Cardiovascular and Respiratory Sciences, School of Clinical and Experimental Medicine

Adrenaline increases ventilation via a β-receptor and carotid body-mediated mechanism: a role in the hyperventilation of hypoglycaemia?

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Abstract

A role for the carotid body (CB) in glucoregulation has been proposed but the evidence is conflicting. Hypoglycaemia *in vivo* induces a CB-dependent hyperventilation, but it is not agreed whether this reflects a direct action of reduced blood glucose on the CB, or an indirect effect of adrenaline. We therefore investigated the effects of adrenaline and hypoglycaemia upon ventilation.

Ventilation (V_E) was recorded during infusions of adrenaline or insulin (to induce hypoglycaemia) in anaesthetized male Wistar rats. CB-mediated effects were determined by application of hyperoxia at each dose. This was repeated during propranolol infusion. Hypercapnia was applied at control and at the end of adrenaline or insulin infusion.

Adrenaline and hypoglycaemia evoked increases in V_E, without an associated change in P_aCO_2. Hyperoxia reduced baseline V_E and offset the ventilatory responses. Propranolol reduced baseline V_E and abolished the hypoglycaemia-mediated ventilatory increase, but an increased P_aCO_2 occurred. Both hypoglycaemia and adrenaline increased the hypercapnic ventilatory response, which was blocked by propranolol.

These data suggest that adrenaline may underlie the increased V_E seen in hypoglycaemia via a β-mediated, O_2 independent pathway within the CB. It also suggests that the increased V_E during hypoglycaemia is a hyperpnoea that is appropriate to the increased metabolism.

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1. Introduction

1.1 Ventilation and its control

Ventilation is the volume of air entering the lungs per minute (V_E, $ml.min^{-1}$), and is equal to the product of respiratory frequency (R_f – number of breaths per minute) and tidal volume (V_t – mls of air entering lungs with each inspiration). V_E is controlled by three parts of the respiratory system; the controller residing in the CNS, the effector respiratory muscles and the afferent sensory input from chemoreceptors and other receptors. The central controller system is located in 2 regions: the pons and medulla of the brainstem, and the cortex. The brainstem is responsible for automatic breathing via three groups of respiratory neurones, which generate the respiratory rhythm. The cortex allows voluntary control over breathing and can override the central pattern generator of the brainstem up to a point. The respiratory muscles are controlled by these central regions to co-ordinate in the action of breathing. The respiratory sensors detect changes in the chemical environment of the blood/ECF. They provide information to the central controller to make necessary changes in ventilation and receive feedback from the effector muscles. The sensors can be divided into three groups: central chemoreceptors, peripheral chemoreceptors and other (largely mechanical) receptors (West, 2012). The central chemoreceptors are located in the medulla near to the respiratory neurones, and respond to changes in the $[H^+]$ of the ECF surrounding them. A decrease in pH leads to increased V_E. The ECF composition is mainly determined by the CSF, which is separated from the blood by the blood brain barrier. Therefore central chemoreceptors cannot respond directly to blood $[H^+]$. However CO_2 can diffuse from the cerebral vessels across the barrier, leading to an increase in $[H^+]$ (according to the Henderson-Hasselbach equation). This stimulates the chemoreceptors to induce hyperventilation, decreasing CO_2 and therefore $[H^+]$, and correcting pH. In this way, P_aCO_2 (partial pressure of CO_2 in the arterial blood) is the major controller of V_E via its effect on the pH of the CSF, and the central chemoreceptors are the most important respiratory sensors in the breath-by-breath control of V_E (West, 2012).

Peripheral chemoreceptors: The carotid body

The peripheral chemoreceptors are present in two locations; the carotid bodies (CBs) and the aortic bodies. The aortic bodies are present above and below the aortic arch, but their contribution is not as significant as that of the carotid bodies in humans. The CBs are located bilaterally at the bifurcation of the common carotid artery to the internal and external carotid arteries (Kumar and Prabhakar, 2012) (Figure 1).

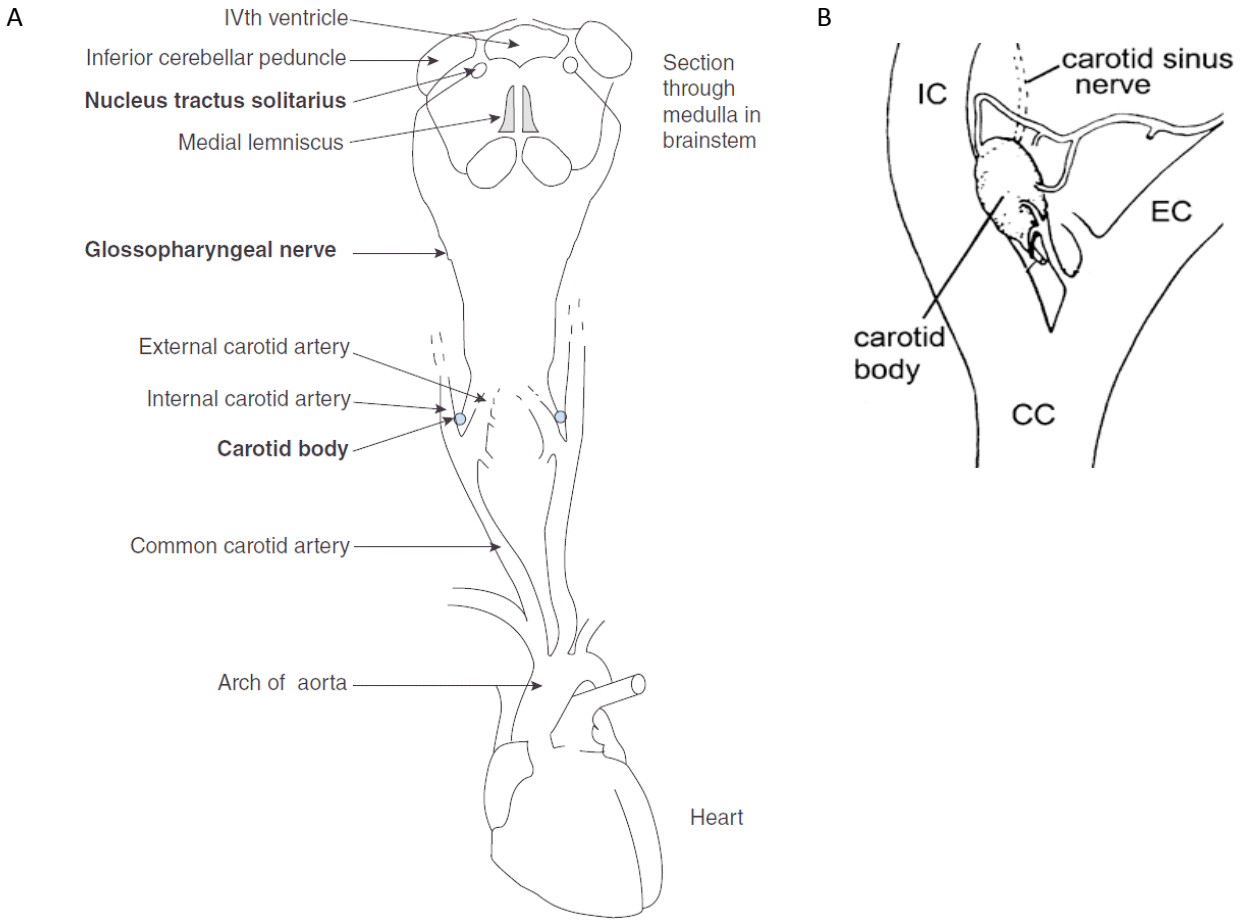

Figure 1: A - A diagram showing the anatomical location of the carotid body (CB). They are positioned bilaterally at the bifurcation of the common carotid (CC) into the internal (IC) and external carotid (EC) arteries. They are afferently innervated by the glossopharyngeal (IXth cranial) nerve, along which chemodischarge is transmitted to the medulla (NTS), which contains cardiorespiratory controller neurone groups. This allows integration of the chemosensory information from the CB, into the characteristic cardiorespiratory reflex response via autonomic and respiratory motorneurones. Image taken from (Kumar, 2007). B - A diagram showing a closer view of the location of the CB. Here it can be seen that the afferent innervation is actually provided by the carotid sinus nerve, a small branch of the glossopharyngeal. Image accessed at http://journals.cambridge.org.

