have been implicated. After a critical analysis of the methods used to measure the vascular responses to acute mental stress (Chapter 2), this thesis examined the effects of acute inflammation on resting cardiac and mood measures (Chapter 3), and on the vascular responses to stress in a healthy population (Chapters 4 - 6). Two different protocols (vaccination and eccentric exercise) induced inflammation. Inflammation did not alter resting cardiac function or mood (Chapter 3). Despite no alteration in resting vascular function, acute inflammation did attenuate stress-induced vasodilation (Chapter 4 and 6). The effects of inflammation on stress-induced vasodilation were more prominent at the site of inflammation, indicating a localised impact of inflammation on stress-induced vasodilation. These findings suggest a possible interaction between inflammation and the vascular responses to mental stress, which could be a mechanism for the triggering of a myocardial infarction through mental stress. Further work is needed to identify the exact mechanisms through which this attenuation occurs, with a view to enhancing the vascular responses to stress in chronically inflamed populations.

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LIST OF PAPERS AND ABSTRACTS

This thesis incorporates the four following papers, which corresponding to four of the five empirical studies:

- **Paine N.J.,** Ring C., Bosch J.A., McIntyre D., & Veldhuijzen van Zanten J.J.C.S The Effect of Acute Mental Stress on Limb Vasodilation is unrelated to Total Peripheral Resistance (In submission)
- **Paine N.J.,** Ring C., Bosch J.A., Drayson M.T., & Veldhuijzen van Zanten J.J.C.S. The time course inflammatory response to *Salmonella typhi* vaccination (In submission)
- **Paine N.J.,** Ring C., Aldred, S., Bosch J.A., Drayson M.T., & Veldhuijzen van Zanten J.J.C.S. Vaccine-induced inflammation impairs the vasodilatory response to mental stress (In submission)
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In addition, the following abstracts arose from presentations of material from this thesis

- **Paine N.J.,** Bosch J.A., McIntyre D., Ring C., & Veldhuijzen van Zanten J.J.C.S. (2012a). Effects of Mental Stress on Peripheral Vascular Resistance is unrelated to Systemic Vascular Resistance. *Psychosom.Med.* **74[3]**, A-57.

- **Paine N.J.,** Bosch J.A., Aldred S., Ring C., Drayson M, & Veldhuijzen van Zanten J.J.C.S. (2012b). The Effect of Inflammation on Vascular Responses to Mental Stress. *Psychosom.Med.* **74[3]**, A-67.
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- **Paine N.J.,** Bosch J.A., & Veldhuijzen van Zanten J.J.C.S. (2012). Inflammation and Vascular Responses to Acute Mental Stress: Implications for the Triggering of Myocardial Infarction. *Current Pharmaceutical Design* **18**, 1494-1501.
- Edwards K.M., Campbell J.P., Ring C., Drayson M.T., Bosch J.A., Downes C., Long J.E., Lumb J.A., Merry A., **Paine N.J.,** & Burns V.E. (2010). Exercise intensity does not influence the efficacy of eccentric exercise as a behavioural adjuvant to vaccination. *Brain, Behavior, and Immunity* **24**, 623-630.

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LIST OF ABBREVIATIONS

MI – Myocardial Infarction

MSII – Mental Stress Induced Ischemia

CVD – Cardiovascular Disease

RA – Rheumatoid Arthritis

CAD – Coronary Artery Disease

BH_4 – tetrahydrobiopterin

BH_2 - dihydrobiopterin

NO – Nitric Oxide

eNOS – endothelial nitric oxide synthase

nNOS – neuronal nitric oxide synthase

iNOS – inducible nitric oxide synthase

L-NMMA - NG-monomethyl-L-arginine

SMTC - S-methyl-l-thiocitrulline

IL- - interleukin

TNF-α – tumor necrosis factor alpha

CRP – C - reactive protein

WBC – white blood cells

GR - granulocytes

CK – creatine kinase

VO_2 Max – maximal oxygen uptake

HR – heart rate

PEP – pre-ejection period

HRV – heart rate variability

rMSSD - root mean square successive Difference

CO – cardiac output

SV – stroke volume

TPR – total peripheral resistance

SBP – systolic blood pressure

DBP – diastolic blood pressure

MAP – mean arterial blood pressure

FBF – forearm blood flow

FVR – forearm vascular resistance

FVC – forearm vascular conductance

CBF – calf blood flow

CVR – calf vascular resistance

CVC – calf vascular conductance

β-adrenergic – beta-adrenergic

α-adrenergic – alpha-adrenergic

SNS – sympathetic nervous system

CHAPTER ONE:

GENERAL INTRODUCTION

1.1 CARDIOVASCULAR DISEASE AND ACUTE MENTAL STRESS AS A TRIGGER FOR MYOCARDIAL INFARCTION

Cardiovascular Disease (CVD) is the most reported cause of death in the United Kingdom with a estimated cost to the economy of approximately £30 billion per year (Scarborough *et al.*, 2010). Myocardial infarction (MI) is a form of CVD, with an annual incidence of approximately 124,000 new MI cases in the UK (Scarborough *et al.*, 2010). Both the effects of chronic stress and acute stress have been explored in the context of CVD; however, the focus of this thesis will be on physiological responses to acute mental stress. Mental stress can be defined as 'events or situations that challenge a person's psychological and/or physiological homeostasis' (pg 3, Carroll, 1992).

The effects of acute mental stress are observable in multiple physiological systems. The physiological features of stress that have been researched intensively include the autonomic nervous system (ANS) and hypothalamus-pituitary-adrenal (HPA) axis (Hellhammer & Schubert, 2011). ANS and HPA activation is induced by the release of noradrenaline in the limbic areas, such as the hippocampus and the amygdale via local release of corticotrophin releasing hormone (CRH) (Chrousos, 2007). Central activation is rapidly translated into enhanced activation of the aforementioned peripheral neuroendocrine systems as well as other central arousal systems (e.g., locus coerulus) (Chrousos, 2007). Such neuro-endocrine perturbations affect the activity of multiple organ systems, including the cardiovascular system (McEwen, 2007), the metabolic system and the immune system (Glaser & Kiecolt-Glaser, 2005). For example, stress reliably causes increases in blood pressure (Obrist, 1981; Turner, 1994), which can be driven by increased total peripheral resistance (TPR) or cardiac output (CO) (Turner, 1994). Likewise, glucocorticoid elevations that result

from HPA activation influence the setpoint of inflammatory responses, e.g., by up-regulation of receptors for inflammatory cytokines on endothelial cells. For a more detailed description of such neuro-endocrine interactions see reviews (Black & Garbutt, 2002; Chrousos, 2009; McEwen, 2007).

Anecdotal evidence and epidemiological studies have identified mental stress as a trigger for MI (Strike & Steptoe, 2005). Survivors of MI have reported emotional stress to have preceded their MI (Newby, 2010; Strike & Steptoe, 2005). For example, feelings of anger (Mittleman *et al.*, 1995; Möller *et al.*, 1999) and emotional upset have been reported prior to an MI (Tofler *et al.*, 1990). In line with this, stressful natural disasters coincide with increases in MI incidence. The incidence of MI has been examined in relation to the start of war or terrorist attacks. Increases in MI were observed during periods of missile attacks in Tel Aviv during the Gulf war (Meisel *et al.*, 1991) and in Zagreb during the war in Croatia (Bergovec *et al.*, 1992). However, no increases in the incidence of or mortality as a result of MI were observed after the terrorist attacks in New York City on September 11 2001 (Chi *et al.*, 2003a; Chi *et al.*, 2003b).

Similarly, the prevalence of an earthquake also is linked to increases in MI, as demonstrated by a variety of earthquakes. In the three days after the Athens earthquake of 1981, an increase in the number of cardiac related deaths was reported, with no alterations in the number of deaths from other causes were observed in relation to a control period (Trichopoulos *et al.*, 1983). Likewise, increases in MI admissions have been associated with incidence of the Hanshin-Awaji earthquake in Japan (Suzuki *et al.*, 1995) and the Northridge earthquake in Los Angeles (Leor *et al.*, 1996a; Leor *et al.*, 1996b). Despite the increases in MI

observed on the day of the Northridge earthquake, a decrease in the following days was observed in comparison to those days in previous years (Kloner *et al.*, 1997). Interestingly, examination of the number of admissions in relation to the proximity to the earthquake epicentre revealed bigger increases were observed in the MI admissions that were closer to the epicentre of the earthquake (and therefore, where the stress stimuli may have been stronger) (Leor *et al.*, 1996a).

In addition to natural disasters, losing important sporting events has been linked to MI. MI incidence and mortality amongst Dutch men and women was higher on the day the Dutch national football team lost a penalty shoot out in comparison to the other days examined (Witte *et al.*, 2000). Similar effects were observed in the number of MI admission during and for 2 days following the English national football team losing a penalty shoot out to Argentina at the 1998 Football World Cup (Carroll *et al.*, 2002), with a 25% increase in MI admissions during the 48 hour period following the match, with no increases in admissions for stroke, road traffic related injuries, self-harm or excessive amounts of admission for any other diagnosis (Carroll *et al.*, 2002). This effect is not restricted to national football teams. A study of Northern England football clubs demonstrated that over a 5 year period from 1994-99, the number of deaths from MI amongst men was higher when their local football team lost at home in comparison to losing or winning away from home (Kirkup & Merrick, 2003). However, supporting a successful football team can also have positive benefits, with lower MI-related mortality amongst French men reported on the day France won the Football World Cup (1998), in comparison to control days (Berthier & Boulay, 2003).

Whilst the above provides evidence for an association between stress and MI, they do not inform about the underlying mechanisms through which mental stress can lead to MI. One epidemiological study examined potential mechanisms by exploring serological markers of inflammation and vasoconstriction in those admitted for MI during the Football World Cup. To allow for a comparison between MI during a stressful period and a non-stressful period, this study also examined those admitted for MI during a reference period when the World Cup was not taking place. Inflammatory markers (such as TNF-α), vasoconstrictor endothelin-1 (ET-1), as well as adhesion molecules (such as sVCAM-1) were higher in those who experienced an MI during the World Cup compared to the reference group (Wilbert-Lampen *et al.*, 2010). Thus, these findings from this field-based study suggest a role for inflammation and vascular factors in the triggering processes.

The underlying mechanisms through which mental stress can lead to MI have been explored in more detail in controlled laboratory based studies. In those studies, mental stress-induced myocardial ischaemia is used as a laboratory proxy measure for stress-related MI. In patients with coronary artery disease (CAD), mental stress elicits myocardial ischaemia in up to 89% of patients depending on the sensitivity of the method used to quantify ischaemia (Strike & Steptoe, 2003). Significantly, the incidence of myocardial ischaemia during acute laboratory stress is a predictor of ambulatory ischaemia (Blumenthal *et al.*, 1995) as well as future cardiac events (Babyak *et al.*, 2010; Jain *et al.*, 1995; Krantz *et al.*, 1999; Sheps *et al.*, 2002). One study even provided evidence that mental stress-induced ischaemia is a better predictor for all cause mortality than the results of the commonly used exercise stress test (Sheps *et al.*, 2002).

In line with the epidemiological study, that revealed increased levels of serological vasoconstriction (Wilbert-Lampen *et al.*, 2010), mental stress-induced ischaemia has been associated with poorer peripheral vascular responses to mental stress. For example, those patients with CAD who displayed mental stress-induced ischaemia showed greater increases in total peripheral resistance (TPR) during mental stress compared to those without mental stress-induced ischaemia (Goldberg *et al.*, 1996; Jain *et al.*, 1998). Peripheral vasoconstriction when measured by peripheral arterial tonometry has also been observed in CAD patients who displayed mental stress-induced ischaemia (Burg *et al.*, 2009).

In addition to highlighting that vascular factors might have a role in triggering an MI, inflammation was also demonstrated to be a possible mechanism (Wilbert-Lampen *et al.*, 2010). The role for inflammation has also been confirmed in laboratory studies, which revealed that CAD patients who displayed mental stress-induced ischaemia had elevated levels of inflammation (Shah *et al.*, 2006). The appearance of ischemic heart disease has been demonstrated to be greater in patients with rheumatoid arthritis (an inflammatory joint disease) in comparison to patients with osteoarthritis (joint disease not characterised by inflammation) by up to 50% (Banks *et al.*, 2000), further highlighting the links between inflammation and ischaemia. Also, associations have been reported between MI and inflammation in RA patients (Södergren *et al.*, 2007). The link between mental stress-induced ischaemia and high basal inflammation is interesting, particularly as CRP has been reported as a risk factor for developing CVD and MI (Wennberg *et al.*, 2012), as well as predicting coronary heart disease (Emerging Risk Factors Collaboration *et al.*, 2010). In fact, even acute transient increases in inflammation, such as during an acute upper-respiratory tract infection, are linked with a greater risk for heart attacks (Meier *et al.*, 1998). In sum, whilst the

responses to mental stress and its role of as a trigger for MI is well established, the underlying pathways are not well characterised. Two possible pathways are hypothesised to be of particular importance for the triggering of MI; inflammation and the vascular responses to stress.

1.2 INFLAMMATION

Inflammation is an adaptive response of the immune system to infection and tissue damage (Kumar *et al.*, 2003; Majno & Joris, 2004; Medzhitov, 2008). Two characteristic features of this response are rapid infiltration of leukocytes and the release of inflammatory cytokines (Kumar *et al.*, 2003; Majno & Joris, 2004; Medzhitov, 2008). These increases can be perceived both locally at the site of infection or damage, as well as systemically in the circulation. However, whilst acute inflammation is deemed protective (Medzhitov, 2008), dysregulated and subsequently chronic levels of inflammation, which is characteristic of diseases such as rheumatoid arthritis (Lee & Weinblatt, 2001) and CVD (Tracy, 1998), result in systemic inflammation, which could lead to an 'overdrive' in inflammatory markers (Stevens *et al.*, 2005) and is considered harmful (Medzhitov, 2008).

Inflammation has been implicated in the development of atherosclerosis (Libby, 2006). Indeed, chronic inflammation has been implicated in the aetiology of cardiovascular disease (CVD) (Hansson, 2005; Libby, 2006), as well as an increased risk for MI (Levy *et al.*, 2008; Ridker *et al.*, 2000). Systemic inflammation can be serologically assessed in both serum and plasma by several soluble markers that are the causes of or responses to inflammation such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) or C-reactive protein (CRP), as well as increased cell numbers such as white blood cells, granulocytes and neutrophils.

TNF-α is synthesised by a range of immune cells including macrophages and mast cells (Medzhitov, 2008), and has a vital role to play as a mediator of the acute phase inflammatory response (Medzhitov, 2008; Möller & Villiger, 2006). TNF-α can induce adhesion molecules (e.g., ICAM-1, VCAM-1) expression on the vascular wall, and resulting in leukocyte margination at the site of inflammation (Moldoveanu *et al.*, 2001). IL-6 is synthesised in response to elevations in other inflammatory cytokines such as TNF-α (Möller & Villiger, 2006). IL-6 can be produced through a variety of cells, including monocytes, macrophages and endothelial cells (Ekstrom *et al.*, 2008) and is an important mediator of the acute inflammatory phase response (Moldoveanu *et al.*, 2001). The argument to whether IL-6 is pro-inflammatory or anti-inflammatory is ongoing, as IL-6 is secreted in response to pro-inflammatory cytokines such as TNF-α and IL-1 (Möller & Villiger, 2006). However, IL-6 also induces anti-inflammatory effects, such as increasing the production of C-reactive protein (CRP) (Ekstrom *et al.*, 2008; Möller & Villiger, 2006). Therefore the appearance of IL-6 in the blood is often earlier than that of CRP (Zakynthinos & Pappa, 2009). CRP is an acute phase protein which is synthesised by the liver and is a commonly examined indicator of systemic inflammation, as its appearance is indicative of the presence of other pro-inflammatory markers (Pepys & Hirschfield, 2003). CRP has been shown to be associated to the risk of Coronary Heart Disease (Emerging Risk Factors Collaboration *et al.*, 2010) and is often used as an index of chronic inflammation (Libby, 2006).

1.3 THE ENDOTHELIUM

The endothelium is a tissue that lines the whole vascular system, with its cells responsible for controlling factors that affect platelet aggregation, thrombosis and inflammation (Galley & Webster, 2004). When the production of these factors is in balance,

the endothelium is atheroprotective but disruption of this equilibrium results in damaged endothelium (Sandoo *et al.*, 2010). This can then lead to endothelial dysfunction, which is an early indicator of atherosclerosis (Lerman & Zeiher, 2005). Endothelial function is commonly assessed by measuring vasodilatory responses to a standardised stimulus such as sudden increases in blood flow or vasodilatory drugs (e.g., acetylcholine). A healthy endothelium is able to vasodilate in response to such stimuli, whereas an attenuated vasodilatory response is indicative of endothelial dysfunction (Sandoo *et al.*, 2010). The endothelium is responsible for vascular tone (Trepels *et al.*, 2006), such that increases in localised blood flow, shear stress or vascular resistance induce vasodilation in a healthy endothelium (Sandoo *et al.*, 2010). The endothelium regulates vasodilation by releasing nitric oxide (NO), which acts as a direct relaxant to smooth muscle cells (Galley & Webster, 2004). NO can be synthesised through three different isoforms of nitric oxide synthase (NOS): endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Given the greater quantity of research examining the roles of eNOS and nNOS in relation to mental stress, the focus will be on the production of NO through eNOS and nNOS, and how inflammation may alter these substrates.

Figure 1.1 illustrates the processes through which eNOS can result in NO production. Endothelial nitric oxide synthase (eNOS) produces NO by converting L-arginine to L-citrulline (van Zonneveld *et al.*, 2010; Zhang, 2008), and decreases in L-arginine can result in reduced eNOS activation (Darley-Usmar *et al.*, 1995; Förstermann & Münzel, 2006; Morris, 2000). Increases in shear stress up-regulate eNOS (Nishida *et al.*, 1992). The importance of eNOS in vasodilation is demonstrated in studies with eNOS knockout mice, which are incapable to dilate fully in response to shear stress or NO agonists (Gödecke *et al.*, 1998; Huang *et al.*, 1995; Scotland *et al.*, 2002). As shown in Figure 1.1, L-arginine conversion can

only occur in the presence of eNOS cofactor tetrahydrobiopterin (BH_4) (Delp *et al.*, 2008; Sindler *et al.*, 2009). BH_4 is critical to NO production (Cosentino & Katusic, 1995; Pierce & LaRocca, 2008) with adequate levels of BH_4 allowing for the transference of electrons resulting in 'coupled' eNOS, subsequent production of NO and the by-product dihydrobiopterin (BH_2) (Pierce & LaRocca, 2008). However, when BH_4 levels are limited, the electron transfer from eNOS becomes 'uncoupled', such that functional eNOS is converted to superoxide (O_2^-) rather than NO (Bevers *et al.*, 2006; Crabtree *et al.*, 2009; Förstermann & Li, 2011; Schmidt & Alp, 2007). The increases in superoxide can then lead to increases in oxidative stress (Wadley *et al.*, 2012).

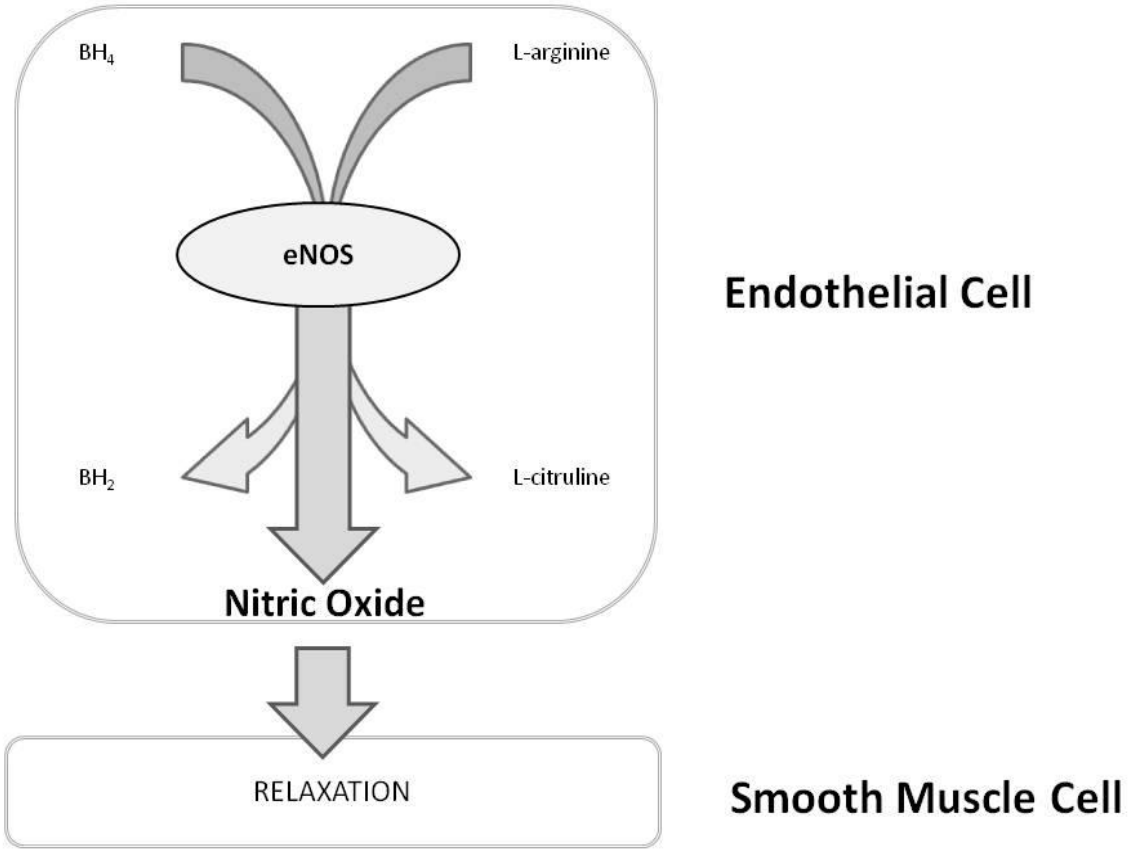

Figure 1.1. Diagram illustrating the production of Nitric Oxide through eNOS activity in the endothelial cell.

1.4 INFLAMMATION AND ENDOTHELIAL FUNCTION

Human *in vivo* studies have assessed the links between inflammation and vascular function. In patients with coronary heart disease (CHD), CRP is associated with an attenuation in NO-dependent blood flow (Sinisalo *et al.*, 2000). Inflammation independently predicted NO bioavailability and was also inversely correlated with L-NMMA induced decreases in resting vascular function (Fichtlscherer *et al.*, 2004a). Inflammation has also been associated with poorer endothelial function in patients with inflammatory disorders, such as rheumatoid arthritis (RA) patients (Vaudo *et al.*, 2004). Decreases in inflammation with medication in patients with cardiogenic shock revealed improvements in endothelial function (Hermansen *et al.*, 2011). Similarly, successful reduction in inflammation through anti-TNF-α treatment in RA patients resulted in an improvement in endothelial function, such that endothelial function in RA patients was similar to healthy control participants (Cardillo *et al.*, 2006). Likewise, anti-TNF-α treatment in RA patients has also resulted in transient improvements in microvascular endothelial-dependent function, which coincided with reductions in HDL cholesterol levels (Sandoo *et al.*, 2012).

While the studies assessing clinical populations are interesting and of merit, the complex nature of the diseases often makes it difficult to ascertain the independent impact of inflammation. For example, in a study by Vita and colleagues, associations were demonstrated between inflammatory markers and endothelial function, but the associations were no longer seen after controlling for age, gender and traditional cardiovascular risk factors (Vita *et al.*, 2004). Likewise, associations have been weakened when factors such as age, gender and lipid concentration are accounted for in the analysis (Cleland *et al.*, 2000). These particular studies suggest that additional factors which are associated with both

inflammation and endothelial function may be responsible for the relationships observed. Therefore, given that NO is critical to maintaining and regulating vascular function, studies have examined the relationship between inflammation and NO *in vitro* to eliminate these confounding factors that appear in clinical human populations. Additionally, administering an inflammatory stimulus and examining vascular function in a healthy population is an alternative method that has been utilised.

1.5 THE RELATIONSHIP BETWEEN INFLAMMATION AND NITRIC OXIDE *IN VITRO*

In vitro studies have revealed an association between inflammation and nitric oxide (NO) parameters through its effect on NOS activity. This *in vitro* relationship may explain the link between inflammation and impaired endothelial function *in vivo* (Hung *et al.*, 2010). It appears that the association between inflammation and NO is reciprocal. For example, NO reduces the activity levels of nuclear factor kappa beta (NF-κβ) (an early step in the inflammatory cascade) (van Zonneveld *et al.*, 2010), as well as subsequent production of inflammatory markers such as TNF-α (De Martin *et al.*, 2000). In a healthy endothelium, NO prevents exaggerated leukocyte adhesion, with reductions in NO resulting in increases in leukocyte adhesion *in vitro* (Kubes *et al.*, 1991) and in animals (Gidday *et al.*, 1998). Conversely, inflammatory cytokines can induce a decrease in NO levels by either reducing NO production through reductions in eNOS activation (Goodwin *et al.*, 2007), increased eNOS uncoupling (Rabelink & van Zonneveld, 2006) or enhancing NO removal (Gao *et al.*, 2007). TNF-α is particularly important as it is responsible for decreasing the availability of L-arginine and therefore suppressing the capacity of eNOS to produce NO (Goodwin *et al.*, 2007). Indeed, a reduction in TNF-α levels in mice resulted in an increase in NO dependent dilation (Gao *et al.*, 2007). Inflammatory markers, such as IL-6, can also inhibit eNOS

activation as demonstrated in human endothelial cells (Hung *et al.*, 2010). The increases in IL-6 result in elevations in the levels of caveolin-1, which results in decreases eNOS activation by reducing Ser1177 phosphorylation (Hung *et al.*, 2010). Similarly, acute phase proteins, such as CRP, reduced NO production in endothelial cells through the down-regulation of eNOS (Verma *et al.*, 2002). In addition, inflammation increases oxidative stress, which in turn reduces BH_4 availability (Cosentino & Luscher, 1999; Vásquez-Vivar *et al.*, 1998), and therefore eNOS uncoupling. As reduced BH_4 results in eNOS uncoupling and consequently reduced NO, an incapacity to produce BH_4, can lead to a worsening effect of inflammation on vascular function *in vitro* as well as *in vivo* (Antoniades *et al.*, 2011). Increases in inflammation can also lead to increases in oxidative stress, with systemic inflammation has resulting in increased generation of reactive oxidative species (ROS) in the vascular wall (Forstermann, 2008) which would result in increases in direct scavenging (Gao *et al.*, 2007). However, despite the strong evidence *in vitro* supporting the links between inflammation and NO, human studies have explored if the relationship between inflammation and endothelial function is also evident *in vivo*.

1.6 THE RELATIONSHIP BETWEEN INFLAMMATION AND NITRIC OXIDE *IN VIVO*

One of the earliest studies in this field used a direct infusion of a combination of inflammatory cytokines such as TNF-α, IL-6 and IL-1. This infusion of IL-6, TNF-α and IL1-beta for one hour was used to increase levels of these inflammatory cytokines (Bhagat & Vallance, 1997). Participants received either each cytokine individually or a combination of these cytokines to assess their impact upon endothelial function. Interestingly, it appeared that the combination of TNF-α, IL-6 and IL-1 produced the most prolonged endothelial

dysfunction in comparison to single administration of TNF-α, IL-6 and IL-1. This study is of particular interest as it highlighted the temporary and transient nature of the impact of inflammation, as administration of a high dose of aspirin 2 hours prior to cytokine administration prevented endothelial dysfunction in response to cytokine administration (Bhagat & Vallance, 1997). Unfortunately, this study did not take a direct measure of inflammation when assessing endothelial function, and therefore it is not known how substantial the increases in inflammation were. Others have used endotoxin administration as a method to examine the impact of inflammation on endothelial function. Endotoxin administration 1 hour prior to endothelial function assessment resulted in endothelial dysfunction, but again, no direct assessment of inflammation was taken, such that the exact levels of inflammation induced remain unknown (Bhagat *et al.*, 1996).

The most commonly used inflammatory stimulus to examine the effects of inflammation on endothelial function is the *Salmonella typhi* Capsular Polysaccharide vaccine, with a transient attenuation in vascular function seen up to 8 hours post vaccination (Antoniades *et al.*, 2011; Hingorani *et al.*, 2000). Similarly, healthy participants experienced a temporary blunting in vascular function 8h post vaccination when participants were in a state of reduced NO (achieved as a result of administration of NO inhibitor L-NMMA), which was not evident in response to a non-NO driven vasodilatory stimulus, indicating that inflammation may have a key role in NO induced dilation (Clapp *et al.*, 2004). Nevertheless, it should be noted that others have not demonstrated endothelial dysfunction after vaccination administration, despite increases in inflammation (Chia *et al.*, 2003). Interestingly, increases in CRP have been demonstrated at 32h post vaccination, but were not associated with the observed endothelial dysfunction (Clapp *et al.*, 2005). The reversible nature of the effects of

acute inflammation on endothelial function has also been demonstrated through administration of oral aspirin two hours prior to vaccination administration. Those who did not receive aspirin revealed endothelial dysfunction in response to typhoid vaccination, whereas those who did receive aspirin demonstrated endothelial function comparable to pre-vaccination levels (Kharbanda *et al.*, 2002). Interestingly, in addition to the effects of inflammation on endothelial function, acute vaccine-induced inflammation has also negatively impacted on large-artery stiffness (Vlachopoulos *et al.*, 2005).

In sum, inflammation is known to affect endothelial function in populations with chronic inflammation, as well as in response to an acute inflammatory stimulus. These findings could be expected, given that poor vascular function and inflammation are important prognostic markers for future acute coronary events (Fichtlscherer *et al.*, 2004b). NO is important for regulating vascular tone at rest (Galley & Webster, 2004; Sandoo *et al.*, 2010), and increases in inflammation decrease NO bioavailability, leading to endothelial dysfunction (Clapp *et al.*, 2004; Trepels *et al.*, 2006).

1.7 THE VASCULAR RESPONSES TO ACUTE MENTAL STRESS

As described previously, mental stress induces a variety of physiological changes; the following will describe the response to mental stress in the vasculature, which have been assessed using a variety of methods, including coronary assessments and peripheral vascular responses. Mental stress elicits increases in both coronary and peripheral blood flow. Increases in myocardial blood flow have been demonstrated in a healthy population (Schoder *et al.*, 2000). Healthy smooth coronary arteries dilate in response to mental stress to accommodate increases in cardiac demand (Dakak *et al.*, 1995), but stenosed arteries either

fail to dilate (Dakak *et al.*, 1995), or vasoconstrict (Yeung *et al.*, 1991). In patients with CAD, coronary blood flow was attenuated in response to mental stress in comparison to non-CAD patients (Kop *et al.*, 2001). In contrast to earlier studies, this study also reported that non-stenotic arteries constricted in response to stress, which was not evident in stenotic arteries (Kop *et al.*, 2001). These coronary assessments involve invasive measurements which carry substantial risk, whereas the peripheral circulation is more accessible for assessment. The associations between coronary function and peripheral vascular responses have been explored both at rest and in response to stress. At rest, vasodilatory responses in the brachial artery have been demonstrated to reflect coronary responses to acetylcholine in CAD patients (Takase *et al.*, 1998; Takase *et al.*, 2005) as well as in a healthy population (Anderson *et al.*, 1995). This finding has been replicated in patients with angiographically normal coronary arteries, where brachial endothelial function was associated with coronary endothelial function in response to acetylcholine (Teragawa *et al.*, 2005). The vasodilatory response of the brachial artery to acetylcholine could even be used as a surrogate predictor for coronary endothelial function in those with suspected CAD (Takase *et al.*, 2006). Likewise, coronary circulation is also associated with resting skin blood flow in healthy populations (Khan *et al.*, 2008). Peripheral vascular responses to stress have also been linked to coronary function. As already mentioned before, CAD patients who displayed mental stress induced ischaemia have also demonstrated in increases in vascular resistance during stress (Goldberg *et al.*, 1996; Jain *et al.*, 1998) as well as attenuated peripheral dilation (Burg *et al.*, 2009). As these studies suggest an association between the coronary and peripheral responses at rest and during stress, it seems justified to assess the peripheral responses to stress as an indicator of coronary artery function during stress.

1.7.1 PERIPHERAL BLOOD FLOW RESPONSES TO MENTAL STRESS

The most common peripheral assessment of vascular function during stress is forearm blood flow (FBF). Mental stress reliably induces an increase in FBF in healthy participants (Joyner & Dietz, 2003; Joyner & Halliwill, 2000; Klein *et al.*, 2010; Pike *et al.*, 2009), with increases to up to 10 times compared to resting blood flow (Blair *et al.*, 1959; Dietz *et al.*, 1994). These increases are also reported immediately post stress (Huang *et al.*, 2010) and can be influenced by posture (Kuipers *et al.*, 2008). As well as increases in FBF in response to mental stress changes in forearm vascular resistance are often seen, with decreases in forearm vascular resistance (FVR) indicative of vasodilation (Carter *et al.*, 2005a). In comparison, attenuations in stress-induced blood flow are found in populations at risk for MI. For example, obese participants (Hamer *et al.*, 2007) and patients with heart failure (Middlekauff *et al.*, 1997) display an attenuation in FBF during stress, whereas other reports of patients with heart failure (Santos *et al.*, 2005) or cardiogenic shock (Hermansen *et al.*, 2011) found no change in FBF during stress.

Several mechanisms have been suggested to be responsible for the vasodilation of the vasculature during mental stress which may, either independently or in collaboration, be responsible for increases in blood flow during stress. Early work examined the role of nerve activation and mechanisms in stress-induced increases in blood flow (Blair *et al.*, 1959). This study concluded that cholinergic vasodilator nerves contributed towards stress-induced vasodilation, as nerve blockade resulted in reduced vasodilation (Blair *et al.*, 1959). Other studies have explored the role of sympathetic nerve activity on blood flow by directly measuring muscle nerve activity. These studies showed no increases in muscle sympathetic

nerve activity in the forearm in response to mental stress (Andersen *et al.*, 1987; Carter *et al.*, 2005b; Carter *et al.*, 2008), and thus may not be a mechanism for stress-induced vasodilation.

The effect of blocking regional adrenergic receptors has also been explored. Early work found stress-induced vasodilation to be unaltered by blocking local alpha (α)-adrenergic receptors, but was attenuated through a stellate ganglion blockade when administered either locally or via surgical sympathectomy (Barcroft *et al.*, 1960; Blair *et al.*, 1959; Blair *et al.*, 1960). More recent work also supports the notion that stress-induced vasodilation is unaffected by blockade of the α-adrenergic receptors (Halliwill *et al.*, 1997). Interestingly, this study also assessed the influence of nerve activation on FBF by a stellate ganglion blockade, showing that FBF response was enhanced as a result of this blockade (Halliwill *et al.*, 1997), and thus the stellate ganglion may also not be a mechanism through which stress-induced vasodilation may occur. Nevertheless, Halliwill and colleagues did reveal that blockade of β-adrenergic receptors in the forearm in conjunction with the stellate ganglion blockade did reduce stress-induced vasodilation (Halliwill *et al.*, 1997) in comparison to a control arm with no blockade administration. Administration of a β-blockade alone resulted in a decrease of 21% in forearm blood flow compared to a control arm without blockade (Lindqvist *et al.*, 1997), providing support for the suggestion that β-adrenergic vasodilation caused by increases in adrenaline might play an important role in vasodilation.

Indeed, activation of the SNS can induce adrenaline production through the adrenal medulla (Black & Garbutt, 2002) and, therefore, may also lead to changes in vascular tone (Newby, 2010). Likewise, infusion of adrenaline (which acts on β-adrenergic receptors) has resulted in increases in resting blood flow (Kjeldsen *et al.*, 1993). A stepwise increase in

adrenaline infusion can result in similar increases in FBF as those found during mental stress, providing support that the adrenaline produced during stress may contribute toward vasodilation (Kjeldsen *et al.*, 1993). In line with this are the reported positive associations between adrenaline and forearm blood flow responses to mental stress (Liu *et al.*, 2006). It is worth mentioning though that the levels of adrenaline during mental stress are lower than the exogenous levels of adrenaline that were necessary to raise the same FBF response in the absence of stress (Lindqvist *et al.*, 1996). The contribution of adrenaline to stress-induced increases in blood flow was calculated by plotting the concentration of adrenaline to the blood flow values during stress. Comparing the blood flow responses to stress to those induced by adrenaline infusion revealed that only up to 30% of the adrenaline that was exogenously infused could be attributed to the increases in blood flow observed (Lindqvist *et al.*, 1996), suggesting that the adrenaline produced during stress may not singularly be able to produce the vasodilatory response seen during mental stress. Indeed, it has been demonstrated that roughly 60% of adrenaline overflow in the forearm could be attributed to increases in stress-induced blood flow, when plotting the blood flow values against the corresponding values for resting forearm adrenaline at rest and in response to stress (Lindqvist *et al.*, 1999).

However, it appears that the strongest and most consistent evidence for a mechanism of stress-induced vasodilation is NO (Cardillo *et al.*, 1997; Dietz *et al.*, 1994; Dietz *et al.*, 1997; Joyner & Halliwill, 2000; Joyner & Casey, 2009). As described earlier, endothelial cells produce NO via endothelial nitric oxide synthase (eNOS), which is responsible for controlling vascular tone (Sandoo *et al.*, 2010). Increased strain on the vessel wall as a result of mental stress causes an increase in shear stress, which in turn leads to increases in eNOS production (Nishida *et al.*, 1992). However, due to the methodological difficulties in directly

measuring nitric oxide in the blood and urine due to its highly transient nature *in vivo* (Beckman *et al.*, 1990; Wadley *et al.*, 2012), the effect of NO in stress-induced vasodilation has been examined by administering NO inhibitors.

Administration of a NO inhibitor (L-NMMA) reduces the FBF response during stress substantially in comparison to a saline infusion (Cardillo *et al.*, 1997) and a no infusion control arm (Dietz *et al.*, 1994). Interestingly, infusion of sodium nitroprusside (endothelium-independent vasodilator, i.e. non-NO driven dilation) had similar effects on blood flow in response to mental stress during the saline and L-NMMA infusion, highlighting further the role of NO in stress-induced vasodilation (Cardillo *et al.*, 1997). However, whilst L-NMMA reduces both basal NO release and NO released in response to a stimulus, it does not alter the structural or functional capacity of the blood vessels to vasodilate (Dietz *et al.*, 1997). Indeed, examination of NO-dependent and non-NO dependent dilation found that administration of a smooth muscle relaxant (a non-NO dependent dilation) during mental stress resulted in more vasodilation (Sarabi & Lind, 2001) than when methacholine (NO-dependent dilation) was administered. This suggests that NO might be the limiting factor in the vasodilatory response to stress (Sarabi & Lind, 2001). In sum, these studies provide evidence for a crucial role for NO in the vasodilatory response to mental stress (Joyner & Dietz, 2003; Joyner & Casey, 2009). Significantly, β-adrenergic driven vasodilation is attenuated when NO production is inhibited, therefore suggesting NO also plays a key role in β-adrenergic driven vasodilation (Dawes *et al.*, 1997).

Interestingly, a study by Sherwood and colleagues examined the relationships between resting endothelial function and the systemic vascular resistance responses to stress. This

study revealed that the vascular responses to acute mental stress were associated with resting endothelial function, such that those who displayed endothelial dysfunction also displayed increased total peripheral resistance (Sherwood *et al.*, 1999). This study is important as it highlights that poor endothelial function can lead to poorer vascular responses to mental stress, as indexed by increases in total peripheral resistance during stress. Therefore, if inflammation can attenuate resting endothelial function, it is hypothesised that inflammation may also attenuate the vascular responses to stress.

1.8 INFLAMMATION AND THE VASCULAR RESPONSES TO ACUTE MENTAL STRESS

This area of research has not been examined extensively, and to our knowledge only one study has investigated the effects of inflammation on vascular responses to mental stress in patients with rheumatoid arthritis (Veldhuijzen van Zanten *et al.*, 2008). In this cross-sectional study, patients with high levels of inflammation displayed the greatest increase in vascular resistance during mental stress (Veldhuijzen van Zanten *et al.*, 2008), which indicates poor vasodilation. This suggests that inflammation may influence the vascular responses to stress in this clinical population. However, other confounding factors associated with RA (e.g., obesity, high blood pressure) may have contributed towards this poorer vascular response. Given the lack of data in this area, the mechanisms through which inflammation may influence the vascular responses to stress can only be inferred from the studies reported above, and must currently remain speculative. Figure 1.2 illustrates the relationships between inflammation, resting endothelial function, nitric oxide and the vascular responses to stress in a healthy population, with Figure 1.3 providing a hypothesis on how inflammation may influence the vascular responses to stress.

Figure 1.2 demonstrates the relationship between inflammation, NO, endothelial function and vascular responses to mental stress. In a healthy population, NO and inflammatory levels are mutually dependent (see Figure 1.2; 1, 2) and, at low levels of inflammation, allow adequate NO availability which is needed for both healthy endothelial function at rest (3) and appropriate vascular responses to stress (4). When in equilibrium, adequate NO results in a downregualtion of inflammatory cytokines (1), and this reduction in inflamamtory cytokines allows for NO production (2). NO levels enhances endothelial function at rest (3) as well as vascular responses to mental stress (4). There is a positive relationship between resting endothelial function and vascular responses to mental stress (5); poorer endothelial function is linked to poorer vascular responses to mental stress.

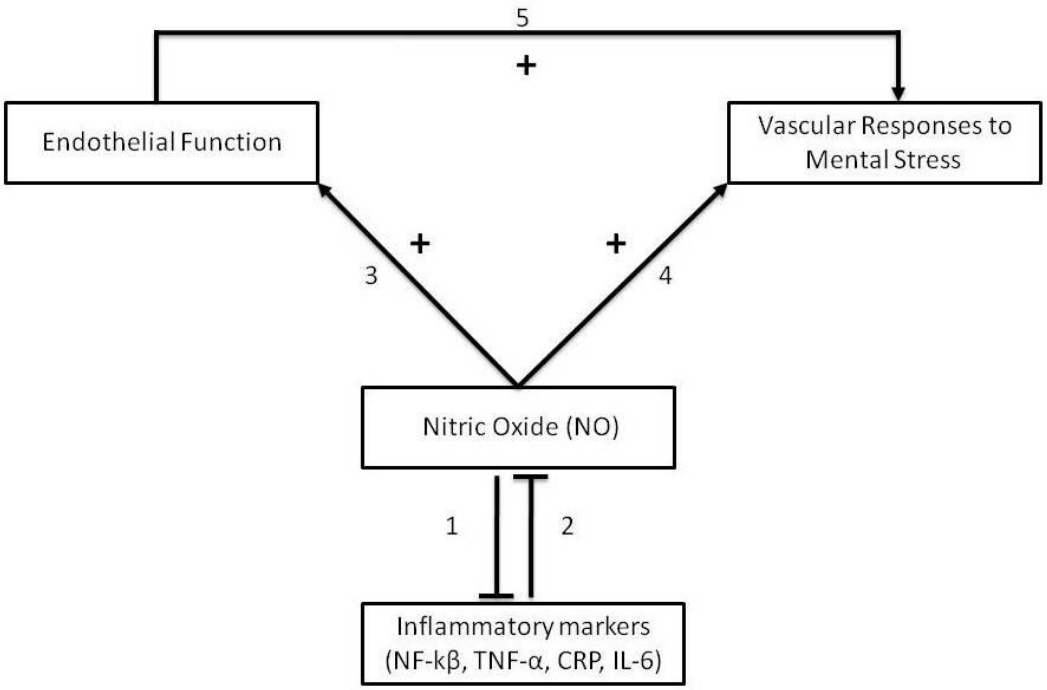

Figure 1.2. Schematic diagram showing the relationships between inflammatory cytokines, nitric oxide, endothelial function and the vascular responses to stress, when there are low levels of inflammation.