They receive afferent innervation from the carotid sinus nerve (CSN), a branch of the glossopharyngeal (IXth cranial) nerve, which projects from the medulla, allowing conversion

of chemosensory discharge information to a graded characteristic autonomic and cardiorespiratory reflex (Kumar, 2007).

They are highly vascularised organs with a rich supply of capillaries, and receive up to 15 times the blood flow of the brain, relative to size. The efferent innervations of the CB (by sympathetic fibres of the superior cervical ganglion) are thought to impact upon the chemoafferent output by modulating blood flow and hence the level of stimulus detected. The high blood flow results in a small arterial-venous PO_2 (partial pressure of O_2) difference in spite of a fairly high metabolic demand, facilitating their response to blood gas status (Kumar, 2007).

The CBs are formed of thousands of type I (glomus) cells arranged in 3-5 cell clusters (glomeruli). A single type II cell, which are fewer in number than type I, associates with each cluster. The whole organ is surrounded by protective connective tissue. It is generally accepted that the type I cells are the chemosensory unit, as they make synaptic connections with afferent CSN fibres, and also possess gap junctions with other type I cells, whilst the type II cells have a supportive role much like glia (Kumar and Prabhakar, 2012).

Stimuli of the CB are decreased P_aO_2 (hypoxaemia), decreased pH (acidosis) and increased P_aCO_2 (hypercapnia). Hypoxaemia is the primary stimulus, although it must be recognised that the type I cells will sense tissue PO_2, rather than P_aO_2 directly. This is therefore dependent upon the inspired level of O_2, haemoglobin concentration (O_2 carrying capacity), the structure of the vasculature and therefore the blood flow reaching the cells, as well as the local metabolic demands and diffusion (West, 2012). As already discussed, the high blood flow of the CB is entirely adequate to meet its O_2 consumption, and reasonable microvascular PO_2s have been reported (Rumsey, 1991) (Whalen, 1973), therefore a low tissue PO_2 cannot explain its sensitivity. This suggests that a cellular mechanism for sensing O_2 must exist, which has been shown to lead to type I cell membrane depolarisation and a subsequent Ca^{2+}-dependent

neurosecretion. The upstream sensing mechanism for this pathway is debated (see review (Kumar and Prabhakar, 2012)). The CB response is completely responsible for the increase in V_E seen in response to hypoxaemia in humans, therefore CB resection (CBR) can lead to a loss of the hypoxic V_E response. The CB will also respond to ischaemic/stagnant hypoxia and histotoxic hypoxia as caused by metabolic poisons (Kumar, 2007).

The response of the CB to P_aCO_2 accounts for only a small proportion of the total V_E response to hypercapnia; the majority is mediated via the central chemoreceptors. However the peripheral response is much faster and therefore may be useful in situations of sudden increases in P_aCO_2, increasing V_E to restore isocapnia. The CB also responds to a decrease in arterial pH, which can be caused by metabolic or respiratory factors. The reflex increased V_E increases excretion of CO_2, helping to correct pH. The amount of CO_2 in the blood has significant implications for the pH of the blood, as described by the Henderson-Hasselbach equation. V_E therefore allows a large amount of control over acid-base status, although some control also comes from excretion of bicarbonate by the kidneys. The ratio between bicarbonate and PCO_2 must be maintained to hold pH at 7.4, and this relationship is represented by the Davenport diagram. The ratio and therefore pH can be disturbed in 4 ways; an acidosis or alkalosis, caused by either respiratory or metabolic factors. Of relevance to hypercapnia is respiratory acidosis, whereby an increased PCO_2 leads to a decreased bicarbonate/PCO_2 ratio and therefore a decreased pH (West, 2012). For this reason it has been questioned whether CO_2 directly acts on the CB, or if the response is mediated by a change in pH, although CO_2 has been shown experimentally to have an independent effect when pH is maintained (Biscoe *et al*, 1970). As the central and peripheral chemoreceptors both respond to CO_2/H^+, but are separated by the blood brain barrier, they may actually be sensing different environments and appear to be out of phase with one another; however this interaction ensures a smooth response to hypercapnia (Schwartzstein and Parker, 2006).

Other respiratory receptors also exist, for example pulmonary stretch receptors. These other receptors tend to be stimulated by mechanical and noxious stimuli (West, 2012).

As a decreased PO_2 is the main stimulus for increased CB activity, it follows that above normoxic levels of PO_2 i.e. hyperoxia, would ablate activity of the CB, although tonic firing has still been reported *in vivo* at P_aO_2s of 400-600mmHg (Kumar, 2007, Biscoe *et al*, 1970). However, despite this, hyperoxia is often used as an experimental tool, allowing a 'chemical denervation' e.g. in humans where one cannot perform a carotid sinus nerve section (CSNX), or in animals where use of CSNX or CBR can be technically difficult and hyperoxia provides the advantage of reversibility.

1.2 Carotid body responses: hypoxia and hypercapnia

The PO_2 in inspired air (at sea level barometric pressure) is 150mmHg, which drops to around 100mmHg at the alveoli (P_AO_2) as determined by the balance between the metabolic demand of the tissues and the level of V_E. In a healthy lung, arterial O_2 (P_aO_2) is only slightly different to P_AO_2, and so this level of O_2 is present in the systemic circulation reaching the tissues. This difference is contributed to by shunted blood e.g. bronchial and coronary circulation, which becomes O_2 depleted before entering the systemic circulation (West, 2012).

As O_2 diffuses to the mitochondria in the tissues, PO_2 is lowered further to 25-30mmHg and below. PCO_2 in the air is negligible, but P_ACO_2 (and hence P_aCO_2) is around 40mmHg. Clearly any change in V_E will affect the alveolar and hence arterial levels of O_2 and CO_2, for example hypoventilation causes PO_2 to fall and PCO_2 to rise (explained by the alveolar V_E equation, as metabolic CO_2 production will continue). The PO_2 of blood can also be affected by the relationship between V_E and perfusion, where a mismatch i.e. perfusion of a poorly ventilated region, can lead to a decreased PO_2 and an increased PCO_2. Hypoventilation, diffusion limitations, shunt and V_E-perfusion mismatch are all physiological causes of

hypoxaemia and can also cause hypercapnia (increased P_aCO_2) although this tends to be quickly corrected by an increase in V_E driven by chemoreceptors (West, 2012).