Figure 1.3 presents a hypothesis regarding the influence of inflammation on vascular responses to stress, which is based on the well-known association between inflammation and

endothelial function at rest. Increases in inflammation disrupt the equilibrium between NO and inflammatory cytokines, as described in Figure 1.2. This increase in inflammation results in a decrease in NO availability (6), which can cause further increases in inflammatory cytokines (7). Decreases in NO can also facilitate leukocyte adhesion, and as such reducing the capacity of eNOS to produce NO (Kubes *et al.*, 1991). As described above, low levels of NO have been associated with poorer endothelial function (8) and are hypothesised to result in an attenuated vasodilatory response to mental stress (9). The relationship between endothelial function and vascular responses to mental stress (10) is supportive of this hypothesis, as it has been shown that those who have poorer endothelial function also have poorer vascular responses to mental stress (Sherwood *et al.*, 1999). Therefore, it is possible that this association could be driven by inflammation and NO.

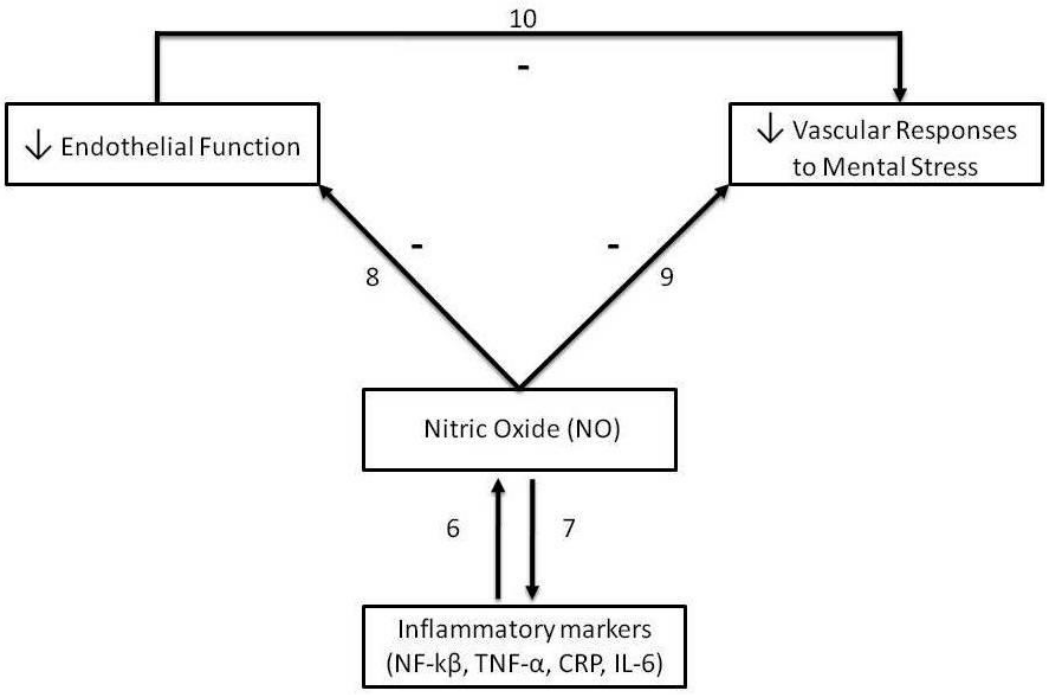

Figure 1.3. Schematic diagram showing the relationships between inflammatory cytokines, nitric oxide, endothelial function and the vascular responses to stress, in the presence of high levels of inflammation.

There is circumstantial evidence to support this proposed model, as populations studied with diseases associated with inflammation show poorer vascular responses to stress (Veldhuijzen van Zanten *et al.*, 2008). It is also worth noting that elevations in resting inflammation can augment the inflammatory response to stress (Kop *et al.*, 2008; Veldhuijzen van Zanten *et al.*, 2005). Therefore, these exacerbated stress-induced levels of inflammation could result in an even further attenuation of the vasodilatory response to mental stress by reducing NO bioavailability. Indeed, neurogenic inflammation as a result of activation of the SNS may also be an important point to consider when examining the effects of inflammation on the vascular responses to mental stress. Cytokines, such as IL-6, are produced by both autonomic and sensory nerves (Nordlind *et al.*, 1995), which innervate in the blood vessel wall (Black & Garbutt, 2002), and thus may also have a role in the vascular responses to mental stress.

In summary, there is ample evidence that mental stress can trigger an MI, however, the underlying mechanisms are not fully understood. The associations between inflammation and vascular function at rest provide provisional evidence that these factors could also influence the effects of mental stress on the vascular responses to stress. In particular, the interaction between inflammation and vascular responses are hypothesised to play an important role. However, more research is necessary to test this hypothesis and, importantly, the detailed mechanisms through which inflammation can influence vascular responses to mental stress.

Therefore, this thesis aims to examine the influences of inflammation on the vascular responses to acute mental stress, by using a healthy population and inducing acute, transient, systemic inflammation. This allows examination of the sole influence of inflammation, as

previous work has examined clinical populations where, in addition to high levels of inflammation, other factors may influence the vascular responses to mental stress. Methods used to induce an acute inflammatory response have included vaccination and eccentric exercise.

1.9 METHODS TO INDUCE INFLAMMATION IN A HEALTHY POPULATION

In order to isolate the influence of inflammation on the vascular responses to mental stress, the ability to induce an increase in inflammation in a healthy population is of paramount importance. Therefore, suitable interventions must be designed and implemented so that adequate levels of inflammation can be induced. Different methods have been used, such as infusion of inflammatory cytokines, administration of a vaccine, and eccentric exercise. These methods are described in more detail below. Depending on the method used, the inflammatory response to the stimulus is most frequently assessed by changes in IL-6, CRP, IL-1 and TNF-α.

1.9.1 INFLAMMATORY RESPONSE TO DIRECT CYTOKINE INFUSION

One method that can be used to induce inflammation is through a direct infusion of cytokines (Bhagat *et al.*, 1999; Bhagat & Vallance, 1997). Unfortunately, these studies have not reported the levels of inflammation induced. Therefore, the precise time course and increases in inflammation in response to cytokine administration is difficult to determine.

1.9.2 INFLAMMATORY RESPONSE TO ENDOTOXIN ADMINISTRATION

Injection of Salmonella *abortus equi* endotoxin is another method to induce systemic inflammation, which can be characterised by increases in IL-6, TNF-α, IL-1ra and white

blood cells (Reichenberg, 2001). Also, the use of *Escherichia coli* endotoxin (lipopolysaccharide (LPS)) has also demonstrated increases in IL-6, TNF-α, IL-1ra and WBC (Eisenberger *et al.*, 2010; Pleiner *et al.*, 2002). Few studies have examined the time course inflammatory response to endotoxin. However, those that have, revealed peak increases in TNF-α and IL-6 at 2-3 hours and 4-5 hours respectively, peak IL-1ra at 4 hours and increases in body temperature at 4-5 hours post vaccination (Eisenberger *et al.*, 2009; Eisenberger *et al.*, 2010; Reichenberg, 2001).

1.9.3 INFLAMMATORY RESPONSE TO VACCINATION

By administrating a vaccine, a small dose of pathogen is injected into the body. In addition to the immune response which results in development of antibodies to protect the person from future infection, the body responds with an inflammatory reaction to combat this infectious pathogen that is injected into the body. Toll-like receptors recognise the infection and trigger the innate immune system (Medzhitov, 2008). Recognition of the pathogen by macrophages and mast cells results in the synthesis and release of inflammatory cytokines (Medzhitov, 2008). The release of inflammatory cytokines leads to neutrophil activation, causing the 'killing' the invading pathogen via releasing of the toxic contents of granules and leading to increases in oxidative stress (Medzhitov, 2008). One such inflammatory mediator is TNF-α. TNF-α is synthesized by several immune cells including macrophages and mast cells (Medzhitov, 2008) and has a vital role as a mediator of the acute phase inflammatory response (Medzhitov, 2008; Möller & Villiger, 2006). IL-6 is synthesised in response to elevations in other inflammatory cytokines such as TNF-α (Möller & Villiger, 2006), and can be produced by a variety of cells, including monocytes, macrophages and endothelial cells (Ekstrom *et al.*, 2008). Given the role of monocytes and macrophages in instigating the inflammatory

response to vaccination, it is assumed that they are responsible for a greater IL-6 production response to vaccination (Medzhitov, 2008). As already mentioned before, IL-6 can also induce increases in production of C-reactive protein (CRP) (Ekstrom *et al.*, 2008; Möller & Villiger, 2006).

A wide range of vaccinations have been used to increase inflammatory levels, including yellow fever and polio, but influenza and typhoid vaccination have been used most commonly. One study that examined the inflammatory response of CRP, IL-6 and fibrinogen to a Yellow Fever vaccination, over an 8 day period post vaccination demonstrated peak CRP responses at 7days post vaccination, IL-6 peak responses at 5 days post vaccination and fibrinogen increases at 2 and 5 days post vaccination (van der Beek *et al.*, 2002). Administration of a polio vaccination in young healthy participants revealed increases in CRP and IL-6 2 days post vaccination but no impact on TNF-α or IL-10 responses to vaccination (Yousfi *et al.*, 2005).

1.9.3.1 INFLUENZA VACCINATION

One of the more commonly used vaccinations in research studies is the influenza vaccine. The time points of inflammatory assessment in response to influenza vaccination often range from 24 hours post vaccination to 3 days. Often populations studied that examine the inflammatory effect of vaccination are elderly (Glaser, 2003) or suffering from diseases (Carty *et al.*, 2006). Populations with high basal levels of inflammation (prior to vaccination) have been investigated to examine the inflammatory response to vaccination. Examination of the inflammatory effect of the influenza vaccination in a population of carotid artery disease patients in comparison to a healthy control group examined IL-6, CRP and serum amyloid-a

(SAA) responses at 24h post vaccination (Carty *et al.*, 2006), revealing increases in all markers for both populations studied. Interestingly, the relative changes in IL-6, CRP and SAA were higher in men with CAD when adjusted for classic CAD risk factors (Carty *et al.*, 2006). A few studies have measured the inflammatory response to the influenza vaccination in healthy populations. Assessment of plasma CRP, IL-6, and TNF-α at baseline, 1 day, 3 days and 7 days post vaccination in a young healthy population revealed small increases in IL-6 at 1 day post vaccination and increases in CRP at both 1 day and 3 days post vaccination, with no changes in TNF-α at any of the time points (Tsai *et al.*, 2005). These modest increases in IL-6 at 24 hours post influenza vaccination have been replicated elsewhere (Edwards *et al.*, 2010). However, no effect on IL-6 was seen 60 minutes post vaccination (Edwards *et al.*, 2006). In sum, it appears that the influenza vaccination produces a mild inflammatory response in comparison to other vaccines, particularly the typhoid vaccination. Indeed, combining an influenza vaccination with a pneumococcal vaccination resulted in a greater inflammatory response 2 days post vaccination as indexed by CRP (Posthouwer *et al.*, 2004).

1.9.3.2 SALMONELLA TYPHI (TYPHOID) VACCINE

The most commonly used vaccine to induce acute inflammation in a healthy population is the *Salmonella typhi* vaccination, which produces a more substantial inflammatory response than the other vaccinations described earlier. Administration has revealed increases in inflammatory markers to be evident over a variety of time points. Increases in IL-6 in response to vaccination frequently demonstrated, ranging from 2 hours (Brydon *et al.*, 2009), 3 hours (Wright *et al.*, 2005), 4 hours (Kharbanda *et al.*, 2002), 6 hours (Chia *et al.*, 2003; Padfield *et al.*, 2010), 8 hours (Antoniades *et al.*, 2011; Clapp *et al.*, 2004; Ekstrom *et al.*, 2008; Vlachopoulos *et al.*, 2007) and even 12 hours (Antoniades *et al.*, 2011)

post vaccination. However, variability exists for TNF-α increases, with some reporting increases at 4 hours post vaccination (Kharbanda *et al.*, 2002), and others demonstrating no changes (Hingorani *et al.*, 2000; Wright *et al.*, 2005). Nevertheless, increases in CRP are more consistently seen at 24 hours post vaccination (Antoniades *et al.*, 2011; Padfield *et al.*, 2010), with marginal increases in CRP reported at 8 hours post vaccination (Vlachopoulos *et al.*, 2007). Interestingly, IL-1 and IL-1ra elevations have also been seen at 3 hours post vaccination by some (Hingorani *et al.*, 2000; Kharbanda *et al.*, 2002) but not all (Wright *et al.*, 2005). Typhoid vaccination did not cause changes in IL-8 and IL-10 (Kharbanda *et al.*, 2002). The variability in increases in inflammatory cytokines may vary in part due to the design of these studies and the time points chosen for assessment. In addition to revealing large increases in inflammation, the vast majority of work using these models to induce acute, systemic, transient inflammation has examined the impact of inflammation on vascular function at rest or psychological factors such as mood.

1.9.4 EXERCISE INTERVENTIONS AS A METHOD TO INDUCE INFLAMMATION

Acute systemic inflammation can also be induced through an exercise protocol. It has been demonstrated that exercise induces increases in inflammatory markers, with studies typically using either concentric or eccentric exercises to induce increases in inflammation. The most commonly examined inflammatory marker in response to exercise is IL-6 due to its more distinct appearance in the blood after exercise, and that it heralds the emergence of other inflammatory markers (Febbraio & Pedersen, 2002). IL-6 production can be stimulated by physiological stress (Pedersen *et al.*, 2001; Steensberg *et al.*, 2002). Recently, IL-6 has started

to be referred to as a ‘myokine’, because of its production by skeletal muscle (Mathur & Pedersen, 2008).

One type of exercise that can result in increases in inflammatory markers is concentric exercise. Concentric exercise can be defined as exercise that involves a shortening of the muscle in length as it contracts (Faulkner *et al.*, 1993). Concentric exercise tasks induce substantial increases in inflammatory cytokines such as IL-6 (for review, see (Pedersen *et al.*, 2001). The presence of plasma IL-6 in the circulation is dependent on several factors such as the type of exercise, exercise duration, and the exercise intensity (Febbraio & Pedersen, 2002). For example, concentric tasks of shorter length induce increase in inflammatory cytokines (Edwards *et al.*, 2006; Starkie *et al.*, 2005), which are not as substantial as during prolonged exercise (Pedersen *et al.*, 2001; Pedersen & Hoffman-Goetz, 2000). While concentric exercise induces substantial increases in inflammatory cytokines, it also causes increases in other factors that could alter the responses to stress. For example, it was suggested that increases in exercise-induced IL-6 were linked to the sympathoadrenal response to exercise (Nehlsen-Cannarella *et al.*, 1997; Nieman *et al.*, 1998; Rhind *et al.*, 2001). Given that β-adrenergic mechanisms are reported to play a role in stress-induced vasodilation, the larger exercise-induced increases in adrenaline seen during concentric exercise could alter the vasodilatory responses to stress. Therefore, the use of concentric exercise as a method to induce inflammation is not suitable for the current research question.

1.9.4.1 ECCENTRIC EXERCISE

The alternative type of exercise task can induce increases in inflammatory cytokines is eccentric exercise. Eccentric exercise is performed when tension over a muscle increases as it

lengthens, resulting in damage to the structure of the skeletal muscle (Proske & Morgan, 2001). Examples of this eccentric action include downhill running (Proske & Morgan, 2001), eccentric cycling (Toft *et al.*, 2002) or lowering a weight whilst trying to resist the forces of gravity (Edwards *et al.*, 2007).

1.9.4.2 MECHANISM OF ECCENTRIC EXERCISE INDUCED INFLAMMATORY RESPONSE

Eccentric exercise results in a localised inflammatory response as a result of myofibril damage and sarcomere disruption (Nosaka *et al.*, 2002; Proske & Morgan, 2001), which is greater in those who are unaccustomed to eccentric exercise (Sorichter *et al.*, 2006). The process of eccentric exercise induced muscle damage starts with sarcomere disruption, causing damage to the sacromere, impairment of excitation-contraction coupling and calcium (Ca^{2+}) infiltration into the sarcoplasm (Peake *et al.*, 2005a; Proske & Allen, 2005; Proske & Morgan, 2001). This activates proteolytic pathways related to muscle repair (Proske & Allen, 2005). The nature of this response would be endogenous (Rock *et al.*, 2005) and subsequent activation of an inflammatory response via neutrophil invasion to the site of injury. The release of proteases from the infiltrated neutrophils reduces the cellular debris secreted due to muscle damage (Medzhitov, 2008). The subsequent macrophage invasion increases inflammatory cytokines such as IL-6 in response to muscle damage (Medzhitov, 2008). Even though these general mechanisms are known, it should be acknowledge that the exact mechanism of inducing an inflammatory response to eccentric exercise must remain in part speculative.

Despite studies showing large increases in inflammation as a result of concentric exercise (Petersen & Pedersen, 2005), eccentric exercise can result in a more extensive, localised, inflammatory response than the concentric exercise (Bruunsgaard *et al.,* 1997; Willoughby *et al.,* 2003). In contrast to the immediate increases in IL-6 that are observed after concentric exercise, eccentric exercise produces a delayed inflammatory response. It has been suggested that this delay supports the idea that cytokine production post exercise is related to muscle damage (Bruunsgaard *et al.,* 1997). However, others have not supported this, with no relationship demonstrated between markers of muscle damage (e.g., CK) and inflammatory markers such as IL-6 (Croisier *et al.,* 1999; Ostrowski *et al.,* 1998b; Ostrowski *et al.,* 1999). It has been proposed that in addition to the site of muscle damage and subsequent inflammatory response as a source of IL-6, the exercising muscle itself may act as a source of IL-6 (Croisier *et al.,* 1999; Febbraio & Pedersen, 2002; Ostrowski *et al.,* 1998b; Ostrowski *et al.,* 1999). In addition to the muscle damage that will lead to increases in IL-6 levels, a greater amount of IL-6 will be produced directly via skeletal muscle contractions (Febbraio & Pedersen, 2002). Interestingly, it has been reported in mice that muscle myofibres express IL-6 in response to eccentric exercise within the first 12 hours of exercise completion, but after this time point, IL-6 is also expressed in proliferating satellite and inflammatory cells (Tomiya *et al.,* 2004).

1.9.4.3 LOCALISED RESPONSES TO ECCENTRIC EXERCISE

Data from studies examining the local responses (i.e. within the skeletal muscle) are pro-inflammatory (Peake *et al.,* 2005a), with increases in pro-inflammatory cytokine such as IL-1 and TNF-α seen within the muscle up to 5 days post exercise (Peake *et al.,* 2005a). In addition, neutrophil invasion occurs within the first few hours of eccentric exercise, with macrophages present from 24 hours post exercise (for review see Peake *et al.,* 2005a).

However, due to its ease of assessment, more is known about the systemic inflammatory responses to eccentric exercise, where in contrast to the local pro-inflammatory responses within the muscle, a greater anti-inflammatory response is seen when assessed in blood (Peake *et al.*, 2005a; Smith, 2000; Toft *et al.*, 2002).

1.9.4.4 SYSTEMIC RESPONSES TO ECCENTRIC EXERCISE

The vast majority of studies examining the effects of eccentric exercise tend to focus their outcome measures on markers of muscle damage such as reduced muscle strength and range of motion, increased muscle soreness, oedema and increased levels of blood based markers of muscle damage such as creatine kinase or myoglobin (MacIntyre *et al.*, 2001; Malm *et al.*, 2004; Nosaka *et al.*, 2002; Paulsen *et al.*, 2005; Peake *et al.*, 2005a; Smith *et al.*, 2000) and therefore the data examining the inflammatory response to exercise is not as extensive. However, studies have demonstrated increases in inflammatory markers (Croisier *et al.*, 1999; Depner *et al.*, 2008; Jackman *et al.*, 2010; Miles *et al.*, 2008; Steensberg *et al.*, 2000; Steensberg *et al.*, 2002; Willoughby *et al.*, 2003). Variations in the inflammatory response to eccentric exercise occur and are dependent on the exercise protocol, including the amount of exercising mass, duration and intensity of the exercise (Febbraio & Pedersen, 2002). For example, the inflammatory responses associated with eccentric exercise are larger when a larger muscle mass is used, e.g. such as comparing an eccentric exercise task in the legs to arms (Peake *et al.*, 2005a). However, due to methodological differences and the timing of inflammatory assessment between published studies, it is difficult to determine when the peak inflammatory response to eccentric exercise occurs.

In contrast to the small changes in response to concentric exercise (Pedersen *et al.*, 1998; Starkie *et al.*, 2001; Toft *et al.*, 2002), equivocal results exist for the TNF-α increases in response to eccentric exercise when assessed systemically (Peake *et al.*, 2005a). TNF-α is produced from a variety of sources, including endothelial cells (Pedersen *et al.*, 1998), but crucially not by skeletal muscle (Steensberg *et al.*, 2002). Indeed, this could be one reason for the lack of systemic changes in TNF-α in response to eccentric exercise is the fact that exercising human skeletal muscle is not a source of TNF-α (Steensberg *et al.*, 2002), which is in contrast to IL-6 (Ostrowski *et al.*, 1998a).

1.9.4.5 ECCENTRIC EXERCISE PROTOCOLS

Studies have examined the impact of downhill running in animals, allowing for detailed examination of potential mechanisms. A two hour period of downhill running in rats has demonstrated increases in systemic TNF-α and CK which persisted up to 24 hours post exercise (Liao *et al.*, 2010). Indeed, a 2.5 hour bout of downhill running has resulted in increased IL-6 and IL-1β at 24 hours and even 48 hours post exercise (Davis *et al.*, 2007). A slightly shorter period of 90 minute downhill running revealed a link between the nuclear factor kappa beta signalling cascade and NOS expression within skeletal muscle (Lima-Cabello *et al.*, 2010). These studies provide evidence for the association between eccentric exercise and early inflammatory cascade responses to exercise.

Human studies have confirmed these findings from animal studies. There is wide variety in the protocols which have been used, and these are summarised in Table 1.1-1.4. Eccentric running tasks (Table 1.1) have been used to increase inflammatory and muscle damage markers, resulting in increases in IL-6 (Buford *et al.*, 2009; Peake *et al.*, 2005b;

Petersen *et al.*, 2001; Sacheck *et al.*, 2006), CRP (Sacheck *et al.*, 2006), and CK (Buford *et al.*, 2009; Hamada *et al.*, 2004; Malm *et al.*, 2004; Peake *et al.*, 2005b; Petersen *et al.*, 2001) displayed across a variety of time points. Others have used eccentric cycling (Table 1.2) to induce increases in inflammation and muscle damage. A 60 minute cycling task at 60 rpm induced increases in IL-6 4 hours post exercise and a progressive increase in CK over 5 days, but no changes in TNF-α (Toft *et al.*, 2002). Peak muscle damage (assessed by CK) were also seen at 96h, highlighting substantial muscle damage as a result of this task (Hellsten *et al.*, 1997).

Table 1.1. Downhill Running Eccentric Exercise Protocols

	Participant details	**Exercise Task**	**Time points and markers assessed**	**Results**
(Sacheck *et al.*, 2006)	N=16	45 min (3 x 15 min blocks, separated by 5 min rest) downhill run @75% $VO_{2\ max}$	IL-6, sIL-6R, CRP @ pre-, immediately post-, 6 h, 1 d, 3 d post-exercise	IL-6: Peak increase seen at 6h, returned to baseline by 1 d. IL-6sR: No changes. CRP: Increase at 1d post exercise.
(Peake *et al.*, 2005b)	N=10	-10% gradient for 45 min at 60% $VO_{2\ max}$	IL-6, CK, Myoglobin, Neutrophil and leukocyte count @ pre-, immediately post-, 1 h, 1 d post exercise.	IL-6: Immediate (peak) increases and at 1h post exercise. Returned to baseline at 1d. CK: Peak increase at 1d. Myoglobin: Peak response at 1h post exercise.
(Hamada *et al.*, 2004)	N=15	-16% descent for 45 min (3 x 15 min blocks, separated by 5 min rest) at 75% $VO_{2\ max}$	CRP and CK measured in blood lactate, cytokine (IL-6, TNF-α, IL-1β) transcription (mRNA) factors from muscle biopsies @ pre-, immediately post-, 1 d, 3 d post exercise.	CRP: No change. CK: Increases at 1 d post exercise, returned to baseline by 3 d. IL-6 mRNA: Increases at 3d TNF-α mRNA: Increases at 3d
(Malm *et al.*, 2004)	N=25 men, 3 females	45 min at respective $VO_{2\ max}$, speeds and gradients. Group 1 (N=5): -4 degree gradient @ 50% $VO_{2\ max}$; Group 2 (N=9): -8 degree gradient @ maximum attainable speed; Group 3 (N=5): +4 degree gradient @ 75% $VO_{2\ max}$; Group 4: Control	CK, White blood cell subsets, CRP, IL-6, IL-1β @ pre-, immediately post-, 6 h, 1 d, 2 d, 4 d and 7 d post-exercise	CK: Greatest responses in -8 degrees group; peak response at 1 d. IL-6 & IL-1β: No change in muscle biopsy samples taken 2d post exercise. Granulocytes: Peak responses at 6h (all groups)

(Buford *et al.*, 2009)	N=29	-17.5% descent for 45 min at 60%max VO2 consumption	Serum CK, muscle mRNA expression of IL-6, IL-8, IL-12, IL-1β and TNF-α @pre-exercise, 3 h post and 1 day post exercise	CK: Elevated at 3 h; Peak response at 1d post exercise. IL-6 mRNA: Peak transcription at 3 h; elevation still at 1 d. IL-8 mRNA: Increased at 3 h. TNF-α, IL-1β, or IL-12 mRNA: No increases.
(Petersen *et al.*, 2001)	N=20	90 min at -5% gradient (75% VO2 intensity)	IL-6, IL-1ra,CK @ pre-, immediately post-, 1 h, 2 h, 1 d, 2 d, 7 d post exercise	IL-6: Increases at 1 h & 2 h; peak response immediately post-exercise. Return to baseline at 1 d & 2 d. CK: Increases at all time points post exercise; peak response at 1 d. IL-1ra: Increases immediately post, 1 h, 2 h (peak response) and 1 d post-exercise.

Only younger participants not receiving additional intervention (e.g., supplementation) are listed. Only blood based markers or inflammation and/or muscle damage reported. All are young men, unless otherwise stated; h refers to hour; d refers to day. IL- refers to Interleukin-; sIL-6R – refers to soluble interleukin-6 receptor; TNF-α refers to tumor necrosis factor alpha; CK refers to Creatine Kinase; CRP refers to high sensitivity C - reactive protein.

Table 1.2. Eccentric Cycling Exercise Protocols

	Participant details	**Exercise Task**	**Time points and markers assessed**	**Results**
(Toft *et al.*, 2002)	N=10	60 min eccentric cycling task (cadence of 60 rpm), broken into 6 intervals: 0–6 min at 50%, 6–12 min at 75%, 12–20 min at 100%, 20–25 min at 130%, 25–40 min at 100%, and 40–60 min at 75% VO_2 max.	TNF-α, IL-6, IL-1ra, and sTNF-R1 @ 0 h (immediately after), 1 h, 2 h, 3 h, 4 h, 1d, 2d, and 5 d post exercise	IL-6: Peak response at 4 h post exercise. CK: progressive increase over 5 d. TNF- α: No changes.
(Hellsten *et al.*, 1997)	N=7	Five bouts of 1 legged eccentric cycling. Each bout lasted 5 min with 4 min rest. Task involved resisting motion of rod rotating at 30rpm, maximal eccentric contractions	Plasma IL-6, IL-1β, CK @ pre-exercise, 45 min, 90 min, 1 d, 2 d, 4 d post exercise	IL-6: Peak increases at 90 min post exercise; Elevations still seen at 1 d, 2 d and 4 d. CK: Significant increases from 1 d, peaking at 4 d post exercise. IL-1β: No changes.

Only younger participants not receiving additional intervention (e.g., supplementation) are listed. Only blood based markers or inflammation and/or muscle damage reported. All are young men, unless otherwise stated; h refers to hour; d refers to day. IL- refers to Interleukin-; sIL-6R – refers to soluble interleukin-6 receptor; TNF-α refers to tumor necrosis factor alpha; CK refers to Creatine Kinase; CRP refers to high sensitivity C - reactive protein.

In addition to eccentric running and cycling, isolated eccentric muscle contractions have been used to induce an inflammatory response. Eccentric exercise of a lower intensity, but a longer exercise duration has revealed increases in IL-6, as demonstrated through a one leg dynamic extensor exercise (40% peak power output) which lasted for 5 hours (Steensberg *et al.*, 2000). Likewise a 3 hour bout of a 2 legged knee extensor exercise (at 55% peak power output) resulted IL-6 increases at 30, 90 & 180 minutes post exercise, with the greatest increase being 100 fold and occurring at 180 minutes (Steensberg *et al.*, 2002).

Studies that have used the arm as the exercising limb tend to focus on elbow flexors for the exercise task (see Table 1.3). As with other modes of eccentric exercise, intensity and duration appear to be critical factors for determining the amount of muscle damage and the inflammatory response to exercise, with greater intensities of exercise resulting in greatest amounts of muscle damage when indexed by CK or myoglobin (Chen *et al.*, 2007; Edwards *et al.*, 2010). Likewise, the greatest number of repetitions results in the most extensive muscle damage (Nosaka *et al.*, 2002). Unfortunately, no inflammatory markers were assessed in either study, thus it is difficult to determine whether the highest inflammatory response would also have been seen at this intensity and duration of task. Eccentric exercise of the elbow flexors have demonstrated increases in IL-6 from 3 hours to 8 hours post exercise (Depner *et al.*, 2008; Miles *et al.*, 2007; Miles *et al.*, 2008; Peake *et al.*, 2006), with no changes seen immediately post exercise (Phillips *et al.*, 2003). Changes in IL-6 return to baseline at 24 hours post exercise despite elevations remaining in CK (Barnes *et al.*, 2010). Interestingly, in order to overcome the smaller muscle mass used when exercising the elbow flexors, studies have chosen to use eccentric portions of a lateral raise to maximise the inflammatory responses to exercise (Edwards *et al.*, 2010).

Table 1.3. Eccentric Exercise Protocols for isolated muscle contractions (arm muscle mass)

	Participant details	Exercise Task	Time points and markers assessed	Results
(Nosaka *et al.*, 2002)	N=110	12, 24 or 60 maximal eccentric contractions of elbow flexors. Each contraction had 15 seconds rest between each contraction	Plasma CK @ pre-exercise, immediately post, 1 d, 2 d, 3 d and 4 d post exercise	CK: No increases at 1 d; Steady increases until peak at 4 d. Peak CK response in 60 contraction group.
(Hirose *et al.*, 2004)	N=10	6 sets of 5 repetitions of eccentric elbow flexors at 40% maximum effort	Plasma CK activity, myoglobin, IL-1β, IL-1, IL-1ra, IL-4, IL-6, IL-8, IL-10, and TNF-α @ pre-exercise, immediately post-, 1 h, 3 h, 6h, 1 d, 2 d, 3 d, 4 d	IL-1β, IL-1ra or IL-6: No changes. TNF-α: decreases at 1 d, 3 d and 4 d post exercise. IL-8: decreased at 6 h to 4 d post exercise. CK: peak at 4d. Myoglobin: peak at 4d.
(Chen & Hsieh, 2001)	N=12	3 sets of 10 repetitions of eccentric elbow flexors. Maximal intensity	Plasma CK, serum IL-6, IL-1β and white blood cells @ pre-exercise, 2 h, 1 d, 3 d, 4 d, 6 d and 7 d post-exercise	WBC (including neutrophils, monocytes or lymphocytes): No change. CK: Increases at 3 d, 4 d (peak) and 6 d post exercise. IL-1β: Peak response 2h post exercise. IL-6: Small non-significant increase.
(Miles *et al.*, 2008)	N=51 (29 men, 22 women)	3 sets of 15 repetitions of eccentric elbow flexors. Maximal effort. 5 min rest between sets, with each repetition lasting 15 seconds.	CK, CRP, IL-6 @ pre-exercise, 4 h, 8 h, 12 h, 1 d, 2 d and 4 d post exercise	CK: Increase at 1 d, 2 d and 3 d. IL-6: Increase at 4 h; peak at 8 h post exercise; still elevated at 12 h. CRP: No changes.

(Barnes *et al.*, 2010)	N=27, assigned to either leg or arm exercise task.	Protocol 1: bilateral leg press at 110% of concentric maximum. 6 sets of 10 repetitions. Protocol 2: 2 sets of 20 repetitions of eccentric elbow flexors (intensity not reported)	Serum CRP, IL-6 and CK (protocol 2 only) @ pre-exercise, immediately post, 1 d, 2 d and 3 d post-exercise	*Protocol 1:* IL-6: No increases. CRP: Increases at 1d and 2d. *Protocol 2:* IL-6: No change. CRP: No change. CK: Increases, but no absolute values reported.
(Phillips *et al.*, 2003)	N=20	3 sets of 10 repetitions eccentric arm curl exercise performed at 80% concentric maximum	CK, serum IL-6, CRP @ pre-exercise, immediately post-, 3 d & 7 d post-exercise	IL-6: Increases at 3 d. CK: Increases at 3 d. CRP: No change.
(Chen *et al.*, 2007)	N= 52	30 eccentric elbow flexor contractions performed at either 100%, 80%, 60% or 40% of each individual's concentric maximum	CK and myoglobin @ pre-exercise, immediately post-, 1 d, 2 d, 3 d, 4 d and 5 d post exercise	CK: Graded response - 100% intensity producing greatest responses; gradual response with peak at 5 d post exercise. Myoglobin: Peak at 4 d post-exercise
(Miles *et al.*, 2007)	N=8 (5 men, 3 women)	3 sets of 15 repetitions of eccentric elbow flexor exercise. Maximal effort. 5 min rest between sets, with each repetition lasting 15 seconds	IL-6, CRP, CK @ pre-exercise, 4 h, 8 h, 12 h, 1 d, 2 d and 5 d post exercise	IL-6: Peak increase at 8 h post-exercise; return to baseline at 1 d. CRP: No changes. CK: No changes.
(Edwards *et al.*, 2010)	N= 158 (50% male)	5 sets of 10 repetitions of eccentric bicep curl and 5 sets of 10 repetitions of eccentric lateral raise, undertaken at either 110%, 85% or 60% of concentric maximum	Plasma CK and IL-6 @ pre-exercise, 1 d post exercise	CK: Greatest increase at 110% intensity; No differences observed for 85% and 60% compared to control. IL-6: No increases (probably due to vaccine administration post-exercise)

(Depner *et al.*, 2008)	N=27, male and female.	3 sets of 15 repetitions of eccentric elbow flexor exercise performed at maximal effort. 5 min rest between sets, with each repetition lasting 15 s.	CRP, IL-6, IL-6 soluble receptor (sIL-6R) @ pre-exercise, 4 h, 8 h, 12 h post exercise	IL6: Increased at 8 h. sIL-6R: Increased at 4 h and 8 h (in both conditions).
(Peake *et al.*, 2006)	N=10	Submaximal task = 10 sets of 60 reps, one arm at 10% maximal isometric strength. Maximal task = 10 sets of 3 lengthening contractions, where participants were asked to maximally resist the lengthening motion of the dynamometer	CRP, CK, IL1-ra, IL-6, TNF-α, IL-10 @ pre-, immediately post-, 3 h, 1 d, 2 d, 3 d and 4 d after exercise	IL-6: Increases for both tasks; IL-6 elevated at 3 h in sub-maximal task, which was not evident for maximal contractions. CK: Increased after both tasks; peak at 4 d. CRP, IL1-ra, TNF-α or IL-10 : No changes

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Conversely, studies have used leg exercises involving the quadriceps to induce an inflammatory response to exercise (Table 1.4). Typically, no changes in TNF-α or TNF-α mRNA expression are seen in response to eccentric exercise (Ross *et al.*, 2010; Steensberg *et al.*, 2002). However, increases in CK are reliably demonstrated from 24-96 hours post exercise, dependent on exercise protocol and study methodologies (Paulsen *et al.*, 2005; Robson-Ansley *et al.*, 2010; Sorichter *et al.*, 2006; Stupka *et al.*, 2000; Stupka *et al.*, 2001). The reported increase in IL-6 in response to eccentric exercise tasks using the legs are summarised in Table 1. The increases in IL-6 have been demonstrated as early as 1 hour post exercise (Jackman *et al.*, 2010), and can remain elevated up to 2 days post exercise (Philippou *et al.*, 2009). Typically the peak response is observed between 6-8 hours post exercise, depending on the protocol utilised (Croisier *et al.*, 1999; Jackman *et al.*, 2010; Paulsen *et al.*, 2005; Willoughby *et al.*, 2003). However, others have demonstrated no changes in IL-6 responses to eccentric exercise, highlighting the importance of selecting an appropriate intensity, quantity and population to use (Sorichter *et al.*, 2006). Most studies tend to assess IL-6 responses to eccentric exercise, however CRP changes have also been examined with elevations in CRP observed at both 24 hours and 48 hours post eccentric exercise (Barnes *et al.*, 2010; Paulsen *et al.*, 2005).

Table 1.4. Eccentric Exercise Protocols for isolated muscle contractions (leg muscle mass)

	Participant details	**Exercise Task**	**Time points and markers assessed**	**Results**
(Ross *et al.*, 2010)	N=10	5 sets of 10 repetitions of a unilateral leg press, undertaken at 120% of the concentric maximum. Sets were separated by a 2 min rest period	Serum myoglobin, IL-8, IL-10, IL-1ra, TNF-α, CRP, IL-6 @ 0, 30, 60, 90, 120, 150, and 180 min post exercise	Myoglobin: Increases from 60 min to 180 min. IL-6: Increased at 180 min. IL-8, IL-10, IL-1ra, TNF-α or CRP: No change
(Paulsen *et al.*, 2005)	N=11	10 sets of 30 repetitions of unilateral, maximal eccentric contractions with m. Quadriceps, with 30 s rest between sets	CK, CRP, IL-6, Neutrophil, Leukocyte and Monocyte cell counts @1.5 h pre-exercise, 1 min, 30 min, 1 h, 6 h and 23 h post exercise. Additional CK and CRP samples obtained at 2-7 d post exercise	IL-6: Gradual increase, peak at 6 h post-exercise. CRP: Peak increase at 2 d. CK: Peak increase at 4 d. Neutrophil, leukocyte and monocytes: Peak response at 5 h post exercise
(Philippou *et al.*, 2009)	N=10	2 sets of 25 repetitions of eccentric leg extension exercise, performed on both legs. Each contraction lasted 4s, with 15 s between each repetition. Maximal resistance of weight	Serum IL-6 @ pre-exercise, immediately post-, 6 h, 2 d, 5 d and 16 d post-exercise	IL-6: Increases immediately post-exercise; peak at 6 h post exercise; elevations still at 2 d post exercise
(Croisier *et al.*, 1999)	N=5	3 sets of 30 repetitions of eccentric knee flexor exercise, conducted on both legs at maximal load	Serum IL-6 and myoglobin @ pre-exercise, 30 min, 2 d, 3 d and 4 d post exercise	IL-6: peak increases at 30 min post-exercise. Myoglobin: peak at 2 d, elevations still at 3 d & 4 d post-exercise.

(Jackman *et al.*, 2010)	N=12	12 sets of 10 repetitions, unilateral eccentric leg extension exercise at 120% concentric maximum. 5 s between each repetition, with 1 min rest between sets.	Serum IL-6, CK and myoglobin @ pre-exercise, 1 h, 8 h, 24 h post exercise	IL-6: Gradual increases from 1 h; peak at 8 h; returned baseline at 24 h. CK: Increases at 8 h and 24 h Myoglobin: Increases at 1 h and 8 h post-exercise.
(Robson-Ansley *et al.*, 2010)	N=18	6 sets of 10 repetitions of unilateral eccentric knee flexor exercise (induce hamstring damage).	Plasma IL-6, sIL-6R, CRP, CK @ pre-exercise, 1 d, 2 d, 3 d post-exercise	CRP: No changes. CK: Elevated at 1 d, 2 d; peak at 3 d. IL-6: No change. sIL-6R: Reductions at 2 d and 3 d.
(Steensberg *et al.*, 2000)	N=6	1 leg dynamic knee extensor exercise, for 5 h at intensity of 25W (40% of peak power output).	Arterial IL-6 @ pre-exercise, and every hour during exercise (1-5)	IL-6: Increases over 5 h; peak IL-6 at 5 h.
(Steensberg *et al.*, 2002)	N=6	3 h of 2 eccentric knee extensor exercise, undertaken at 55% peak power output.	Muscle IL-6 and TNF-α mRNA, plasma IL-6 and TNF-α @ pre-exercise, 30 min, 90 min and 180 min post exercise	IL-6: Increases at 30 min, 90 min and 180 min; Peak at 180min. IL-6 mRNA: Increases at 30 min post-exercise. TNF-α: No changes. TNF-α mRNA: No changes.
(Sorichter *et al.*, 2006)	N=14.	Concentric exercise (60min run at 80% VO2max) and eccentric exercise (6 sets of 10 repetition eccentric leg press).	CK, CRP and IL-6 @ pre-exercise, 1 h, 6 h, 1 d, 3 d and 7 d post exercise	*Concentric exercise:* IL-6: peak at 1 h post exercise CRP: peak response at 1 d. CK: No changes. *Eccentric exercise:* IL-6: No changes. CRP: No changes. CK: Peak at 3 d post-exercise.

(Stupka *et al.*, 2001)	N=16 (8 men, 8 women).	36 eccentric repetitions of unilateral leg press and 100 eccentric knee extension repetitions, undertaken at 120% concentric maximum.	CK @ pre-exercise, 1 d, 2 d 4 d and 7 d post-exercise	CK: Peaks at 2 d and 4 d post exercise; elevations still at 7 d post-exercise
(Stupka *et al.*, 2000)	N=16 (8 men, 8 women)	3 sets of 12 repetitions of an eccentric leg press and 9 sets of 12 repetitions of eccentric leg extensions @ 120% concentric maximum.	CK and plasma granulocyte @ pre-exercise, 1 d, 2 d and 6 d post-exercise	CK: Increases at 2 d and 6 d post-exercise Granulocytes: Elevated at 2 d post-exercise
(Willoughby *et al.*, 2003)	N=8	7 sets of 10 repetitions of eccentric knee extensor exercise, conducted at 150% of concentric maximum	IL-6, IL-6 mRNA @ pre-exercise, immediately post-, 2 h, 4 h, 6 h, 1 d, 2 d, 4 d post exercise	IL-6: Increases at 4 h after exercise; peak response at 6 h. IL-6 mRNA: Peak at 2 h post-exercise; still elevated at 4 h.
(Peterson *et al.*, 2003)	N=8	10-14 sets of 10 repetitions of eccentric knee extensor contractions, at 120% concentric maximum.	$CD15^+$ (Neutrophils), $CD68^+$ (Macrophages) @ pre-exercise and 1d post-exercise	$CD15^+$: No increase. $CD68^+$: Elevations at 1 d.
(MacIntyre *et al.*, 2001)	N=12	30 sets of 10 repetitions eccentric knee flexor exercise, with each set starting at the beginning of each min for a 30 min period.	Plasma IL-6 @ pre-exercise, immediately post-, 2 h, 4 h, 6 h, 20 h, 1 d, 2 d, 3 d, 6 d, 8 d post-exercise	IL-6: Graded increases up to 6 h post-exercise (peak response); still elevated at 1 d post exercise.

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In addition to blood based measures, inflammation can be characterised by elevated levels of localised blood flow to the site of injury, increased vascular permeability and immune cells invading the site of injury (Hirose *et al.*, 2004; MacIntyre *et al.*, 1996; Proske & Morgan, 2001). Therefore, in addition to direct measures of inflammation in blood, indirect measures can be taken to indicate inflammation. Previous work has shown increases in limb circumference to be indicative of swelling and oedema following eccentric exercise (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004). Likewise, self reported pain in the exercised muscle has been used as a measure of muscle damage (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004; Jackman *et al.*, 2010).

1.10 OVERVIEW OF THESIS

There is a body of evidence that inflammation can attenuate resting vascular function, through reductions in NO (Clapp *et al.*, 2004). As NO is critical to stress-induced vasodilation (Dietz *et al.*, 1994; Dietz *et al.*, 1997; Joyner & Dietz, 2003; Joyner & Casey, 2009), this provides a potential pathway through which inflammation could also attenuate the vascular responses to mental stress. This thesis will focus on and examine the functional outcomes on the vascular responses to stress during a state of high inflammation

Thus, this thesis aims to examine this hypothesis through the following experimental chapters. Firstly, Chapter 2 examines the relationships between the peripheral (forearm blood flow) and the whole body (total peripheral resistance) responses to mental stress tasks of differing length. This is important given the links between the presence of mental stress-induced ischaemia and poor vascular responses to stress as indexed by increases in TPR and

reduced peripheral vasodilation. Despite the vast work examining the role of vaccination-induced inflammation on vascular function at rest, as far as we are aware no studies have investigated the time course inflammatory response to this stimulus. Thus, Chapter 3 examines the time course inflammatory response to the Salmonella typhi vaccination. The impact of acute inflammation on mood changes and cardiovascular measures are also examined in this Chapter. Chapter 4 studies the influence of acute vaccine-induced inflammation on the vascular responses to acute mental stress, 6 hours after administration of the Salmonella typhi vaccination, which is based on the findings in Chapter 3. As far as we are aware, only one study has used eccentric exercise as an acute inflammatory stimulus and examined the effects on the vasculature. Barnes *et al*. (2010) examined the effects of eccentric exercise either in the leg or arm on resting carotid-femoral pulse wave velocity (cfPWV), 24 hours and 48 hours post exercise. They demonstrated that after either leg or arm eccentric exercise cfPWV was elevated, and that the markers of muscle damage were associated with the increase in cfPWV 48 hours post exercise. This study highlighted the potentially unfavourable, yet transient, effects of eccentric exercise on resting vascular function. Therefore, Chapter 5 and 6 examine the role of eccentric exercise in attenuating the vascular responses to mental stress. Chapter 5 investigates the effects of an acute eccentric exercise task undertaken in the arm, and the impact of this on the vascular responses to mental stress when examined 24 hours after the exercise task. Chapter 6 investigates the effects of an acute eccentric exercise task undertaken in the leg, and its impact on the vascular responses to mental stress when 6 hours post exercise.

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CHAPTER TWO:

THE EFFECT OF ACUTE MENTAL STRESS ON LIMB VASODILATION IS UNRELATED TO TOTAL PERIPHERAL RESISTANCE

2.1 ABSTRACT

Mental stress can trigger myocardial infarction, and impaired vascular responses to mental stress have been implicated as an underlying mechanism. Vascular function in response to stress can be assessed with different methods, two of which are total peripheral resistance (TPR), which is a measure of the systemic (whole body) vascular response, and forearm blood flow (FBF), which is a more localised vascular response measure. Little is known about how these different vascular assessments are correlated, or how task duration influences these different vascular responses to stress. Therefore these aspects were examined in two separate studies. Healthy male participants (Study 1: N= 29, Study 2: N= 23) completed a 20 minute rest period and a mental arithmetic task (Study 1: 8 min; Study 2: 16 min). Forearm blood flow (FBF) was measured by strain gauge venous occlusion plethysmography, with Total Peripheral Resistance (TPR) assessed using impedance cardiography, as was Heart Rate (HR). Mean arterial blood pressure (MAP) was determined using a Finometer. In both studies, HR, MAP and FBF increased in response to stress. Whereas in study 1 (shorter) no changes in TPR were seen, increases in TPR in response to stress were found during study 2 (longer). FBF was not associated with TPR at rest or during stress (all *p's*>.05). It appears that limb and whole body vascular responses to stress do not yield the same information about impairments of the vascular system. Further, task duration is a determinant of TPR responses to stress. These findings are relevant to the interpretation of prior research findings and the design of future studies on stress and vascular responses.

2.2 INTRODUCTION

Anecdotal and epidemiological studies have demonstrated that psychological stress may trigger a Myocardial Infarction (MI). This has been found for emotional distress caused by an earthquake (Katsouyanni *et al.*, 1986; Suzuki *et al.*, 1995), war (Bergovec *et al.*, 1992; Meisel *et al.*, 1991), and a football penalty shootout (Carroll *et al.*, 2002). Laboratory studies assessing mental stress-induced ischaemia have implicated poor peripheral vascular responses to stress as a possible contributing mechanism (Burg *et al.*, 2009; Goldberg *et al.*, 1996; Jain *et al.*, 1998). Significantly, non-invasive peripheral assessments (assessed by brachial artery endothelial function) of the vasculature can be used as a proxy measure for coronary artery endothelial function (Anderson *et al.*, 1995; Takase *et al.*, 1998; Takase *et al.*, 2005), highlighting the relevance of examining the peripheral vascular responses to stress. The commonly used assessments to examine the peripheral vascular responses to stress are forearm blood flow (FBF) and total peripheral resistance (TPR).

Blood Flow

First described in the 1950's (Whitney, 1953), FBF is typically assessed by venous occlusion plethysmography which measures the total blood flow of a limb. By inflating a wrist cuff to supra-systolic pressure and inflating an upper arm cuff to below diastolic yet above venous pressure, arterial inflow still occurs while venous outflow is blocked. The subsequent changes in limb (arm/leg) circumference as detected by the strain gauge are indicative of blood flow (Joyner *et al.*, 2001; Newton *et al.*, 2001). In this method, 70% of the measured flow is reflective of muscle blood flow and the remainder indicative of skin blood flow (Cooper *et al.*, 1955).

Up to 10-fold increases in FBF in response to mental stress have been demonstrated in healthy populations (Blair *et al.*, 1959; Dietz *et al.*, 1994), with strong reproducibility (Hamer *et al.*, 2006). Significantly, FBF during mental stress does not increase in heart failure patients, in contrast to healthy controls (Middlekauff *et al.*, 1997; Santos *et al.*, 2005) and is impaired in those at higher risk of MI (Hamer *et al.*, 2007), thus, suggesting the role of the peripheral vasculature as a potential trigger for MI.

Several mechanisms have been postulated that might, either singularly or in combination, be responsible for increases in FBF in response to stress. Firstly, the vascular responses to stress were considered to be neurally driven (Blair *et al.*, 1959), however, associations between muscle sympathetic nerve activity and blood flow responses to stress have not been consistently demonstrated (Carter *et al.*, 2005a; Carter *et al.*, 2005b; Carter *et al.*, 2008). Beta (β)-adrenergic mechanisms such as adrenaline have also been speculated to be the reason for stress-induced increases in blood flow (Kjeldsen *et al.*, 1993; Lindqvist *et al.*, 1997). However, there appears to be more consistent support for the role of nitric oxide (NO) (Dietz *et al.*, 1994; Halliwill *et al.*, 1997; Joyner & Dietz, 2003; Joyner & Casey, 2009). Importantly, β-adrenergic driven vasodilation is attenuated when NO production is inhibited, suggesting NO plays an important role in β-adrenergic driven vasodilation, as without NO reductions in dilation through an alternative mechanism are seen (Dawes *et al.*, 1997). In addition to increases in forearm blood flow as a result of mental stress, other indicators of vasodilation (such as decreases in forearm vascular resistance (FVR) and increases in forearm vascular conductance (FVC)) may also occur (Pike *et al.*, 2009). FVC is calculated as (FBF/Mean Arterial Pressure) × 100, whereas FVR is calculated as Mean Arterial

Pressure/FBF (Pike *et al.*, 2009). Therefore, these assessments allow a measure of resistance through the limb, in addition to blood flow.

Total Peripheral Resistance

Total peripheral resistance (TPR) reflects whole-body vascular resistance, and can be assessed using impedance cardiography. Impedance cardiography is a technique that assesses the resistance of the thorax to an electrical current, which is in part determined by the amount of fluid in the aorta. Ejection of blood into the thoracic cavity during the systolic phase of contraction is associated with a reduction in impedance. Impedance cardiography identifies the start of the systole, the peak blood flow velocity and when blood stops flowing to calculate stroke volume (SV, ml) using the Kubicek Formula (Kubicek *et al.*, 1974). SV can then be used to derive cardiac output and subsequently TPR. TPR is measured in dynes-s/cm^{-5}, and can be calculated as (Mean Arterial Pressure/Cardiac Output) × 80 (Sherwood *et al.*, 1990). Cardiac Output is calculated as Heart Rate × Stroke Volume.

Stress-induced changes in TPR are important to measure from a clinical standpoint, because stress-induced myocardial ischemia is associated with stress-induced increases in TPR in those with coronary artery disease (Goldberg *et al.*, 1996; Jain *et al.*, 1998). However, research of the effects of mental stress on TPR yield inconsistent results. Increases in TPR, as assessed by impedance cardiography, have been demonstrated in healthy populations in response to singular (Bacon *et al.*, 2010), as well as successive (Gillin *et al.*, 2008; Ottaviani *et al.*, 2007) acute stress tasks. Examination of both normotensive and mildly hypertensive individuals has demonstrated no changes in TPR in response to stress (Tsai *et al.*, 2003). Increases in TPR have been speculated only to occur after a more prolonged stress task, as

gradual increases in TPR have been observed during a 32 minute stress task (Ring *et al.*, 2002a).

The FBF and TPR responses to mental stress have been studied extensively, but to our knowledge, no studies have simultaneously examined these two measures at rest and in response to mental stress. Cardiovascular measurements have been associated with measures of vascular function. Pike *et al*. (2009) showed a positive correlation between heart rate and both FBF and FVC, plus a negative association between HR and FVR. Even though FVC, FVR and FBF are known to associate with heart rate (Pike *et al.*, 2009), little is known about the relationship between TPR and HR responses to stress. Therefore, this study aims to examine the relationships and interactions between these measurements at rest and in response to mental stress in a healthy population. This association is examined in two separate studies with different task duration in order to explore the potential effects of task duration on the vascular responses to mental stress.

2.3 METHODS

2.3.1 Study 1

Participants

Twenty nine male university students were recruited (mean age ± SD = 19.2 ± 0.7 years, mean body mass index ± SD = 22.2 ± 2.1 kg/m^2). None of the participants were suffering from an acute illness or infection, reported a history of inflammatory, cardiovascular or auto-immune disorders, had taken any anti-inflammatory medication in the last 4 weeks or any regular prescribed medication. Participants reported to the laboratory after an overnight fast and having refrained from vigorous exercise and over-the-counter medication for at least

24 hours and from alcohol for at least 12 hours. The study was approved by the local research ethics committee. All participants all gave written informed consent.

Procedure

The session started between 6.30am-9am and was performed in a temperature (18°C) controlled environment. Upon arrival, the participant was familiarised with the procedures. Measurements of height and weight were taken, and the participant was instrumented for the impedance cardiography assessment. The participant was then placed into a supine position on a bed, where he remained throughout the session. After the blood pressure and blood flow equipment was attached, the participant rested for 20 minutes (baseline rest period) while watching a nature documentary (*Planet Earth*; BBC). During minutes 13, 15, 17 and 19 blood flow measurements were taken, impedance cardiography and blood pressure were analysed for these minutes. After explanation and practice, participants completed an 8 minute mental stress task. During minutes 1, 3, 5 and 7, blood flow was recorded and impedance cardiography and blood pressure were analysed for these minutes. Hereafter, these minutes will be referred to as minutes of assessment for baseline and stress accordingly.

Mental Stress Task

The mental stress task was the paced auditory serial addition test (PASAT). Participants were presented with a series of single digit numbers, which were delivered using a CD player. They were required to add each number they heard to the number presented previously, while retaining the last number to add it to the next number they heard (Gronwall, 1977; Ring *et al.*, 2002b). The numbers were delivered in four consecutive two minute blocks, becoming progressively more challenging whereby the numbers were presented every 2.4 s,

then 2.0 s, 1.6 s and 1.2 s, respectively. The experimenter, who sat 1 meter adjacent to the participant, checked their responses against the correct answers. Participants were alerted by a loud, aversive noise after an incorrect response or once during every 10 additions in instances where no error was made. Further, the participant was filmed and asked to view the live recording on a screen while performing the task. If the participant looked away the aversive noise again sounded as a reminder. The participants were informed that the recording would be analyzed by two senior academics for body and facial composure (in actuality, no such analysis was undertaken). They were truthfully told that a £10 gift voucher would be awarded for the best performance on the task and a leader board with the highest five scores achieved by the participants was displayed. The addition of elements of social evaluation, competition, punishment and reward have been shown to enhance the provocativeness and physiological responsiveness to the task (Veldhuijzen van Zanten *et al.*, 2004).

Physiological Measurements

Cardiovascular measures

Beat-to-beat arterial blood pressure was recorded continuously during both baseline and stress task using a Finometer (Finapres Medical Systems). A small cuff was placed around the middle finger of the dominant hand of each participant. The hand was placed at heart level and inside a heated pad (Dreamland Thermo Therapy; model C1701) to ensure that a stronger and more consistent reading was taken. From this output, continuous data was recorded via a Power1401 (CED) connected to a computer programmed in Spike2 (CED). Mean arterial pressure was calculated between successive diastolic blood pressure points, and then averaged over the minute of assessment.

Indices of cardio-dynamic activity were recorded continuously using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) (de Geus *et al.*, 1995; Willemsen *et al.*, 1996). This system used six Ag/AgCl spot electrodes (Invisatrace, ConMed Corporation) to record electrocardiography (ECG) and impedance cardiography (ICG), in line with published guidelines (Sherwood *et al.*, 1990). The ECG was recorded using three electrodes, with two active electrodes placed at the jugular notch of the sternum between the collar bones and below the left breast (4 cm under the nipple) while the ground electrode was placed on the right side of the body, between the two lower ribs. The R-wave of the ECG signal was detected with an automated level adjustment detector and the raw interbeat intervals (IBI) were stored.

The ECG electrode on the jugular notch also served as the upper impedance-measuring electrode. The second measuring ICG electrode was placed at the xiphoid process of the sternum. The two current electrodes were placed on the back; one at least three cm above and below the horizontal plane of the upper and lower measuring electrode respectively. A current (50 kHz, 350 mA) was applied to the current electrodes, and a voltage recorded from the measuring electrodes, which was proportional to the change in impedance. Ejection of blood during the systolic phase of contraction into the thoracic cavity is associated with a reduction in impedance; with this reduction being proportional to blood flow. The first derivative (dZ/dt, Ohms/s) of trans-thoracic impedance (Z_0, Ohms) was used to identify the commencement of systole (B-point) when blood begins to be ejected from the heart, the maximal value of dZ/dt during systole (dZ/dt_{max}) when blood flow velocity peaks, and the closing of the semilunar valves (X-point) when blood stops flowing.

The ECG signal was amplified, with an input impedance of 1 MΩ and a common mode rejection ratio of 70 dB, and led through a bandpass filter of 17 Hz (Q=33). The ICG signal was amplified and led to a precision rectifier, after which the signal was low pass filtered at 72 Hz) to give Z_0. Subsequently, high pass filtering Z_0 at 0.1 Hz supplied ΔZ, which in turn was high pass filtered at 3 Hz to determine dZ/dt. dZ/dt was sampled at 250 Hz, while Z_0 was sampled at 10 Hz. The R-wave locked dZ/dt data blocks were ensemble averaged over 10 seconds; ensemble averaging was used to remove respiratory artefact from the ICG. An interactive scoring program provided with the VU-AMS (AmsImp) was used to identify the B-point, dZ/dt_{max}, and the X-point in each 10 second ensemble (Sherwood *et al.*, 1990). R-R interbeat interval was used to calculate heart rate (HR (bpm) = 60000 / IBI). Left ventricular ejection time (LVET, ms), defined as the time between the B-point and the X-point. Stroke volume (SV, ml) was calculated as $SV = \rho \times (L/Z_0)^2 \times LVET \times dZ/dt_{max}$ (Kubicek *et al.*, 1974), where ρ is blood resistivity (assumed to be 135 Ohms/cm) and L is the distance between the recording electrodes. Cardiac output (CO, l/min) was equated to equal SV × HR. Finally, Total Peripheral Resistance (TPR dyne-s/cm^{-5}) was computed as TPR = (MAP/Cardiac Output) × 80.

Forearm Blood Flow

Venous occlusion plethysmography, with a mercury-in-silastic-strain gauge was utilized to measure forearm blood flow (FBF), with changes in limb circumference indicative of changes in blood flow (Joyner *et al.*, 2001; Newton *et al.*, 2001). A strain gauge was fitted around the widest part of the dominant forearm. One congestion cuff was placed around brachial region of the upper arm (SC12, Hokanson) and another at the wrist (TMC7, Hokanson). The strain gauge was connected to a plethysmograph (EC6, Hokanson), which

produced a calibrated output voltage proportional to forearm circumference with a frequency response of 0 – 25 Hz. The plethysmograph signal was digitized at 100 Hz, via a Power1401 (CED) connected to a computer programmed in Spike2 (CED). The brachial cuff was inflated for 5s to above venous pressure (40 mmHg), using a rapid cuff inflator (E20, Hokanson) attached to an automated air source (AG101, Hokanson). Following 15 seconds rest, the brachial cuff was inflated again. This was repeated three times during each minute of assessment. Throughout each minute of assessment, the wrist cuff was manually inflated by Sphygmomanometer (S300, Hokanson) to supra-systolic blood pressure (>200 mmHg), to prevent any blood flow into the hand. Calibration and forearm blood flow analysis was undertaken offline using Spike2 (CED). Increases in forearm circumferences associated with inflation of the upper arm cuff were identified and for each of these, the slope was measured between the upstroke of first two pulses following cuff inflation. The slope was assessed using a least squares fit to the data to minimise the effects of outlying data points. Three measurements of forearm blood flow occurred per minute, with these mean averaged to give an estimate of forearm blood flow per minute. Forearm vascular resistance (FVR) was calculated as MAP/FBF, with forearm vascular conductance (FVC) was calculated as FBF/MAP × 100.

Data reduction and statistical analysis

For all cardiovascular and vascular variables, the average of the four measurements taken during rest was computed to obtain a baseline value. The four minutes of assessment during stress are referred to as Stress 1, Stress 2, Stress 3 and Stress 4. To examine the responses to stress, a series of 5 time (Baseline, Stress 1, Stress 2, Stress 3, Stress 4) repeated measures Analysis of Variance (ANOVA) were conducted on FBF, FVR, FVC, HR, TPR and

MAP. Greenhouse-Geisser correction was applied for all ANOVAs (Vasey & Thayer, 1987). Eta squared (η^2) was used as a measure of effect size. Where appropriate, Newman-Keuls post-hoc comparisons are reported. Reactivity scores for all cardiovascular and vascular measures were calculated as the difference between the average stress value (calculated as the average of Stress 1, Stress 2, Stress 3 and Stress 4) minus the baseline value. Subsequently, Pearson correlations were computed to examine the associations between the responses at rest, during stress and the stress-induced differences between the cardiovascular and vascular variables assessed. Occasional missing data are reflected in the reported degrees of freedom.

2.3.2 Study 2

Participants

Twenty three male university students were recruited (mean age ± SD = 19.5 ± 0.9 years, mean body mass index (BMI) ± SD = 24.6 ± 2.8kg/m^2). The participants met the same inclusion and adherence criteria as described in Study 1. The study was approved by the local Research Ethics Committee, and all participants all gave written informed consent.

Procedure

All sessions started between 2pm and 6pm and were performed in a temperature controlled environment. The procedures were the same of those of Study 1 with the exception of a longer 16 minute stress task, which was chosen because TPR changes have been reported in response to a longer stress task (Ring *et al.*, 2002a). Assessments were taken during minutes 1, 3, 5 and 7 of the first part of the task, and minutes 9, 11, 13 and 15 of the second part of the task, blood flow was recorded and impedance cardiography and blood pressure were analysed for these minutes.

Mental Stress Task

As with Study 1, the mental stress task was the PASAT. However, this version of the task lasted 16 minutes, and was divided in two 8 minute tasks. For each 8 minute block, the numbers were delivered in four consecutive two minute blocks, the numbers presented every 3.2 s, then 2.8 s, 2.4 s and then 2.0 s for the first task, with numbers presented every 2.4 s, then 2.0 s, 1.6 s and then finally 1.2 s for the latter of the two tasks. A one minute rest period separated the two tasks.

Assessments

Forearm blood flow, blood pressure and cardiovascular measures were taken as described in study 1.

Data reduction and statistical analysis

For all cardiovascular and vascular variables, the average of the four measurements taken during rest was derived to obtain a baseline value. Four measurements were taken during each part of the stress tasks, with the four minutes of assessment in the first stress task referred to as Stress 1-4 (as in study 1). The second part of the stress tasks took an additional four minutes of assessment during minute 9 (Stress 5), minute 11 (Stress 6), minute 13 (Stress 7) and minute 15 (Stress 8). The same statistical analyses were conducted in this study, with the exception that the Time factor of the analyses involved 9 levels instead of 5, to reflect the longer stress task.

2.4 RESULTS

2.4.1 Study 1

Overall responses to stress

Figure 2.1 illustrates the vascular and cardiovascular responses to the 8-min mental stress task. ANOVAs revealed significant time effects for FBF (F (4, 23) = 20.2, $p < .001$, $\varepsilon = .75$, $\eta^2 = .44$), FVR (F (4, 23) = 7.03, $p < .001$, $\varepsilon = .65$, $\eta^2 = .31$) and FVC (F (4, 23) = 9.68, $p < .001$, $\varepsilon = .77$, $\eta^2 = .28$), HR (F (4, 23) = 26.31, $p < .001$, $\varepsilon = .43$, $\eta^2 = .70$) and MAP (F (4, 25) = 27.6, $p < .001$, $\varepsilon = .46$, $\eta^2 = .76$). No time effects were observed for TPR (F (4, 20) = .96, $p = .67$, $\varepsilon = .55$, $\eta^2 = .02$). As highlighted in Figure 2.1 post-hoc analyses showed that FBF, FVC, HR and MAP all increased in response to stress, whereas FVR decreased in response to mental stress. There was no change in TPR in response to mental stress.

Correlation Analysis

Correlation analyses were undertaken to examine the extent to which the baseline, responses to stress or stress-induced changes in vascular and cardiovascular responses to stress were associated. These correlations are presented in Table 2.1. Baseline FBF was negatively correlated with baseline FVR ($p < .001$) and baseline FVC ($p < .001$). Baseline MAP was also negatively correlated with baseline FVR ($p < .05$) and baseline FVC ($p < .001$). TPR was not correlated with either baseline FBF, FVR, FVC ($p > .05$), or baseline HR or MAP ($p > .05$). As with the baseline values, FBF during stress was negatively correlated with stress FVR ($p < .001$) and stress FVC ($p < .001$). Stress MAP was also correlated with stress FVR ($p<.05$) and negatively correlated with stress FVC ($p < .001$). Again, stress TPR was not correlated with either stress FBF, FVR, FVC ($p > .05$) nor with HR or MAP during stress ($p > .05$). Stress-induced changes in FBF were correlated in stress-induced changes in

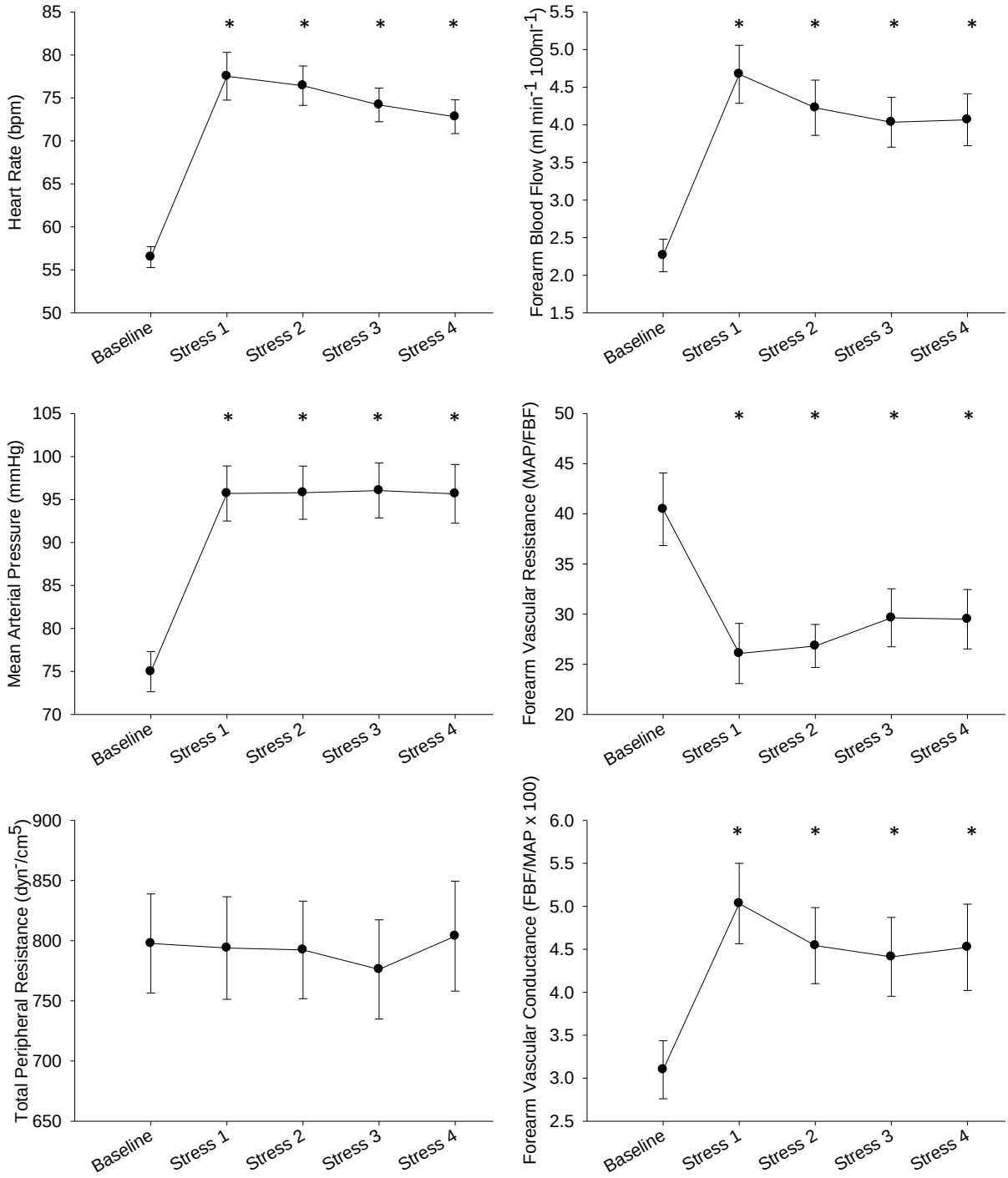

Figure 2.1. Mean ± SE heart rate, mean arterial pressure, total peripheral resistance, forearm blood flow, forearm vascular resistance and forearm vascular conductance responses to 8 minute mental stress (Study 1). * indicates significantly different to baseline (p<.05)

FVC ($p < .001$) and changes in FVC were negatively correlated with stress-induced changes in FVR ($p < .05$). Stress induced changes in FBF were associated with stress induced changes

in HR (p = .005) and MAP (p < .05). Again, stress-induced changes in TPR was not correlated with either stress-induced changes in FBF, FVR, FVC (p > .05) nor stress-induced changes in HR or MAP (p > .05).

Table 2.1. Pearson correlation coefficients between vascular measures at rest, during stress, and as stress-induced changes (Δ = baseline – stress) for Study 1 (8 minute stress task)

	Baseline FBF	Baseline FVR	Baseline FVC	Baseline HR	Baseline MAP
Baseline FBF	-	-.81**	.94**	.03	-.14
Baseline FVR	-	-	-.84**	-.02	.42*
Baseline FVC	-	-	-	-.09	-.43*
Baseline TPR	.17	-.11	.15	-.22	-.06

	Stress FBF	Stress FVR	Stress FVC	Stress HR	Stress MAP
Stress FBF	-	-.89**	.89**	.36	-.12
Stress FVR	-	-	-.88**	-.17	.40*
Stress FVC	-	-	-	.07	-.51**
Stress TPR	-.04	.10	-.06	-.10	-.07

	Δ FBF	Δ FVR	Δ FVC	Δ HR	Δ MAP
Δ FBF	-	-.37	.86**	.52**	.40*
Δ FVR	-	-	-.40*	-.19	-.05
Δ FVC	-	-	-	.23	.02
Δ TPR	-.06	.05	-.13	.23	.19

Note: * p < .05, ** p < .001.

2.4.2 Study 2

Overall responses to stress

Figure 2.2 displays the vascular and cardiovascular responses to the 16 minute mental stress task. ANOVAs revealed significant time effects for FBF (F (8, 12) = 12.42, p < .001, ε = .44, η^2 = .40), FVR (F (8, 12) = 2.74, p < .001, ε = .42, η^2 = .29), FVC (F (8, 11) = 6.94, p = .002, ε = .37, η^2 = .24) and TPR (F (8, 10) = 2.78, p < .05, ε = .41, η^2 = .14), HR (F (8, 12) =

41.54, $p < .001$, $\varepsilon = .24$, $\eta^2 = .69$) and MAP (F (8, 15) = 10.95, $p < .05$, $\varepsilon = .28$, $\eta^2 = .14$). Post hoc analyses identified increases in FBF, FVC, HR, MAP, and a decrease in FVR in response to stress, which remained different to baseline throughout (see Figure 2.2).

Correlation Analysis

Correlation analyses were undertaken to examine whether the baseline, responses to stress or stress-induced changes in vascular and cardiovascular responses to stress were associated. These correlations are in Table 2.2. Baseline FBF was negatively correlated with baseline FVR ($p < .001$) and baseline FVC ($p < .001$). TPR was not correlated with either baseline FBF, FVR, FVC, HR ($p > .05$) but was associated with MAP ($p = .02$). As with the baseline values, FBF was negatively correlated with stress FVR ($p < .001$) and stress ($p < .001$). As with study 1, stress TPR was not correlated with either stress FBF, FVR, FVC ($p > .05$), neither HR nor MAP during stress ($p > .05$). Only stress-induced changes in FBF were correlated with changes in FVC ($p < .001$). Stress-induced changes in TPR were not correlated with stress-induced changes in FBF, FVR, FVC ($p > .05$). However, stress-induced increases in TPR were correlated with stress-induced changes in HR ($p < .02$) and MAP ($p < .05$).

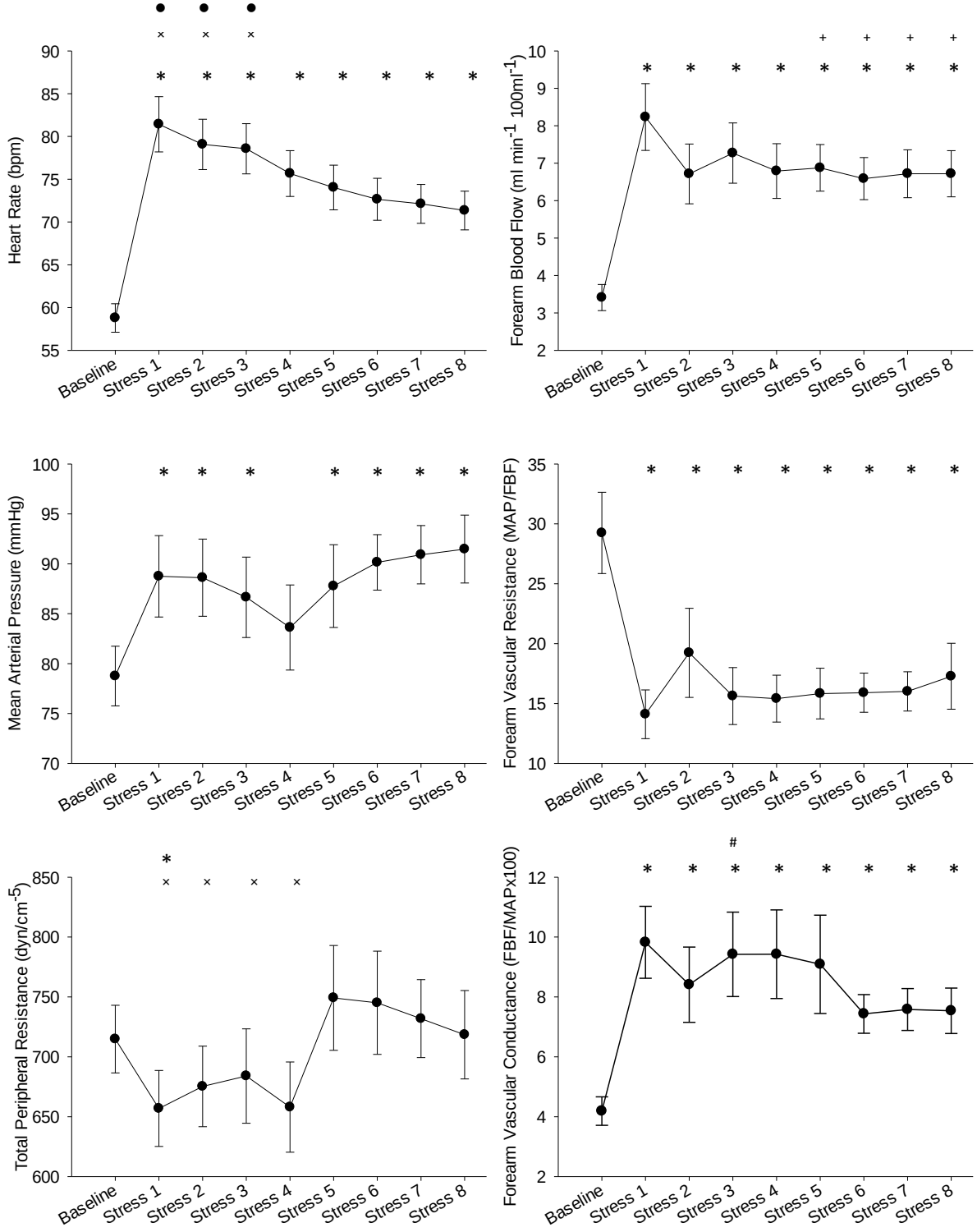

Figure 2.2. Mean ± SE heart rate, mean arterial pressure, total peripheral resistance, forearm blood flow, forearm vascular resistance and forearm vascular conductance responses to 16 minute mental stress (Study 2). * indicates significantly different to baseline ($p < .05$), $^{+}$ indicates significantly different to stress 1, $^{\#}$ indicates significantly different to stress 2, $^{\times}$ indicates significantly different to stress 5-8, • indicates significantly different to stress 4 ($p < .05$)

Table 2.2. Pearson correlation coefficients between vascular measures at rest, during stress, and as stress-induced changes (Δ = baseline – stress) for Study 2 (16 minute stress task)

	Baseline FBF	Baseline FVR	Baseline FVC	Baseline HR	Baseline MAP
Baseline FBF	-	-.83**	.93**	.06	.05
Baseline FVR	-.83**	-	-.87**	-.00	.22
Baseline FVC	.93**	-.87**	-	.04	-.27
Baseline TPR	.21	.02	-.00	-.20	.50*

	Stress FBF	Stress FVR	Stress FVC	Stress HR	Stress MAP
Stress FBF	-	-.83**	.92**	.13	.06
Stress FVR	-.83**	-	-.90**	.14	.34
Stress FVC	.92**	-.90**	-	-.05	-.29
Stress TPR	.20	.11	-.05	.24	.41

	Δ FBF	Δ FVR	Δ FVC	Δ HR	Δ MAP
Δ FBF	-	-.27	.91**	.24	.11
Δ FVR	-.27	-	-.33	-.26	-.11
Δ FVC	.91**	-.33	-	.03	-.23
Δ TPR	.34	-.02	.12	.34*	.44*

Note: * $p < .05$, ** $p < .001$.

2.5 DISCUSSION

This study examined the associations among various assessments of cardiovascular responses to mental stress. During the 8 minute task (study 1) increases in HR, MAP, FBF, FVC and decreases in FVR were seen in response to stress. During the longer 16 minute task (study 2) similar responses to mental stress were found, even though the responses tended to diminish as the task progressed. Gradual increases in TPR were seen only during the longer stress task. No associations were found between FBF and TPR regardless of task length at rest, during stress or when assessed in terms of stress-induced changes. In sum, despite the different responses seen to the stress task, it appears that there are no links between the

vascular responses when assessing whole body vascular responses (TPR) and the vascular responses of a limb (FBF).

This study demonstrated that the TPR responses at rest, during stress or as measure of stress-induced difference was not associated to the FBF, FVR or FVC responses at any of these time points. Indeed, these results were displayed regardless of task length, and may be due to differences in the method of assessment or the mechanisms which underlie these responses. TPR is a measure of constriction of the vasculature, which is derived from cardiac output and MAP and reflects the overall constriction in the body, whereas FBF is a direct measure of dilation in one particular limb. The TPR responses to stress have been reported to be more variable and dependent on other factors (e.g., time (Ring *et al.*, 2002a)), and may even be due to other factors associated with calculation of this measurement, such as changes in cardiac output and blood pressure in response to stress (Kelsey *et al.*, 2007). We also found this to be the case, in that the TPR response to stress was not as consistent as the other limb-related variables assessed.

However, it has been shown that differences in limb blood flow as a result of stress do not always reflect the whole body vasodilatory changes, or even responses between different anatomical locations. For example, in response to mental stress increases in calf and forearm blood flow are evident when measured by plethysmography (Carter *et al.*, 2005b), but correlations between calf and forearm blood flow were not seen. Further correlations between forearm vascular resistance or conductance and the vascular resistance in calf are not demonstrated (Carter *et al.*, 2005b). Differences in the forearm and calf responses to mental stress have been observed (Carter *et al.*, 2005b; Hjemdahl *et al.*, 1989; Rusch *et al.*, 1981),

with stress-induced increases in forearm blood flow consistently seen whereas the results for calf blood flow are equivocal (Kuipers *et al.*, 2008). Myogenic differences (i.e., differences in blood pressure due to posture) have been put forward as a potential reason for these stress-induced differences between limb (Imadojemu *et al.*, 2001), however given the supine position observed in this study this is unlikely to be a reason for the differences between FBF and TPR responses to stress. Importantly, assessment of FBF focuses on a small area of the vasculature, which is in contrast to TPR assessment which gives an indication of vascular function over a larger area. Assessments of the relationship between the micro-vasculature and macro-vasculature have shown inconsistent findings with some showing associations between micro- and macro-vascular function in rheumatoid arthritis patients (Foster *et al.*, 2010) and others showing no association (Arosio *et al.*, 2007; Sandoo *et al.*, 2011), highlighting differences in larger and smaller vessel function. Likewise, increases in renal vasoconstriction in response to mental stress have been demonstrated (Hayashi *et al.*, 2006), insinuating how different vascular responses to stress can occur depending on the vascular beds. Therefore, different organs and vascular beds might respond differently to stress.

It may be surprising that there are no associations between the responses of FBF and TPR at rest, to stress and as a stress-induced change, given the links between the mechanisms which are responsible for vasodilation. Vasodilation of the vasculature can occur through NO, which is regulated by increases in shear stress on the side of the vessel wall (Nishida *et al.*, 1992). Inhibition of NO has resulted in elevations in resting vascular resistance (TPR), as well as attenuated FBF responses to mental stress (Dietz *et al.*, 1994). Further, other mechanisms have links to both TPR and FBF responses to stress with beta (β)-adrenergic mechanisms impacting upon both TPR and FBF responses to stress. For example, in addition to the

altering TPR responses via increases in adrenaline resulting in increases in HR (McEwen, 2007), and subsequently CO and TPR (Turner, 1994), adrenaline can also influence the vasculature directly. Adrenaline, which is secreted into the blood by the adrenal medulla, can lead to vasodilation by acting upon the β-adrenergic receptors resulting in relaxation of the smooth muscles around the blood vessel (Widmaier *et al.*, 2006). Increases in β-activation have been shown to cause increases in vasodilation and decreases in TPR (Joyner & Dietz, 2003). Interestingly, increases in β-activation could, at least, be partially responsible for mental stress-induced increases in FBF (Lindqvist *et al.*, 1997; Lindqvist *et al.*, 1999). In line with this, increases in adrenaline (a β-activated mechanism) are reported to be associated with increases in stress-induced blood flow (Lindqvist *et al.*, 1996; Lindqvist *et al.*, 2004). Nevertheless, the levels of adrenaline produced in response to stress are lower than external levels of adrenaline needed to induce the same amount of FBF response in the absence of mental stress (Lindqvist *et al.*, 1996), suggesting that adrenaline is not solely responsible for stress-induced vasodilation. It is worth noting that a reduction in dilation through β-adrenergic mechanisms has been demonstrated when NO production was inhibited (Dawes *et al.*, 1997), highlighting the evidence for NO playing a more prominent role in stress-induced vasodilation.

Despite these similar mechanisms, the lack of an association between TPR and FBF might also be accounted for by differential adrenergic regulation. However, without direct assessments, the influence of alpha (α)- or β-adrenergic activation must remain speculative. Adrenaline can also act on the α-adrenergic receptors located on the vessel wall, which can cause vasoconstriction, resulting in increases in blood pressure (Widmaier *et al.*, 2006). FBF appears to be unaltered by α-adrenergic blockade (Halliwill *et al.*, 1997), whereas TPR

increases with α-adrenoreceptor activation (Joyner & Dietz, 2003). Therefore, it may be possible that the differences in TPR and FBF seen in this study might be as a result of changes in α-adrenoreceptor activation. The initial decrease of TPR can occur as a result of either increased β-adrenoreceptor activation or decreases in α-adrenoreceptor activation (Joyner & Dietz, 2003), with increases in TPR seen towards the end of the task considered partly as a result of increases in α-adrenergic activation (Joyner & Dietz, 2003). The blood pressure responses to stress remain stable, initially as a result of increases in cardiac output, which is then followed by increases in TPR as a result of α-adrenoreceptor activation (Ring *et al.*, 2002a). In line with this, studies of longer duration report increases in TPR seen towards the end of the task (Hamer & Steptoe, 2007; Ring *et al.*, 2002a), with constrictive influences on blood pressure and vascular resistance reported elsewhere with prolonged exposure to stress (Kelsey *et al.*, 1999; Miller & Ditto, 1991) highlighting the role of task length in the TPR responses to mental stress.

It should be noted that circadian variation has been demonstrated for FBF, with lower FBF demonstrated in the morning and attributed to lower α-adrenergic sympathetic activation (Panza *et al.*, 1991). Few studies have examined circadian variation of TPR finding lower TPR during night in comparison to daytime (Guo & Stein, 2003). However, given that blood pressure (which is used to calculate TPR) is higher in the morning (Guo & Stein, 2003), it could be assumed that there would be differences in TPR in the morning compared to the afternoon. However, this focus of the current study is not to examine and compare the circadian differences in the responses to stress, but rather to examine the relationships between FBF and TPR within each study. Therefore, as these factors ought to be equal and consistent, these differences in circadian variation should be irrelevant.

One criticism of using a longer mental stress task is that habituation to the task may occur. However, despite mild attenuation, HR and BP remained significantly elevated during the stress period above the baseline values reported. The continuous increase in task difficulty ensured that participants remained engaged and stressed throughout the task, which is in line with previous use of this task from our laboratory (Ring *et al.*, 2002a; Ring *et al.*, 2002b; Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009). This version of the PASAT has been proven to consistently perturb cardiovascular activity throughout versions of the task of up to 32 minutes (Ring *et al.*, 2002a; Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009). Further, self reported perceptions of the stress task were not different despite the differences in task length, such that the task was perceived to be as difficult, stressful in nature and engaging despite task length.