Hypoxia

As discussed, hypoxia (low O_2 content) or hypoxaemia (low O_2 specifically in the blood) is the primary and most powerful stimulus of the CB, and so has received much research attention. As tissue PO_2 decreases and tissue becomes hypoxic, the CB type I cells respond by increasing Ca^{2+}-dependent neurosecretion leading to a rapid and sustained increase in neural discharge, the frequency of which increases exponentially with the stimulus (reviewed by Kumar and Prabhakar, 2012). An example raw trace showing the increased discharge in the carotid sinus nerve in response to transition from hyperoxia to hypoxia, and back again, as well as superimposed single action potentials, are shown below in Figure 2.

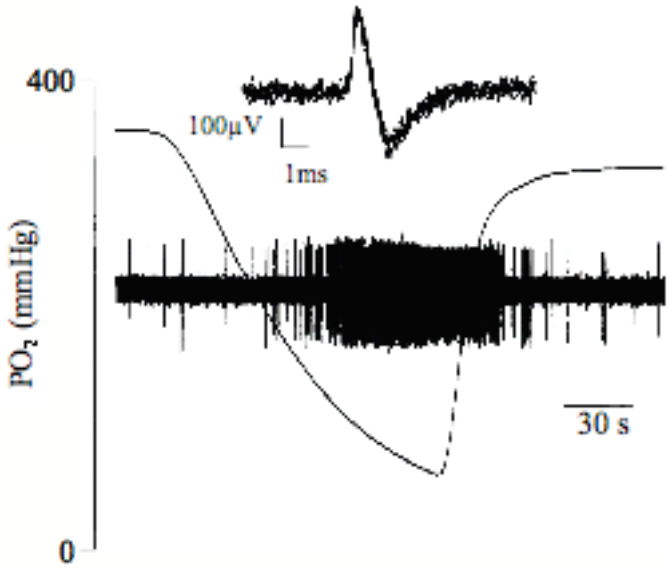

Figure 2: A raw trace showing *in vitro* chemodischarge of the CSN. Discharge increased upon decreasing PO_2 from hyperoxic to hypoxic levels, which was reversed on return to hyperoxia. Pepper *et al* (1995).

A gradually increasing discharge is seen between P_aO_2s of 400-140mmHg. At around 100mmHg the slope of this response increases, reaching a maximum at ~30mmHg. The transmission of this discharge to the medulla results in a graded cardiorespiratory response including hyperventilation (an increased V_E above metabolic demand, primarily via an

increased R_f), which decreases P_ACO_2, increasing P_AO_2 and hence P_aO_2. Bradycardia, decreased cardiac output, peripheral vasoconstriction and an increased adrenomedullary output can also be triggered, although these can often be hidden by hyperventilation induced feedback responses e.g. by pulmonary stretch receptors (see review Marshall, 1999). In very severe hypoxia, discharge can decrease and the reflex response begins to fail (Kumar, 2007).

Hypercapnia

As previously mentioned, the CB can also respond to hypercapnia. The contribution of the CB to this response is generally thought to be small, with most of the response being mediated by central chemoreceptors. However it has been reported that the CBs may account for 30-50% of the hypercapnic ventilatory increase, even with a concurrent hyperoxia (Bruce and Cherniack, 1987, Heeringa *et al*, 1979, Nattie, 1999), although some of this response may be due to the CB reflex response leading to an increased central sensitivity (Blain *et al*, 2010). It has also been shown that dogs with isolated CBs perfused to maintain basal activity, show an increase in the time taken to respond to hypercapnia (Smith *et al*, 2006). Despite this significant contribution by the CB to the response to CO_2, the majority of research has focused on the transduction of the O_2 signal. In contrast to O_2, where basal discharge persists even in hyperoxia, if P_aCO_2 falls below 18-25mmHg this basal discharge is stopped (Bartels *et al*, 1956, Eyzaguirre and Lewin, 1961). Reduction in P_aCO_2 can also abolish discharge induced by hypoxia, although this may just be as a consequence of a non-physiological pH (Kumar and Prabhakar, 2012).

CB chemoafferent discharge increases linearly up to around a P_aCO_2 of 65mmHg, after which it decreases and plateaus (Biscoe *et al*, 1970), however the amplitude of this discharge is smaller than that seen during hypoxia (Fitzgerald and Parks, 1971, Lahiri and Delaney, 1975, Pepper *et al*, 1995), which may again be due to an affect of the decreased pH on the type I cell secretory processes (Rocher *et al*, 2009).

The response to P_aCO_2 is faster than that to P_aO_2, which suggests that fluctuations in P_aCO_2 may cause oscillating discharges in CB activity, presenting a candidate for the transmission of information to the brain during exercise (or situations of increased metabolism) (Band *et al*, 1978, 1980, Kumar *et al*, 1988). This may be in association with some other blood-borne mediator such as a hormone (Bin Jaliah *et al*, 2005, Maskell *et al*, 2006), or via changes in $[K^+]$, as blood gas tension oscillations alone cannot fully explain V_E changes in exercise and the reliability of signalling in this manner via the blood to the CB is questioned (Nye, 1994).

As described, the three stimuli of the CB are responded to individually, potentially via a range of different sensors and transduction processes, but producing the same cardiorespiratory reflex response. The stimuli can also interact, potentiating this response beyond the simple sum of individual responses (Nielsen and Smith, 1952).

As discussed, the V_E response to a decrease in P_aO_2 is mediated solely by peripheral chemoreceptors, and is investigated by inhalation of hypoxic mixtures. Within this mixture PCO_2 can be kept constant and under this condition PO_2 has to fall to ~50mmHg before a ventilatory response is seen. However if PCO_2 is increased in combination with hypoxia, V_E will increase at a PO_2 ~100mmHg. This is why CO_2 is the major controller of V_E, whilst PO_2 becomes more important in pathological conditions such as chronic lung disease where CO_2 is chronically retained, or at altitude where decreased barometric pressure decreases the partial pressure of gases resulting in chronic hypoxia. P_aCO_2 is therefore held within a tight range. The ventilatory response to CO_2 is usually investigated by inhalation of a gas mixture containing CO_2 or a re-breathing technique, and in humans it has been shown that V_E increases 2-3L.min^{-1} for each 1mmHg increase in PCO_2. As mentioned, most of this response is mediated by the central chemoreceptors, but peripheral chemoreceptors respond more rapidly (West, 2012).

1.3 Glucoregulation

Glucose is the main substrate utilised in metabolism to produce ATP and the only energy source utilised by the brain, and as such there is a requirement for a system by which plasma glucose levels are monitored and maintained within a set range (~4-7mmol/L). This is achieved by the existence of glucose sensitive sites, which are able to induce a neuroendocrine reflex response when stimulated by a change in plasma glucose levels. The main glucose-sensing sites that trigger this counter-regulatory response are peripheral; the pancreatic α and β-cells (Burcelin *et al*, 2008), the liver (Donovan *et al*, 1991), and the hepatic portal vein (Hevener *et al*, 1997), although some central centres are also sensitive to glucose (Burdakov *et al*, 2005). In response to hypoglycaemia, insulin secretion is halted to decrease uptake of glucose by e.g. skeletal muscle. Glycogenolysis (the breakdown of glycogen to glucose in the liver and skeletal muscle) and gluconeogenesis (production of glucose from the breakdown of glycerol and glutamine in adipose tissue (lipolysis) and the kidney respectively) can be stimulated to increase appearance of glucose in the blood. These pathways are regulated hormonally, for example catecholamines (adrenaline and noradrenaline) are released in response to low glucose. They can increase the rate of glycogenolysis by action at glucagon releasing α-pancreatic cells, and reduce insulin production and release by β-cells. Glucagon decreases the synthesis, and increases breakdown, of glycogen (the storage form of glucose). Both catecholamines and glucagon can also increase gluconeogenesis and glucose delivery to the blood in exercise or stress. Cortisol is also released in response to hypoglycaemia, and can potentiate the effects of glucagon, as well as increasing gluconeogenesis. Another of these counter-regulatory mediators is growth hormone, which acts to decrease tissue glucose uptake and increase both gluconeogenesis and lipolysis (Yeo and Sawdon, 2007).