There are limitations and other considerations that should be acknowledged. Venous occlusion plethysmography is a technique that has been widely used and displays good reproducibility (Roberts *et al.*, 1986), however, this technique is very sensitive to movement artefacts. To counteract this, three blood flow assessments per minute of blood flow were taken, and movement artefacts deleted after careful inspection of the data, in order to calculate a more reliable average of blood flow for the minutes of assessment. Also, the nature of impedance cardiography involves an element of subjective scoring when determining the B-point, dZ/dt_{max}, and X-point of each impedance trace, and therefore, it is important that all the assessments and analysis are conducted by the same experimenter to minimise the amount of subjectivity involved.

Associations between peripheral and coronary vascular responses at rest have been demonstrated (Anderson *et al.*, 1995; Takase *et al.*, 1998; Takase *et al.*, 2005), and both exaggerated TPR responses to mental stress as well impaired limb vasodilatory responses to mental stress have been associated with mental stress-induced ischaemia in CAD patients (Burg *et al.*, 2009; Goldberg *et al.*, 1996; Jain *et al.*, 1998). However, in the current studies (study 1 and 2) no associations between the local (FBF) and systemic (TPR) responses to mental stress were found, indicating that the vascular responses to mental stress may not be uniform across the whole of the vascular system. In view of this, questions remain about which peripheral assessment is the best predictor. Indeed, to our knowledge no mental stress-induced ischaemia studies have assessed both TPR and FBF, therefore it is currently not known which measurement is the most appropriate peripheral predictor of myocardial responses to mental stress.

In conclusion, this study examined the relationships between the cardiovascular responses to mental stress in a short (8 minute) and longer (16 minute) mental stress task. It appears that associations exist between some of the β-adrenergic driven responses to mental stress; however these associations appear to be dependent on task length. The differences in the nature of the assessments may also play a contributory role towards the lack of associations seen. As TPR was not associated with any of the limb vasodilatory responses to stress (FBF, FVR, FVC) it remains to be determined which peripheral assessment may be the best predictor of the myocardial responses to stress.

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CHAPTER THREE:

THE TIME COURSE INFLAMMATORY RESPONSE

TO *SALMONELLA TYPHI* VACCINATION

3.1 ABSTRACT

The *Salmonella typhi* vaccination has been used as an inflammatory stimulus to induce acute, transient increases in inflammation, demonstrating increases in interleukin-6 (IL-6) from 2-12 h post-vaccination, at 24 h for C-reactive protein (CRP), with variable results for tumor necrosis factor-alpha (TNF-α). Additionally, acute inflammation is associated with negative mood and reduced heart rate variability in mice. However, no study has examined the time course of the inflammatory response to this vaccine and its impact on measures of mood, cardiac function or physical symptoms. Using a blinded crossover design, eight participants received the *Salmonella typhi* vaccine (inflammation condition) and a saline (control condition) injection on two separate days. Blood samples and measures of mood (Profile of Mood States) were taken at baseline (0 h), 4 h, 5 h, 6 h, 7 h, 8 h and 24 h post-injection. Physical symptoms and pain were assessed at 4 h, 5 h, 6 h, 7 h, 8 h and 24 h post-injection, with cardiovascular function assessed over the first 8 hours. Repeated measures ANOVAs and polynomial trend analyses were conducted to compare the patterns of response in both conditions. In comparison to the saline control condition, vaccination resulted in increases in TNF-α, IL-6, CRP and white blood cells (WBC), with peak IL-6, TNF-α and WBC responses at 6-8 h and peak CRP responses at 24 h (all *p's* < .05). No increases were observed in the control condition. No effects of inflammation were apparent for any mood or cardiovascular measures. Participants tended to report more pain in the vaccinated limb in the inflammation condition ($p < .07$). This study revealed increases in the inflammatory markers and localised pain at the site of vaccination, but no effects on mood or cardiovascular function.

3.2 INTRODUCTION

Inflammation is an adaptive response of the immune system to infection and tissue damage (Medzhitov, 2008). Two characteristic features of this response are rapid infiltration of leukocytes and the release of inflammatory cytokines (Medzhitov, 2008). Acute inflammation is deemed protective whereas a dysregulated and chronic inflammatory response is considered more harmful (Medzhitov, 2008), that could lead to an 'overdrive' in inflammatory markers (Stevens *et al.*, 2005). Indeed, chronic inflammation has been implicated in the aetiology of cardiovascular disease (CVD) (Hansson, 2005; Libby, 2006), as well as an increased risk for myocardial infarction (Levy *et al.*, 2008; Ridker *et al.*, 2000). The aim of this study was to characterise the time course of the inflammatory response to *Salmonella typhi* vaccination and investigate its impact on mood, symptoms, and cardiovascular function over a 24 h period.

Inflammation can be serologically assessed by elevations in inflammatory markers, such as C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), as well as increased cell numbers, such as white blood cells. Infection (i.e., an invasion by and subsequent multiplication of a pathogenic microorganism within a body tissue or part (Sanz *et al.*, 2011)) or vaccination (an injection of a small dose of a pathogen) initiates an inflammatory response as well as an immune response. The infection is recognised by macrophages and mast cells, which results in the synthesis of inflammatory cytokines (Medzhitov, 2008). One such inflammatory cytokine is TNF- α. TNF- α is synthesized by a number of immune cells, including macrophages and mast cells (Medzhitov, 2008), and plays a vital role as a mediator of the acute phase inflammatory response (Medzhitov, 2008; Möller & Villiger, 2006). Another such cytokine is IL-6, which is synthesised in response to

elevations in other inflammatory cytokines, like TNF- α (Möller & Villiger, 2006), by a variety of cells, including monocytes, macrophages and endothelial cells (Ekstrom *et al.*, 2008). IL-6 can also induce increases in C-reactive protein (CRP), an important marker of the acute phase response (Ekstrom *et al.*, 2008; Möller & Villiger, 2006).

The relationship between inflammation and other variables, such as mood, has often been examined in populations with high levels of inflammation, such as acute coronary syndromes (Lesperance *et al.*, 2004) and rheumatoid arthritis (Zautra *et al.*, 2004). However, in addition to the elevated inflammation in these populations, other classical risk factors for these diseases are observed which can confound the associations seen (Cleland *et al.*, 2000; Vita *et al.*, 2004). To overcome any possible confounding with other factors in such patients, acute inflammatory stimuli have been used to examine the unique influence of inflammation on the variable of interest. The *Salmonella typhi* vaccination is one such method of stimulation. This vaccination elicits increases in inflammatory markers across a range of time points. Increases in IL-6 following vaccination have been demonstrated at 2 h (Brydon *et al.*, 2009), 3 h (Wright *et al.*, 2005), 4 h (Kharbanda *et al.*, 2002), 6 h (Chia *et al.*, 2003; Padfield *et al.*, 2010), 8 h (Antoniades *et al.*, 2011; Clapp *et al.*, 2004; Ekstrom *et al.*, 2008; Vlachopoulos *et al.*, 2007) and 12 h (Antoniades *et al.*, 2011) post vaccination. However, there is less consistent evidence for TNF- α, with some reporting increases at 4 hours post vaccination (Kharbanda *et al.*, 2002), and others not finding any changes (Hingorani *et al.*, 2000; Wright *et al.*, 2005). Finally, increases in CRP are consistently seen at 24 h post vaccination (Antoniades *et al.*, 2011; Padfield *et al.*, 2010). In sum, the time course of the inflammatory response varies depending upon the cytokine under investigation.

In addition to the inflammatory effects of vaccination, this vaccine has been used to examine the effects of inflammation on other factors, such as mood. Assessment of mood involves assessing the emotional state of a participant and a given time, which can reflect both positive and negative mood states. One common method used to assess mood is to use the Profile of Mood States (POMS) questionnaire pack (McNair *et al.*, 1981), which consists of six mood constructs: tension-anxiety, vigour-activity, depression-dejection, fatigue-inertia, anger-hostility and confusion-bewilderment. These assessments are important as mood can influence bodily functions and is linked to the prevalence of CVD and CVD-linked mortality (Baune *et al.*, 2012). In addition, mood can influence the immune and inflammatory responses to vaccination. For example, a study that assessed IL-6 and depression (which can include symptoms of fatigue, anxiety and negative mood (Miller *et al.*, 2009)) in response to an influenza vaccination in seniors, found that higher depression at baseline resulted in higher IL-6 at baseline and in response to vaccination as well as no effect of vaccination on mood (Glaser, 2003). Interestingly, depressive symptoms have been shown to predict an exaggerated macrophage inhibitory factor response to influenza virus vaccination during pregnancy, demonstrating an enhanced inflammatory response during pregnancy (Christian *et al.*, 2010). Conversely, positive mood is predictive of an improved antibody response to vaccination (Marsland *et al.*, 2006).

Mood and inflammation are inversely related in a variety of populations (Raison *et al.*, 2006). Greater depressive symptoms have been reported in clinical populations with elevated levels of inflammatory cytokines, such as acute coronary syndromes (Lesperance *et al.*, 2004) and rheumatoid arthritis (Zautra *et al.*, 2004), with the greatest symptoms reported in those with higher inflammatory markers (Lesperance *et al.*, 2004; Zautra *et al.*, 2004). Interestingly,

elevations in inflammation are also seen in populations that are only mildly depressed (Irwin & Miller, 2007; Raison *et al.*, 2006). The relationship between inflammation and depression has also been examined in healthy populations, in whom acute, transient inflammation has been induced. Studies that have administered endotoxin found transient increases in negative mood constructs, such as anxiety and depressed mood (Eisenberger *et al.*, 2009; Eisenberger *et al.*, 2010; Reichenberg, 2001). Studies that used a milder inflammatory stimulus, such as the *Salmonella typhi* vaccination, have found more equivocal effects on mood. Positive mood changes have been observed in response to a saline placebo injection which was not evident in participants who received a typhoid vaccination; suggesting that inflammation might lead to negative mood (Strike *et al.*, 2004). Positive correlations between IL-6 and negative mood evident following vaccine administration have been found (Wright *et al.*, 2005). Conversely, negative mood did not increase as a result of vaccination administration (Brydon *et al.*, 2009), however, this was examined at only 2 hours post vaccination administration, whereas the aforementioned studies assessed mood at a later time point. Therefore, it is possible that changes in mood occur when the inflammatory response is greatest, and as such the time course of any changes in mood warrants investigation. To date, one study has examined the time course of mood changes in response to vaccination, but did not report any data detailing the inflammatory response or any links between cytokines and mood (Strike *et al.*, 2004).

One benefit of using the *Salmonella typhi* vaccination as an inflammatory stimulus is that it does not induce feelings of malaise or fever (Hingorani *et al.*, 2000; Strike *et al.*, 2004; Wright *et al.*, 2005), which is in contrast to more potent inflammatory stimuli, such as endotoxin (Reichenberg, 2001). However, other physical responses to *Salmonella typhi* vaccination have been noted (Brydon *et al.*, 2009; Wright *et al.*, 2005), including aching

joints and headache, with either no (Brydon *et al.*, 2009) or small changes (Wright *et al.*, 2005) in physical symptoms observed. However, these physical symptoms were typically assessed at only one or two time points as a manipulation check, and as such, no time course effects of inflammation on physical symptoms have been reported.

In addition to characterizing the psychological and inflammatory responses to vaccination, the current study will also determine any associated changes in cardiovascular activity. Previous research has documented no effects of induced inflammation on blood pressure and heart rate (Chia *et al.*, 2003; Padfield *et al.*, 2010; Strike *et al.*, 2004; Vlachopoulos *et al.*, 2005), as well as cardiac output and total peripheral resistance (Vlachopoulos *et al.*, 2005). However, a study has revealed that inflammation reduced heart rate variability in mice (Hajiasgharzadeh *et al.*, 2011), as well as lipopolysaccharide induced increases in inflammation leading to alterations in heart rate variability (Kox *et al.*, 2011), supporting a link between inflammation and the autonomic nervous system (Libert, 2003). However, as far as we are aware, no studies have examined the influence of vaccine induced inflammation on cardiovascular indices of sympathetic and parasympathetic activity. This is surprising, given evidence that decreased heart rate variability has been identified as a potential mechanism linking depression with elevated cardiac mortality in patients who have suffered a myocardial infarction (Carney *et al.*, 2001). Therefore, given the evidence supporting the association between depression and inflammation (Raison *et al.*, 2006), and the evidence that acute inflammation induces changes in negative mood (Eisenberger *et al.*, 2009; Eisenberger *et al.*, 2010; Reichenberg, 2001; Strike *et al.*, 2004; Wright *et al.*, 2005), it is possible that alterations in the degree of inflammation may also influence cardiovascular parameters, such as heart rate variability.

Despite research examining these constructs separately, or in combination but over a shortened time frame, no study has reported the time course of inflammatory, psychological (mood, symptoms) and physiological (cardiovascular) responses to vaccination. Therefore, the current study has several aims. Our primary aim was to examine the inflammatory response to *Salmonella typhi* vaccination over a 24 hour period. The secondary aims were to determine the effects of this inflammatory stimulus on cardiovascular function and psychological mood. It was hypothesised that vaccination would induce an inflammatory response, with increases in IL-6 peaking at six hours and CRP peaking at 24 hours post-vaccination. It was also hypothesised that inflammation would increase negative mood, and blunt cardiac function, as indexed by reduced heart rate variability.

3.3 METHODS

Participants

Eight (N = 8) male participants (mean age ± SD = 26.63 ± 8.07 years, mean body mass index ± SD = 23.21 ± 1.80 kg/m^2) were recruited. At the time of testing, none were suffering from an acute illness or infection, reported a history of inflammatory, cardiovascular or auto-immune disorders, or had taken any medication in the last 4 weeks. None had vaccine-related allergies or had received a typhoid vaccination in the last 12 months. Participants reported to the laboratory having refrained from vigorous exercise for at least 24 hours, from alcohol for at least 12 hours and food or caffeine in the 2 hours prior to the start of testing. The study was approved by the local research ethics committee, and all participants gave written informed consent.

Procedure

Participants completed an inflammatory condition (vaccination) and a control condition (saline) on two separate days, at least 7 days apart, in a counter-balanced blinded design. All participants reported to the laboratory between 8:30 and 9:00 am, and after written consent was obtained, questionnaires were completed and instrumentation for cardiovascular assessment undertaken. Blood pressure was recorded before the first blood sample was taken (0 h). Next, the injection was administered and participants waited 20 min to check for any adverse events. A blood sample, questionnaires, and cardiovascular measurements were taken at each of the following time points (4 h, 5 h, 6 h and 7 h post injection) until the final time point (8 h post injection). Participants returned the following day to complete questionnaires and provide a final blood sample and blood pressure reading. All participants repeated the procedure in the other condition. Participants were asked to replicate their diet on both testing days, in an attempt to negate the effects of diet upon the responses to vaccination. There was at least a 45 minute period between eating and a blood sample.

Vaccination

On each day, participants received one of two injections: 0.5 ml *Salmonella typhi* capsular polysaccharide vaccine (0.025 mg in 0.5 ml, Typhim Vi, Sanofi Pasteur UK) or a saline placebo (0.5 ml) via intra-muscular injection into the deltoid muscle of the non-dominant arm, in a counterbalanced, blinded crossover design.

Questionnaires

Subjective ratings of physical responses to vaccination and mood were assessed by questionnaire at 4 h, 5 h, 6 h, 7 h, 8 h and 24 h post injection. Participants were asked to rate their physical symptoms related to the vaccination. These included general physical

symptoms they had experienced since injection, including malaise, sore throat, watery eyes or eye strain, upset stomach, sneezing, congested nose, persistent cough, headaches, neck ache, shortness of breath, feverishness, chills, nasal discharge, and ringing in the ears using a 5 point Likert scale, with anchors of 0 (not at all) and 5 (severe). Participants were also asked to rate joint pain and pain at the site of the injection using a 10-point scale, with anchors of 1 (very mild) and 10 (very severe). The specific physical symptoms and general reactions were aggregated to give an overall score at each time point of assessment. The Cronbach alphas for the physical symptoms and pain ratings at the site of vaccination aggregate scales ranged from 0.79-0.96.

Mood was assessed with a modified version of the Profile of Mood States (POMS) questionnaire (McNair *et al.*, 1981), from which the following 6 constructs were calculated; tension-anxiety, anger-hostility, vigor-activity, fatigue-inertia, confusion-bewilderment, and depression-dejection. Participants were asked to rate on a 5-point scale (0 = not at all, 4 = extremely), how they felt at that precise moment. The Cronbach alpha's for each subscale ranged from 0.43 - 0.98 in the saline condition and from 0.68 - 0.96 in the vaccination condition. The low reliability score in the saline condition was due to of the absence of anger; the remaining coefficients were above 0.65.

Physiological Measurements

Cardiovascular measures

Resting blood pressure was assessed at each blood draw using an Omron 705CP (HEM-705CP-E) blood pressure monitor. Systolic (SBP) and diastolic (DBP) blood pressure was used to calculate mean arterial pressure (MAP). Indices of cardio-dynamic activity were recorded continuously using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS, Amsterdam, The Netherlands) (de Geus *et al.*, 1995; Willemsen *et al.*, 1996). This

system used six Ag/AgCl spot electrodes (Invisatrace, ConMed Corporation) to record electrocardiography (ECG) and impedance cardiography (ICG), in line with published guidelines (Sherwood *et al.*, 1990). Sixty second ensemble averages were calculated and used to determine Heart Rate (HR, bpm), Pre-Ejection Period (PEP; ms) and Root Mean Square Successive Difference (rMSSD) as a measure of Heart Rate Variability (HRV, ms).

Blood Sampling

The blood draws at baseline (0 h) and 24 h were taken using a 21 gauge butterfly needle (Becton Dickinson, UK), to reduce the amount of time the participant was cannulated for and the risk of the cannula becoming blocked. The remaining draws (4 h, 5 h, 6 h, 7 h, 8 h) were taken using a cannula (18 gauge, Insyte, Becton Dickinson), which was inserted into an antecubital vein in the participant's dominant arm. On each draw, the first 3 ml of blood was collected in a syringe and discarded. The timing of cannula insertion remained consistent across both conditions. Blood was collected into two 4 ml and one 2 ml vacutainers containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton–Dickinson, UK). The 4 ml samples were stored on ice until centrifugation (1500 × g for 10 min at 4 degrees C) and plasma was stored at -80 degrees C for later assessment of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and C - reactive protein (CRP). The 2 ml samples were placed on a roller until analyzed for full blood cell count (total lymphocyte, monocyte and granulocyte count) using a Coulter Analyzer (Beckman Coulter, Inc.).

Assays

Plasma IL-6 and TNF-α were measured in duplicate using high-sensitivity ELISAs (Quantikine HS Human IL-6 ELISA and Quantikine HS Human TNF-α ELISA, both R&D

Systems, UK) in accordance with the manufacturer's instructions. The reported sensitivity of the assays was 0.039 pg/ml and 0.106pg/ml for IL-6 and TNF-α respectively, with recorded intra-assay and inter-assay variations both < 10%. Analysis of high sensitivity C-reactive protein was undertaken at a commercial laboratory (Synlab, Leinfelden, Germany) by immunonephelometry using a Behring Nephelometer II. The detection limit for CRP was 0.015 mg/L (High Sensitivity CRP, Dade Behring), with all samples assayed in the same run, yielding a within-assay CV% of < 4.5%.

Data reduction and analysis

For all inflammatory and mood responses to vaccination, a series of 2 Condition (Control, Inflammation) by 7 Time (0, 4, 5, 6, 7, 8 and 24 hours post injection) repeated measures analyses of variance (ANOVAs) were conducted. Physiological responses to vaccination (HR, PEP, HRV and MAP) were examined by a series of 2 Condition (Control, Inflammation) by 6 Time (0, 4, 5, 6, 7, and 8 hours post injection) repeated measures ANOVAs. For all ANOVAs, Greenhouse-Geisser correction was applied where appropriate (Vasey & Thayer, 1987). Eta squared (η^2) was used as a measure of effect size. Occasional missing data are reflected in the reported degrees of freedom. Polynomial trend analyses were conducted to examine the time course of response to vaccination for each of the variables assessed (inflammatory, physiological and psychological).

The six POMs constructs (tension-anxiety, anger-hostility, vigor-activity, fatigue-inertia, confusion-bewilderment, and depression-dejection) were averaged at each time point to yield a mean value between 0 (not at all) and 4 (extremely). Total negative mood was calculated by combining all negative constructs together as described elsewhere (Wright *et al.*, 2005). Additionally, each individual physical symptom and general reaction to the

injection was aggregated to give an overall score for general physical symptoms and pain at each time point of assessment.

3.4 RESULTS

Inflammatory response to vaccination

Figure 3.1 illustrates the inflammatory response to vaccination in terms of changes in the numbers of lymphocytes, monocytes and granulocytes as well as the concentrations of IL-6, TNF- α and CRP. A series of 2 Condition x 7 Time ANOVAs yielded condition effects for granulocytes (F (1, 7) = 30.48, p =.001, ε = 1.00, η^2 = .81), and IL-6 (F (1, 7) = 6.60, p =.037, ε = 1.00, η^2 = .49), with values higher for vaccination than the saline control condition. Time effects were noted for lymphocytes (F (2.38, 16.62) = 4.37, p = .025, ε = .40, η^2 = .38), granulocytes (F (3.22, 22.51) = 15.32, p < .001, ε = .54, η^2 = .69), and IL-6 (F (2.63, 18.44) = 7.14, p = .003, ε = .44, η^2 = .51). Importantly, condition by time interaction effects emerged for granulocytes (F (3.02, 21.12) = 12.07, p = .002, ε = .50, η^2 = .50) and IL-6 (F (3.05, 21.35 = 11.24, p = .042, ε = .51, η^2 = .32), with monocytes revealing partial interaction effects (F (2.53, 15.12) = 1.09, p = .098, ε = .42, η^2 = .30). To characterize the time course of the inflammatory response in the vaccination and placebo conditions, we computed polynomial trend analyses on the immune variables. These analyses revealed quadratic effects for time (granulocytes = F (1, 7) = 40.11, p < .001, η^2 = .85; IL-6 = F (1, 7) = 15.64, p = .006, η^2 = .69) and, importantly, condition by time (monocytes = F (1, 6) = 13.06, p = .011, η^2 = .69; granulocytes = F (1, 7) = 14.73, p = .006, η^2 = .68; IL-6 = F (1, 7) = 6.00, p = .044, η^2 = .46). Cubic time effects were observed for lymphocytes (F (1, 7) = 11.45, p = .012, η^2 = .62). As can be seen in Figure 3.1, granulocytes, IL-6 and monocytes only increased following

vaccination, peaking around 6-7 hours post inoculation, before returning to baseline levels after 24 hours.

Analysis of hsCRP responses by a 2 condition x 7 time ANOVA yielded no main condition, time or condition by time interaction effects (*p's* >.05). However, trend analyses revealed a significant linear condition by time effect (F (1, 7) = 8.483, p = 0.027, η^2 = .59), with a gradual increase in CRP in the inflammatory condition, and a gradual decrease in CRP in the control condition, peaking after 24 hours post vaccination. No condition, time, or time by condition interaction effects were observed for TNF-α (*p's* >.05). Trend analyses revealed a significant cubic condition by time effect for TNF-α (F (1, 7) = 6.92, p = .034, η^2 = .50), with steady increases in TNF-α observed in the inflammatory condition, plateauing 6-8 h post vaccination. During the same time period in the placebo condition, decreases in TNF-α were observed.

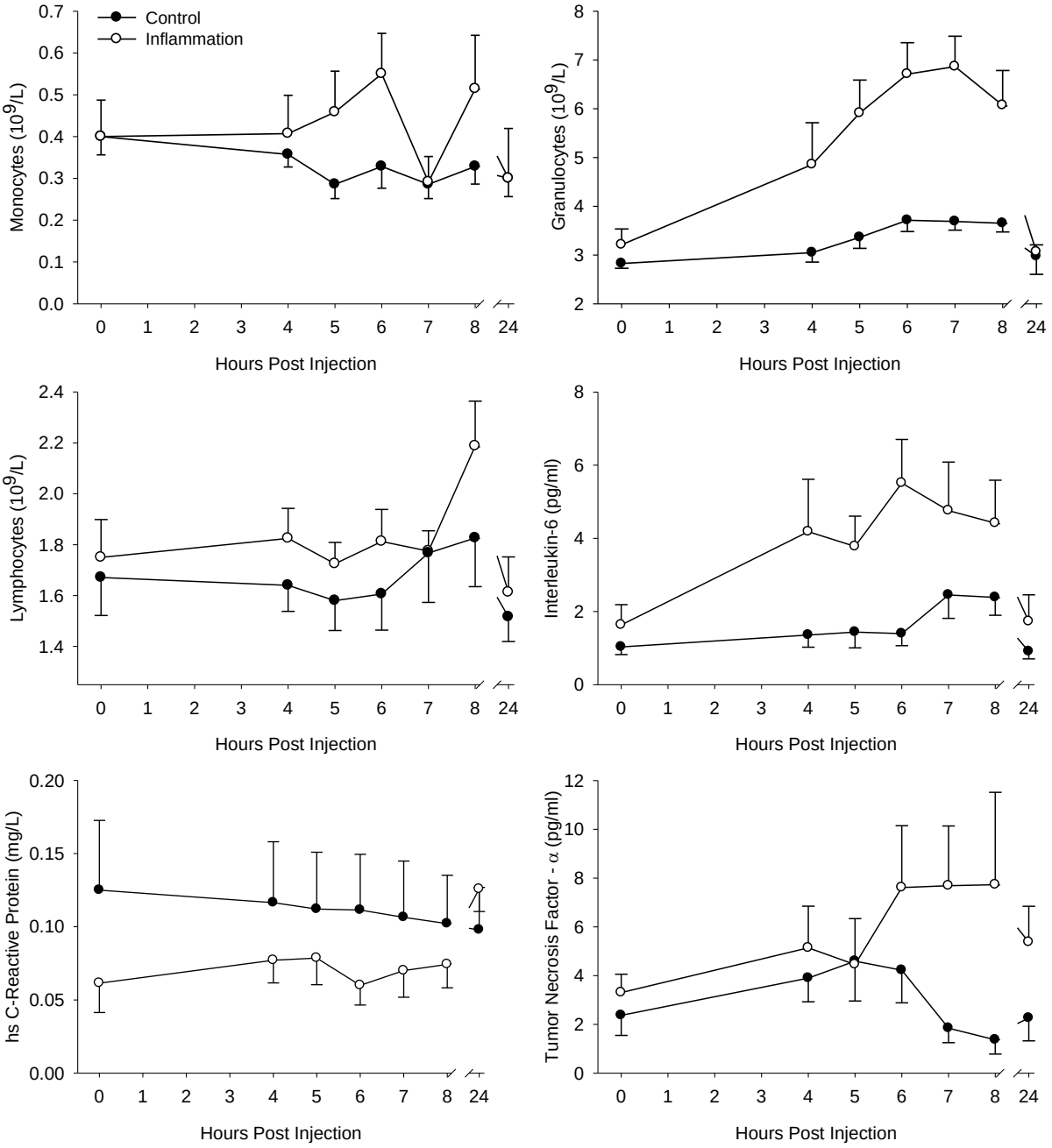

Figure 3.1. Mean ± SE inflammatory responses to vaccination for Monocytes, Lymphocytes, hsCRP, Granulocytes, IL-6, and TNF-α. Shaded circles indicate the control condition, with the clear circles indicative of the inflammation condition.

Physical symptoms in response to vaccination

Analysis of all reported physical symptoms revealed no main time or time by condition interaction effects (*p's* > .05), as revealed by Table 3.1. There were no differences in the physical symptoms reported in the vaccinated condition (*p's* > .05). There was a tendency for pain at the site of vaccination to be elevated in the vaccine condition, (F (1, 7) = 4.77, p = .065, ε =1.00, η^2 = .41). No time or time by condition interaction effects were observed (*p's* > .05).

Table 3.1. Mean (SD) physical symptoms to injection on a scale of 0-10 for physical symptoms, and 0-5 for pain

Time post-injection (Hours)	**Physical Symptoms (0-10)**		**Pain (0-5)**	
	Control condition	Inflammation condition	Control condition	Inflammation condition
4	0.13 (0.35)	0.75 (1.49)	0 (0)	1.06 (0.82)
5	0 (0)	0.50 (1.41)	0 (0)	1.56 (1.97)
6	0.25 (0.71)	0 (0)	0 (0)	1.14 (1.89)
7	0.13 (0.35)	0 (0)	0 (0)	0.88 (0.92)
8	0 (0)	0 (0)	0 (0)	1.00 (1.71)
24	0.25 (0.71)	0.13 (0.35)	0 (0)	1.25 (2.02)

Note: A score of 0 indicates 'Not at all', with 5/10 indicating 'Severe'. Aggregated physical symptoms refer to the aggregate of all physical symptoms, with pain referring to localised pain at the site of vaccination.

Physiological responses to vaccination

Figure 3.2 captures the time course of heart rate, heart rate variability, pre-ejection period and mean arterial pressure in the hours following inoculation. It is notable that there were no significant condition or condition by time effects (all *p's* >.05). Exploratory polynomial trend analyses confirmed there to be no linear, quadratic or cubic time effects for any variable (all *p's* >.05), with one exception being that there was an overall cubic trend for time for heart rate (F (1, 7) = .11, p = .006, η^2 = .74), which revealed a small decrease in heart rate at 4 h, before rising to be slightly elevated at 5-6 h before returning to baseline values at the 8 h time point.

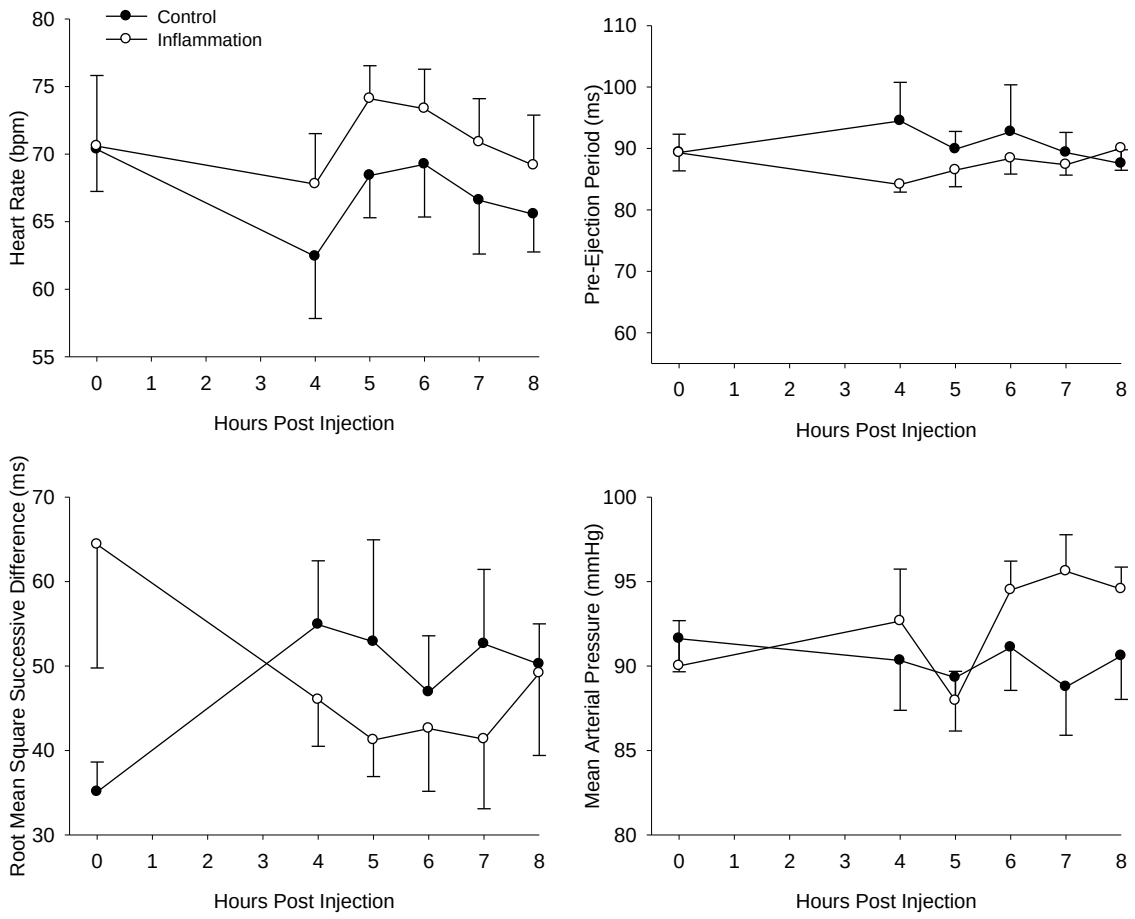

Figure 3.2. Mean ±SE cardiovascular responses to vaccination for HR, rMSSD, PEP and MAP. Shaded circles indicate control condition, clear circles indicative of inflammation condition.

Psychological responses to vaccination

Changes in the negatively-valenced moods experienced by participants in the two conditions are depicted in Figure 3.3. Examination of each individual POMS construct (tension, anger, fatigue, confusion and depression) and total negative mood (by combining all negative constructs to give one aggregate score) was conducted by a series of 2 condition x 7 time ANOVAs. These analyses yielded no condition, time, or condition by time interaction effects (*p's* >.05). Thus, there were no differences in mood between conditions, nor changes in mood over time.

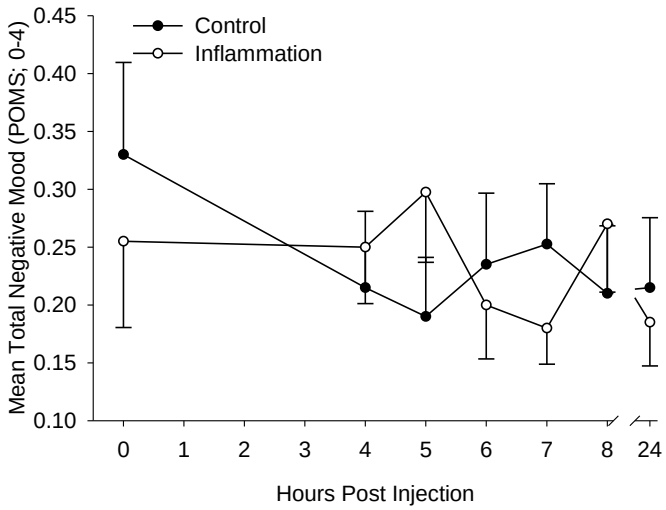

Figure 3.3. Mean ±SE total negative mood responses over the 24h period post injection, in both the control (black) and inflammation (white) conditions.

3.5 DISCUSSION

In broad agreement with our predictions, the administration of a 0.5 ml dose of the *Salmonella typhi* vaccine increased the concentrations of two cytokines, IL-6 and TNF-α, as well as the numbers of immune cells, white blood cells and granulocytes, in the blood, with responses peaking (at more than double the initial values) approximately 6-7 hours post vaccination and returning back to basal levels at 24 hours post vaccination. CRP also

increased following vaccination, with only a small rise characterizing the first 8 hours, and then with values increasing to more than double the basal levels after 24 hours post vaccination. In addition, participants reported more physical symptoms and pain at the site of the injection following vaccination. However, no effects of vaccination on mood and cardiovascular activity were observed**.**

Effects of vaccination on inflammatory markers

This study was the first, as far as we aware, to examine the time course of the inflammatory response to a vaccination in comparison to a saline injection, over a 24 hour period. Vaccination administration results in a small dose of bacterium being injected into the body, causing both inflammatory and immune responses to the pathogen. The pathogen is recognised by macrophages and mast cells, resulting in synthesis and release of inflammatory cytokines (Medzhitov, 2008). The findings of the current study are in line with the pathway through which this inflammatory response occurs. Significant increases in IL-6 levels were observed in response to vaccination, with trend analyses revealing a peak at 6 hours post-vaccination. This pattern of response is identical to the ones demonstrated for the numbers of white blood cells and granulocytes in the circulation, with the increases in these immune cells providing further evidence of the ongoing inflammatory response to vaccination. No such increases were observed in the control condition. However, this specific vaccination results in only injecting capsular polysaccharide rather than the whole bacteria, and it may be that granulocytes do not directly recognize capsular polysaccharide. However, granulocytes are indicative of neutrophil number (Kusumanto *et al.*, 2003), which is important because neutrophils are activated to 'kill' the pathogen in the vaccine by releasing their granular cellular content (Medzhitov, 2008). Therefore, the maintenance of the peak (6-7 h) elevations

in white blood cells and granulocytes are most likely due to the elevations in IL-6 which are seen at 6 h post vaccination. We obtained no direct measure of macrophage activity, and as no interaction effects were observed for monocyte responses to inoculation, thus the exact timing of the putative increase in macrophages must remain speculative. However, given that inflammatory markers are already elevated at 4 h post-vaccination, it is probable that macrophage increases would have been observed before the first post-vaccination blood sample at 4 h. A previous study examined the response to a double dose (i.e., 1 ml) of the vaccination used in the current study, revealing the peak IL-6 response to be at 10 h post-vaccination (Bennermo *et al.*, 2004). However, the larger quantity of vaccine used might be one reason for the delayed peak IL-6 response, and given that more pathogen would have been within the vaccine, a more substantial and prolonged inflammatory response is likely to have occurred.

In a similar pattern to the IL-6 response observed in this study, changes in TNF- α were observed in the vaccine condition. TNF- α increases would have been observed as a consequence of the macrophage response to vaccination, and would also be responsible for synthesising IL-6 (Möller & Villiger, 2006). There appears to be great variability in the TNF-α response to this particular vaccination, with studies reporting increases (Kharbanda *et al.*, 2002) while others demonstrated no changes (Harrison *et al.*, 2009; Hingorani *et al.*, 2000; Wright *et al.*, 2005). The variety in the time points of assessment might be one reason for this or the amount of vaccination (1 ml vs 0.5 ml) (Bennermo *et al.*, 2004; Hingorani *et al.*, 2000; Kharbanda *et al.*, 2002; Wright *et al.*, 2005). The trend analyses show that CRP increased selectively at 24 h in the vaccine condition. This is in line with other studies where increases in CRP were observed 24 h post-vaccination (Antoniades *et al.*, 2011; Padfield *et al.*, 2010).

Given the relationship between IL-6 and CRP (Ekstrom *et al.*, 2008; Möller & Villiger, 2006), it is anticipated that the increases in CRP observed in the inflammation condition are indicative of increases as a result of the other inflammatory responses to vaccination.

The effect of vaccination on physical symptoms

In addition to the inflammatory response to vaccination, we also examined the contemporaneous self-reported symptoms. The current study found no significant differences in the physical symptoms reported in response to vaccination in contrast to the saline injection. This is in line with previous work that has demonstrated that the *Salmonella typhi* vaccination does not induce feelings of malaise or fever (Hingorani *et al.*, 2000; Strike *et al.*, 2004; Wright *et al.*, 2005), or feelings of aching in the limb in which the vaccination was administered (Brydon *et al.*, 2009). However, it should be acknowledged that participants in the vaccination condition tended to report localised pain in the limb that was vaccinated compared to the saline inoculation. This tendency may be reflective of relatively small changes in physical symptoms, which have also been reported elsewhere (Wright *et al.*, 2005) or due to the small sample size used in this study, which is more likely given the effect size of .41 revealed in this study (Cohen, 1992). Taken together, these results suggest that in addition to the systemic response to vaccination, as indexed by increases in inflammatory markers, a localised, as indexed by elevated muscular pain, response at the site of vaccination might also be evident.

Effect of vaccination on mood

Despite the increases in inflammatory markers observed in this study, no effects of vaccination-induced inflammation were observed on mood, either when mood was assessed

as a series of individual constructs, or as a total aggregate negative mood score. This finding is in contrast to previous a study reporting that increases in inflammation (indexed by IL-6) were positively correlated with negative mood assessed by the POMS (Wright *et al.*, 2005). Other studies have revealed similar results, with increases in positive mood in response to a saline vaccination not observed in response to a typhoid vaccination (Strike *et al.*, 2004). Importantly, it is worth noting that this latter study did not find negative mood to be induced by vaccination *per se,* but rather reported there to be no vaccine-related increases in positive mood. Interestingly, in response to stress tasks, increases in task-related anger and anxiety have been positively associated with increases in IL-6, highlighting further the links between negative mood and inflammatory responses (Carroll *et al.*, 2011). However, similarly to the current study, others have also not observed increases in negative mood two hours after vaccination administration (Brydon *et al.*, 2009), indicating that this vaccination may not induce negatively-valenced feelings. Indeed, there is more consistent evidence for increases in negative mood following endotoxin administration (Eisenberger *et al.*, 2009; Eisenberger *et al.*, 2010; Reichenberg, 2001), hinting that increases in negative mood might only occur in response to more substantial inflammatory response. Pro-inflammatory cytokines (such as TNF-α and IL-1) can signal the brain either by activating the primary afferent neurons which are situated at the site of inflammation, or by a slow diffusing process of cytokines from the circumventricular organs to the amygdaloid complex (Dantzer, 2001). This can result in sickness symptoms that may include negative and depressed affect (Dantzer, 2001; Dantzer *et al.*, 2008).

Interestingly, other studies have demonstrated that negative mood, as indexed by depression ratings, may be associated with elevated inflammatory levels (Stewart *et al.*,

2009). Depressive symptoms in older adults predicted IL-6 levels six years later, rather than inflammation predicting the appearance of depressive symptoms (Stewart *et al.*, 2009). This finding was replicated in a coronary heart disease population where depression predicted the levels of IL-6 and hsCRP (Duivis *et al.*, 2011). These two particular studies are in contrast to others which reveal inflammation to predict the appearance of depressive symptoms (Gimeno *et al.*, 2009). It is apparent from these studies that the relationship between inflammation and depression may be bi-directional.

Effect of vaccination on cardiac function

Similar to the mood responses to vaccination, with the exception of the overall (and non-specific) trend for heart rate to decrease over time which has been reported elsewhere (Strike *et al.*, 2004), no effects of vaccination were observed on cardiovascular activity. No effects of acute inflammation have been observed on blood pressure and heart rate (Chia *et al.*, 2003; Padfield *et al.*, 2010; Strike *et al.*, 2004; Vlachopoulos *et al.*, 2005), or cardiac output and total peripheral resistance (Vlachopoulos *et al.*, 2005). Thus, acute vaccine-induced inflammation had no effect on the cardiovascular parameters assessed. Nevertheless, endotoxin administration, a more potent inflammatory stimulus, resulted in reduced left ventricular function in a healthy population (Suffredini *et al.*, 1989), and therefore a more substantial inflammatory stimulus may impair cardiovascular function. Indeed, there is evidence that chronic inflammation can affect cardiovascular activity, with elevations in inflammatory markers such as IL-6 and CRP inversely associated with heart rate variability, in healthy populations and those with cardiovascular diseases (Haensel *et al.*, 2008; Sajadieh *et al.*, 2004; von Känel *et al.*, 2011) .

Future work and limitations

The current study might have implications regarding the vaccinating of individuals who already have elevated levels of inflammation, such as those at risk of CVD, stroke, myocardial infarction or even an aging population. Analyses of studies that have examined the risk of CVD and myocardial infarction after vaccination have revealed no increased risk of myocardial infarction (Smeeth *et al.*, 2004) and small reductions in the risk of incidence of cardiovascular death (Warren-Gash *et al.*, 2009). However, these studies examined vaccines, such as influenza, that induce weaker inflammatory responses and as such, the effects of a vaccine that induces a greater inflammatory response, such as the typhoid vaccination, might warrant closer examination. Likewise, given the known increases in risk of myocardial infarction as a result of infection (Meier *et al.*, 1998), the effects of vaccination as a trigger for myocardial infarction in a healthy population might be worth further consideration. Indeed, given the known effects of acute inflammation on resting vascular function (Antoniades *et al.*, 2011; Clapp *et al.*, 2004; Hingorani *et al.*, 2000), examination of the time course effects of inflammation on vascular function also justifies further consideration.

This study did not examine the immune response to vaccination, as indexed by antibody titres or other immune markers. Future studies may wish to assess these parameters after several weeks and months to examine whether the acute inflammatory response to vaccination might predict the antibody response to vaccination. Interestingly, previous research has demonstrated associations between antibody titres at 4 weeks post vaccination and IL-6 (Edwards *et al.*, 2006), however, the increases in IL-6 were elicited by a mental stress task prior to vaccination and not as a result of vaccination itself. Indeed, given the role of inflammatory markers in initiating the immune response to vaccination, it might be

interesting to examine whether the extent of the inflammatory response might be reflective of the extent of the immune response to vaccination.

Conclusion

To our knowledge, this is the first study to examine the inflammatory time course effects of the *Salmonella typhi* vaccination, and its impact on mood and cardiovascular activity. It appears that the *Salmonella typhi* vaccination is a potent inflammatory stimulus resulting in increases in IL-6 and TNF-α, which peaked at 6-7 h post vaccination. Elevations in white blood cells and granulocytes were also demonstrated in the vaccination condition. CRP also increased following vaccination, with largest increases seen at 24 h post vaccination. There was a tendency for more physical symptoms and pain at the site of vaccination in the vaccination condition, but no effects of inflammation were observed on mood or any cardiovascular measure.

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CHAPTER FOUR:

VACCINE-INDUCED INFLAMMATION IMPAIRS THE VASODILATORY RESPONSE TO MENTAL STRESS

4.1 ABSTRACT

Both inflammation and vascular responses to mental stress have been implicated in triggering myocardial infarction. It is known that inflammation is associated with poorer vascular function at rest. To extent this literature, this study examined the effects of vaccine-induced inflammation on the vascular responses to mental stress. Eighteen healthy male participants completed two afternoon stress reactivity sessions: inflammation and control, scheduled at least one week apart. Inflammation was induced by administering a *Salmonella typhi* capsular polysaccharide (typhoid) vaccination in the morning, six hours prior to afternoon stress testing session. Blood samples were taken at the start of each session to assess cytokine levels. Blood flow, blood pressure, heart rate and cardiac output, were assessed at rest and in response to mental stress. Tumor necrosis factor alpha and interleukin-6 were higher (*p*'s < .001) in the inflammation condition than the control condition confirming that the vaccine elicited the expected inflammatory state. Mental stress induced increases in forearm blood flow, blood pressure, heart rate, and cardiac output in both conditions (all *p*'s < .001). Importantly, forearm and calf blood flow responses to stress were attenuated in the inflammation condition compared to the control condition (*p*'s < .05). These findings show that vaccine-induced inflammation can attenuate the vascular responses to mental stress. The impairment in the vasodilatory responses to stress by increased levels of inflammation suggests a mechanism through which inflammation can increase the risk for myocardial infarction.

4.2 INTRODUCTION

There is accumulating evidence that acute mental stress precedes myocardial infarction (MI). For example, survivors of MI have identified emotional stress, such as episodes of anger, as a trigger for their MI (Strike & Steptoe, 2005). In addition, epidemiological studies have reported increases in MI incidence in areas struck by an earthquake (Leor *et al.*, 1996; Suzuki *et al.*, 1995), war zones (Bergovec *et al.*, 1992; Meisel *et al.*, 1991), and even following major football matches (e.g., Carroll *et al.*, 2002; Wilbert-Lampen *et al.*, 2010). Further evidence has implicated both inflammation and the vasculature in mental stress-induced MI. For instance, serological markers of inflammation and vasoconstriction were found to be elevated in patients admitted for MI during the Football World Cup, compared to those admitted for MI during a non-eventful period (Wilbert-Lampen *et al.*, 2010). Similarly, laboratory studies have revealed that cardiac patients exhibiting mental stress-induced ischaemia have higher levels of inflammation at rest (Shah *et al.*, 2006), as well as poorer peripheral vascular responses to mental stress (Burg *et al.*, 2009; Goldberg *et al.*, 1996; Jain *et al.*, 1998). These studies therefore highlight the potential roles of both inflammation and the vasculature in myocardial infarction as well as mental stress induced ischemia.

Endothelial dysfunction is considered an indicator of atherosclerosis (Lerman & Zeiher, 2005), a process which is in large driven by inflammation (Hansson, 2005; Libby, 2006). The association between inflammation and vascular function has been explored in various populations, with impaired endothelial function found in patients with increased levels of inflammation including coronary artery disease (Fichtlscherer *et al.*, 2004) and rheumatoid arthritis (Vaudo *et al.*, 2004). However, given that patients with these diseases

need to be medicated and often present with multiple cardiovascular risk factors that can also influence vascular function, it is not possible to isolate the influence of inflammation on endothelial function. To avoid these limitations, the influence of inflammation on endothelial function has been studied in healthy populations using a variety of experimental manipulations designed to mimic or induce transient systemic inflammation. Specifically, direct infusion of inflammatory cytokines (Bhagat & Vallance, 1997) and administration of a *Salmonella typhi* capsular polysaccharide vaccine (Clapp *et al.*, 2004; Hingorani *et al.*, 2000; Kharbanda *et al.*, 2002) can impair endothelial function.

Both *in vivo* and *in vitro* studies provide evidence that inflammation affects the vasculature by reducing NO bioavailability. Endothelial function is assessed measuring the vasodilatory response of the blood vessels to a standardised stimulus (Sandoo *et al.*, 2010). This stimulus (e.g., shear stress) enhances the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS), resulting in the synthesis of nitric oxide (NO) (van Zonneveld *et al.*, 2010; Zhang, 2008). NO acts as a direct relaxant to the smooth muscle cells surrounding the blood vessel, resulting in dilation (Galley & Webster, 2004; Sandoo *et al.*, 2010). NO has been implicated as a mechanism through which inflammation causes endothelial dysfunction (Clapp *et al.*, 2004; Hingorani *et al.*, 2000; Kharbanda *et al.*, 2002). This association between inflammation and NO bioavailability has been substantiated by in vitro studies, which revealed reductions in NO production through increases in inflammatory markers such as tumor necrosis factor alpha (TNF-α) (Goodwin *et al.*, 2007), interleukin-6 (IL-6) (Hung *et al.*, 2010) and C-reactive Protein (CRP) (Verma *et al.*, 2002). Interestingly, there is evidence for a reciprocal relationship between NO and inflammation, with NO resulting in decreases in inflammatory markers such as TNF-α (De Martin *et al.*, 2000). In

sum, both *in vivo* and *in vitro* studies provide evidence that inflammation affects the vasculature by reducing NO bioavailability.

In healthy participants, mental stress reliably causes vasodilation, as shown by increases in blood flow (Blair *et al.*, 1959; Dietz *et al.*, 1994; Joyner & Halliwill, 2000). This stress-induced vasodilation is attenuated in those at risk for cardiovascular disease (Hamer *et al.*, 2007) and patients with heart failure (Middlekauff *et al.*, 1997; Santos *et al.*, 2005). Several mechanisms may underlie the stress-induced vasodilation, including neural (Carter *et al.*, 2005a; Carter *et al.*, 2005b; Carter *et al.*, 2008) and beta (β)-adrenergic stimuli (Lindqvist *et al.*, 1997; Lindqvist *et al.*, 1999), however, stress-induced vasodilation is most consistently associated with NO bioavailability (Joyner & Dietz, 2003; Joyner & Casey, 2009; Sarabi & Lind, 2001). As described above, in a healthy endothelium, shear stress stimulates eNOS to produce NO. It is thought that stress-induced increases in blood pressure result in increased shear stress, which induces NO production and therefore enhances vasodilation. The role of NO is emphasised by studies showing that inhibiting NO caused a substantial reduction in FBF during stress (Cardillo *et al.*, 1997; Dietz *et al.*, 1994). Unfortunately, direct measurement of NO in blood is difficult and results are often unreliable (Wadley *et al.*, 2012), as it is an extremely transient substance (Beckman *et al.*, 1990). While indirect measures are possible, results provide very limited information.

Only one study, to our knowledge, has examined the influence of inflammation on vascular responses to mental stress: rheumatoid arthritis patients with high-grade systemic inflammation displayed an increase in vascular resistance in response to mental stress, which was not evident in patients with low-grade inflammation (Veldhuijzen van Zanten *et al.*,

2008). Given the complex nature of rheumatoid arthritis, it was not possible to determine the unique influence of inflammation on these vascular responses to stress. Therefore, the overall aim of the present study was to investigate the role of inflammation on the vascular responses to mental stress in a healthy population. Inflammation was induced by administering a *Salmonella typhi* polysaccharide vaccine. Given the association between inflammation and resting endothelial function, it was hypothesised that inflammation would lead to an attenuated vasodilatory response to mental stress (Paine *et al.*, 2012a).

4.3 METHODS

Participants

Eighteen male participants were recruited from the University of Birmingham (mean age ± SD = 19.5 ± 0.9 years, mean body mass index ± SD = 24.6 ± 2.8 kg/m^2) and completed the inflammation intervention. A further five male participants (mean age ± SD = 19.6 ± 1.34 years, mean body mass index ± SD = 25.3 ± 4.6 kg/m^2) completed a placebo intervention to enable us to determine the effects of repeated testing on cardiovascular reactivity to mental stress. None of the participants suffered from an acute illness or infection, reported a history of inflammatory, cardiovascular or auto-immune disorders, or had taken any medication in the last 4 weeks. None had vaccine-related allergies or had received a typhoid vaccination in the last 12 months. Participants reported to the laboratory having refrained from vigorous exercise for at least 24 hours, from alcohol for at least 12 hours and food or caffeine in the 2 hours prior to testing. The study was approved by the local research ethics committee and all participants gave written informed consent.

Procedure

Participants completed 2 sessions scheduled seven days apart (Figure 4.1); a control stress session (condition 1; control condition) and an intervention stress session (condition 2; intervention condition). In the intervention condition, a typhoid vaccination (inflammation intervention) or saline injection (placebo intervention) was given 6 hours before the start of the intervention stress session (condition 2). This time was chosen in order for the session to start at the peak inflammatory (IL-6) response to the vaccine (Paine *et al.,* in submission; Padfield *et al.,* 2010). All testing was performed in a temperature controlled laboratory (18°C). Both stress sessions took place in the afternoon and began at the same time for each participant, with start times between 2:00 pm and 5:00 pm.

Stress sessions

Upon arrival at the laboratory, the participant's height and weight were recorded, and they were instrumented for the cardiovascular assessment. The participant then assumed a supine position on a bed, where he remained throughout the session. An 18 gauge canula (Insyte, Becton Dickinson) was inserted into an antecubital vein of the dominant arm of each participant. After the blood pressure device (Finometer Medical Systems, Amsterdam) and blood flow equipment was attached, they rested for 20 minutes (baseline rest period) and watched a nature documentary (*Life*; BBC) on a television screen. During minutes 13, 15, 17 and 19 blood flow was measured. Continuous recordings of impedance cardiography and blood pressure were taken, and minutes 13, 15, 17 and 19 were assessed. A resting blood sample was taken at the end of the baseline rest period. After practice of the metal stress task, participants completed two 8 minute blocks of the mental stress task, with 1 minute rest in

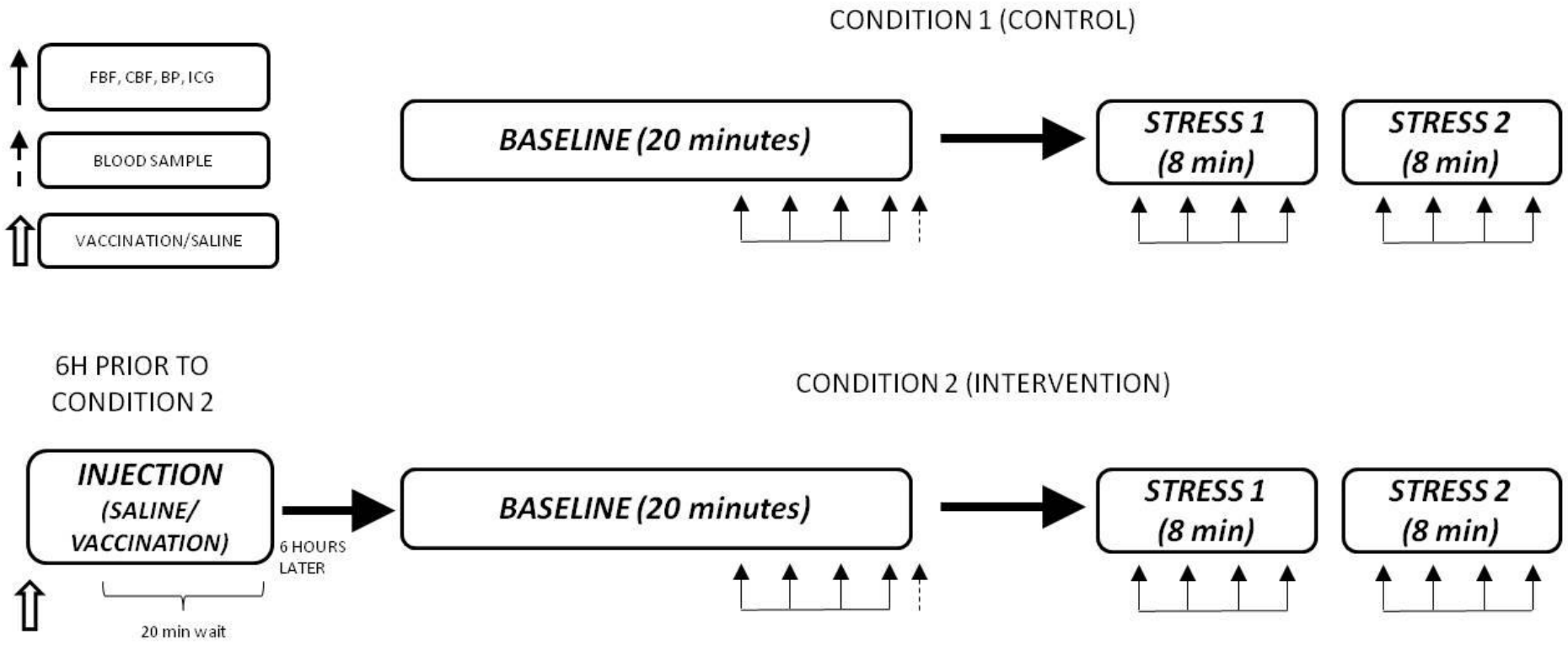

Figure 4.1. Flow chart of the study protocol

between. During minutes 1, 3, 5 and 7 of each block, blood flow was measured, the impedance cardiography and blood pressure recordings were analysed for those minutes.

Vaccination/Saline administration

The experimental participants received 0.5 ml *Salmonella typhi* capsular polysaccharide vaccine (0.025 mg in 0.5 ml, Typhim Vi, Sanofi Pasteur, UK) whereas the control participants received a 0.5 ml saline injection in a blinded design via intra-muscular injection into the deltoid muscle of the non-dominant arm, by a registered nurse. Participants remained in the laboratory for 20 minutes post-injection for observation. All participants returned to the laboratory 6 hours later to complete the intervention session. At the start of this session, they rated the extent of any potential physical symptoms related to receiving the earlier vaccination or saline injection, such as malaise, sore throat, watery eyes or eye strain, upset stomach, sneezing, congested nose, persistent cough, headaches, neck ache, shortness of breath, feverishness, chills, nasal discharge, ringing in the ears and muscle ache in the limb, using a 5 point Likert scale (0 = *not at all*, 5 = *severe*). General reactions such as joint pain and pain at the site of the injection were also assessed using a 10 point scale (1 = *very mild*, 10 = *very severe*).

Mental Stress Task

The paced auditory serial addition test (PASAT) was used as mental stress task. The PASAT has been widely demonstrated to repeatedly produce a stressful stimulus, even when the task is repeated several times on separate days (Veldhuijzen van Zanten *et al.*, 2005; Willemsen *et al.*, 1998). During the PASAT, participants were presented with a series of single digit numbers, delivered using a CD player, and were required to add each new number

to the number presented previously (Gronwall, 1977; Ring *et al.*, 2002). The task lasted a total of 16 minutes, split into two eight minute tasks (referred to as Stress 1 and Stress 2 respectively), separated by one minute rest. The numbers were delivered in four 2-minute blocks, with the numbers respectively presented every 3.2 s, 2.8 s, 2.4 s and 2.0 s for the first task, and every 2.4s, 2.0s, 1.6s and 1.2s for the second task. This resulted in a progressive increase in task difficulty. The experimenter, who sat 1 metre adjacent to the participant, checked their responses against the correct answers.

To enhance the provocativeness of the task several features were added that had shown to enhance stress responses in previous studies (Veldhuijzen van Zanten *et al.*, 2004). Participants heard a loud aversive noise once in each block of 10 numbers, with the noise presented after the participants' first incorrect response, or if they had not made an error, the noise was delivered at the end of the 10 number block. Participants were filmed and were asked to look at their faces displayed on a television screen while performing the task. If participants looked away from the screen, they heard the aversive noise and were reminded to continue to watch the screen. Participants were also informed that the recording would be analyzed by two senior academics for body and facial composure during the task. In reality, no such analysis occurred. They were truthfully told that a £10 gift voucher would be awarded for the best performance on the task and a leader board with the highest five scores achieved by the participants was displayed, to compare participant scores. Another £10 voucher given to the participant recorded the greatest improvement in score between the two sessions. However, the latter of these rewards was only revealed before the start of the test in the second (i.e., intervention) session, to eliminate the risk of a poor performance in the first (i.e., control) session. These elements of social evaluation, competition, punishment and reward have been shown to enhance the provocativeness of the task (Veldhuijzen van Zanten *et al.*,

2004). At the end of the stress task, participants were asked to rate the task in terms of perceived difficulty, arousal, stressfulness, engagement and perceived performance using a 7 point Likert scale, with anchors of 0 (not at all) and 6 (extremely).

Physiological Measurements

Cardiovascular measures

Beat-to-beat arterial blood pressure was recorded continuously during both baseline and stress tasks using a Finometer (Finapres Medical Systems; Amsterdam, The Netherlands). A small cuff was placed around the middle finger of the dominant hand of each participant. From this output, continuous data was recorded via a Power1401 (CED) connected to a computer programmed in Spike2 version 6 (CED). Cardiovascular parameters were derived from the blood pressure waveform obtained from the recorded output and this was then analysed offline. Mean systolic (SBP) and diastolic (DBP) blood pressure were obtained in periods of assessment and used to calculate mean arterial pressure (MAP).

Indices of cardio-dynamic activity were recorded continuously using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS, Amsterdam, The Netherlands) (de Geus *et al.*, 1995; Willemsen *et al.*, 1996). This system used six Ag/AgCl spot electrodes (Invisatrace, ConMed Corporation) to record electrocardiography (ECG) and impedance cardiography (ICG), in line with published guidelines (Sherwood *et al.*, 1990). Ten second ensemble averages were calculated and used to determine the following measures: Heart Rate (HR, bpm), Pre-Ejection Period (PEP; ms), Root Mean Square Successive Difference as a measure of heart rate variability (r-MSDD; ms) and Cardiac Output (as a product of stroke volume and HR) (CO; l/min). In addition, Total Peripheral Resistance (TPR; dyn-s/cm^{-5}) was calculated as (MAP / CO) × 80.

Blood Flow

Venous occlusion plethysmography, using a mercury-in-silastic-strain gauge, was utilised to measure forearm blood flow (FBF) and calf blood flow (CBF). Venous occlusion plethysmography is a reliable technique used to measure blood flow over repeated cardiac cycles (Joyner *et al.*, 2001). One strain gauge was fitted around the widest part of the non-dominant forearm and another was fitted around the calf. One congestion cuff was placed around brachial region of the upper arm (SC10, Hokanson) and thigh (SC12, Hokanson), one around the wrist, and another around the ankle (TMC7, Hokanson). The strain gauges were connected to a plethysmograph (EC6, Hokanson), which produced a calibrated output voltage proportional to limb circumference with a frequency response of 0 – 25 Hz. The plethysmograph signal was digitized at 100 Hz with 16-bit resolution, via a Power1401 (CED) connected to a computer programmed in Spike2 version 6 (CED). The brachial and femoral cuffs were inflated for 5 seconds to above venous pressure (40 mmHg), using a rapid cuff inflator (E20, Hokanson) attached to an automated air source (AG101, Hokanson). This procedure allows for arterial inflow, while preventing venous return. After 15 seconds, the brachial and femoral cuffs were inflated again. This was repeated three times per minute. Throughout the minute of assessment, the wrist and ankle cuff was manually inflated by Sphygmomanometer (S300, Hokanson) to supra-systolic blood pressure (> 200 mmHg), to prevent any blood flow into the hand and foot. Calibration and blood flow analysis was undertaken offline using Spike2 (CED). Increases in limb circumferences associated with inflation of the cuffs were identified and for each of these, the slope was measured between the upstroke of first two pulses following cuff inflation. The slope was assessed using a least squares fit to the data to minimise the effects of outlying data points. Three measurements of blood flow (slopes in response to cuff inflation) occurred per minute, with these averaged to

give a mean estimate of blood flow per minute. Vascular resistance (forearm – FVR; calf – CVR) was calculated as Mean Arterial Pressure/Blood Flow, with vascular conductance (forearm – FVC; calf – CVC) calculated as Blood Flow/Mean Arterial Pressure × 100.

Blood Sampling

Blood samples were taken at the end of the baseline period and were collected into three 6 ml and one 2 ml vacutainers containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton–Dickinson, UK). The 6 ml samples were stored on ice until centrifugation (1500 × g for 10 min at 4°C) and plasma was stored at -80°C for later assessment of IL-6 and TNF-α. The 2 ml samples were placed on a roller until analysis for full blood cell count using a Coulter Analyzer (Beckman Coulter, Inc.).

Assays

Plasma IL-6 and TNF-α were measured in duplicate using high-sensitivity ELISA (Quantikine HS Human IL-6 ELISA and Quantikine HS Human TNF-α ELISA, both R&D Systems, UK) in accordance with the manufacturer's instructions. The reported limit of detection of the assays was 0.039 and 0.106 pg/ml respectively, with recorded intra-assay and inter-assay variations both < 10% for IL-6 and TNF-α.

Data reduction

For all cardiovascular variables, the 4 measurements taken during baseline, stress 1, and stress 2 were averaged to establish a single Baseline, Stress 1 and Stress 2 value, respectively. The overall stress value was calculated as a mean average of stress 1 and stress 2 to give an overall average for the whole 16 minutes of the stress task. For the experimental

participants, Analyses of Variance (ANOVAs) were conducted to examine differences between baseline values in the control and inflammation sessions. Two Condition (Control, Inflammation) by two Time (Baseline, Stress) repeated measures ANOVAs were conducted on cardiovascular and vascular measurements. Where interaction effects were observed, further analysis was conducted to explore the interaction effect in more detail by examining each minute of assessment during the stress task (e.g., minute 2, minute 4, minute 6, minute 8, minute 10, minute 12, minute 14 and minute 16) using a 2 condition by 9 time (baseline, each minute of assessment during the stress task) ANOVA, with Greenhouse-Geisser correction (Vasey & Thayer, 1987). For all ANOVAs, eta squared (η^2) was used as a measure of effect size, and, where appropriate, Newman-Keul post-hoc comparisons are reported.

Reactivity scores for all cardiovascular and vascular measures were calculated as the difference between the average stress value (calculated as the average between Stress 1 and Stress 2) and the baseline value. Subsequently, Pearson correlations were conducted to examine the associations between resting inflammatory markers (IL-6, TNF- α, WBC) and vascular as well as cardiovascular reactivity. These analyses were conducted for the control and inflammation intervention condition separately. Occasional missing data are reflected in the reported degrees of freedom.

4.4 RESULTS

Inflammatory response to vaccination (Salmonella typhi polysaccharide vaccine)

Table 4.1 displays the measures of inflammation at baseline in the intervention and control conditions in the inflammation intervention group. A series of 2 Condition (Control, Intervention) ANOVAs confirmed that the vaccination induced an inflammatory response in those who received the inflammation intervention: TNF-α, IL-6, and granulocytes were all

elevated during the inflammatory intervention condition compared to the control condition (Table 4.1). Data from our laboratory demonstrated no significant increases in IL-6 after saline administration in comparison to a *Salmonella typhi* vaccination (Paine *et al.*, in submission). Finally, in comparison to those who received saline injection, participants subjected to the inflammation intervention reported greater muscle ache (Inflammation: 2.22 ± 1.35, Saline: 0.2 ± 0.48; F (1, 21) = 13.04, p = .002, η^2 = .38), and localised pain at the site of the injection (Inflammation: 3.17 ± 1.92, Saline: 0.2 ± 0.48; F (1, 21) = 13.18, p = .002, η^2 = .39) at the start of the stress testing session, six hours following the vaccination.

Table 4.1. Mean (SD) inflammatory markers for the inflammation intervention group only (N = 18) during the control condition and the intervention condition.

Variable	Control Condition	Intervention Condition	ANOVA
IL-6 (pg/ml)	0.90 (1.03)	2.97 (1.58)	F (1, 15) = 16.23, p = .001, η^2 = .52
TNF-α (pg/ml)	4.93 (4.12)	16.94 (8.11)	F (1, 15) = 27.90, p < .001, η^2 = .65
Granuloctyes (10^9/L)	3.92 (1.16)	6.89(1.23)	F (1, 17) = 106.53, p < .001, η^2 = .86

Task impact ratings

Table 4.2 presents the task impact ratings for in both the control and intervention conditions for both the placebo and inflammation group. There were no condition differences in perceived levels of stress, arousal, engagement or performance of the task, for either intervention group.

Table 4.2. Mean (SD) task ratings of the mental stress task in the for the inflammation group (N=18) and the placebo group (N=5)

Inflammation Intervention	Control Condition	Intervention Condition	ANOVA
Perceived stress	4.56 (0.71)	4.44 (0.71)	F (1, 17) = .49, p > .05, η^2 = .03
Perceived arousal	3.22 (1.35)	3.39 (1.50)	F (1, 17) = 1.00, p > .05, η^2 = .06
Perceived performance	2.28 (0.96)	2.44 (1.04)	F (1, 17) = .55, p > .05, η^2 = .02
Perceived engagement	3.78 (1.35)	3.67 (1.28)	F (1, 17) = .21, p > .05, η^2 = .01

Placebo Intervention	Control Condition	Intervention Condition	ANOVA
Perceived stress	4.80 (0.84)	4.60 (0.55)	F (1, 4) = .29, p > .05, η^2 = .07
Perceived arousal	2.60 (0.55)	2.60 (0.89)	F (1, 4) = 1.00, p > .05, η^2 = .00
Perceived performance	2.40 (1.14)	3.00 (1.00)	F (1, 4) = 2.25, p > .05, η^2 = .36
Perceived engagement	3.60 (1.14)	3.80 (1.64)	F (1, 4) = .17, p > .05, η^2 = .04

Note: Ratings ranged from 0 (not at all) to 6 (extremely)

Physiological Responses to mental stress - Inflammation group

Cardiovascular responses

Figure 4.2 depicts the HR, CO, PEP, rMSSD, SBP and DBP at rest and in response to mental stress in the control and intervention condition. A series of 2 Condition (Control, Intervention) by 2 Time (Baseline, Stress) repeated measures ANOVA yielded main effects for time for HR (F (1, 14) = 58.84, p < .001, η^2 = .81), CO (F (1, 14) = 14.69, p = .002, η^2 = .51), PEP (F (1. 14) = 9.56, p = .008, η^2 = .41), rMSSD (F (1, 14) = 7.72, p = .015, η^2 = .36), SBP (F (1, 14) = 27.59, p < .001, η^2 =.66) and DBP (F (1, 14) = 26.92, p < .001, η^2 = .66). Newman-Keuls post hoc analyses revealed that HR, CO, SBP, and DBP increased in response

to stress, whereas PEP and rMSSD decreased in response to stress. No condition effects or condition by time interaction effects were seen for CO, HR, PEP, rMSSD, SBP and DBP (all *p's* > .05).

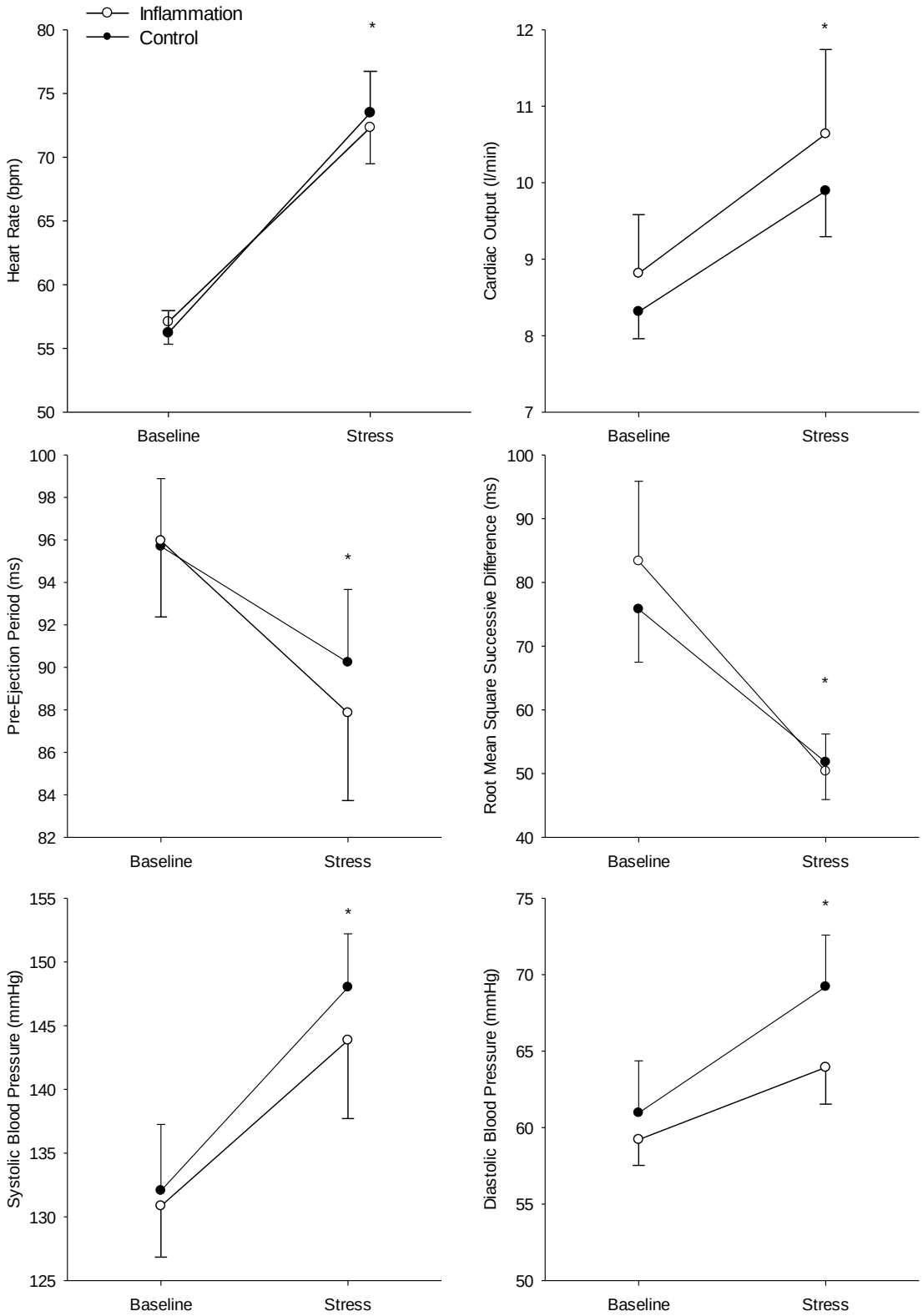

Figure 4.2. Mean ± SE responses to mental stress for HR, CO, PEP, rMSSD, SBP and DBP in the inflammation group (N = 18). Note: * significantly different from baseline ($p < .05$)

Vascular responses

Forearm and calf vascular function at rest and in response to mental stress are illustrated in Figure 4.3. A series of 2 Condition x 2 Time ANOVAs revealed significant main effects for time for forearm vascular responses: FBF (F (1, 17) = 140.24, $p < .001$, $\eta^2 = .90$), FVR (F (1, 16) = 54.46, $p < .001$, $\eta^2 = .79$), FVC (F (1, 16) = 58.46, $p < .001$, $\eta^2 = .79$). Newman-Keuls post hoc analyses confirmed that FBF and FVC increased whereas FVR decreased in response to stress. No interaction or condition effects were evident for FVR and FVC, nor was there a condition effect for FBF. Importantly, there was evidence for a condition by time interaction effect for FBF (F (1, 17) = 3.37, $p = .08$, $\eta^2 = .17$), whereby the stress-induced vasodilation appeared to be attenuated in the inflammation intervention condition. To explore this interaction, a more temporally detailed follow-up was conducted examining the blood flow responses at rest to mental stress for each 2 minute block of the mental stress task, to give 8 time points for assessment during the stress task. Thus, analysis using a 2 condition by 9 time (baseline, min 2 of the task, min 4, min 6, min 8, min 10, min 12, min 14, min 16) ANOVA was conducted. This analysis confirmed a main time effect (F (8, 7) = 13.88, $p < .001$, $\varepsilon = .44$, $\eta^2 = .50$) and a condition by time interaction effect (F (8, 7) = 12.8, $p = .006$, $\varepsilon = .51$, $\eta^2 = .22$). The mean difference between baseline blood flow and the blood flow recorded during each 2 minute period of mental stress (e.g., minute 2, minute 4 and so on until the end of all 16 minutes) was calculated to assess the changes in stress-induced blood flow from baseline to each 2 minute period of the stress task, and is presented in Table 4.3. These differences were calculated for both conditions (e.g., control and intervention), and then compared between condition through a 2 condition repeated measures ANOVA.

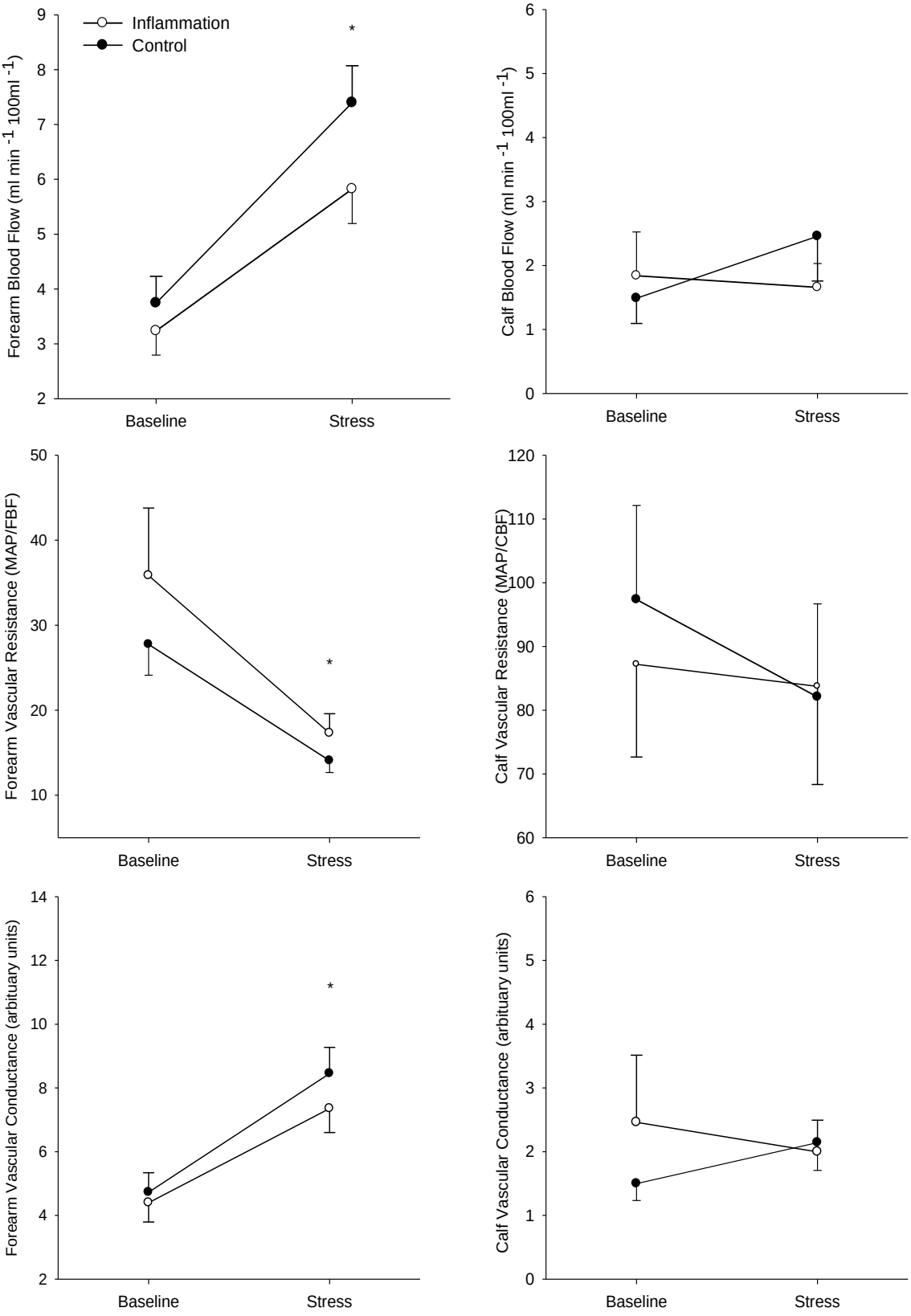

Figure 4.3. Mean ± SE responses to mental stress for FBF, FVR, FVC, CBF, CVR, CVC in the inflammation group (N = 18). Note: * significantly different from baseline ($p < .05$)

Table 4.3. Mean difference (SD) forearm blood flow responses from baseline to each two minute period of mental stress in each condition for the inflammation group (N=18). Calculated as Δ 2 minute period of stress – baseline blood flow.

Forearm Blood flow (ml min^{-1} $100ml^{-1}$)	Control Condition	Intervention Condition
Δ Baseline - minute 0-2 of mental stress	5.19 (3.86)	2.46 (1.56)*
Δ Baseline - minute 2-4 of mental stress	3.45 (2.74)	2.38 (1.60)
Δ Baseline - minute 4-6 of mental stress	4.11 (2.74)	2.72 (1.54)
Δ Baseline - minute 6-8 of mental stress	3.47 (2.43)	2.29 (1.02)
Δ Baseline - minute 8-10 of mental stress	3.64 (2.10)	2.58 (1.74)
Δ Baseline - minute 10-12 of mental stress	3.11 (2.06)	2.50 (1.43)
Δ Baseline - minute 12-14 of mental stress	3.82 (2.35)	3.21 (2.32)
Δ Baseline - minute 14-16 of mental stress	3.30 (2.22)	3.00 (1.48)

* indicates significantly different between condition (p = .01)

These analyses revealed significant differences between the increases in stress-induced blood flow from baseline, in response to the first 2 minutes of the stress task (e.g., the difference in blood flow between baseline and minutes 0-2 of mental stress) (F (1, 16) = 8.64, p = .01, η^2 = .35). No other differences were observed between conditions in the stress-induced increases in blood flow during the remaining 2 minute periods (p's > .05). These analyses indicate that the initial vasodilation in response to mental stress was attenuated during the inflammation condition, with no differences in the stress-induced increases in blood flow (at any other time point during the stress task) from baseline. There was however a hint of reduced vasodilation during minute 8-10 of the stress task, which occurred after a one minute break in the task.

For the vascular measurements in the calf, the condition by time ANOVAs yielded no condition (p's > .05) or time effects for any variable: CBF (F (1, 15) = 2.24, p = .16, η^2 = .13), CVR (F (1, 14) = 1.62, p = .22, η^2 = .10) and CVC (F (1,14) = 0.74, p = .78, η^2 = .01). Critically, a condition by time interaction effect was noted for CBF (F (1, 15) = 6.34, p =

.024, η^2 = .30). Follow-up analysis using a 2 condition by 9 time ANOVA confirmed an interaction effect (F (8,5) = 2.86, p = .013, ε = .36, η^2 = .26), with post hoc analyses showing that the calf vasodilatory response in the inflammation condition are smaller compared to that in the control condition. Table 4.4 displays the mean differences between baseline calf blood flow and the calf blood flow recorded during each 2 minute period of mental stress revealed significant differences between conditions during minutes 0-2 (F (1,17) = 8.15, p = .011, η^2 = .32), minutes 2-4 (F (1,17) = 4.83, p = .042, η^2 = .22), minutes 4-6 (F (1,14) = 8.58, p = .011, η^2 = .38), minutes 6-8 (F (1,15) = 5.77, p = .03, η^2 = .28), minutes 8-10 (F (1,15) = 6.42, p = .023, η^2 = .30), minutes 12-14 of the stress task (F (1,15) = 6.45, p = .023, η^2 = .30). No differences were observed between any other time points of the stress task.

Table 4.4. Mean difference (SD) calf blood flow responses from baseline to each two minute period of mental stress in each condition for the inflammation group (N=18). Calculated as Δ 2 minute period of stress – baseline blood flow.

Calf Blood flow (ml min^{-1} $100ml^{-1}$)	**Control Condition**	**Intervention Condition**
Δ Baseline - minute 0-2 of mental stress	1.20 (1.54)	-0.02 (1.12) *
Δ Baseline - minute 2-4 of mental stress	0.80 (1.16)	-0.06 (1.24) *
Δ Baseline - minute 4-6 of mental stress	1.20 (1.39)	-0.29 (1.78) *
Δ Baseline - minute 6-8 of mental stress	0.84 (1.46)	-0.31 (1.64) *
Δ Baseline - minute 8-10 of mental stress	0.99 (1.36)	-0.22 (1.35) *
Δ Baseline - minute 10-12 of mental stress	0.99 (1.43)	-0.00 (1.86)
Δ Baseline - minute 12-14 of mental stress	1.05 (1.46)	-0.13 (1.44) *
Δ Baseline - minute 14-16 of mental stress	0.79 (1.16)	-0.16 (1.96)

* indicates significantly different between condition (p < .05)

Finally, analysis of TPR (Figure 4.4) by 2 Condition by 2 Time repeated measures ANOVA yielded no time, (F (1, 11) = 0.77, p = .40 η^2 = .07), condition (F (1, 11) = 0.006, p = .94 η^2 = .00), or time by condition interaction effect (F (1, 11) = 0.62, p = .62, η^2 = .02).

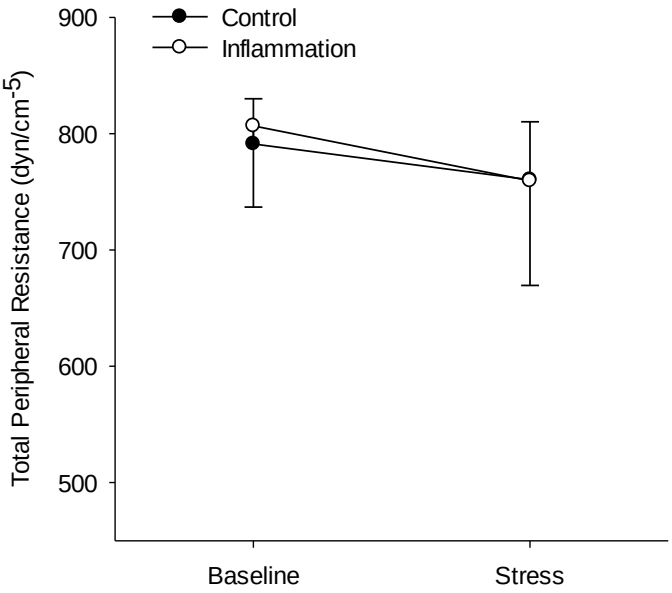

Figure 4.4. Mean ± SE responses to mental stress for TPR in the inflammation group (N = 18).