Insulin is the major hormone responding to increased blood glucose, and acts to increase expression of glucose transporters (e.g. GLUT-4), increase the synthesis of glycogen, with a

corresponding decrease in glucagon production, decrease in glucneogenesis and lipolysis and increase in triglyceride synthesis (another form of glucose storage) (Yeo and Sawdon, 2007).

Figure 3 shows these major hormonal control pathways of plasma glucose:

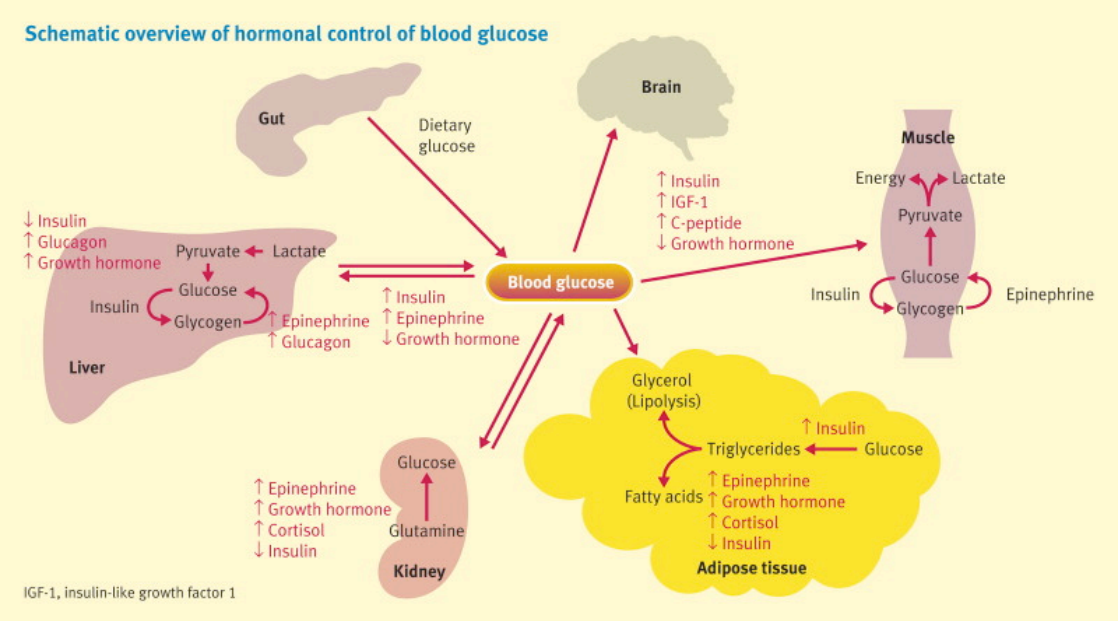

Figure 3: An overview of the hormonal control of plasma glucose levels. Image taken from Yeo and Sawden, (2007).

1.4 Role for the CB in glucoregulation

As well as the above established sites of glucose sensing, there is also a suggested role for the CB as a glucosensor. Due to the high blood flow and metabolic rate of the CB, it is well suited for a role in sensing a metabolic substrate such as glucose. However discrepancies in the data are present between species and preparation types and controversy exists over low glucose as a direct CB stimulus.

For example, Almaraz *et al* (1984) showed no increase in neural discharge in response to low superfusate glucose concentration in an isolated cat CB preparation, or even a decrease in neurotransmitter release/discharge frequency in zero glucose for around 2 hours. After this time a small increase in activity has been seen, before tissue death (Conde *et al*, 2007). Another *in vitro* intact cat CB study showed that removal of glucose for 12 mins had no effect

on absolute release of neurotransmitters, also supporting no direct role for low glucose (Fitzgerald *et al*, 2009).

García-Fernández *et al* (2007) demonstrated the presence of GLUT-1, GLUT-3 and GLUT-4, but not the GLUT-2 glucose transporter in the rat CB, which has been shown to be essential for glucose sensitive cells via GLUT-2 -/- mice (Thorens, 2001). CB cells have also been shown to express no glucokinase enzyme or specific K_{ATP} channel subunits that are expressed in liver, pancreatic cells and some central glucose sensitive neurons (Thorens, 2001). The fact that the CB does not possess these characteristics typical of other glucosensors, coupled with the discussed *in vitro* data, disagrees with any role for the CB in sensing low glucose directly.

However there is also substantial evidence for a direct action of low glucose on the CB. *In vitro* studies have shown that low and zero glucose could elicit a dose-dependent increase in neurotransmitter secretion in rat and mouse thin CB slice preparations (Pardal & Lopez-Barneo, 2002, Lopez-Barneo, 2003, Garcia-Fernadez, 2007), and co-cultures of rat CB type I cells (Zhang *et al*, 2007).

An *in vivo* infusion of glucose into the vascularly isolated cat CB sinus reduced activity by 20% and increased the threshold for the hypoxic response (Alvarez-Bullya, 1988). This group also showed that direct CB stimulation by sodium cyanide (a metabolic inhibitor) increased glucose output from the liver and increased brain retention of glucose, via an adrenal and sinus nerve mediated mechanism (Alvarez-Bullya, 1988, 1997). Intracarotid injections of glucose have also been shown to reduce the normal counter-regulatory response to hypoglycaemia in dogs (Frizzell, 1993).

More recent *in vivo* studies have utilised the hyperinsulinaemic hypoglycaemic clamp protocol to investigate the role of the CB in glucoregulation. Koyama *et al* (2000) performed hypo- and euglycaemic clamps in conscious dogs. They showed that an increased glucose infusion was required to clamp plasma glucose after CBR (Figure 4) and that glucagon and cortisol counter-

regulatory responses were significantly reduced. The adrenaline response was also reduced by 50%, but not significantly.

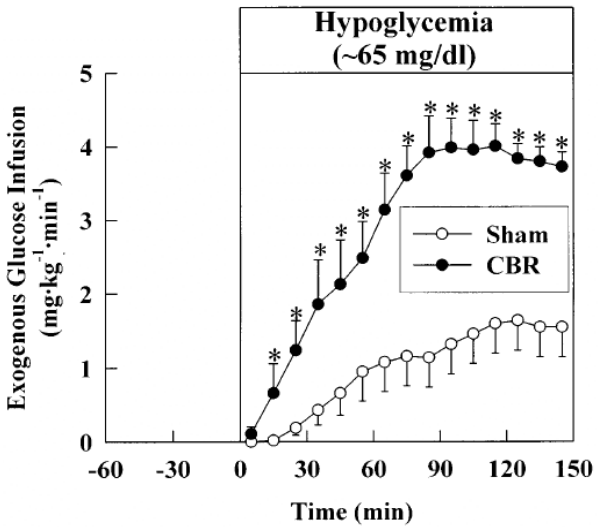

Figure 4: A graph showing the rate of glucose infusion required to maintain a hypoglycaemic plasma glucose clamp in conscious dogs. CBR (carotid body resected) animals required a higher infusion rate compared to shams, indicating a role for the CB in glucoregulation. Taken from Koyama *et al* (2000).