Associations between inflammation and physiological responses to mental stress

Pearson correlation analyses were undertaken to examine the relationship between the markers of inflammation and the physiological responses to mental stress. In the inflammation condition TNF-α was positively correlated with stress-induced changes in PEP (r (16) =.50, p < .05). During the control condition, resting levels of inflammation were not associated with stress-induced changes in the physiological responses to mental stress.

Physiological responses to mental stress - Placebo group

Figures 4.5-4.7 (a-f) depict the HR, CO, PEP, rMSSD, SBP and DBP, FBF, FVR, FVC, CBF, CVR, CVC and TPR responses to mental stress during the control and intervention conditions in the participants who received the placebo intervention. A series of 2 Condition (Control, Intervention) by 2 Time (Baseline, Stress) repeated measures ANOVAs yielded time effects for HR (F (1, 3) = 89.6, p = .003, η^2 =.97), FBF (F (1, 4) = 15.3, p = .017, η^2 =.79), FVR (F (1, 4) = 16.1, p = .016, η^2 =.80) and FVC (F (1, 4) = 10.5, p = .032, η^2 =.72). No effect of time (F (1, 2) = 0.20, p = .90, η^2 =.01), condition (F (1, 2) = 1.13, p = .40, η^2 =.36) or condition by time (F (1, 2) = 1.02, p = .42, η^2 =.34) interaction effects were observed for TPR. No other condition, time or condition by time interaction effects were observed (p's > .05). In sum, these control analyses indicate that stress reactions were similar in the first and second testing sessions.

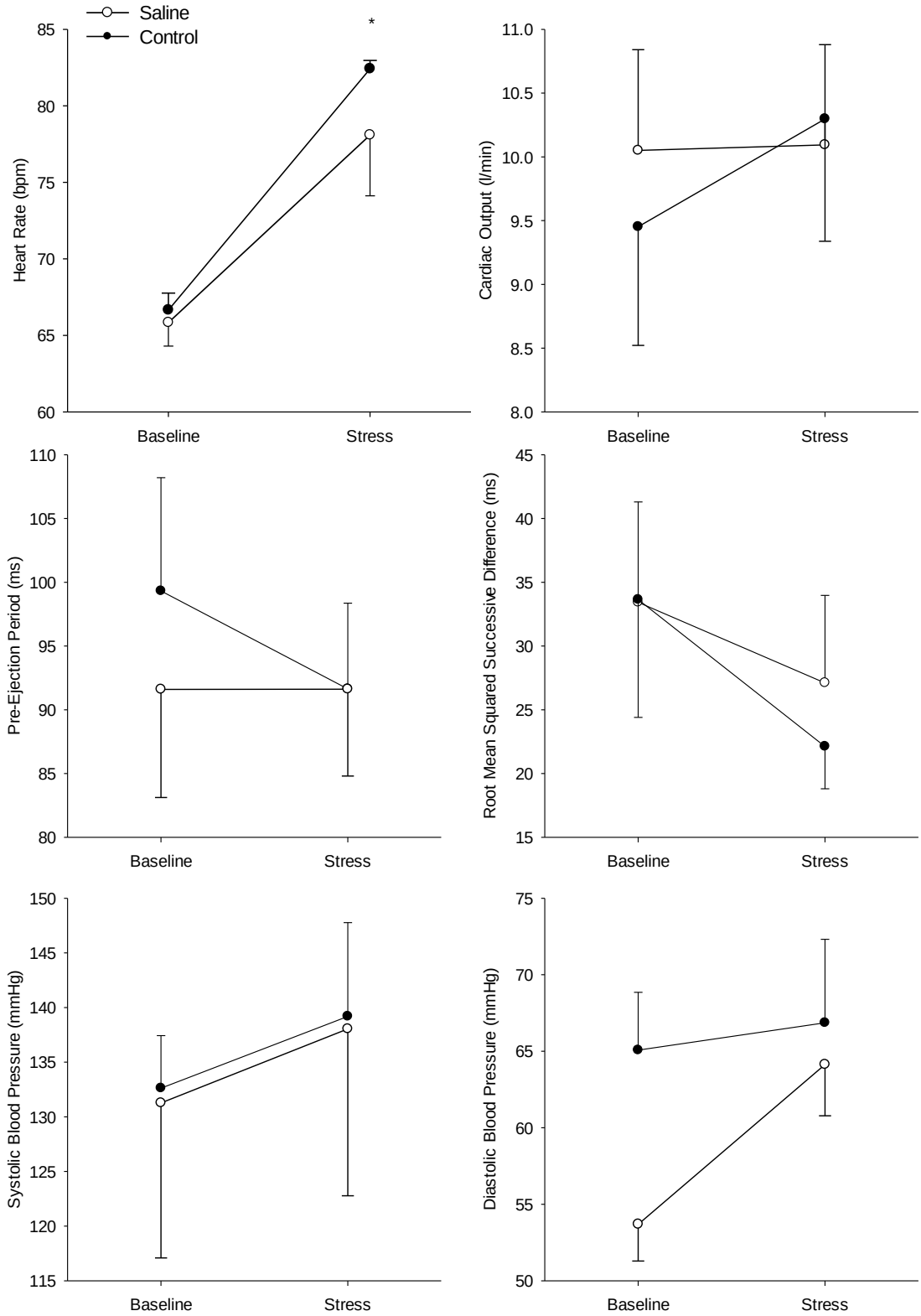

Figure 4.5. Mean ± SE responses to mental stress for HR, CO, PEP, rMSSD, SBP and DBP in the placebo group (N = 5). Note: * significantly different from baseline ($p < .05$)

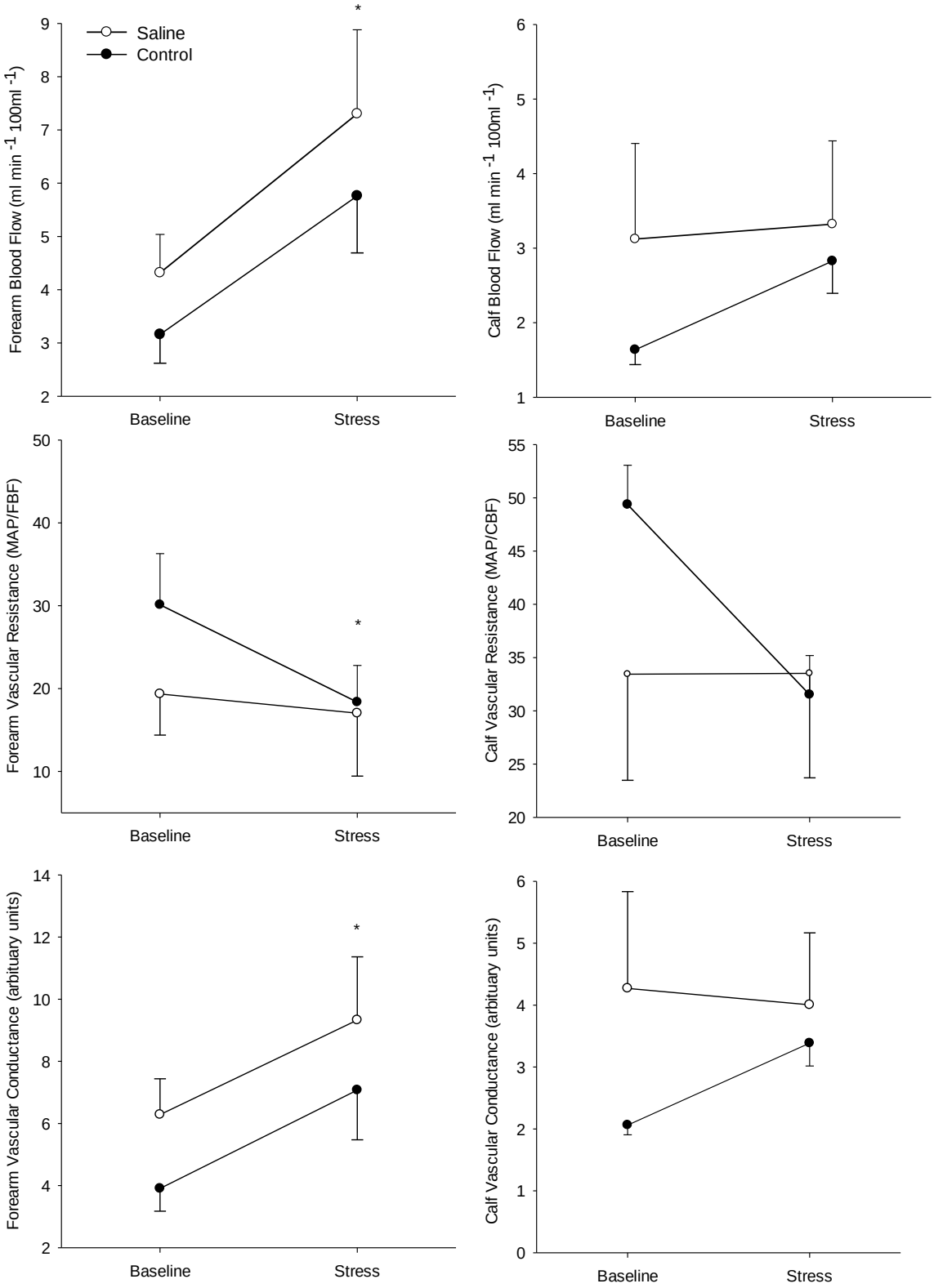

Figure 4.6. Mean ± SE responses to mental stress for FBF, FVR, FVC, CBF, CVR, CVC in the placebo group (N = 5). Note: * significantly different from baseline ($p < .05$)

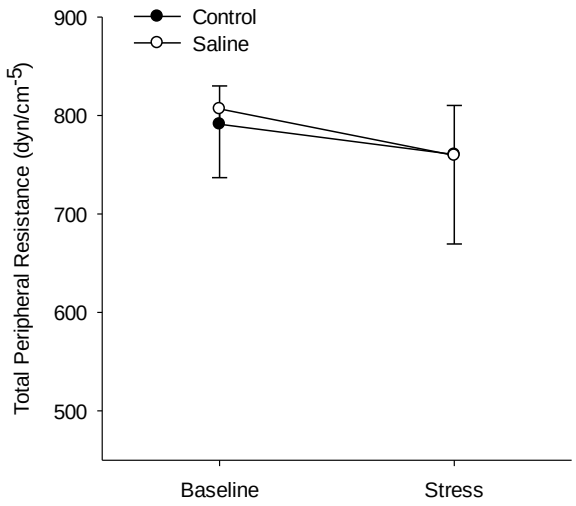

Figure 4.7. Mean ± SE responses to mental stress for TPR in the placebo group (N = 5).

4.5 DISCUSSION

The aim of this study was to examine the impact of inflammation on the vascular responses to a mental stress task. The *Salmonella typhi* polysaccharide vaccination successfully induced a systemic inflammatory response, as evidenced by increases in TNF-α, IL-6 and granulocytes. We found that this inflammatory stimulus caused attenuation in the vasodilatory response to mental stress. Further, the attenuation in the vascular responses to stress was most pronounced in the limb where the vaccination was administered. The effect of inflammation on cardiovascular function was specific to the vascular response to mental stress, as no influence of inflammation was found on the cardiac responses to stress or resting levels. Importantly, not only was stress-induced vasodilation attenuated in the inflammation condition, the attenuation was evident during the first two minutes of the stress task. This is of particular interest given that mental stress-induced ischaemia is also evident during the two minutes of a stress task (Blumenthal *et al.*, 1995).

The vaccination model used in the current study was chosen to assess the influence of inflammation on the vasculature in a population without any underlying pathologies, therefore allowing assessment of the individual impact of inflammation. Importantly, it should be noted that despite using a healthy population, the increases in inflammation in this study were substantial and comparable to populations at risk for myocardial infarction. For example, IL-6 concentrations of 2.58 ± 0.39 pg/ml have been reported in patients with coronary artery disease (Kop *et al.,* 2008) and concentrations of 1.81 pg/ml reported in men who had experienced a MI (Ridker *et al.,* 2000). Therefore, our levels of 2.97 ± 1.58 pg/ml are similar to those of clinical populations, demonstrating that the vaccination model used in this study was able to successfully mimic the inflammatory levels of those at risk for MI.

The reduction in stress-induced vasodilation observed in the inflamed state could have occurred via several mechanisms. Stress-induced vasodilation has been shown to be dependent on NO bioavailability (Joyner & Dietz, 2003; Joyner & Casey, 2009), most likely through the upregulation of eNOS as a result of increases in shear stress (Nishida *et al.*, 1992). Using the same vaccination model as the current study, a previous study found that inflammation led to endothelial dysfunction at rest through a reduction in NO bioavailability (Clapp *et al.*, 2004). Therefore, it is suggested that the attenuated stress-induced vasodilation seen in the current study during inflammation is also due to a reduction in NO. As direct measurement of NO in blood is difficult and are often unreliable (Wadley *et al.*, 2012), the reasons for a reduction in NO production must remain speculative. NO bioavailability could be reduced as a result of decreased eNOS activation (Goodwin *et al.*, 2007), eNOS

uncoupling (Rabelink & van Zonneveld, 2006), or enhanced NO removal through increases in oxidative stress (Gao *et al.*, 2007).

eNOS activity is highly dependent on L-arginine availability as it converts L-arginine to L-citruline to produce NO (van Zonneveld *et al.*, 2010; Zhang, 2008). Therefore, eNOS activation can be reduced as a result of decreases in L-arginine (Darley-Usmar *et al.*, 1995; Förstermann & Münzel, 2006; Morris, 2000). Given that inflammation can reduce L-arginine availability (Goodwin *et al.*, 2007), it is possible that inflammation could reduce NO production through this pathway. Indeed, the reciprocal relationship between inflammation and L-arginine availability leads to L-arginine suppressing inflammatory markers such as Nuclear Factor Kappa Beta (Kagemann *et al.*, 2007) and TNF-α (De Martin *et al.*, 2000). Indeed, decreases in L-arginine as a result of inflammation are important because L-arginine is an early step in the process of NO production (van Zonneveld *et al.*, 2010; Zhang, 2008). However, studies that have administered L-arginine supplementation to reverse the effects of inflammation on endothelial function revealed no improvement in endothelial function (Clapp *et al.*, 2004), indicating that L-arginine deficiency alone may not be responsible for decreases in NO.

Similarly, the co-factor tetrahydrobiopterin (BH_4) may be another important factor in the attenuation of the vascular response to stress, via reduced NO. BH_4 is another factor critical to NO production (Cosentino & Katusic, 1995; Pierce & LaRocca, 2008) and limited BH_4 has been shown to uncouple eNOS, resulting in production of superoxides rather than NO (Bevers *et al.*, 2006; Crabtree *et al.*, 2009). Indeed, inflammation increases oxidative stress, which in turn reduces BH_4 availability (Cosentino & Luscher, 1999; Vásquez-Vivar *et*

al., 1998). Moreover, the inability to produce BH_4 results in more detrimental effect of inflammation on vascular function (Antoniades *et al.*, 2011). Even though L-arginine is a critical factor for eNOS activation, L-arginine conversion can only occur in the presence of BH_4 (Delp *et al.*, 2008; Sindler *et al.*, 2009), highlighting the importance of the availability of both these substrates in NO production. In sum, both L-arginine and BH_4 have been found important factors for NO production through eNOS and inflammation has been associated with a reduction in both.

Finally, high levels of inflammation may also result in elevations in oxidative stress, with systemic inflammation has resulting in increased generation of reactive oxidative species (ROS) in the vascular wall (Forstermann, 2008). Therefore, acute inflammation, as induced in this study, might also reduce NO levels through oxidative stress pathways and result in increases in direct scavenging (Gao *et al.*, 2007). In sum, inflammation may alter NO production by both influencing the availability of L-arginine and subsequent levels of eNOS activation, as well as BH_4 availability and resulting decreases in eNOS uncoupling. However, due to methodological issue with measuring NO in blood (Wadley *et al.*, 2012), the reasons for a reduction in NO production must remain speculative.

Sympathetic activation has also been shown to be a factor related to stress-induced vasodilation. However, it is unlikely that the attenuated vasodilation in the current study can be attributed to the influence of inflammation on sympathetic activation or parasympathetic withdrawal, as the changes in sympathetic activation (assessed by a reduction in PEP) and parasympathetic withdrawal (assessed by reduced rMSSD) were not different between the control and the intervention condition for those who received the inflammation intervention.

Further, even though, the change in heart rate variability was associated with vasodilation in the control condition for those who received the inflammation intervention, this association was not evident in the inflammation condition. Thus, the attenuation in FBF cannot be attributed to changes in sympathetic and parasympathetic activation, and is more likely to be due a direct effect of inflammation on the vasculature.

Even though the vasodilatory response to mental stress was attenuated in the inflammation condition, there were no correlations between the inflammatory markers and the vascular assessments. This is in contrast to the only other study that used a vaccination to assess the effects of inflammation on responses to mental stress, where IL-6 responses to a vaccine were positively correlated with systolic blood pressure in response to a speech task (Brydon *et al.*, 2009). However, the timing of the stress task relative to the vaccination was different; whereas Brydon and colleagues assessed stress reactivity only 2 h post vaccination, in the current study this was done 6 h post vaccination to coincide with peak cytokine response to vaccination (Paine *et al.*, in submission; Padfield *et al.*, 2010). It is also worth noting that, in line with our study, Brydon and colleagues also found no differences in the HR and BP responses to mental stress as a result of vaccination (Brydon *et al.*, 2009). Unfortunately, vascular responses to stress were not assessed in their study.

TNF-α and IL-6 were assessed because of their known role in altering NO availability (Goodwin *et al.*, 2007; Hung *et al.*, 2010) and the influence of inflammation on vascular function at rest (Clapp *et al.*, 2004; Hingorani *et al.*, 2000). Unfortunately, these previous studies have not reported analyses on associations between inflammatory markers and endothelial function at rest. The lack of associations in the current study could be due to

sample size. Alternatively, it is possible that neither TNF-α or IL-6 are directly responsible for the attenuation in vascular responses to stress, but merely indicative of an inflamed state. Therefore, other cytokines such as IL-8 or IL-10 may be directly responsible for the attenuation in blood flow seen during stress. It is worth noting that CRP is commonly assessed to explore the associations between vascular function and inflammation in observational studies (e.g., Verma *et al.*, 2002; Verma *et al.*, 2004). However, given the known delayed increase in CRP changes in response to the vaccination used in this study (Clapp *et al.*, 2005; Padfield *et al.*, 2010; Vlachopoulos *et al.*, 2005; Paine *et al.*, in submission), it is thought that CRP was not increased at the time of the stress task.

Albeit reduced, there was still evidence of vasodilation in the forearm during stress, whereas stress induced no change in calf blood flow during the inflammation condition. It should be recognised that even in the control condition for those who received the inflammation intervention; the changes in CBF were smaller in comparison to the FBF changes (CBF – 55%, FBF – 122%). Although FBF and CBF responses to mental stress were both attenuated during inflammation, there was no association between the changes in CBF or FBF in either the control or the intervention condition for those who received the inflammation intervention. This lack of association between the forearm and calf vascular responses to stress has been reported before (Carter *et al.*, 2005b). Indeed, while increases in FBF in response to mental stress are repeatedly found (Dietz *et al.*, 1994; Dietz *et al.*, 1997; Joyner & Halliwill, 2000), stress produces inconsistent responses in the calf (Blair *et al.*, 1959; Carter *et al.*, 2005b; Hjemdahl *et al.*, 1984; Hjemdahl *et al.*, 1989; Kamiya *et al.*, 2000; Kuipers *et al.*, 2008; Rusch *et al.*, 1981). Differences in the reproducibility in blood flow between the forearm and calf have been speculated to be possibly attributable to myogenic

differences (Imadojemu *et al.*, 2001). It is also possible that the difference between blood flow responses in the calf and forearm were due to the fact that the vaccine was given in the arm where the forearm blood flow responses were assessed, and despite a systemic effect of inflammation, the localised inflammatory effect may have been greater and thus could be the reason for the greater attenuation in the vascular responses to stress in the forearm.

Interestingly, mild inflammation in the current study did not influence TPR responses to stress. The most likely explanation for this seemingly contradictory finding is the differences in methods of assessment. Examination of blood flow allows a direct assessment of vasodilation in one limb, whereas TPR is derived from CO and blood pressure, and is reflective of whole body changes. Therefore, the lack of change in TPR responses during inflammation could the result of cardiac output and blood pressure not being altered by inflammation. This could be supported by the changes in FVR seen in response to stress that are not seen when assessing TPR, indicating that the changes seen in examining the vasculature in a more direct manner might not be reflective of the changes found when the vascular assessment is derived from the whole body. Indeed, this may also highlight the differences in the responses to stress of different vascular beds.

As a limitation it should be acknowledged that the control and intervention conditions were not counterbalanced. Therefore, it is possible that the attenuated vasodilatory response to mental stress could be attributable to habituation to the stress task. However, previous studies using the same stress task as in this study have reported no attenuation when the stress task was repeated several times either within or between sessions (Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009; Willemsen *et al.*, 1998). In addition, there was no

attenuating effect on any of the other physiological responses, such as heart rate or blood pressure, or psychological responses. Only the vascular responses to mental stress were affected during inflammation, and therefore, this high level of physiological selectivity is not consistent with mere habituation. Additionally, it is important to note that in the group who received the placebo intervention there were no significant differences in the responses of any of the physiological parameters assessed between the control and the intervention conditions, and thus the vascular attenuation observed in the inflammation intervention group should not be attributed to task habituation.

Given the temporary and reversible effects of inflammation on resting vascular function in healthy populations (Bhagat & Vallance, 1997), future work examining the effects of reducing inflammation (e.g., drug therapy or behavioural interventions) on the vascular responses to stress should be strongly considered. Equally, the impact of inflammation on eNOS has been examined more thoroughly than the impact of inflammation on other nitric oxide synthase isoforms such as nNOS and iNOS, particularly in the context of mental stress and this should be considered for further work. Likewise, the impact of inflammation on the vasculature in terms of coronary outcome measures such as myocardial ischaemia should be investigated.

In sum, this study observed an attenuation of the vascular responses to mental stress during a state of elevated inflammation. This interaction between inflammation and the vascular responses to mental stress might be one mechanism through which mental stress could trigger a myocardial infarction. Future work should focus on the associations between

different inflammatory markers, and focus on how reductions in inflammatory levels may reduce the potential risks associated with the vascular responses to mental stress.

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CHAPTER FIVE:

ARM ECCENTRIC EXERCISE DOES NOT ATTENUATE THE FOREARM BLOOD FLOW RESPONSES TO MENTAL STRESS

5.1 ABSTRACT

There is evidence that mental stress can trigger myocardial infarction, with both inflammation and vascular responses to stress implicated as potential mechanisms. Stress-induced vasodilation is evident in healthy participants, but is impaired in those at risk for cardiovascular disease and inflammation is associated with poorer vascular function at rest, but little is known about how inflammation affects vascular responses to mental stress. Eccentric exercise has been previously used to induce inflammation in healthy participants, and thus the aim of this study was to assess the effects of eccentric-exercise induced inflammation on the vascular responses to stress in a healthy population. Healthy male participants (N = 20) completed two sessions: control and inflammation. Forearm blood flow (FBF), heart rate (HR), cardiac output (CO) and blood pressure (BP) was assessed during rest (20 min) and in response to mental arithmetic stress (8 min). On day 1, they completed the control session, and on day 2, they completed the inflammation session. At the end of the control session, inflammation was induced by an eccentric arm exercise task; participants completed 50 eccentric portions of a bicep curl and 50 eccentric portions of a lateral raise (5 × 10 sets; 110% of their concentric maximum). Measures of arm limb circumference were taken pre and immediately post exercise task. Participants returned 24 h later to complete the inflammation session. Forearm and bicep limb circumference increased (p's < .05) from pre to post exercise task, indicative of inflammation. FBF, HR, CO, and BP all increased (p's < .001) from rest to stress; however, the stress-induced cardiovascular responses did not differ between the control and inflammation conditions. Thus, it appears that eccentric exercise did not influence vascular function or the vascular responses to mental stress, 24 h post exercise.

5.2 INTRODUCTION

Acute mental stress can lead to myocardial infarction (MI), with survivors of MI identifying emotional stress (e.g. episodes of anger) as a trigger for their MI (Strike & Steptoe, 2005). Likewise, earthquakes (Leor *et al.*, 1996a; Leor *et al.*, 1996b; Suzuki *et al.*, 1995), war (Bergovec *et al.*, 1992; Meisel *et al.*, 1991), and watching sport events (e.g., Carroll *et al.*, 2002; Wilbert-Lampen *et al.*, 2010) have been associated with an increased incidence in MI. Evidence suggests a role for inflammation and the vasculature as potential mechanisms through which this triggering process could occur. For example, serological markers of inflammation and vasoconstriction were increased in patients admitted for MI during the 2006 FIFA World Cup compared to those admitted for MI at other times (Wilbert-Lampen *et al.*, 2010). Additionally, cardiac patients who manifest mental stress-induced ischaemia also have elevated resting inflammation levels (Shah *et al.*, 2006), and poorer vascular responses to mental stress (Burg *et al.*, 2009; Goldberg *et al.*, 1996; Jain *et al.*, 1998). Therefore, inflammation and vascular responses to mental stress have been suggested to contribute to mental stress induced MI (Paine *et al.*, 2012b).

Both acute (Meier *et al.*, 1998) and chronic (Levy *et al.*, 2008; Ridker *et al.*, 2000) inflammation are associated with an increased risk of MI and implicated in the aetiology of cardiovascular disease (CVD) (Hansson, 2005; Libby, 2006). The impact of inflammation on resting vascular function has been examined in a number of studies. The evidence has revealed negative associations between vascular function and inflammation in patients with cardiovascular disease and rheumatoid arthritis (Fichtlscherer *et al.*, 2004; Vaudo *et al.*, 2004). In addition, inducing inflammation in healthy participants causes transient endothelial dysfunction (Bhagat & Vallance, 1997; Clapp *et al.*, 2004; Hingorani *et al.*, 2000; Kharbanda

et al., 2002), highlighting the pure influence of inflammation on vascular function. However, little is known about the effects of inflammation on the vascular responses to mental stress.

In a healthy population, mental stress induces a vasodilatory response, characterised by increases in blood flow (Blair *et al.*, 1959; Dietz *et al.*, 1994; Joyner & Halliwill, 2000). Interestingly, stress-induced vasodilation is reduced in individuals at risk for CVD (Hamer *et al.*, 2007) and patients with heart failure (Middlekauff *et al.*, 1997; Santos *et al.*, 2005). Different mechanisms cause vasodilation in response to mental stress, with stress-induced dilation most consistently depending on nitric oxide (NO) bioavailability (Cardillo *et al.*, 1997; Dietz *et al.*, 1994; Joyner & Dietz, 2003; Joyner & Casey, 2009; Sarabi & Lind, 2001). To date, few studies have examined the influence of inflammation on vascular responses to mental stress. A study by (Veldhuijzen van Zanten *et al.*, 2008) demonstrated that rheumatoid arthritis patients with high-grade systemic inflammation displayed an increase in vascular resistance in response to mental stress, which was not evident in patients with low-grade inflammation. In addition, a study by Paine *et al.*, (2012a) has demonstrated an attenuation in stress-induced increases in blood flow were seen following vaccine-induced inflammation in healthy participants, with the effects more pronounced in the limb where the vaccination was administered (Paine *et al.*, 2012a). It has been thought that reductions in NO through inflammation could be one mechanism through which inflammation could attenuate the vascular responses to stress (Paine *et al.*, 2012b).

Acute eccentric exercise can induce acute and systemic inflammation in response to the associated muscle damage (Peake *et al.*, 2005). Eccentric exercise is performed when tension over a muscle increases as it lengthens, which occurs when slowly lowering a weight

whilst trying to resist the forces of gravity (Edwards *et al.*, 2007; Proske & Morgan, 2001). Eccentric exercise initiates a localised inflammatory response, as a result of myofibril damage in the muscle (Nosaka *et al.*, 2002; Proske & Morgan, 2001). This form of exercise results in a more extensive, localised, inflammatory response at the site of muscle damage compared to concentric exercise (Bruunsgaard *et al.*, 1997; Willoughby *et al.*, 2003). However, the pattern of inflammatory response to eccentric exercise differs between inflammatory cytokines. For example, IL-6 increases are evident as early as 2 hours post exercise (Petersen *et al.*, 2001), whereas increases in CRP have been demonstrated at 24 hours post exercise (Barnes *et al.*, 2010; Paulsen *et al.*, 2005). Oedema (indexed as increases in limb circumference) and muscle pain have been used previously as indirect markers of inflammation as a result of exercise (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004). Previous work has used eccentric exercise as an inflammatory stimulus to induce increases in resting arterial stiffness 48 hours after completion of eccentric exercise of the elbow flexors (Barnes *et al.*, 2010), highlighting the potential impact of eccentric exercise-induced inflammation on the vasculature.

The aim of the present study is to investigate the role of inflammation, induced by a bout of acute eccentric exercise in the bicep and deltoid muscles of the non-dominant arm (Campbell *et al.*, 2010; Edwards *et al.*, 2010), on the vascular responses to mental stress in a healthy population. It was hypothesised that inflammation will result in reduced vascular function at rest and attenuated vascular responses to mental stress (Paine *et al.*, 2012b).

5.3 METHODS

Participants

Twenty male participants were recruited from the University of Birmingham (mean age ± SD = 19.2 ± 0.7 years, mean body mass index ± SD = 22.2 ± 2.1 kg/m^2). None of the participants were suffering from an acute illness or infection, reported a history of inflammatory, cardiovascular or auto-immune disorders, had taken any anti-inflammatory medication in the last 4 weeks or any regular prescribed medication. None of the participants smoked or undertook regular resistance training. Participants reported to the laboratory having refrained from vigorous exercise and over-the-counter medication for at least 24 hours, from alcohol for at least 12 hours and following an overnight fast. The study was approved by the local research ethics committee. All volunteers gave written informed consent to participate.

Eccentric Exercise Task

An eccentric exercise protocol was used to induce inflammation in the non-dominant arm of each participant (Edwards *et al.*, 2010) as increased damage and inflammatory responses are greater in those who are unaccustomed to eccentric exercise (Sorichter *et al.*, 2006). Prior to the commencement of the task, each participant's one repetition maxima (1RM) of the lateral raise and biceps were determined (concentric 1 repetition maximum: Bicep Curl: 16.52 ± 2.62 kg; Lateral Raise: 10.69 ± 2.16 kg). Participants performed the eccentric portions of the bicep curl and lateral raise exercises at an intensity of 110% of their 1RM, lowering the weight in a controlled manner, at a constant velocity over a five second period, as described previously (Edwards *et al.*, 2010). Participants completed a set of lateral raises, followed by 15 seconds rest before completing a set of bicep curls. Thirty seconds rest occurred before the next set of lateral raises. The length of the task was approximately 20

minutes. This cycle was completed 10 times; thus, participants performed a total of 50 repetitions of each contraction (10 sets of five repetitions). Limb circumference measurements of the bicep and forearm were taken from the non-dominant arm (i.e. exercised arm) of each exercising participant before and after exercise to assess changes in limb circumference. Participants were asked to rate muscle pain immediately post-exercise using a visual analogue scale, with anchors of 0 (no pain) and 100 (worst pain imaginable). Participants rated the pain when the exercised limb rested by their side (resting arm pain) as well as the pain when they performed the exercise actions (movement arm pain). They also rated muscle pain 24 hours post-exercise. These measurements of limb circumference and muscle pain have been used previously as indirect markers of an inflammatory response (Edwards *et al.*, 2010).

Mental Stress Task

The mental stress task was the paced auditory serial addition test (PASAT). Participants were presented with a series of single digit numbers, which were delivered using a CD player. They were required to add each number they heard to the number presented previously, while retaining the last number to add it to the next number they heard (Gronwall, 1977; Ring *et al.*, 2002). The numbers were delivered in four consecutive two minute blocks, with the numbers initially presented every 2.4 s, then 2.0 s, 1.6 s and then finally 1.2 s in the resulting two minute blocks. Consequently the task increased in difficulty as the task progressed. Elements of social evaluation, competition, time pressure and punishment were included to enhance the provocative nature of the task (Veldhuijzen van Zanten *et al.*, 2004). The PASAT elicits a stress response, with no physiological habituation, even when task-related learning effects occur (Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009; Willemsen *et al.*, 1998). Thus, the same PASAT was used on both days. At the end

of the stress task, participants were asked to rate the task in terms of perceived difficulty, arousal, stressfulness, engagement and perceived performance using a 7-point scale, with anchors of 0 (not at all) and 6 (extremely).

Physiological Measurements

Cardiovascular measures

Beat-to-beat arterial blood pressure was recorded continuously during both baseline and stress tasks using of a Finometer (Finapres Medical Systems; Amsterdam, The Netherlands). A small cuff was placed around the middle finger of the dominant hand of each participant. From this output, continuous data was recorded via a Power1401 (CED) connected to a computer programmed in Spike2 (CED). Cardiovascular parameters were scored offline from the blood pressure waveform. Measures of systolic (SBP) and diastolic (DBP) blood pressure were obtained as mean averages from the blood pressure waveforms recorded in periods of assessment. Mean arterial pressure (MAP) was then calculated.

Indices of cardiodynamic activity were recorded continuously using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS, Amsterdam, The Netherlands) (de Geus *et al.*, 1995; Willemsen *et al.*, 1996). This system used six Ag/AgCl spot electrodes (Invisatrace, ConMed Corporation) to record electrocardiography (ECG) and impedance cardiography (ICG), in line with published guidelines(Sherwood *et al.*, 1990). Ten second ensemble averages were calculated and used to determine the following measures: Heart Rate (HR, bpm), Pre-Ejection Period (PEP; ms), Root Mean Successive Squared Difference as a measure of Heart Rate Variability (rMSSD; ms), and Cardiac Output (as a product of stroke

volume and HR) (CO; l/min). In addition, Total Peripheral Resistance (TPR; dyn-s/cm^{-5}) was derived from MAP and CO.

Blood Flow

Venous occlusion plethysmography, using a mercury-in-silastic-strain gauge, was used to measure forearm blood flow (FBF) in the dominant limb of each participant. A strain gauge was fitted around the widest part of the dominant forearm. One congestion cuff was placed around brachial region of the upper arm (SC12, Hokanson) and another at the wrist (TMC7, Hokanson). The strain gauges were connected to a plethysmograph (EC6, Hokanson), which produced a calibrated output voltage proportional to limb circumference with a frequency response of 0 – 25 Hz. The plethysmograph signal was digitized at 100 Hz with 16-bit resolution, via a Power1401 (CED) connected to a computer programmed in Spike2 (CED). The brachial cuffs were inflated for 5s to above venous pressure (40 mmHg), using a rapid cuff inflator (E20, Hokanson) attached to an automated air source (AG101, Hokanson). This procedure allows for arterial inflow, while preventing venous return. Following 15 seconds, the brachial cuffs were inflated again. This was repeated three times per minute. Throughout the minute of assessment, the wrist cuff was manually inflated by Sphygmomanometer (S300, Hokanson) to supra-systolic blood pressure (> 200 mmHg), to prevent any blood flow into the hand. Calibration and blood flow analysis was undertaken offline using Spike2 (CED). Increases in limb circumferences associated with inflation of the cuffs were identified and for each of these, the slope was measured between the upstroke of first two pulses following cuff inflation. The slope was assessed using a least squares fit to the data to minimise the effects of outlying data points. Three measurements of blood flow (slopes in response to cuff inflation) occurred per minute, with these mean averaged to give

an estimate of blood flow per minute. Forearm Vascular Resistance (FVR) was calculated as Mean Arterial Pressure/Blood Flow, with Forearm Vascular Conductance (FVC) calculated as BF/MAP × 100.

Procedure

All sessions started between 6.30 am and 9:00 am. Participants completed two sessions on consecutive days, such that the sessions were conducted 24 h apart. All procedures were performed in a temperature controlled environment (18°C). Upon arrival, all participants were familiarised with the procedures and were instrumented. Measurements of height and weight were taken, and participants rested in a supine position for the duration of all procedures and watched a nature documentary (*Planet Earth*; BBC) during rest periods. After instrumentation, endothelial function was assessed (data not reported due to methodological issues) following which, a 20 minute baseline period followed during which FBF was measured during minutes 13, 15, 17 and 19. In addition, ICG, ECG and blood pressure were analysed for these minutes. After the explanation and practice participants completed the 8 minute PASAT. During minutes 1, 3, 5 and 7, FBF was recorded and ICG, ECG and blood pressure were analysed for these minutes. Once completed, the participant was asked to report how difficult, stressful, arousing and engaging they found the PASAT, as well as how well they thought they performed. Next, participants stayed to complete the eccentric exercise task. All participants returned the following day (24 hours later), when all procedures were repeated with the exception of the exercise task.

Data reduction and statistical analysis

For all cardiovascular variables, the average of the four measurements taken during rest was derived to obtain a mean baseline value. Similarly, the four measurements taken during stress task were averaged to obtain a mean task value. A series of 2 Condition (Control, Inflammation) by 2 Time (Baseline, Stress) repeated measures Analyses of Variance (ANOVAs) were conducted on the cardiovascular and vascular measurements. To provide a more conservative estimate of significance the Greenhouse-Geisser correction was applied (Vasey & Thayer, 1987). Two Condition ANOVAs were conducted to examine differences in the task impact ratings. For all ANOVAs, eta squared (η^2) was used as a measure of effect size, and Newman-Keuls post-hoc comparisons are reported. To examine difference in limb circumference as a result of exercise, 2 Time (pre-task, post-task) ANOVAs were conducted. Differences in reported pain (both limb stationary and mimicking exercise action) immediately post-exercise and 24 hours post exercise was also examined using this analysis. To examine whether differences in stress-induced cardiovascular or vascular responses as a result of exercise occurred, reactivity scores for all cardiovascular and vascular measures were calculated as the difference between the average stress value and the baseline value. To examine whether there were any associations between the exercise induced changes and the cardiovascular and vascular assessments, Pearson correlations were conducted, examining the associations between the self reported pain scores at 24 h, changes in limb circumference as a result of the exercise task and the cardiovascular responses to stress. These analyses were conducted for the inflammation session only. Occasional missing data are reflected in the reported degrees of freedom.

5.4 RESULTS

Responses to exercise

Table 5.1 depicts the changes in response to the exercise task. Assessment of changes in limb circumference as a result of exercise revealed significant increases in bicep circumference from 29.0 ± 2.2cm to 29.6 ± 2.3 cm, and increases in forearm limb circumference from 25.9 ± 1.7 cm to 26.9 ± 1.9 cm. There was no difference in the pain reported when mimicking the exercise action in the exercised limb in comparison to not moving the exercised limb immediately post exercise (movement arm pain 39.7 ± 21.8, resting arm pain 34.9 ± 21.9). At 24 hours post exercise, there were reductions in the pain reported when not mimicking the exercise action in the exercised limb (immediately post exercise 34.9 ± 21.9, 24 h post exercise 14.9 ± 16.1), but no changes in pain when mimicking the exercise action (immediately post exercise 39.7 ± 21.8, 24 h post exercise 33.3 ± 23.0), indicating moderate to high levels of pain and muscle soreness when moving the limb.

Table 5.1. Mean (SD) self-reported pain and limb circumference responses to the eccentric exercise task

	Movement Pain	**Resting Pain**	
Immediately post exercise	39.7 (21.8)	34.9 (21.9)	($F(1, 20) = 1.32$, $p > .05$, $\eta^2 = .06$)
	Immediately post exercise	**24hours post exercise**	
Movement pain	39.7 (21.8)	33.3 (23.0)	($F(1, 20) = 1.10$, $p > .05$, $\eta^2 = .05$)
Resting pain	34.9 (21.9)	14.9 (16.1)	($F(1, 20) = 13.50$, $p = .002$, $\eta^2 = .40$)
	Immediately Pre Exercise	**Immediately Post Exercise**	
Bicep Limb Circumference (cm)	29.0 (2.2)	29.6 (2.3)	($F(1, 20) = 6.27$, $p = .021$, $\eta^2 = .24$)
Forearm Limb Circumference (cm)	25.9 (1.7)	26.9 (1.9)	($F(1, 20) = 78.04$, $p < .001$, $\eta^2 = .80$)

Note: Movement pain refers to mimicking the exercise action; resting pain refers to the arm when resting by the side of the participant. Scale of 0-100; 0=no pain, 100=worst pain imaginable.

Self reported responses to mental stress task

There were no condition differences (*p*'s > .05) in perceived difficulty, stress, arousal or engagement.

Physiological responses to mental stress

Figure 5.1 depicts the HR, CO, PEP, rMSSD, SBP and DBP responses to mental stress during the control and inflammation condition. 2 Condition (Control, Inflammation) by 2 Time (Baseline, Stress) repeated measures ANOVAs yielded time effects for HR (F (1, 18) = 77.61, $p < .001$, $\eta^2 = .81$), CO (F (1, 17) = 17.71, $p = .001$, $\eta^2 = .52$), PEP (F (1, 17) = 18.02, $p = .001$, $\eta^2 = .52$), rMSSD (F (1, 18) = 15.34, $p = .001$, $\eta^2 = .46$), SBP (F (1, 20) = 63.04, $p < .001$, $\eta^2 = .76$) and DBP (F (1, 20) = 93.58, $p < .001$, $\eta^2 = .82$). Post hoc analyses revealed that HR, CO, SBP, and DBP increased whereas PEP and rMSSD decreased in response to stress. No condition effects or condition by time interaction effects were seen for CO, HR, PEP, rMSSD, SBP and DBP (*p*'s > .05).

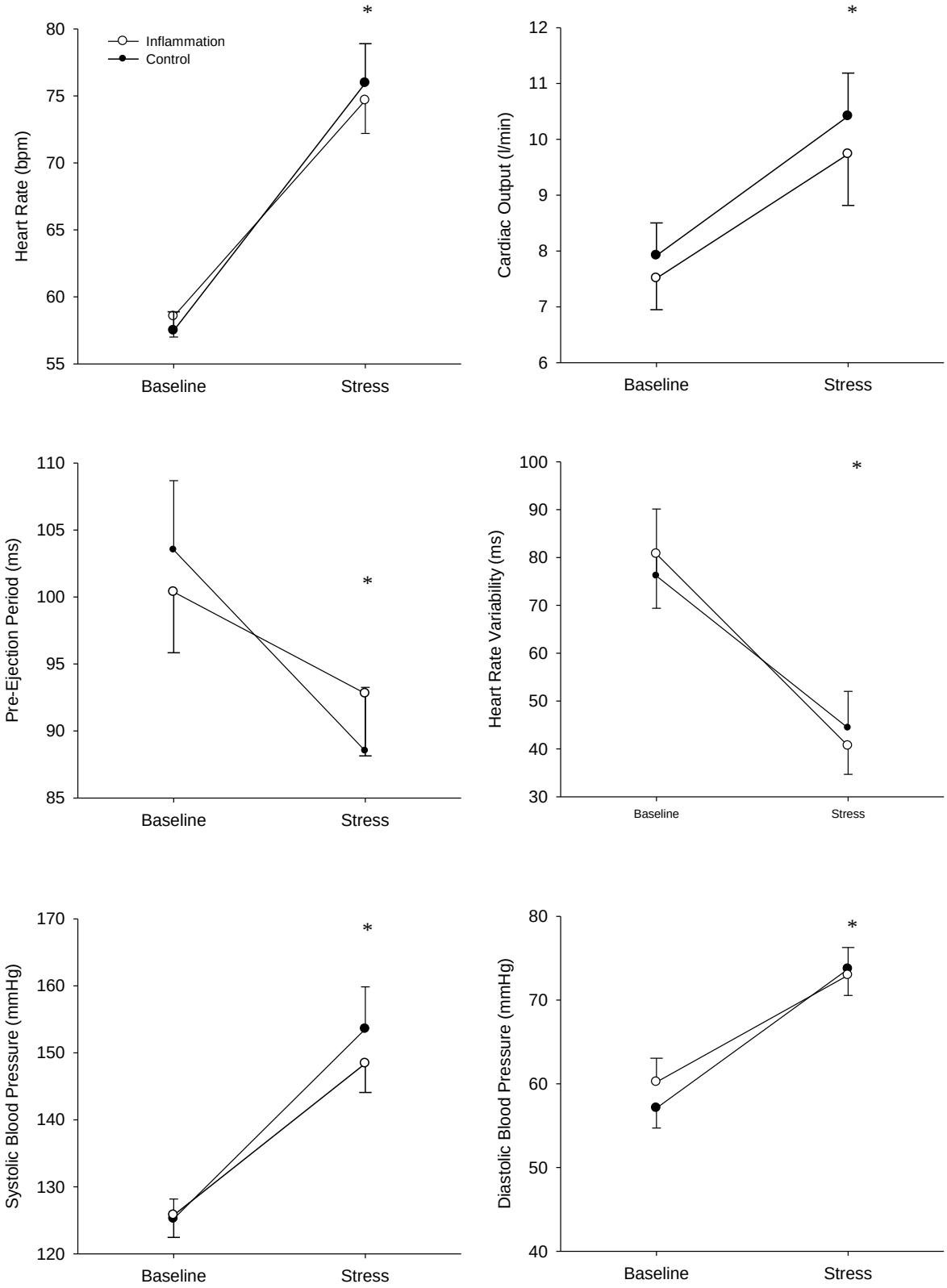

Figure 5.1. Mean ± SE responses to mental stress for HR, PEP, SBP, CO, rMSSD and DBP. * indicates significantly different from baseline ($p < .05$)

The forearm vascular responses to mental stress are shown in Figure 5.2. Separate 2 Condition by 2 Time ANOVAs revealed a significant time effects for FBF (F (1, 19) = 47.03, p < .001, η^2 = .71), FVR (F (1, 19) = 42.32, p < .001, η^2 = .69), and FVC (F (1, 19) = 32.32, p < .001, η^2 = .64). Post hoc analyses revealed increased FBF and FVC as well as decreased FVR in response to stress. No time effect emerged for TPR (F (1, 17) = 0.60, p = . 45, η^2 = .03) (Figure 5.2). There were no condition or condition by time interaction effects for FBF, FVR, FVC and TPR (p's >.05).

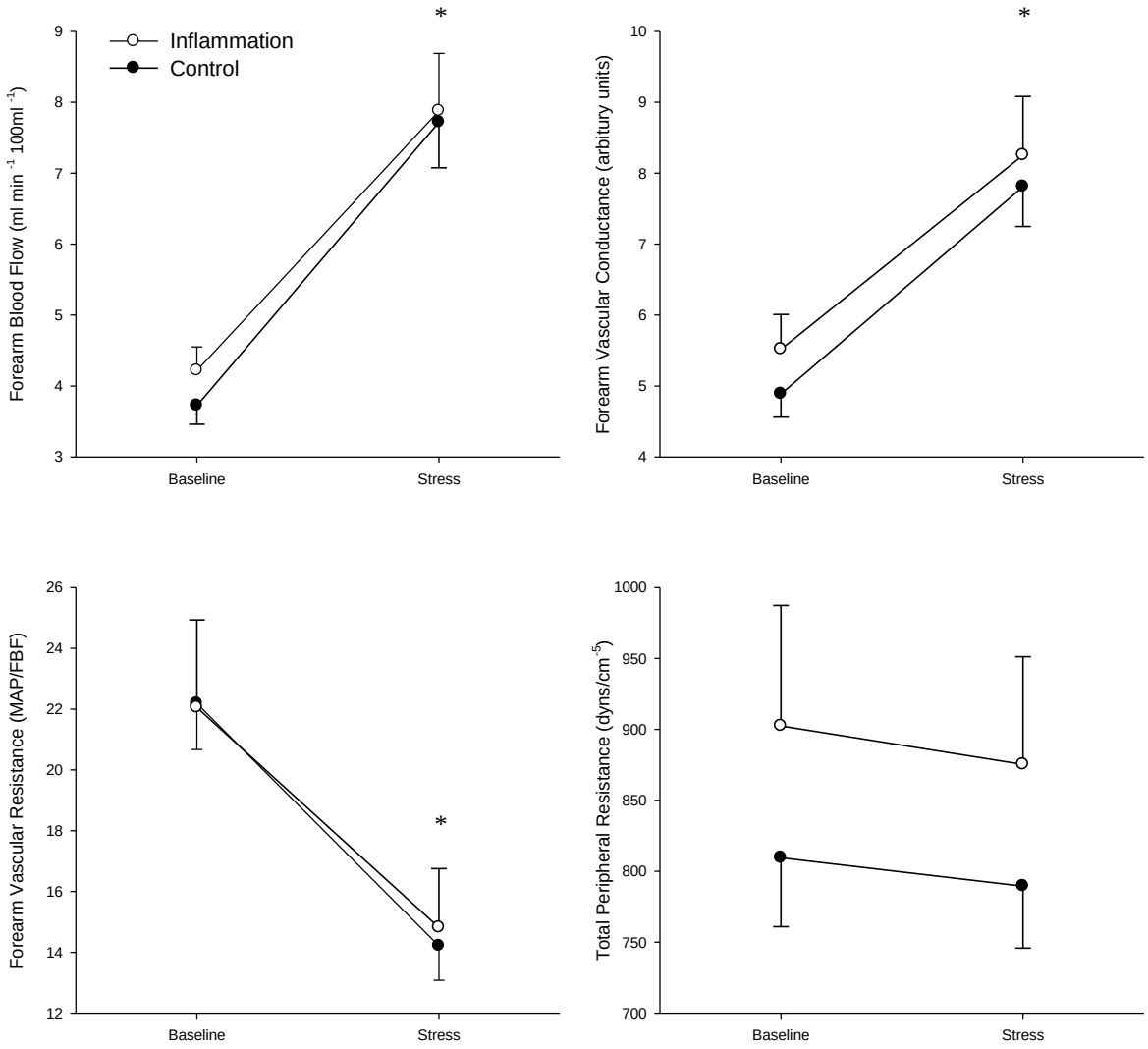

Figure 5.2. Mean ± SE responses to mental stress for FBF, FVR, FVC and TPR
* represents significantly different from baseline (p < .05)

Associations between inflammation and physiological responses to mental stress

Pearson correlation analyses were undertaken to examine the relationships between the measures of inflammation (limb circumference and muscle pain 24 hours post exercise) and the cardiovascular responses to mental stress (see Table 5.2), to determine if there were any relationships between exercise-induced inflammation and the cardiovascular responses to stress. Stress-induced changes in FBF and FVC were positively associated with pain. Self reported pain when not moving the exercised limb was positively associated with increases in TPR ($p < .05$) in response to mental stress.

Table 5.2. Pearson correlation coefficients between the cardiovascular responses to mental stress and the self reported pain 24 h post exercise and the change in limb circumference as a result of exercise

	Resting Pain	**Movement Pain**	**Δ Forearm Limb Circumference change**	**Δ Bicep Limb circumference change**
Δ FBF	.518*	.463*	.137	-.194
Δ FVR	.008	.045	.195	.079
Δ FVC	.541*	.448*	.138	-.206
Δ TPR	.552*	.379	-.078	-.108

Note: * $p < .05$

5.5 DISCUSSION

This study aimed to examine the effects of eccentric-exercise induced inflammation on the vascular responses to mental stress, 24 hours post exercise. The eccentric exercise protocol induced immediate increases in limb circumference in both the bicep and forearm, which are indicative of inflammation (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards

et al., 2010; Hirose *et al.*, 2004). In both the exercise and control conditions stress-induced increases in HR, CO, SBP, DBP, FBF and FVC and decreases in rMSSD, PEP and FVR were evident. Eccentric exercise did not affect either resting or stress-induced cardiovascular function. Nevertheless, stress-induced blood flow and self reported pain in the exercising limb were associated. The reasons for this could be due to a less substantial inflammatory response to eccentric exercise or the examination of vascular function at a site not proximal to the site of inflammation.

Eccentric exercise has been shown to induce a systemic inflammatory response (Bruunsgaard *et al.*, 1997; Nosaka *et al.*, 2002; Proske & Morgan, 2001; Willoughby *et al.*, 2003). Indeed, immediate increases in limb circumference in both the bicep and forearm and elevations in self reported pain were observed in this study, indicating inflammation was induced (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004). However, different inflammatory markers have different patterns of response to eccentric exercise. For example, increases in IL-6 in response to eccentric exercise have been observed between 2-12 h post-exercise depending on the exercise protocol (Miles *et al.*, 2007; Miles *et al.*, 2008; Paulsen *et al.*, 2005; Toft *et al.*, 2002; Willoughby *et al.*, 2003), with IL-6 returning to baseline levels 24 h post exercise (Barnes *et al.*, 2010; Sorichter *et al.*, 2006; Willoughby *et al.*, 2003). However, elevations in CRP have been demonstrated 24-48 h post eccentric exercise (Barnes *et al.*, 2010; Paulsen *et al.*, 2005).

Despite these elevations in CRP at 24 hours post exercise, it should be noted that the increases in CRP described above were in response to eccentric exercise in larger muscle groups than the ones used in the current study. Eccentric exercise tasks using larger muscle

masses have demonstrated CRP increases (Barnes *et al.*, 2010; Paulsen *et al.*, 2005), as well as a greater IL-6 response to exercise (Febbraio & Pedersen, 2002). Therefore, elevations in CRP at 24 h post exercise in the current study may not be as large as in other studies, and the use of a greater muscle mass is something that should be considered in future studies to ensure greater elevations in inflammatory markers such as CRP and IL-6. Nevertheless, previous work that has used this protocol and other high intensity eccentric arm exercise tasks have shown Creatine Kinase (CK), a marker of muscle membrane disruption (Jackman *et al.*, 2010), to be elevated at 24 h post exercise (Chen *et al.*, 2007; Edwards *et al.*, 2010). Increases in resting pulse wave velocity have been correlated with increases in CK as a result of elbow flexor eccentric exercise, and highlighting a potential role for CK and muscle damage in altering vascular function (Barnes *et al.*, 2010). However, no alterations in vascular function (as indexed through forearm blood flow) were seen in this study, and thus it is possible that the levels of CRP and CK induced in this study were not sufficient to induce changes in vascular measures.

The lack of changes in vascular function may conversely be as a result of the location of the inflammation in relation to the site of vascular function that was assessed. The non-dominant arm of each participant was used for the exercise task as it was hoped that as this limb would be less accustomed to eccentric exercise, and therefore the inflammatory response would be greater (Sorichter *et al.*, 2006). However, forearm blood flow was assessed in the non-exercised arm, allowing examination of the impact of eccentric exercise-induced inflammation on the systemic vascular responses to stress. Indeed, the detection of the inflammatory response may be systemic, but muscle damage as a consequence of exercise more localised (Nosaka *et al.*, 2002; Proske & Morgan, 2001). As such, it may be possible

that inflammation has a greater effect on the vasculature that is proximal to the site of the inflammation. Indeed, we have shown that the greatest effect of inflammation on the vasculature has been shown to be in the limb where the inflammatory stimulus was administered (Paine *et al.*, 2012a). Barnes *et al.* (2010) demonstrated relatively small increases in pulse wave velocity when measured between the carotid and femoral artery (~3-5%) in response to eccentric exercise when undertaken in the arm (eccentric elbow flexor exercise task) or leg (an eccentric bilateral leg press exercise task). This again suggests that a greater impairment may have been observed if vascular function was assessed proximally to the site of inflammation. However, as the current study only assessed vascular responses in one limb, the hypothesis must remain speculation.

In line with vaccination-induced inflammation, there were no changes in cardiovascular assessments at rest or in response to stress following eccentric exercise. The current study did have a non-counterbalanced design; however there were no differences in the perception of the stress task in both stress conditions. Indeed, the use of this task has demonstrated no attenuation in the cardiovascular responses to stress in response to multiple repeats of the task (Ring *et al.*, 2002a; Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009). Therefore, the lack of any changes as a result of the exercise is unlikely to be attributable to any habituation to the task. One major limitation of the study is that no direct measurement of inflammation (through blood measures) was taken. However, measurements of limb circumference and self reported pain have been previously used elsewhere as indirect markers of inflammation (Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004), and thus our findings indicate that inflammation was successfully induced.

In sum, it appears that using this particular eccentric exercise protocol to induce inflammation did not influence limb blood flow responses to mental stress. This may be due to the inflammation having mainly a local effect on the vasculature, or could be due to the time that the assessments were taken relative to the eccentric exercise. Future work should therefore address these issues to identify whether eccentric exercise influences the vascular responses to mental stress.

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CHAPTER SIX:

THE EFFECT OF ECCENTRIC-EXERCISE INDUCED INFLAMMATION ON THE VASCULAR RESPONSES TO MENTAL STRESS

6.1 ABSTRACT

Mental stress has been identified as a trigger of myocardial infarction (MI), with both inflammation and the vascular responses to mental stress independently implicated as contributing factors. This study examined a potential moderating effect of inflammation on the vascular responses to mental stress. Eighteen healthy male participants completed a stress task under two counter balanced conditions. In the inflammation condition, release of cytokines was induced by a bout of eccentric exercise (12 × 5 repetitions of unilateral eccentric knee extension at 120% intensity of concentric one repetition maximum), 6 hours prior to an afternoon stress session. In the control condition, participants sat and relaxed for 45 minutes, 6 hours prior to the afternoon stress session. Forearm blood flow, calf blood flow, blood pressure, heart rate and cardiac output were assessed at rest and in response to mental stress. As expected, interleukin-6 was higher (p = .01) 6 hours post-exercise, i.e., at the start of the stress session, compared to the no-exercise control condition. Mental stress increased forearm blood flow, calf blood flow, blood pressure, heart rate, and cardiac output in both conditions (p's < .001). Stress-induced calf blood flow was attenuated in the inflammation condition compared to the control condition (p < .05). This study found that the inflammatory response to eccentric exercise attenuated the vascular responses to mental stress locally at the site of eccentric exercise-induced inflammation. The observed impairment in vascular responses to stress associated with increased levels of inflammation suggests a mechanism through which inflammation might increase the risk for MI.

6.2 INTRODUCTION

Acute mental stress has been identified as a possible of trigger myocardial infarction (MI). For example, survivors of MI have mentioned emotional stress (e.g. episodes of anger) as a trigger for their MI (Strike & Steptoe, 2005). Likewise, earthquakes (Leor *et al.*, 1996a; Leor *et al.*, 1996b; Suzuki *et al.*, 1995), war (Bergovec *et al.*, 1992; Meisel *et al.*, 1991), and even key football matches (e.g., Carroll *et al.*, 2002; Wilbert-Lampen *et al.*, 2010) have been associated with an increased incidence in MI. To explore the mechanisms through which stress might lead to MI, laboratory studies have assessed mental stress-induced ischemia, which is predictive of future cardiac events and mortality (Babyak *et al.*, 2010; Krantz *et al.*, 1999). Mental stress-induced ischemia is associated with higher resting levels of inflammation in cardiac patients (Shah *et al.*, 2006). Also, mental stress-induced ischemia is displayed alongside poorer vascular responses to stress, characterised by either increases in vascular resistance (Goldberg *et al.*, 1996; Jain *et al.*, 1998) or decreased peripheral dilation (Burg *et al.*, 2009). Interestingly, serological markers of inflammation and vasoconstriction were increased in patients admitted to hospital for MI during the Football World Cup compared to those admitted for MI during a non-stressful period (Wilbert-Lampen *et al.*, 2010). Therefore, both inflammation and vascular responses to mental stress might contribute to mental stress induced MI (Paine *et al.*, 2012c).

Both acute (Meier *et al.*, 1998) and chronic (Levy *et al.*, 2008; Ridker *et al.*, 2000) inflammation are associated with increased risk for MI, and play a critical role in development and progression of cardiovascular disease (CVD) (Hansson, 2005; Libby, 2006). The impact of inflammation on resting vascular function has been examined extensively; studies have revealed inverse associations between vascular function and inflammation in clinical

populations (Fichtlscherer *et al.*, 2004; Vaudo *et al.*, 2004). However, the extent of the relationship between the vascular function and inflammation weakens when classic risk factors are controlled for (Cleland *et al.*, 2000; Vita *et al.*, 2004), and thus the sole influence of inflammation may be masked by the other risk factors. Therefore experimentally inducing inflammation in a healthy population is one method used to directly examine the effects of inflammation on vascular function without the presence of potentially confounding factors. These studies have demonstrated that inflammation in healthy participants causes transient endothelial dysfunction at rest (Bhagat & Vallance, 1997; Clapp *et al.*, 2004; Hingorani *et al.*, 2000; Kharbanda *et al.*, 2002).

In healthy participants, mental stress causes vasodilation (Blair *et al.*, 1959; Dietz *et al.*, 1994; Joyner & Halliwill, 2000), which is reduced in populations at risk for cardiovascular disease (Hamer *et al.*, 2007) and patients with heart failure (Middlekauff *et al.*, 1997; Santos *et al.*, 2005). Despite different mechanisms being proposed to be the cause of stress-induced vasodilation, the most consistent evidence is for the role of nitric oxide (NO) (Cardillo *et al.*, 1997; Dietz *et al.*, 1994; Joyner & Dietz, 2003; Joyner & Casey, 2009; Sarabi & Lind, 2001), with reductions in NO leading to an attenuated dilatory response to mental stress (Cardillo *et al.*, 1997; Dietz *et al.*, 1994; Joyner & Dietz, 2003; Joyner & Casey, 2009; Sarabi & Lind, 2001).

The role of NO in stress-induced vasodilation is interesting, given the reciprocal relationship between NO and inflammation at rest (De Martin *et al.*, 2000; Hung *et al.*, 2010; Verma *et al.*, 2002). Thus, given the common link between NO for maintaining vascular function and stress-induced vasodilation, it is hypothesised that inflammation may also

attenuate the vasodilatory response to mental stress (Paine *et al.*, 2012c), particularly given that the reduction in resting endothelial function as a result of increased inflammation has been attributed to a reduction in NO (Clapp *et al.*, 2004). Very few studies have examined the influence of inflammation on stress-induced vasodilation, despite evidence for the association between basal inflammation and mental stress-induced ischemia (Shah *et al.*, 2006). It has been found that rheumatoid arthritis patients with high-grade systemic inflammation displayed an increase in vascular resistance in response to mental stress, which was not evident in patients with low-grade inflammation (Veldhuijzen van Zanten *et al.*, 2008). Interestingly, poorer vascular responses to mental stress have been shown when inflammation was induced in a healthy population using a typhoid vaccination (Paine *et al.*, 2012a).

A potent, but less often utilized method to induce inflammation is eccentric exercise. Eccentric exercise involves increasing the tension over a muscle as it elongates (e.g., gradually lowering a weight against gravity) (Edwards *et al.*, 2007; Proske & Morgan, 2001). Eccentric exercise initiates a localised inflammatory response through ensuing myofibril damage in the muscle (Nosaka *et al.*, 2002; Proske & Morgan, 2001). The extent of the inflammatory response has typically been characterized by the increase in interleukin-6 (IL-6) (Croisier *et al.*, 1999; Depner *et al.*, 2008; Jackman *et al.*, 2010; Miles *et al.*, 2008; Steensberg *et al.*, 2000; Steensberg *et al.*, 2002; Willoughby *et al.*, 2003). IL-6 is a cytokine that is involved in the acute-phase response that occurs as a result of injury (Willoughby *et al.*, 2003). The peak IL-6 response is most commonly reported to be at 6 hours post eccentric-exercise (MacIntyre *et al.*, 2001; Paulsen *et al.*, 2005; Philippou *et al.*, 2009; Willoughby *et al.*, 2003), and the magnitude of the response is dependent on the duration, intensity and size of muscle used, such that a high intensity exercise using a large muscle mass should result in

a more substantial increase in IL-6 (Febbraio & Pedersen, 2002). The exact sources from which IL-6 is released likely involve both resident leukocytes as well as the exercising muscle (Ostrowski *et al.*, 1998; Willoughby *et al.*, 2003).

The aim of the current study was to investigate the effect of inflammation induced by a bout of acute eccentric exercise on the vascular responses to mental stress in a healthy population. To ensure a robust response, exercise was performed in the quadriceps muscle of the non-dominant leg. One previous study has examined the impact of eccentric exercise on resting vascular function, revealing increases in resting arterial stiffness at 48 hours after completion of eccentric exercise of the elbow flexors (Barnes *et al.*, 2010). No study has investigated effects on stress-induced vascular responses, which are of interest given their potential role in the triggering of MI. Therefore, it is hypothesised that eccentric exercise-induced inflammation will attenuate the vascular responses to mental stress, compared to a no-exercise control condition.

6.3 METHODS

Participants

Eighteen male participants were recruited from the University of Birmingham (mean age ± SE = 20.4 ± 1.2 years, mean body mass index (BMI) ± SD = 23.7 ± 0.6 kg/m^2). None of the participants were suffering from an acute illness or infection, reported a history of inflammatory, cardiovascular or auto-immune disorders, or had taken any medication in the last 4 weeks. None of the participants undertook any regular resistance exercise as greater amounts of muscle damage and inflammation have been demonstrated in those who are unaccustomed to eccentric exercise (Sorichter *et al.*, 2006). Participants reported to the

laboratory having refrained from vigorous exercise for at least 24 hours, from alcohol for at least 12 hours and food or caffeine in the 2 hours prior to testing. The study was approved by the local Research Ethics Committee and all participants gave written informed consent.

Eccentric Exercise Task

The eccentric exercise task was undertaken using a Cybex leg extension machine (Cybex International Medway, MA) and was adapted from that used by Jackman *et al.*, (2010). Firstly, measurements of limb circumference of both legs and assessments of muscle soreness were taken. After familiarisation with the equipment, each participant's one repetition maximum (1RM) of their non-dominant leg was determined. Each participant concentrically lifted the weight from a position of knee flexion to knee extension, holding it outstretched for 2 s, before lowering it. Weight was added until participants could no longer lift the weight. Each 1RM was determined within 5 attempts to minimise fatigue. Once this was determined, the eccentric exercise task was explained before participants completed a set at a reduced weight (50% 1RM) to ensure understanding of the action. Participants were seated with their non-dominant leg positioned at 90 degrees to their torso. Two experimenters lifted the weight to its starting position (with the exercising leg extended to approximately 15 degrees of flexion), and the participant lowered the weight until their knee was flexed at approximately 110 degrees. Participants lowered the weight slowly over a 4 second period, resisting the weight at all times. If this was not achieved, the weight was replaced at the starting position and the action repeated. Participants completed 12 sets of 5 eccentric repetitions at 120% of their concentric 1RM. Five seconds rest separated each repetition, with a 1 minute rest at the end of each set. At the end of the task, the 'Borg scale' (1970) was used to assess perception of physical exertion during the exercise task, using a 14 point scale range

from six to 20, with six rated as “no exertion at all” and 20 being rated as “maximal exertion” (Borg, 1970).

Rest Task

Participants rested for 25 minutes and completed a questionnaire pack (data not reported).

Mental Stress Task

The paced auditory serial addition test (PASAT) was used as mental stress task. The PASAT has been widely demonstrated to repeatedly produce a stressful stimulus, even when the task is repeated several times on separate days (Veldhuijzen van Zanten *et al.*, 2005; Willemsen *et al.*, 1998). During the PASAT, participants were presented with a series of single digit numbers, delivered using a CD player, and were required to add each new number to the number presented previously (Gronwall, 1977; Ring *et al.*, 2002). The task lasted a total of 16 minutes, split into two eight minute tasks (referred to as Stress 1 and Stress 2 respectively), separated by one minute rest. The numbers were delivered in four 2-minute blocks, with the numbers respectively presented every 3.2 s, 2.8 s, 2.4 s and 2.0 s for the first task, and every 2.4 s, 2.0 s, 1.6 s and 1.2 s for the second task. This resulted in a progressive increase in task difficulty. The experimenter, who sat 1 metre adjacent to the participants, checked their responses against the correct answers.

To enhance the stressfulness of the performance several features were added that had shown to enhance stress responses in previous studies (Veldhuijzen van Zanten *et al.*, 2004). Participants heard a loud aversive noise once in each block of 10 numbers, with the noise presented after the participants’ first incorrect response, or if they had not made an error, the

noise was delivered at the end of the 10 number block. Participants were filmed with a video camera and were asked to look at their faces displayed on a television screen while performing the task, which they were told was to be analysed. If participants looked away from the screen, they heard the aversive noise and were reminded to continue to watch the screen. They were told that a £10 gift voucher would be awarded for the best performance on the task and a leader board with the highest five scores achieved by the participants was displayed, to compare participant scores. Another £10 voucher given to the participant recorded the greatest improvement in score between the two sessions. However, the latter of these rewards was only revealed before the start of the test in the inflammation session, to eliminate the risk of a poor performance in the control session. These elements of social evaluation, competition, punishment and reward have been shown to enhance the provocativeness of the task (Veldhuijzen van Zanten *et al.*, 2004). At the end of the stress task, participants were asked to rate the PASAT in terms of perceived difficulty, arousal, stressfulness, engagement and perceived performance using a 7- point Likert scale.

Procedure

Participants completed 2 conditions scheduled seven days apart (Figure 6.1), with the order of the conditions counterbalanced. The control condition consisted of a 45 minute rest session in the morning, whereas the inflammation condition consisted of an eccentric exercise task in the morning. Six hours after the morning session, the participant completed the stress session.

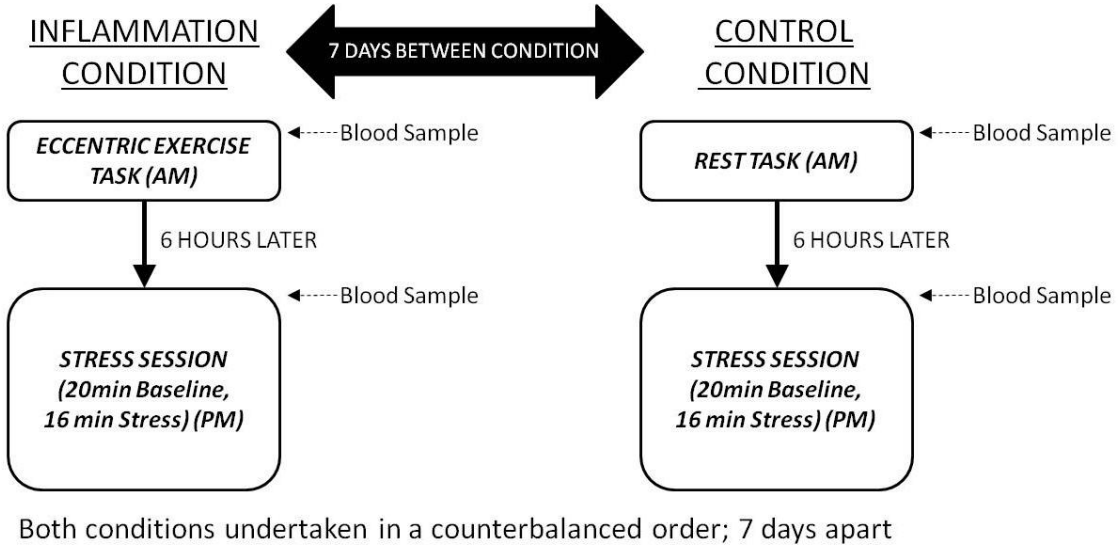

Figure 6.1. Diagram illustrating the protocol that each participant undertook

The time between the morning sessions and the stress reactivity sessions was chosen in order to start the afternoon session close to the peak inflammatory response to eccentric exercise (MacIntyre *et al.*, 2001; Paulsen *et al.*, 2005; Philippou *et al.*, 2009; Willoughby *et al.*, 2003). Indeed, our own data confirmed elevations in granulocytes at this time point (Figure 6.2). All procedures were performed in a temperature controlled (18°c) laboratory. The start times for both sessions was the same for each participant, with morning session start

times between 8:00 am and 10:00 am, and afternoon stress sessions starting between 2:00 pm and 4:00 pm.

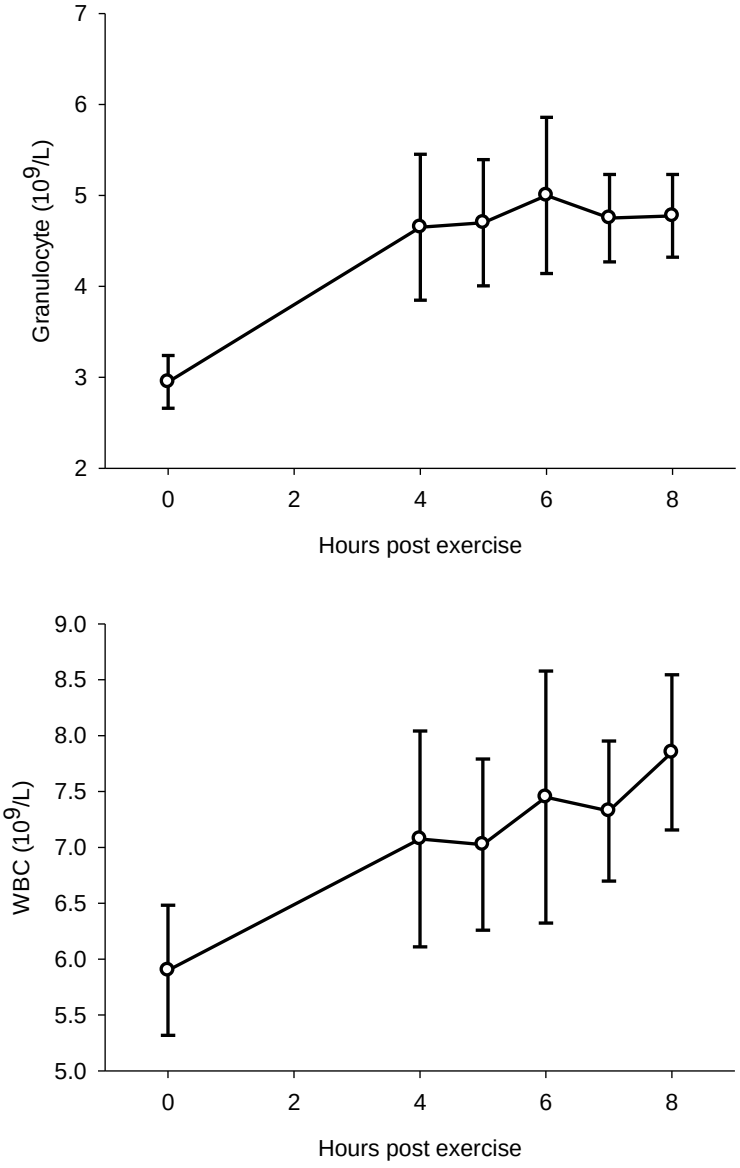

Figure 6.2. Mean ± SE illustrating the white blood cell (WBC) and granulocyte (GR) responses to eccentric exercise task (N=4).

Morning Session

Upon arrival in the laboratory, a blood sample was taken using a 21 gauge butterfly needle (Becton Dickinson, UK). Measures of limb circumference and muscle soreness were obtained and participants completed the eccentric exercise task or the rest task. After completion of these tasks, repeat measures of limb circumference and muscle soreness were taken. Both sessions lasted approximately 45 minutes.

Stress Session

Upon arrival in the laboratory height and weight were measured, and the participants were instrumented with the cardiovascular equipment. The participants were then placed in a supine position on a bed, where they remained throughout the session. Following additional assessments while in supine position (12 minutes), an 18 gauge canula (Insyte, Becton Dickinson) was inserted into an antecubital vein of the dominant arm of each participant. After the Finometer and blood flow equipment was attached, they rested for 20 minutes (baseline rest period) while watching a nature documentary (*Life*; BBC). Blood flow was measured during minutes 13, 15, 17 and 19. A resting blood sample was taken at the end of the baseline rest period. After practice of the metal stress task, participants completed two 8 minute blocks of the mental stress task, with 1 minute rest in between. During minutes 1, 3, 5 and 7 of each block, blood flow was recorded. ICG, ECG and blood pressure were recorded throughout the session, however, off line analyses were conducted on only the minutes during which blood flow was taken.

Cardiovascular measures

Beat-to-beat arterial blood pressure was recorded continuously during both the baseline and stress tasks using a Finometer (Finapres Medical Systems; Amsterdam, The Netherlands). A small cuff was placed around the middle finger of the dominant hand of each participant. From this output, continuous data was recorded via a Power1401 (CED) connected to a computer programmed in Spike2 version 6 (CED). Cardiovascular parameters were derived from the blood pressure waveform obtained from the recorded output and this was then analysed offline. Systolic (SBP) and diastolic (DBP) blood pressure were calculated from each blood pressure waveform recorded during the minute of assessment, and mean averaged for the periods of assessment. Systolic and diastolic blood pressure was then used to calculate mean arterial pressure (MAP). Indices of cardio-dynamic activity were recorded continuously using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS, Amsterdam, The Netherlands) (de Geus *et al.*, 1995; Willemsen *et al.*, 1996). This system used six Ag/AgCl spot electrodes (Invisatrace, ConMed Corporation) to record electrocardiography (ECG) and impedance cardiography (ICG), in line with published guidelines (Sherwood *et al.*, 1990). Ten second ensemble averages were calculated and used to determine the following measures: Heart Rate (HR, bpm), Pre-Ejection Period (PEP; ms), with Root Mean Square Successive Difference calculated as a measure of Heart Rate Variability (rMSSD; ms) and Cardiac Output (as a product of stroke volume and HR) (CO; l/min). In addition, Total Peripheral Resistance (TPR; dyn-s/cm^{-}5) was derived from MAP and CO.

Blood Flow

Venous occlusion plethysmography, using a mercury-in-silastic-strain gauge, was utilised to measure forearm blood flow (FBF) and calf blood flow (CBF). A strain gauge was fitted around the widest part of the non-dominant forearm and calf. One congestion cuff was placed around brachial region of the upper arm and thigh (SC12, Hokanson) and another at the wrist and ankle (TMC7, Hokanson). The strain gauges were connected to a plethysmograph (EC6, Hokanson), which produced a calibrated output voltage proportional to limb circumference with a frequency response of 0 – 25 Hz. The plethysmograph signal was digitized at 100 Hz with 16-bit resolution, via a Power1401 (CED) connected to a computer programmed in Spike2 version 6 (CED). The brachial and femoral cuffs were inflated for 5s to above venous pressure (40 mmHg), using a rapid cuff inflator (E20, Hokanson) attached to an automated air source (AG101, Hokanson). This procedure allows for arterial inflow, while preventing venous return. Following 15 seconds, the brachial and femoral cuffs were inflated again. This was repeated three times per minute. Throughout the minute of assessment, the wrist and ankle cuff was manually inflated by Sphygmomanometer (S300, Hokanson) to supra-systolic blood pressure (> 200 mmHg), to prevent any blood flow into the hand and foot. Calibration and blood flow analysis was undertaken offline using Spike2 (CED). Increases in limb circumferences associated with inflation of the cuffs were identified and for each of these, the slope was measured between the upstroke of first two pulses following cuff inflation. The slope was assessed using a least squares fit to the data to minimise the effects of outlying data points. Three measurements of blood flow (slopes in response to cuff inflation) occurred per minute, with these mean averaged to give an estimate of blood flow per minute. Vascular resistance (forearm – FVR; calf – CVR) was calculated as Mean Arterial Pressure/Blood Flow, with vascular conductance (forearm – FVC; calf – CVC) calculated as Blood Flow/Mean Arterial Pressure × 100.

Limb circumference and muscle soreness

Limb circumference measurements were taken from the non-dominant leg, with participants asked to flex their quadriceps, to mark the widest part of the quadriceps. Participants then relaxed their leg in order to measure limb circumference. On a scale of one to 100, with one being pain-free and 100 being the worst pain imaginable, participants rated how painful the leg was when it was resting (referred to as resting leg pain), and when asked to mimic the exercise actions (referred to as movement leg pain) (Melzack, 1987).

Blood Sampling and analysis

Blood samples were taken at the start of the morning sessions using a 21 gauge butterfly needle (Becton Dickinson, UK) inserted to the antecubital vein of the non-dominant arm. During the stress sessions a blood sample was taken using an 18 gauge canula (Insyte, Becton Dickinson) was inserted into an antecubital vein of the dominant arm. At each time point, blood was collected into two 6ml and one 2ml vacutainers containing potassium ethylene diaminetetraacetic acid (K3EDTA) (Becton–Dickinson, UK). Both 6ml samples were stored on ice until centrifugation (1500 × g for 10 min at 4°C) and plasma was stored at -80°C for later assessment of IL-6. Plasma IL-6 was measured in duplicate using high-sensitivity ELISA (Quantikine HS Human IL-6 ELISA, R&D Systems, UK) in accordance with the manufacturer's instructions. The reported sensitivity of the assays was 0.039 pg/ml, with recorded intra-assay and inter-assay variations both < 10%.

Data reduction and statistical analysis

For all cardiovascular variables, the 4 measurements taken during baseline, stress 1, and stress 2 were averaged to establish a single Baseline, Stress 1 and Stress 2 value, respectively. Due to the skewed distribution of the IL-6 data, IL-6 levels were log transformed and analysis conducted on the $\log_{10}$ values. Analysis of Variance (ANOVAs) was conducted to examine differences between all baseline values in the control and inflammation sessions. Two Condition (Control, Inflammation) by three Time (Baseline, Stress 1, Stress 2) repeated measures ANOVAs were conducted on cardiovascular and vascular measurements, with Greenhouse-Geisser correction (Vasey & Thayer, 1987). For all ANOVAs, eta squared (η^2) was used as a measure of effect size, and, where appropriate, Newman-Keuls post-hoc comparisons are reported. Occasional missing data are reflected in the reported degrees of freedom.

Reactivity (change) scores for all cardiovascular and vascular measures were calculated as the difference between the average stress value (calculated as the average between Stress 1 and Stress 2) and the baseline value. Subsequently, Pearson correlations were conducted to examine the associations between resting inflammatory markers (IL-6) and vascular as well as cardiovascular reactivity. These analyses were conducted for the control and inflammation condition separately.

6.4 RESULTS

Responses to eccentric exercise

Table 6.1 displays the self-reported pain ratings and changes in limb circumference, pre- and post-task for both the rest and exercise conditions. In the inflammation (exercise) condition, increases in limb circumference as a result of exercise were demonstrated (*F* (1,

17) = 22.86, $p < 0.001$, $\eta^2 = .57$), as were higher self reported pain in the exercising leg at rest (F (1, 17) = 20.38, $p < 0.001$, $\eta^2 = .55$) and when mimicking the exercise action (F (1, 17) = 17.33, $p = .001$, $\eta^2 = .51$). The mean average self perceived exertion score for the exercise task was 15.9 ± 2.5, with the mean weight used for the task 72.1 ± 14.7 kg. As expected, no changes in limb circumference or pain were observed in the rest condition (*p's* > .05).

Table 6.1. The mean (SD) self reported pain responses and limb circumference changes to the task for both the exercise and rest condition

EXERCISE TASK				
		Pre- task	Immediately Post- task	
Movement pain	Non-exercising leg	2.44 (3.42)	3.72 (6.98)	F (1, 17) = 1.02, $p > .05$, $\eta^2 = .06$
	Exercising leg	2.83 (3.26)	22.17 (21.30)	F (1, 17) = 17.33, $p = .001$, $\eta^2 = .51$
Resting Pain	Non-exercising leg	1.83 (2.57)	2.44 (5.10)	F (1, 17) = .72, $p > .05$, $\eta^2 = .04$
	Exercising leg	1.83 (2.55)	15.11 (13.92)	F (1, 17) = 20.38, $p < 0.001$, $\eta^2 = .55$
Limb Circumference (cm)		50.27 (4.37)	51.16 (4.20)	F (1, 17) = 22.86, $p < 0.001$, $\eta^2 = .57$

REST TASK				
		Pre- task	Immediately Post- task	
Movement pain	Non-exercising leg	3.44 (4.34)	3.50 (4.18)	F (1, 17) = .07, $p > .05$, $\eta^2 = .00$
	Exercising leg	3.11 (3.58)	3.22 (3.69)	F (1, 17) = .24, $p > .05$, $\eta^2 = .01$
Resting Pain	Non-exercising leg	2.33 (5.31)	2.61 (5.37)	F (1, 17) = 2.03, $p > .05$, $\eta^2 = .11$
	Exercising leg	2.28 (4.96)	2.44 (5.04)	F (1, 17) = .81, $p > .05$, $\eta^2 = .05$
Limb Circumference (cm)		49.62 (4.89)	49.65 (4.80)	F (1, 17) = .46, $p > .05$, $\eta^2 = .03$

Note: Movement pain refers to mimicking the exercise action; resting pain refers to the leg when resting in a stationary position with the knee flexed at 90 degree. Both pain scales rated using a scale of 0-100; 0=no pain, 100=worst pain imaginable.

Figure 6.3 illustrates the changes in IL-6 as a result of exercise and rest. A two condition (control, inflammation) by two time (pre-task, baseline) repeated measures ANOVA revealed a condition by time interaction effect (F (1, 15) = 7.94, p = .013, η^2 = .35). Further interrogation of this effect revealed that IL-6 was higher in the inflammation condition (F (1, 14) = 8.53, p = .011, η^2 = .38) at the baseline period in the stress reactivity session, with no

increases in IL-6 observed in the control (rest) condition. No difference in IL-6 were observed in the blood samples obtained before the start of the exercise and rest tasks (*p's* >.05).

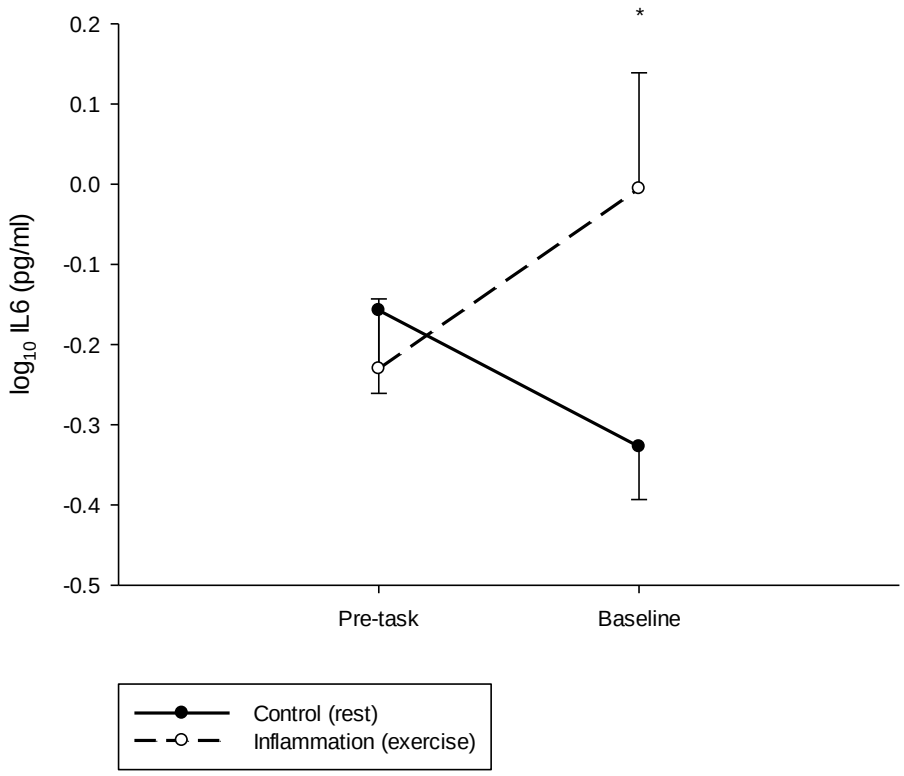

Figure 6.3. Mean ± SE IL-6 responses to the rest and exercise tasks * indicates differences between condition ($p < .02$)

Self-reported Task ratings

No differences in the performance score, perceived performance, perceived levels of stress, arousal, and engagement were observed between conditions (*p's* > .05).