They used the same methods to evaluate the role of the CB in maintaining plasma glucose during exercise (Koyama *et al*, 2001), and saw a mismatch between glucose production and disappearance at the onset of exercise, as well as blunted glucagon and noradrenaline counter-regulatory responses in CBR animals. Although the authors suggested low glucose as the direct stimulus, the effect of the counter-regulatory hormones cannot be discounted.

As hinted at previously, the CB possesses polymodality, whereby any of the stimuli to increase chemoafferent discharge results in the same set of cardiorespiratory reflexes. Based on this, hyperinsulinaemic hypoglycaemia, if acting at the CB, would be expected to induce a hyperventilation and associated hypocapnia. This was investigated by Bin Jaliah *et al* (2004) using eu- and hypoglycaemic clamp protocols in anaesthetised rats. An increased V_E was seen in correlation with the decrease in plasma glucose levels in sham animals; whereas a reduced basal V_E and no increased V_E response to hypoglycaemia was seen in CSNX animals. However a corresponding *in vitro* preparation in the same lab showed no increase in single

fibre discharge in response to low or removed superfusate glucose, suggesting that low glucose was not the direct stimulus (Bin Jaliah, 2004, 2005). Moreover, i*n vivo*, the hyperventilation seen in the sham animals was not accompanied by a hypocapnia, suggesting that the hyperinsulinaemic hypoglycaemia had increased metabolism, resulting in an isocapnic hyperpnoea. This was addressed by a consequent experiment (Bin Jaliah *et al*, 2005) using the same methods, which showed sham animals had an increased CO_2 chemosensitivity in hypoglycaemia, not seen in the CSNX group. Again a corresponding *in vitro* preparation showed opposing results, with a low glucose superfusate blunting the response to increased CO_2 (Bin Jaliah, 2005). These experiments suggest a potential role for the CB in matching V_E to metabolism, posing a potential explanation for what is seen during exercise (as suggested by Band *et al*, 1978, 1980, Kumar *et al*, 1988). V_E rises almost immediately, closely matching the increase in O_2 consumption with increased CO_2 excretion, maintaining arterial PCO_2, O_2 and pH.

As no response was seen *in vitro*, Bin Jaliah *et al* (2005) suggested that this increased peripheral CO_2 sensitivity may be mediated hormonally, for example by adrenaline. This is not a new idea, as adrenaline has previously been suggested to play a role in exercise hyperpnoea (Linton *et al*, 1992), and increased levels of adrenaline are seen in both hypoglycaemia (Vollmer *et al*, 1997) and exercise (Christensen *et al*, 1983). Adrenaline, as well as other adrenoreceptor agonists, have previously been shown to increase ventilation *in vivo* (e.g. Whelan and Young, 1953, Young, 1957, Joels and White, 1968, Heistad *et al*, 1972, Maskell *et al*, 2006), an action which could be blocked by CSNX or application of hyperoxia (Joels and White, 1968, Heistad *et al*, 1972, Maskell *et al*, 2006) which is consistent with a direct action on the CB, rather than an action via the effect of adrenaline on blood flow. Joels and White (1968) showed that this increase in ventilation was accompanied by an increased chemoreceptor discharge. They also showed that a similar increase in ventilation and discharge could be achieved by infusion directly into the carotid

artery, and that this could be ablated by CSNX. Heistad *et al* (1972) and Maskell *et al* (2006) showed that the β-adrenoreceptor blocker propranolol was able to prevent the observed ventilatory responses, suggesting a potential mechanism.

However *in vitro* studies have revealed opposing results; adrenaline doses of 10-220μg, applied via the superfusate onto an isolated CB and sinus nerve, produced either no response or a decrease in chemodischarge (Eyzaguirre and Koyano, 1965). These findings have been replicated by co-workers, who saw a dose-dependent inhibition of afferent fibre discharge at doses of 1μM and above, and no effect at lower doses, in a similar CB preparation (unpublished data, Holmes and Kumar, 2011).

Ward *et al* (2007) carried out hypo and hyperglycaemic clamp experiments in humans and showed that glucagon, cortisol, adrenaline and noradrenaline increased in response to hypoglycaemia (Figure 5), as did basal V_E and the ventilatory response to hypoxia.

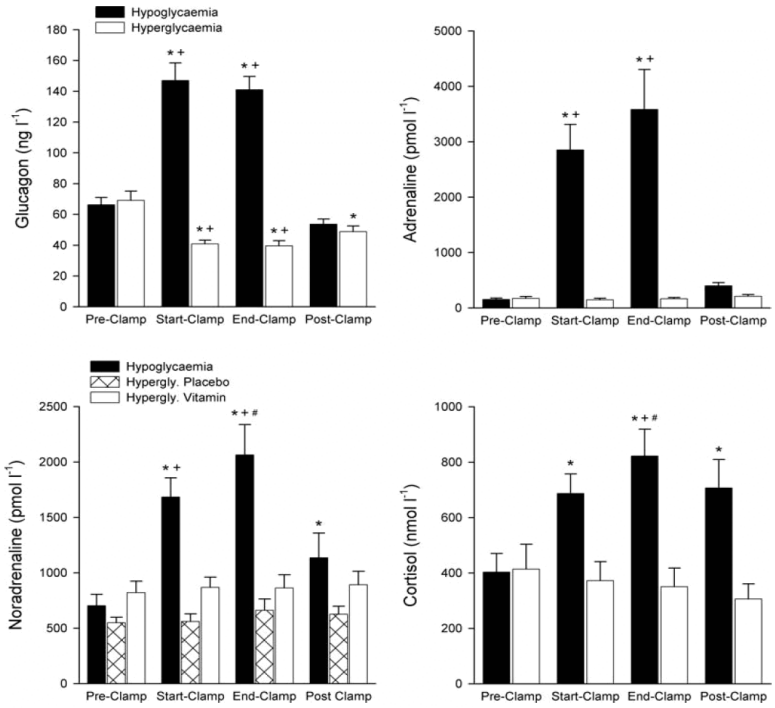

Figure 5: A series of graphs showing that levels of glucagon, adrenaline, noradrenaline and cortisol were all increased in response to hypoglycaemia. Taken from Ward *et al* (2007).

This is consistent with an effect of hypoglycaemia on the CB, but again cannot rule out the possibility of mediation via the release of the counter-regulatory hormones rather than low glucose directly. Wehrwein *et al* (2010) also carried out hypoglycaemic clamps in humans, but under normoxia or hyperoxia to isolate CB involvement. The glucose infusion required to maintain the clamp was 45% higher and the normal counter-regulatory hormonal response was significantly blunted under hyperoxia i.e. without CB input (Figure 6).

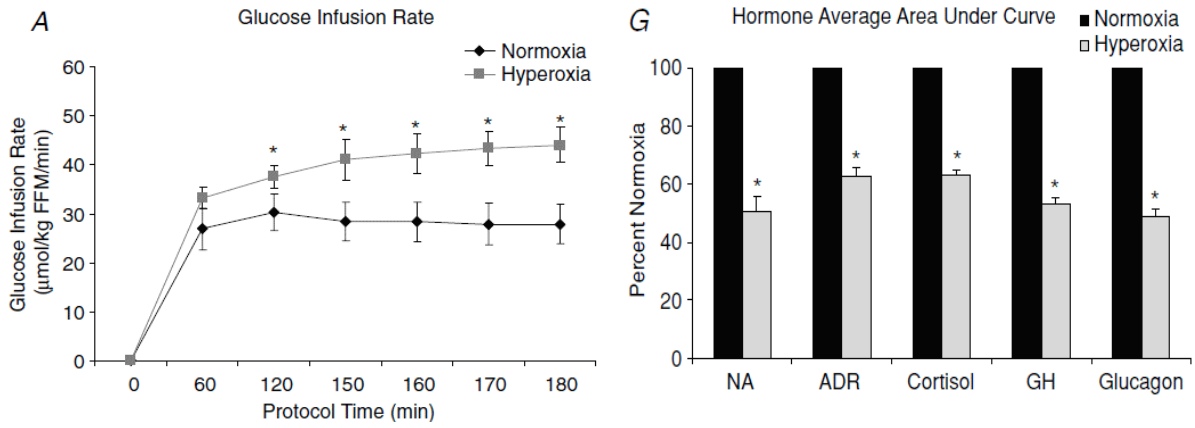

Figure 6: A shows the glucose infusion rate required to maintain a plasma glucose clamp was increased in the presence of hyperoxia i.e. when CB function was ablated. G shows that the amount of counter-regulatory hormone release under hyperoxia was reduced compared to normoxic conditions. Taken from Wehrwein *et al*, (2010).

This supports the hypothesis originally proposed by Koyama *et al* (2000) that intact CB's are required for a normal response to hypoglycaemia, but still does not clarify whether low glucose is the stimulus, or whether it could be adrenaline.

2. Aims and Hypotheses

The CB is a peripheral chemoreceptor responding to changes in arterial gas tensions and pH. It has also been proposed to have a role in glucoregulation, although it is unknown whether the direct stimulus is low glucose or some other blood-borne mediator such as a counter-regulatory hormone.

Based on the current evidence, this project aimed to further investigate the role of the CB in glucoregulation, specifically with respect to how the CB is stimulated in hypoglycaemia e.g. directly by low glucose or by the associated counter-regulatory hormone adrenaline. The interaction between hypoglycaemia/adrenaline and the hypercapnic ventilatory response was also investigated.

The hypotheses were as follows:

- Administration of the hypoglycaemic counter-regulatory hormone adrenaline would activate the CB via β-adrenoreceptors, increasing basal V_E. Hyperoxia and propranolol would ablate CB-mediated increases in V_E.
- Administration of the counter-regulatory hormone adrenaline would increase hypercapnic sensitivity.
- Basal V_E would increase during an insulin-induced hypoglycaemic ramp. Hyperoxia and propranolol would ablate this CB–mediated increase in V_E.
- Hypercapnic sensitivity would be increased during hypoglycaemia.

3. Methods

3.1 Animals

Male Wistar rats were sourced from Charles River Laboratories, with body weights of 260-415g on the day of experimentation. The animals were housed in single sex groups, at a temperature of 21±1°C and humidity of 40-45%, under a 12-hour light/dark cycle (lights on at 7:00am). Food and water was available ad libitum, until food was withdrawn from midnight before experimentation to ensure stable and consistent basal blood glucose levels.

The care of the animals and all procedures performed were carried out by Home Office licensees in line with the Home Office (UK) guidelines, University of Birmingham guidelines on ethical use of animals and the Animals (Scientific Procedures) Act (UK) 1986.

3.2 Anaesthesia and surgery

Anaesthesia was induced with 4% isoflurane in O_2 at 4 L min^{-1} (Merial Animal Health Ltd.). Surgery was then carried out as follows (see Figure 7): the right jugular vein was isolated and cannulated (ID 0.5mm, OD 1.5mm), allowing maintenance of anaesthesia with a 17-20 $mg.kg^{-1}.hour^{-1}$ infusion of Alfaxan® (Vétoquinol UK Ltd.) using a syringe driver (Perfusor® securaFT, B. Braun.), and 0.1ml boluses as necessary. The trachea was isolated and cannulated with a stainless steel T-piece cannula. The right brachial artery was isolated from the brachial plexus and cannulated (ID 0.4mm, OD 0.8mm). The right femoral artery and vein were isolated from the femoral sheath and cannulated (ID 0.58mm, OD 0.96mm, and 3 x ID 0.28mm, OD 0.61mm, each soldered to an ID 0.58mm, OD 0.96mm, respectively). The left femoral artery was isolated.

Plane of anaesthesia was monitored throughout by assessing response to a strong paw pinch. Core body temperature was also monitored and maintained at 37°C throughout, via a rectal temperature probe linked to a homeothermic heat pad system (Harvard Apparatus) the animal was positioned upon. All cannulae were filled with heparinised saline (20 Units/ml Heparin,

LEO® Pharma) and sourced from Portex™, Smiths Medical. Suture was from Look®, Surgical Specialities Corporation and needles (0.5x25, 0.6x30mm and 0.8x40mm, Microlance™) and syringes (1, 2, 5 and 10ml, Plastipak™) were from BD.