Physiological responses to mental stress

Cardiovascular responses to mental stress

Figure 6.4 depicts the HR, CO, PEP, rMSSD, SBP and DBP responses to mental stress in the control and inflammation condition. Two Condition (Control, Inflammation) by 3 Time (Baseline, Stress 1, Stress 2) repeated measures ANOVA yielded main effects for time for HR (F (2, 16) = 31.47, $p < .001$, $\eta^2 = .74$), CO (F (2, 14) = 8.87, $p = .022$, $\eta^2 = .26$), PEP (F (2, 9) = 11.46, $p = .002$, $\eta^2 = .52$), rMSSD (F (2, 14) = 16.15, $p < .001$, $\eta^2 = .63$), SBP (F (2, 16) = 26.47, $p < .001$, $\eta^2 = .61$) and DBP (F (2, 16) =23.06, $p < .001$, $\eta^2 = .54$). Newman-Keuls post hoc analyses revealed that HR, CO, SBP, and DBP increased, whereas PEP and rMSSD decreased in response to stress. No condition effects or condition by time interaction effects were seen for CO, HR, PEP, rMSSD, SBP and DBP (all *p's* > .05).

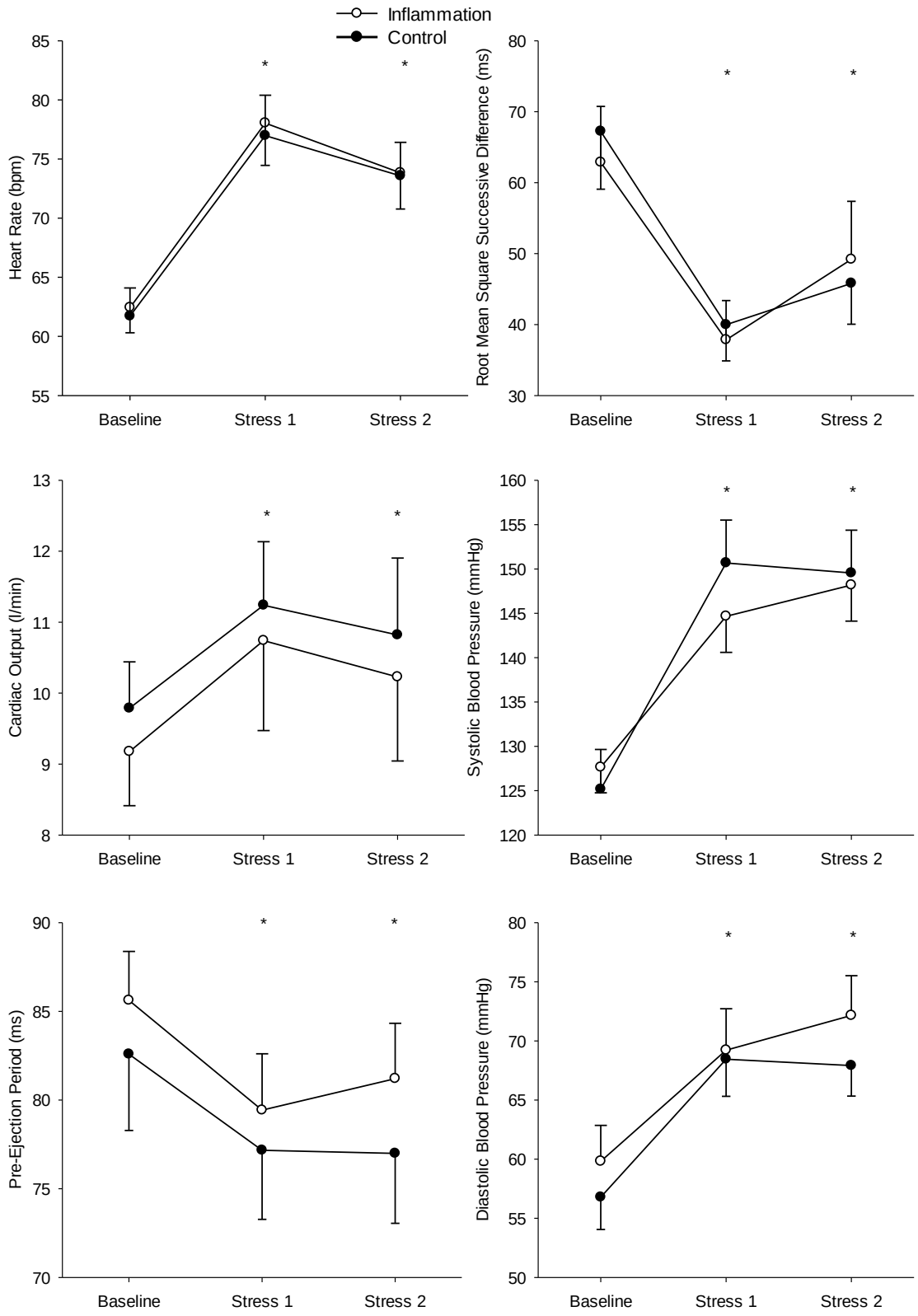

Figure 6.4. Mean ± SE responses to mental stress for HR, CO, PEP, rMSSD, SBP and DBP. * represents significantly different from baseline ($p < .05$)

Vascular responses to mental stress

Forearm and calf blood flow responses to mental stress are illustrated in Figure 6.5. Two Condition x 3 Time ANOVAs revealed significant main effects for time for forearm vascular responses: FBF (F (2, 16) = 21.40, $p < .001$, $\eta^2 = .66$), FVR (F (2, 16) = 13.53, $p < .001$, $\eta^2 = .53$) and FVC (F (2, 16) = 8.53, $p = .001$, $\eta^2 = .42$). Newman-Keuls post hoc analysis revealed increases in FBF and FVC and decreases in FVR in response to stress, as depicted in Figure 6.5. There were no condition effects or condition by time interaction effects for FBF, FVR, FVC (all *p's* > .05).

For the vascular assessments recorded in the calf, there was a main effect for time for CBF (F (2, 15) = 8.08, $p = .004$, $\eta^2 = .31$), but not for CVR (F (2, 13) = 0.89, p = .31, $\eta^2 = .12$) or CVC (F (2, 13) = 0.28, $p = .77$, $\eta^2 = .04$). No condition effects were demonstrated for CBF, CVR or CVC (*p's* > .05). A condition by time interaction effect was evident for CBF (F (2, 15) = 6.83, $p = .002$, $\eta^2 = .32$) and CVR (F (2, 13) = 4.01, $p = .019$, $\eta^2 = .31$). Newman-Keul post hoc analysis of this interaction effect for CBF, revealed a greater stress-induced increase in CBF in the control condition compared to the inflammation condition. Post hoc analysis of the condition by time interaction effect for CVR revealed greater CVR responses during inflammation. Importantly, further analysis revealed that the attenuation in calf blood flow was not affected by the order in which the conditions were undertaken (all *p's* > .05).

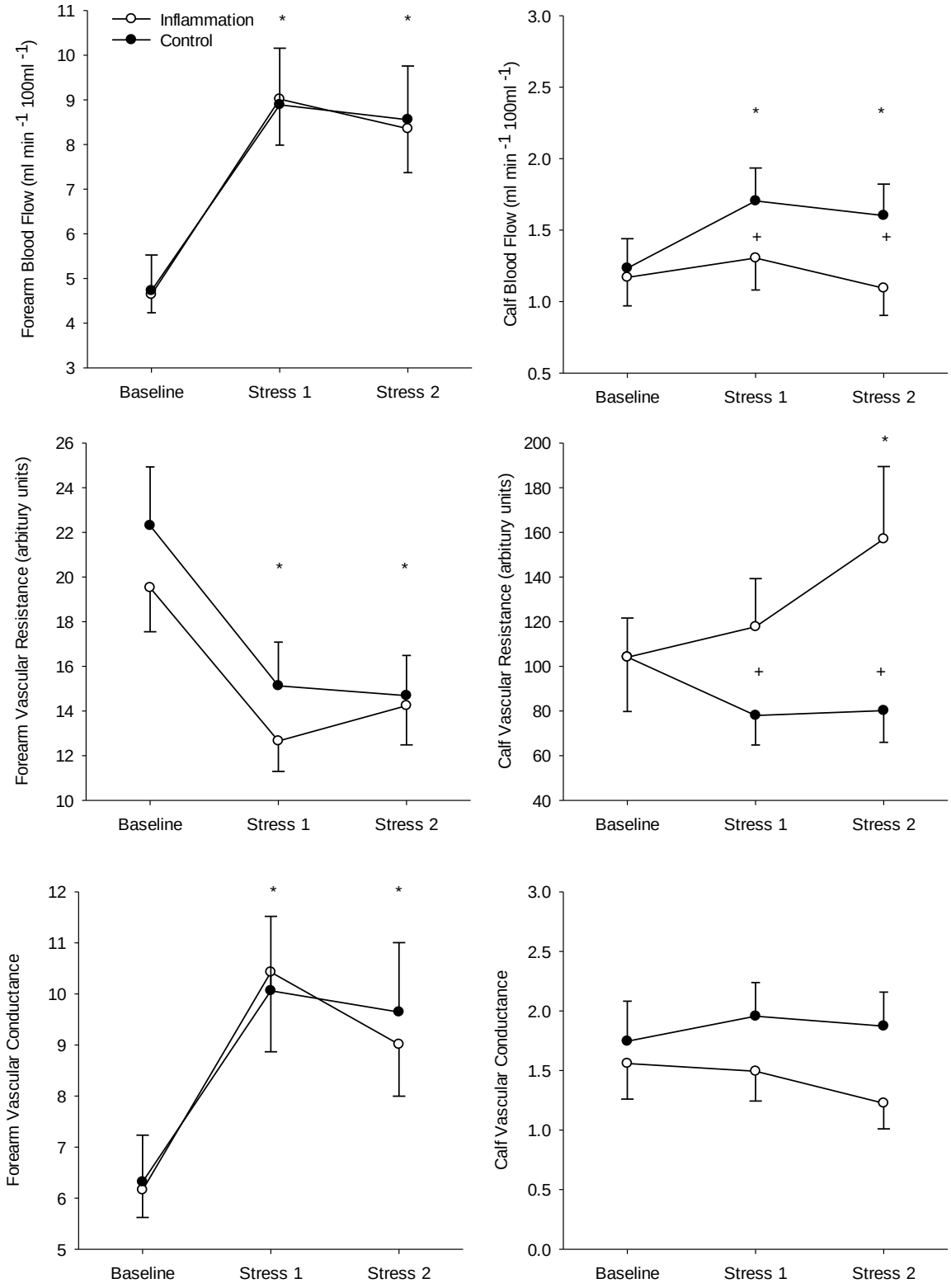

Figure 6.5. Mean ± SE responses to mental stress for FBF, FVR, FVC, CBF, CVR, and CVC. *indicates different from baseline ($p < .05$); + represents significantly different between conditions ($p < .05$)

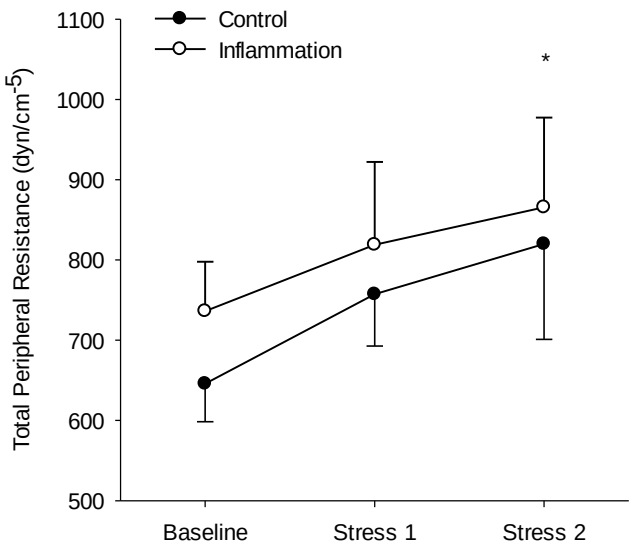

Figure 6.6. Mean ± SE responses to mental stress for TPR. * represents significantly different from baseline ($p < .05$)

Finally, condition by time ANOVA yielded main time effects for TPR (Figure 6.6), (F (2, 14) = 3.96, p = .034, η^2 = .24), but no condition (F (1, 15) = 0.40, p = .54, η^2 = .03), or time by condition interaction effect (F (2, 14) = 0.08, p = .86, η^2 = .01). Post hoc analysis of the time effect revealed differences between TPR at baseline and after 16 minutes of the PASAT (stress 2) (p = .014).

Associations between inflammation and physiological responses to mental stress

Pearson correlation analyses were undertaken to examine the relationships between the markers of inflammation and the physiological responses to mental stress in both the control and inflammation conditions. These analyses revealed no associations between IL-6 and any of the vascular assessments.

6.5 DISCUSSION

The aim of this study was to determine whether inflammation moderated the vascular responses to mental stress, and thus might be a mechanism through which stress could trigger a myocardial infarction. Our eccentric exercise task successfully induced an acute systemic inflammatory response: the eccentric exercise task caused an increase in IL-6 level reaching 2.49 ± 3.40 pg/ml, which is similar to the levels of inflammation induced by a vaccination in healthy participants (Paine *et al.*, 2012a). Importantly, these levels of IL-6 are also similar to those of patients with coronary artery disease (Kop *et al.*, 2008) and myocardial infarction (Ridker *et al.*, 2000). Likewise, increases in limb circumference and self-reported pain immediately post-exercise were demonstrated, providing corroborative evidence for inflammation. However, the data showed no evidence of a systemic effect of inflammation, as no effect of inflammation was observed on the FBF responses to stress or on the cardiac responses to stress. The results did show evidence that was consistent with an effect of local inflammatory processes, as in the inflammation condition, the vasodilatory response to mental stress was attenuated in the calf, i.e. the limb where the exercise was undertaken.

The effect of inflammation appears to be specific to the vascular measures, as no changes in any of the cardiovascular measures (HR, SBP, DBP or CO) were observed. This provides further evidence that inflammation may not alter these variables at rest or in response to stress, as has been observed in other studies (Paine *et al.*, 2012a). Given that the current study design was counter balanced and no differences in the perception of the stress task were observed, the lack of any cardiovascular changes cannot be attributed to habituation to the task. Further, habituation to this stress task has also not demonstrated elsewhere either

(Paine *et al.*, 2012a; Veldhuijzen van Zanten *et al.*, 2005; Veldhuijzen van Zanten *et al.*, 2009; Willemsen *et al.*, 1998).

The attenuated calf blood flow responses to mental stress in the inflammation condition may be attributable to several mechanisms. For example, sympathetic activation has been demonstrated to be involved in the vasodilatory response to mental stress (Lindqvist *et al.*, 1997; Lindqvist *et al.*, 1999). However, no changes in cardiac sympathetic activation (reflected by a reduction in Pre-Ejection Period) or parasympathetic withdrawal (reflected by reduced Heart Rate Variability) were evident between the inflammation and control conditions, and therefore it is unlikely that the attenuated dilation demonstrated here could be attributed to changes in sympathetic activation or parasympathetic withdrawal. In addition, the stress-induced changes in cardiac sympathetic and parasympathetic activity in both the control and inflammation condition were not associated with changes in stress-induced blood flow. However, local sympathetic activation was not measured in this study, and as such the role of local sympathetic activation in stress-induced vasodilation at the site of the vasculature remains to be determined.

Another putative mechanism is NO, as NO availability has been most consistently reported as a mechanism for stress-induced vasodilation (Joyner & Dietz, 2003; Joyner & Casey, 2009) with increases in blood flow in response to mental stress upregulating eNOS in the vascular endothelium via increases in shear stress and consequently NO availability (Nishida *et al.*, 1992). Moreover, inflammation has been shown to reduce endothelial function at rest via reduced NO availability (Clapp *et al.*, 2004). Unfortunately, to test this possibility direct measurements of NO would be needed, which are notoriously difficult and unreliable

(Wadley *et al.*, 2012), and therefore the direct influence of NO must currently remain speculative. However, NO levels may be lowered through enhanced NO removal through increases in oxidative stress (Gao *et al.*, 2007), reductions in eNOS activation (Goodwin *et al.*, 2007), or eNOS uncoupling (Rabelink & van Zonneveld, 2006). Elevations in oxidative stress, as a consequence of systemic inflammation (Forstermann, 2008), would lead to increased synthesis of reactive oxidative species (ROS) in the vascular wall and reduced NO (Forstermann, 2008). Eccentric exercise has previously resulted in increases in markers of oxidative stress such as free radicals, glutathione, lipids and protein oxidation (Nikolaidis *et al.*, 2008; Theodorou *et al.*, 2011), as well as increases in intra-muscular TNF-α (Peake *et al.*, 2005), which contributes to increases in oxidative stress (Zhang *et al.*, 2009). Increases in superoxide production and reactive oxidative species (ROS) can lead to reduction in BH_4 (Cosentino & Luscher, 1999; Sindler *et al.*, 2009; Vásquez-Vivar *et al.*, 1998), which enhances eNOS uncoupling and reduces NO production. To our knowledge, little is known about the effects of acute eccentric exercise with BH_4. However, increases in vaccine-induced increases in inflammation can reduce BH_4 availability, and result in endothelial dysfunction (Antoniades *et al.*, 2011). Therefore, a reduction in BH_4 availability is important, given the roles of BH_4 in maintaining endothelial function and protecting the vasculature from inflammation induced endothelial dysfunction (Antoniades *et al.*, 2011). BH_4 is critical to NO production through other pathways such as eNOS activation, where L-arginine is converted to produced NO (van Zonneveld *et al.*, 2010; Zhang, 2008), can only occur in the presence of BH_4 (Delp *et al.*, 2008; Sindler *et al.*, 2009). Therefore, a reduction in L-arginine would result in decreases in eNOS activation (Darley-Usmar *et al.*, 1995; Förstermann & Münzel, 2006; Morris, 2000). Increases in inflammatory markers in vitro have been demonstrated to reduce L-arginine in aortic endothelial cells, and thus may be a mechanism for reduced NO

(Goodwin *et al.*, 2007; Zhang *et al.*, 2009). However, despite increases in inflammation reducing NO levels through these outline pathways, the methodological issues with measuring NO in blood (Wadley *et al.*, 2012) mean that these must remain speculative, and require further investigation.

In contrast to others who have examined the effects of inflammation on the vascular responses to stress (Paine *et al.*, 2012a), the current study protocol only induced an attenuated vascular response in the calf, and not in the arm. This could be due to the different methods used to induce inflammation; vaccination versus eccentric exercise. The inflammatory response to vaccination occurs in response to the bacterial infection which is injected into the muscle, with the innate immune system instigating the response (Medzhitov, 2008). In contrast, eccentric exercise results in a more substantial localised inflammatory response (Bruunsgaard *et al.*, 1997; Willoughby *et al.*, 2003), than the response that occurs to vaccination (Medzhitov, 2008), and induces a localised inflammatory response as a result of myofibril damage, and disruption of sarcomere excitation-coupling (Nosaka *et al.*, 2002; Proske & Morgan, 2001). Increases in leukocytes in response to leg eccentric exercise have been demonstrated as soon as one hour post exercise (Paulsen *et al.*, 2005), demonstrating a role in the inflammatory response, but as more is known about the inflammatory response to infection than to tissue injury (Medzhitov, 2008), the direct mechanisms through which muscle damage can induce an inflammatory response currently remain speculative.

The current study demonstrated an increase in the markers that indicate a localised inflammatory response (i.e., limb circumference and pain). Thus, the differences in the inflammatory responses to vaccination and exercise may be the reasons for the attenuation in

blood flow, which was only evident at the site proximal to the inflammatory source within this study. However, it should be noted that IL-6 increased, indicating that a systemic response to eccentric exercise was also observed in addition to these local indicators of inflammation. This finding is in line with previous research (Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004).

Indeed, to the best of our knowledge, only one study has examined the effects of acute eccentric exercise on the vasculature. This study revealed that both arm and leg eccentric exercise induced significant increases in pulse wave velocity when measured between the carotid and femoral artery which is indicative of increases in arterial stiffness (Barnes *et al.*, 2010). However, these increases were relatively small (3-5% changes) which may suggest that greater increases could have been seen if vascular assessment was undertaken at a site more proximal to the source of inflammation.

The differences in the calf and forearm blood flow responses to stress may not be the result of the inflammation and other changes evoked by eccentric exercise, as others have noted differences in the FBF and CBF responses to stress (Carter *et al.*, 2005; Paine *et al.*, 2012a). This has been hypothesised to be due to differences between vascular beds, with further evidence of differences in the responses to stress of different organs (Hayashi *et al.*, 2006). Likewise, limb vascular responses are not associated with systemic vascular responses to stress (Paine *et al.*, in submission), and thus the observed differences within the different areas of the vasculature may be an alternative reason for the differences in calf and forearm blood flow observed in this study.

No associations were observed between IL-6 and the vascular responses to stress. IL-6 was chosen as a marker of inflammation due its role in altering NO availability *in vitro* (Hung *et al.*, 2010), as well as its prominent appearance after eccentric exercise (Croisier *et al.*, 1999; Depner *et al.*, 2008; Jackman *et al.*, 2010; Miles *et al.*, 2008; Steensberg *et al.*, 2000; Steensberg *et al.*, 2002; Willoughby *et al.*, 2003). Given that the peak IL-6 response is most commonly reported to be at 6 hours post eccentric exercise (MacIntyre *et al.*, 2001; Paulsen *et al.*, 2005; Philippou *et al.*, 2009; Willoughby *et al.*, 2003), IL-6 and vascular measures taken in this study were examined when IL-6 levels were maximum. The lack of associations between IL-6 and vascular responses to stress have also been reported before (Paine *et al.*, 2012a). Therefore, IL-6 increases may be representative of an inflamed state rather than being a direct cause for the attenuated stress-induced vascular responses.

Another reason for this lack of association may be the choice of inflammatory marker. Previous observational studies have seen associations between inflammation (as indexed by CRP) and vascular function at rest (e.g., Cleland *et al.*, 2000; Verma *et al.*, 2002; Verma *et al.*, 2004). Indeed, the greatest increases in TPR in response to stress were demonstrated in populations with the greatest levels of CRP (Veldhuijzen van Zanten *et al.*, 2008), and basal CRP levels have been linked to the presence of mental stress induced ischemia (Shah *et al.*, 2006). However, elevations in CRP have only been demonstrated at least 24 h post eccentric exercise (Barnes *et al.*, 2010; Paulsen *et al.*, 2005), and therefore we thought that CRP would not have been elevated 6 hours post exercise, which was the time point at which the stress task was conducted. Indeed, IL-6 increases occur more quickly in comparison to CRP and indeed can lead to an stimulation of CRP (Zakynthinos & Pappa, 2009). Whilst CRP is an acute phase protein is has also been used in studies as a marker to indicate more chronic or

long term levels of inflammation and may be more indicative of an inflamed state (Libby, 2006). However, recently studies have questioned the role of CRP as a predictor in cardiovascular research (Emerging Risk Factors Collaboration *et al.*, 2010; Heart Disease Genetics Collaboration, 2011), and thus suitability of examining CRP as a predictor of the responses to stress is still to be determined.

In conclusion, eccentric exercise-induced inflammation induced an attenuation of the vasodilatory response to mental stress, which is similar to a previous finding using a vaccination to cause inflammation (Paine *et al.*, 2012a). The effect on the vasculature was only seen in the limb that performed the exercise, indicating a localised effect of inflammation. These findings indicate that inflammation and its influence on the vascular responses to stress might be attributable to incidence of myocardial infarction when triggered by acute mental stress. Future work should continue to explore the mechanisms through which elevations in inflammation may attenuate the vascular responses to stress. Future work examining the effects of reducing inflammation on the vascular responses to stress is an interesting direction for further research particularly given the reversible effects of acute inflammation on resting vascular function (Bhagat & Vallance, 1997).

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6.7 LIST OF REFERENCES

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CHAPTER SEVEN:

GENERAL DISCUSSION

7.1 OVERALL AIMS OF THIS THESIS

Despite the evidence for acute stressful events acting as a trigger for myocardial infarction (MI), the underlying mechanisms through which this triggering effect occurs remain unclear. Increased inflammation and poor vascular responses to mental stress have been implicated as potential mechanisms, however, little is known about the association or interaction between inflammation and the vascular responses to acute mental stress. Therefore, the overall aim of this thesis was to examine the effects of inflammation on the vascular responses to acute mental stress. Given that factors such as age, gender and other classic cardiovascular risk factors have influenced the relationships between inflammation and other vascular assessments at rest (Cleland *et al.*, 2000; Vita *et al.*, 2004), the studies in this thesis employed an experimental design to induce acute, transient inflammation in a healthy population. This set up was chosen to specifically explore the sole influence of inflammation on vascular response to mental stress.

Following a critical analysis of the methods commonly used to assess the vascular responses to acute mental stress (Chapter 2), the time course of the inflammatory response to a vaccine was examined (Chapter 3). The subsequent chapters assessed the effects of inflammation on the responses to stress. Two different protocols were used to induce inflammation; administration of a vaccine (Chapter 4) and an eccentric exercise task (Chapters 5 and 6).

7.2 THE MODELS USED TO INDUCE INFLAMMATION

Both models used to induce acute systemic inflammation proved successful in increasing inflammatory markers (Chapters 3, 4 and 6). Elevations in IL-6 were demonstrated at 6 hours following the *Salmonella typhi* vaccination (Chapters 3 and 4). The vaccination model (Chapters 3 and 4) also demonstrated increases in TNF-α and white blood cell subsets, as well as elevations in CRP at 24 hours post vaccination (Chapter 3). In addition to the increases in systemic inflammatory markers, the results from the study that determined the time course response to the *Salmonella typhi* vaccination demonstrated that a localised response, as indexed by elevated muscular pain, at the site of vaccination was also evident.

Similarly to the IL-6 response to vaccination (Chapters 3 and 4), IL-6 increases were also evident 6 hours after the leg eccentric exercise task used in Chapter 6. Changes in TNF-α were not assessed in Chapter 6 (leg eccentric exercise task), as systemic increases in TNF-α are not expected following eccentric exercise (Peake *et al.*, 2005a; Ross *et al.*, 2010; Toft *et al.*, 2002). However, CRP increases have reported in the literature 24 hours after eccentric exercise (Barnes *et al.*, 2010; Paulsen *et al.*, 2005), which is similar to the increases in CRP observed in response to vaccination (Chapter 3). Additionally, increases in measures of localised inflammation (such as limb circumference as a measure of oedema and self reported pain) were observed as a result of the eccentric exercise tasks. These localised and indirect measures of inflammation have been reported elsewhere as a result of eccentric exercise (Campbell *et al.*, 2010; Edwards *et al.*, 2007; Edwards *et al.*, 2010; Hirose *et al.*, 2004). Importantly, the levels of inflammation (indexed by IL-6) induced by these two different protocols are comparable to the inflammation experienced by patients with CVD and MI (Kop *et al.*, 2008; Ridker *et al.*, 2000).

7.3 THE EFFECTS OF INFLAMMATION ON THE VASCULAR RESPONSES TO STRESS

The studies reported in this thesis revealed that inflammation affected the vascular responses to mental stress. Inflammation induced by vaccination attenuated both forearm and calf blood flow responses to stress (Chapter 4), whereas inflammation induced by leg eccentric exercise reduced the vasodilatory response to stress in the calf (Chapter 6). However, arm eccentric exercise did not appear to alter blood flow (Chapter 5). In sum, when a substantial amount of inflammation was induced, the vascular responses to mental stress were attenuated.

The findings from Chapter 4, 5 and 6 indicate that, despite similar increases of systemic markers of inflammation, the two inflammatory protocols induced subtle differences in the vascular responses to mental stress. The processes underlying the systemic inflammatory response to exercise and vaccination might be the reason for these differences. The inflammatory response to vaccination occurs in response to the bacterium which is injected into the body, with the innate immune system instigating the response (Medzhitov, 2008). Eccentric exercise, however, induces an inflammatory response to myofibril damage, and disruption of sarcomere excitation-coupling (Nosaka *et al.*, 2002; Proske & Morgan, 2001). Thus, the inflammatory response to eccentric exercise is more localised and endogenous, in comparison to the more systemic and exogenous inflammatory stimuli of vaccination. Therefore, high intensity and long duration eccentric exercise results in a more substantial and localised inflammatory response (Bruunsgaard *et al.*, 1997; Febbraio & Pedersen, 2002; Willoughby *et al.*, 2003) compared to the response that occurs to vaccination (Medzhitov, 2008). Currently, more is known about the processes through which the inflammatory response to infection occurs in comparison to that of tissue injury (Medzhitov,

2008); thus, the direct mechanisms through which muscle damage can induce an inflammatory response currently remain speculative. In sum, the differences in the methods used to induce inflammation may contribute to the differences observed in the findings within this thesis where eccentric exercise-induced inflammation resulted in a localised attenuation (e.g., no change in forearm blood flow), whereas vaccination-induced inflammation induced attenuations at a site which was not proximal to the site of inflammation (e.g., in the calf).

As far as we are aware, the studies that used eccentric exercise to induce inflammation (Chapters 5 and 6) were the first to examine the effect of eccentric exercise induced inflammation on vascular responses to stress, as previous studies have only examined resting vascular function (pulse wave velocity) in relation to eccentric exercise (Barnes *et al.*, 2010). Importantly, Barnes and colleagues only reported small changes (~3-5%) in carotid-femoral pulse wave velocity as a result of exercise which was conducted in the leg or arm, suggesting that the magnitude of the changes may have been greater if the vascular assessment had been conducted closer to the muscles used to perform the exercise task. This is of particular interest, given that only calf blood flow was attenuated as a result of eccentric exercise in the leg (Chapter 6), and no attenuation in blood flow was observed in the dominant forearm as a result of eccentric exercise undertaken in the non-dominant arm (Chapter 5) or the leg (Chapter 6). These studies highlight how eccentric exercise induced inflammation may have a localised effect on the vasculature.

It should be acknowledged that inflammation was not directly associated with the vascular responses to mental stress. This seems to be in contrast to the only previous study that used a vaccination protocol to assess the effect of inflammation on the blood pressure

responses to mental stress, which found a relationship between IL-6 and blood pressure responses 2 hours post vaccination administration (Brydon *et al.*, 2009). The time course study in this thesis revealed sustained elevations in IL-6 from 4 hours post vaccination until the peak response at 6 hours (Chapter 3), it is therefore unlikely that the inflammatory levels were stable or consistent 2 hours post vaccination. Thus, examining the vascular responses to stress 6 hours post vaccination administration allows for examination in a relatively steady inflammatory state. Also, the study by Brydon *et al.* (2009) did not assess vascular function at rest or in response to stress, but focussed on sickness behaviour, and the blood pressure and heart rate responses to stress. In line with the findings in this thesis, Veldhuijzen van Zanten *et al.* (2008), who reported increased TPR responses to mental stress in a cross-sectional study in RA patients with high-grade systemic inflammation, also demonstrated no direct association between inflammation and vascular responses to mental stress. Thus, it appears that there may not be a specific, single inflammatory marker which is a mediator of an attenuated vascular response to stress.

In contrast to Veldhuijzen van Zanten *et al.* (2008), this thesis demonstrated no effect on TPR responses when experimental models of acute inflammation were used in healthy participants. RA patients can be characterised by continuously high levels of inflammation, which will influence not only the vasculature but also impact on the CVD risk factors including lipid concentrations, endothelial dysfunction or even CVD itself (Kitas & Erb, 2003; Sandoo *et al.*, 2012a; Sandoo *et al.*, 2012b). Thus, even though the current experimental protocols used in this thesis allowed for the assessment of the unique effect of inflammation on the vasculature, these models do not reflect the continuous inflammatory overdrive that is experienced by RA patients. The combination of both elevated and chronic levels of

inflammation might have had a specific effect on TPR which was not evident in the current studies. It is worth noting that even though the levels of inflammation induced by the current protocols were lower than those seen in patients with RA, the levels of inflammation were comparable to those experienced by CVD and MI patients (Kop *et al.*, 2008; Ridker *et al.*, 2000)

7.4 INFLAMMATION AND VASCULAR RESPONSES TO MENTAL STRESS – FUTURE WORK

The research in this thesis showed that inflammation affected the vascular responses to mental stress. Future work could examine the mechanisms through which inflammation influences the vascular responses to mental stress. Inflammation affects endothelial function at rest by causing a reduction in NO availability (Clapp *et al.*, 2004; Cleland *et al.*, 2000; Hingorani *et al.*, 2000). Given the role of NO in stress-induced vasodilation (as illustrated by Figure 7.1), it is hypothesised that the reduced NO availability due to inflammation is a mediator of the attenuated vascular responses to stress. Due to the difficulties in directly measuring NO (Wadley *et al.*, 2012), future studies could examine the role of NO by examining the mechanisms through which NO can be produced. This can be investigated through directly examining the substrates and co-factors which synthesis NO, or by inhibiting their function and examining the impact on NO production.

NO can be synthesised through different nitric oxide synthase isoforms (eNOS, nNOS and iNOS), using different substrates including L-arginine and BH_4. Endothelial nitric oxide synthase (eNOS) produces NO by converting L-arginine to L-citrulline (van Zonneveld *et al.*, 2010; Zhang, 2008). L-arginine conversion can only occur in the presence of eNOS co-factor

tetrahydrobiopterin (BH_4) (Delp *et al.*, 2008; Sindler *et al.*, 2009) such that BH_4 is critical to NO production (Cosentino & Katusic, 1995; Pierce & LaRocca, 2008). nNOS is produced in the central nervous system (CNS) (Schuman & Madison, 1994; Toda & Okamura, 2003), as well as in the nucleus of the endothelial cell (Chakrabarti *et al.*, 2012) and vascular smooth muscle cells (Han *et al.*, 2007; Kavdia & Popel, 2004; Yogi *et al.*, 2010).

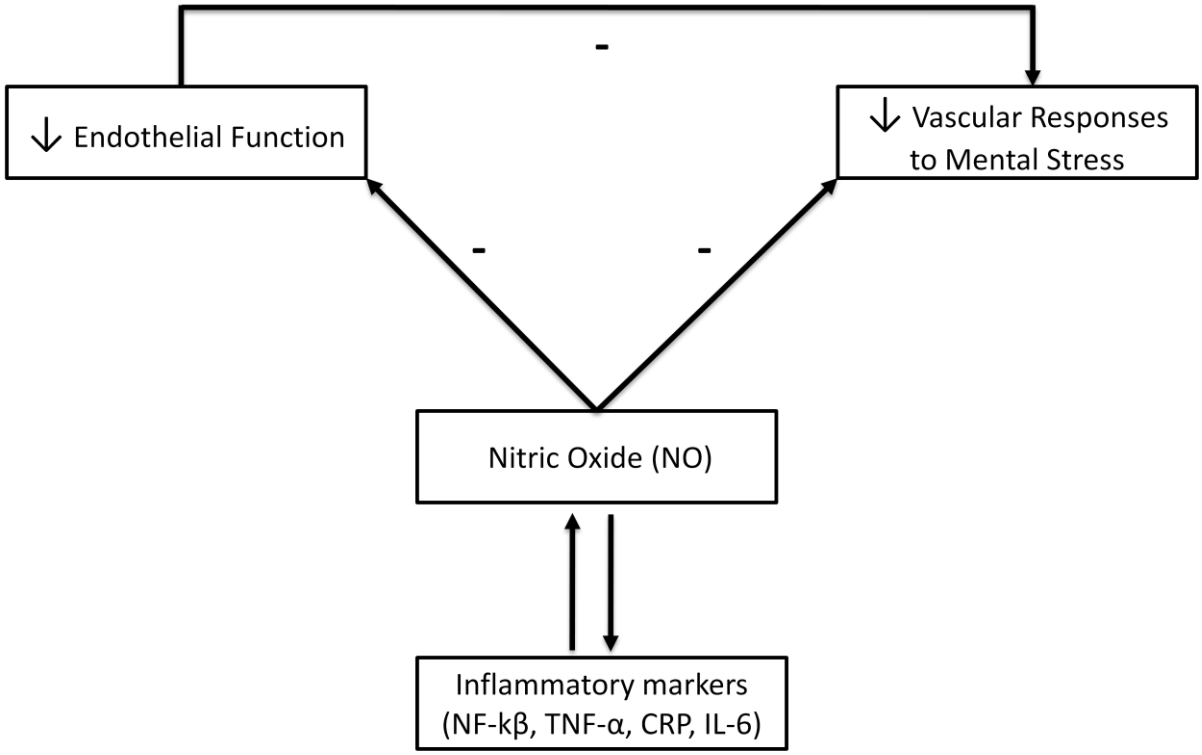

Figure 7.1. Schematic diagram showing the relationships between inflammatory cytokines, nitric oxide, endothelial function and the vascular responses to stress, in the presence of high levels of inflammation.

iNOS in particular is responsible for production of NO during periods of inflammation, usually in combination with oxidative stress (Förstermann & Li, 2011). Increases in iNOS (as is typically seen with aging) can lead to NO production, however, this can be scavenged and lead to increases in oxidative stress, reduced NOS co-factor and substrate availability (Wadley *et al.*, 2012).

Studies in eNOS knockout mice provide evidence that eNOS may play a key role in NO production (Gödecke *et al.*, 1998; Huang *et al.*, 1995; Scotland *et al.*, 2002). Given that in a diseased or chronically inflamed state eNOS expression is often greater than in a healthy population (Förstermann & Li, 2011), this may suggest that the role and availability of co-factors and substrates needed for eNOS activation and NO production (e.g., L-arginine and BH_4) play an important role, which warrants further investigation. Given that inflammation can reduce eNOS activation (Hung *et al.*, 2010), the investigation of co-factor and substrate variability appears to be a sensible area for investigation. Dysregulation of these co-factors and substrates can lead to greater levels of oxidative stress production rather than NO (Bevers *et al.*, 2006; Crabtree *et al.*, 2009; Förstermann & Li, 2011; Schmidt & Alp, 2007).

In line with the findings reported in the context of blood flow at rest (Seddon *et al.*, 2008), stress-induced increases in forearm blood flow appears to be regulated by both eNOS and nNOS, as demonstrated by blunted responses to nNOS inhibitor S-methyl-l-thiocitrulline (SMTC) during mental stress, as well as the non-specific NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) (Seddon *et al.*, 2008). Thus, the studies of Seddon and colleagues suggest that both nNOS and eNOS have a role in vasodilation in at rest and in response to stress (Seddon *et al.*, 2008). It is thought that eNOS and nNOS are similar in terms of structure and the substrates and co-factors that they use to produce NO, however they differ in terms of regulation and subcellular localization (Zhang & Casadei, 2012). Therefore, given the effects of inflammation on eNOS activation and co-factors, it may be pertinent to examine the effects of inflammation on nNOS during mental stress.

The final NOS isoforms that could have a role in the vasodilatory response to mental stress in the context of inflammation is iNOS. Increases in iNOS can lead to NO production, however, this can be scavenged and lead to increases in oxidative stress, reduced co-factor and substrate availability (Wadley *et al.*, 2012). Also, elevations in systemic inflammation often result in increased levels of oxidative stress and enhanced synthesis of reactive oxidative species (ROS) in the vascular wall (Forstermann, 2008). This can lead to reduced NO production due to increases in direct scavenging of peroxynitrite (an oxidising agent of NO) by superoxide (Gao *et al.*, 2007). Therefore, examination of oxidative stress in the context of the vascular responses to mental stress may warrant investigation. Increases in markers of oxidative stress have been demonstrated in response to eccentric exercise (Nikolaidis *et al.*, 2008; Theodorou *et al.*, 2011). Likewise, increases in intra-muscular TNF-α (as seen in response to eccentric exercise) (Peake *et al.*, 2005b), have been linked to elevations in oxidative stress (Zhang *et al.*, 2009). Currently, little is known about the role of oxidative stress, inflammation and iNOS on the vascular responses to mental stress. However, as oxidative stress influences NO availability, and given the links between inflammation and oxidative stress (Wadley *et al.*, 2012), further examination of oxidative stress and the vascular responses to stress needs further investigation. In sum, while it is known that inflammation can reduce eNOS activation (Hung *et al.*, 2010), but little is known about the influence of inflammation on nNOS or iNOS. Therefore, future studies should investigate this area of research.

The studies in the current thesis used an experimental design to assess the effects of inflammation in a healthy population. Future research could also explore the effects of reducing inflammation on the vascular responses to mental stress in populations with high

levels of inflammation. There is evidence that inflammation reducing medication lowers the risk of MI in RA patients (Dixon *et al.*, 2007). In addition, an exercise intervention, which has anti-inflammatory effects, has been shown to reduce the incidence of mental stress-induced ischaemia in patients with CVD (Blumenthal *et al.*, 2005). Therefore, it is likely that a reduction in inflammation also has a positive effect on the vascular responses to mental stress. The studies which have examined the effects of reducing inflammation and improving resting vascular function has yielded variable results, with some reports of improvements in endothelial function after anti-inflammatory treatment (Cardillo *et al.*, 2006; Hürlimann *et al.*, 2002; Raza *et al.*, 2005). Given the relationship between endothelial function and the vascular responses to mental stress (Sherwood *et al.*, 1999), improving endothelial function to enhance the vascular responses to stress would seem to be important. Therefore, it is likely that the reduction in inflammation also has a positive effect on the vascular responses to mental stress. Advancing the knowledge about the mechanisms that underlie the triggering of MI by mental stress may also have implications for medical treatments, as medication can then be developed to specifically alter these mechanisms. Likewise, acute transient increases in inflammation, such as during an acute upper-respiratory tract infection, are linked with a greater risk for myocardial infarction (Meier *et al.*, 1998). Therefore, investigating the influence and the mechanisms of acute inflammation on the vascular responses to mental stress in an popualtion who may already be at risk for MI, such as the elderly, could be investigated.

7.5 IMPLICATIONS & CONCLUSION

Given the evidence for mental stress as a trigger for myocardial infarction, this thesis aimed to examine the effects of inflammation on the vascular responses to acute mental stress,

by using experimental models to induce inflammation in a healthy population. The series of studies revealed that inflammation induced a reduction in stress-induced vasodilation. The influence was specific to the vascular responses to mental stress, as inflammation did not influence mood or cardiac measures. Importantly, the different models used to induce inflammation suggested that the greatest effect of inflammation on the vascular responses to stress were at the site of the inflammation. Thus, inflammation through its impact on the vasculature may be a potential pathway by which mental stress triggers MI. Further work is needed to examine the precise mechanisms through which this occurs, with a potential view to examining the effects of reducing inflammation in clinical populations at risk for MI populations.

The studies within this thesis were the first to successfully demonstrate that experimentally inducing acute inflammation in a healthy population results in an attenuated vascular responses to mental stress. The use of a healthy population allowed for the examination of the sole influence of increased levels of inflammation on the vascular responses to stress. The findings of reduced stress-induced vasodilation in a state of elevated inflammation may have important clinical ramifications. Given the studies that demonstrated elevated basal inflammation or poorer vascular responses to mental stress were evident in populations who also displayed mental stress induced ischemia (Burg *et al.*, 2009; Goldberg *et al.*, 1996; Jain *et al.*, 1998; Shah *et al.*, 2006), this thesis provides evidence that the combination of these factors and the interactions between inflammation and the vasculature in response to mental stress could be an important pathway through which mental stress might trigger an MI, particularly in people who have elevated basal inflammatory levels.

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