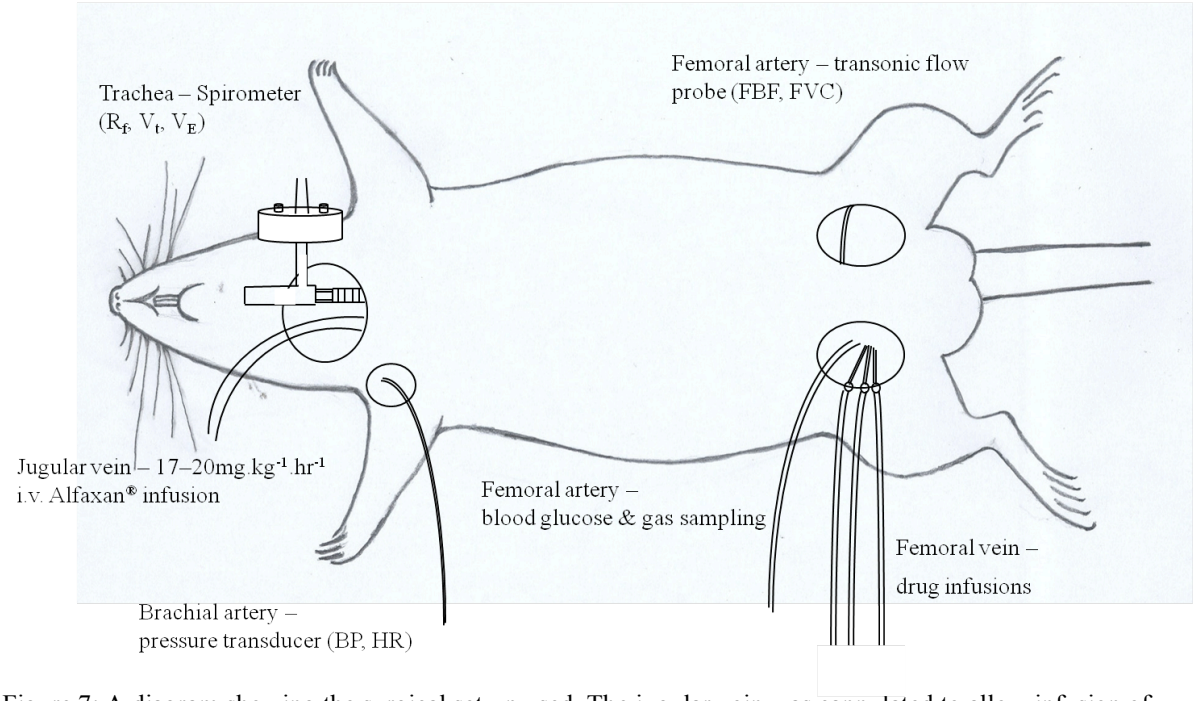

Figure 7: A diagram showing the surgical set-up used. The jugular vein was cannulated to allow infusion of anaesthetic (Alfaxan, 17-20mg.kg^{-1}hr^{-1}). The trachea was cannulated to allow airflow to be monitored via a spirometer, allowing respiratory frequency (R_f) and tidal volume (V_t) to be derived. The brachial artery was cannulated to record mean arterial blood pressure (MABP), from which heart rate (HR) was derived. The right femoral artery was cannulated with a specialised line for taking blood samples. The femoral vein was cannulated with 3 cannulae soldered to larger cannulae, to allow multiple drug infusions. The left femoral artery was isolated to allow measurement of femoral blood flow (FBF) using a flow probe. Femoral vascular conductance (FVC = FBF/MABP) and ventilation ($V_E = R_f \times V_t$) were also calculated.

3.3 Equipment and data

A spirometer (MacLab, ADInstruments) was attached to the tracheal T-piece to measure airflux, allowing calculation of respiratory frequency (R_f), tidal volume (V_t) and minute ventilation ($V_E = R_f \times V_t$). Animals breathed room air, but a plastic tube running from cylinders of N_2, O_2 (Boc) and CO_2 (Murex) could be attached to the spirometer via a t-piece, allowing control of inspired gases by a rotameter (Cole Parmer).

A pressure transducer (Capto) was attached to the brachial cannula to monitor arterial blood pressure (ABP) (via a MacLab Bridge Amp, ADInstruments), from which mean ABP and HR were derived. Blood samples were taken via a specialised femoral artery cannula, which minimised blood loss and ensured a pure sample. The samples were used to monitor blood

glucose (Accu-Chek®, Aviva), blood gases and haematocrit (150µl capillary tubes and Gem® 4000 premier analyser, Instrumentation Laboratory Ltd.). The isolated femoral artery had a flow probe (Transonic Systems Inc.) attached to monitor femoral blood flow (FBF), an index of hindlimb skeletal muscle blood flow, and also allowing calculation of femoral vascular conductance (FVC = MFBF/MABP). The cannulae in the femoral vein were used to give drug infusions (see section 3.4) using syringe drivers (kd Scientific). Data was recorded at sampling frequencies of 100-1000 samples/second, using PowerLab and Labchart software (ADInstruments) on a Mac OS X computer. The experimental set-up is shown in Figure 8 below.

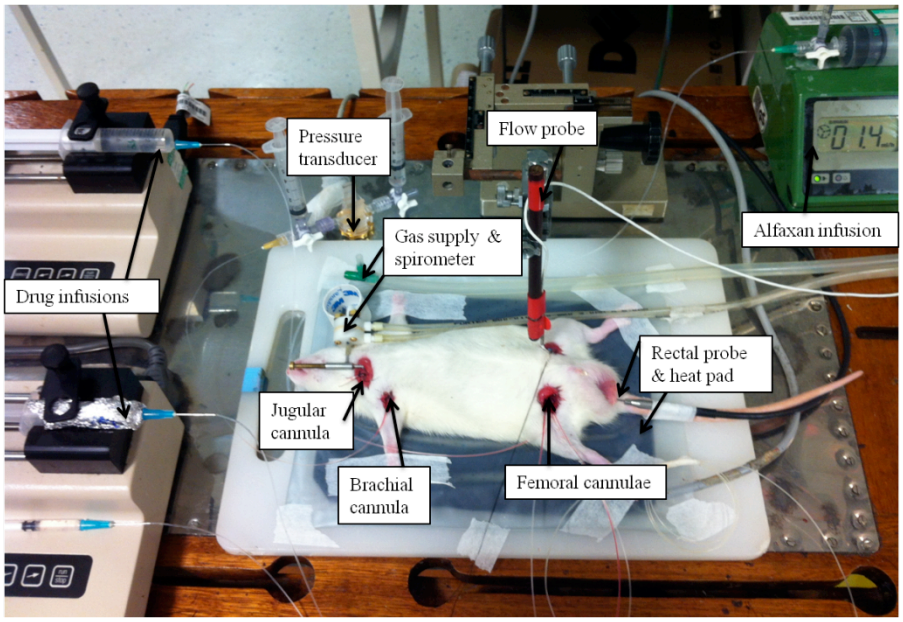

Figure 8: A photograph showing the experimental set-up used. A spirometer was attached to the tracheal T-piece to measure airflux, allowing calculation of respiratory frequency (R_f), tidal volume (V_t) and minute ventilation ($V_E = R_f \times V_t$). A plastic tube provided a gas supply from cylinders of N_2, O_2 and CO_2, which could be attached to the spirometer via a t-piece. A pressure transducer was attached to the brachial cannula to monitor arterial blood pressure (ABP), from which MABP and HR were derived. Blood samples were taken via a specialised cannula in the right femoral artery. The cannulae in the right femoral vein were used to give drug infusions. The isolated left femoral artery had a flow probe attached to monitor femoral blood flow (FBF), also allowing calculation of femoral vascular conductance (FVC = MFBF/MABP). Core body temperature was monitored and maintained at 37°C via a rectal temperature probe linked to a homeothermic heat pad system.

3.4 Drugs

Adrenaline (E4250 - Sigma®) and propranolol (P0884 - Sigma®) were dissolved in saline. Insulin (Hypurin® Bovine Neutral, CP Pharmaceuticals Ltd.) was made up in Gelofusine® plasma expander (4% w/v, Dechra Veterinary Products) and infused using a 10ml glass syringe (81601, Hamilton) to minimise adherence to the equipment.

3.5 Experimental protocols

Before any experiment, 40 minutes post-surgery stabilisation was allowed. A 20-minute baseline was then recorded, during which blood glucose was sampled at 0, 10 and 20 minutes, and a blood gas was taken at 20 minutes. Blood glucose and gas samples were then taken periodically throughout each experiment.

3.5.1 - Group 1: The effect of adrenaline on ventilation

The effect of adrenaline on ventilation was investigated (n=6) using infusions at concentrations between 0.1ng–1µg.kg^{-1}.min^{-1}. 5 minutes was allowed for each dose to reach a steady state, before CB-mediated effects were determined with 30 seconds exposure to 100% O_2 (hyperoxia) which resulted in P_aO_2s > 300 mmHg, and was also carried out at baseline. After recovery from adrenaline, a 0.3mg.kg^{-1}min^{-1} propranolol infusion was given (n=5) for 10 minutes to reach a steady baseline, before a control hyperoxia was carried out. The adrenaline dose-response was then repeated in the presence of propranolol.

Group 2: Hypercapnic ventilatory sensitivity

The effect of adrenaline on the hypercapnic ventilatory response (CO_2 sensitivity = ΔV_E / mmHg P_aCO_2) was determined (n=5) using a constant infusion of 1µg.kg^{-1}min^{-1}. The dose was allowed 5 minutes to reach a steady state, before the animal was exposed to graded levels of hypercapnia: 4 and 8% inspired CO_2 for 5 minutes. Adequate time was allowed between

each hypercapnia for recovery to baseline values. Subsequent blood gas analysis revealed that the presumed 4% level of hypercapnia did not significantly alter P_aCO_2 from basal values, whereas the 8% level gave P_aCO_2 levels of 57 ± 1.3 mmHg, therefore this level was used subsequently. This level of hypercapnia was also carried out at baseline, prior to administration of adrenaline, to obtain a control response. After recovery from adrenaline, propranolol was given as described above (n=2) and the hypercapnic response assessed. The adrenaline infusion was then repeated in the presence of propranolol, and the hypercapnia (61 ± 4.5 mmHg) repeated.

3.5.2 Group 3: Hyperinsulinaemic hypoglycaemic ramp

Insulin was infused at different rates between 2 - 12 $mU.kg^{-1}min^{-1}$ in order to achieve 3 – 4 target levels of hypoglycaemia (blood glucose of 5.7 (basal) to 2.9 mmol/L) (n=6)). Each level was maintained for around 10 minutes, allowing the effect on basal ventilation to be recorded, before 30 seconds exposure to hyperoxia was used to determine CB-mediated input. At the most severe level of hypoglycaemia, the hypercapnic ventilatory response (CO_2 sensitivity) was assessed by exposure to a P_aCO_2 of 58 ± 0.9 mmHg for 5 minutes. Basal ventilation and control responses to hyperoxia and hypercapnia were also recorded at euglycaemia. The protocol was then repeated in the presence of a 0.3$mg.kg^{-1}min^{-1}$ propranolol infusion (n=6). Control responses were carried out after 10 minutes propranolol infusion, before the insulin infusion was restarted (hypercapnia causing P_aCO_2 levels of 56 ± 1 mmHg). Blood samples were taken throughout the ramp protocols with a view to measuring the levels of the counter-regulatory hormone adrenaline (see section 6 - future work).

3.6 Data analysis

All data is presented as the mean ± SEM of the absolute data or as the difference from baseline or corresponding control response. Graphs were plotted in DeltaGraph 5.7.5, and statistics carried out using GraphPad Prism.

The effect of adrenaline on V_E, with hyperoxia and/or propranolol, was plotted as a scatter graph and analysed using repeated measures or one-way ANOVAs as appropriate, with post-hoc Bonferroni tests. Paired or unpaired t-tests were used to compare specific pairs of V_E responses and to analyse the cardiovascular and blood glucose data.

The effect of hypoglycaemia on V_E, with hyperoxia and/or propranolol, was plotted as a scatter graph and had linear regression lines fitted. The slope of each data set was analysed statistically using one sample t-tests (to compare each line to zero) and one-way ANOVA with post-hoc Bonferroni was used to compare appropriate pairs of slopes. Paired t-tests were used to analyse the cardiovascular data.

The hypercapnic ventilatory responses under control conditions, in the presence of adrenaline/hypoglycaemia and with or without propranolol, were also plotted as scatter graphs. The slopes of these lines were plotted as bar charts representing CO_2 sensitivity (ΔV_E/ mmHg P_aCO_2). One-way ANOVAs, with post-hoc Bonferroni tests were carried out to compare the CO_2 sensitivities within each experimental group. Paired and unpaired t-tests were also used to compare specific pairs of data.

The relationship between P_aCO_2 and V_E during adrenaline infusion/hypoglycaemia, and the effect of propranolol on this relationship was plotted as a scatter graph. Linear regression lines were fitted to each. The slopes were compared to zero using one-sample t-tests and to each other using unpaired t-tests. All data was analysed to the 95% significance level i.e. $P < 0.05$ taken as significant.

4. Results

4.1 Group 1

The effect of adrenaline on ventilation

Figure 9 A shows raw traces of ABP, HR, airflux, R_f, V_t and FBF at baseline and during infusion of $1\mu g.kg^{-1}min^{-1}$ adrenaline. The main effects seen were increased R_f and V_t, as well as increased FBF, MABP and decreased HR. The cardiovascular effects of adrenaline are summarised in Table 1, reflecting the changes shown in the traces, although none reached significance. Adrenaline also caused a significant increase in [blood glucose] from a basal value of 6.6 ± 0.5 to $7.3 \pm 0.5 mmol.L^{-1}$ at $1\mu g.kg^{-1}min^{-1}$ (paired t-test, $P < 0.05$).

Figure 9 B shows an adrenaline dose-response curve; adrenaline caused a significant increase in V_E from a basal value of 133.5 ± 4.1 to 151.9 ± 6.8 $ml.min^{-1}$ at the highest dose. However V_E did not increase progressively with each dose tested, only becoming significant at 0.1 and $1\mu g.kg^{-1}min^{-1}$. It was therefore inappropriate to fit a dose-response curve or linear regression line, or to continue repeating the full range of doses, therefore 0, 0.1 and $1\mu g.kg^{-1}min^{-1}$ were used from this point.

The effect of hyperoxia and propranolol

Figure 10 shows raw traces showing the effects of hyperoxia and propranolol, compared to control. Hyperoxia decreased R_f, reflected in the V_t trace. Hyperoxia also significantly decreased HR and FBF. Whilst this trace shows no significant effect on ABP, overall MABP significantly increased, as shown by group mean data in Table 2 (paired t-tests vs control, $P < 0.05$). The major effect of propranolol alone was to cause a significant decrease in HR (cardiovascular data shown in Table 1), as well as a significantly decreased ABP and a slight but non-significant decrease in FVC (as seen in the FBF trace). R_f slightly decreased compared to control, but not by as much as in hyperoxia. There was no significant difference between the HR and MABP with propranolol alone and propranolol with adrenaline infusion.

There was a significant difference in FVC, however the value recorded with propranolol and adrenaline together was not significantly different from the control value (Table 1).

Figure 11 A shows the selected adrenaline doses increasing V_E (i.e. normoxia). Hyperoxia significantly shifted the response down, but V_E still increased with adrenaline. Propranolol also shifted the response down (not as far as hyperoxia), but V_E still increased with adrenaline. However at the top dose, propranolol appears to partly reduce the response, although this did not quite reach significance (unpaired t-test, P = 0.0519). Propranolol did not alter the effect of adrenaline on blood glucose as levels still rose, from 6.9 ± 0.5 basally, to 7.3 ± 0.4 at 1$\mu g.kg^{-1}$ min^{-1} adrenaline, but not significantly. The combination of hyperoxia and propranolol was additive (significances shown on graph from repeated measures ANOVA & post hoc Bonferroni, comparing means of each line).

Figure 11 B shows the effect of hyperoxia, propranolol and the combination, as the difference from the response at each corresponding adrenaline dose. The hyperoxia and combination differences remained fairly stable at each dose, whereas the propranolol difference slightly, but not significantly, increased at the top dose (one-way ANOVAs & post-hoc Bonferroni, P > 0.05) reflecting the reduced response in A.

The effect of adrenaline on P_aCO_2

Figure 12 shows the association between the increased V_E caused by adrenaline (from baseline to1$\mu g.kg^{-1}min^{-1}$), and the P_aCO_2 levels sampled at the corresponding doses. In normoxia i.e. the control response to adrenaline, V_E increased and P_aCO_2 remained constant, showing just a slight decrease. In the presence of propranolol, adrenaline still increased V_E, but not by as much as in control, as seen in Figure 11 A. However P_aCO_2 increased. Statistics could not be carried out on this data as not every animal the ventilation data was drawn from had a corresponding blood gas taken, and vice versa, and so this plot can only suggest a trend from the data available.

4.2 Group 2

The effect of adrenaline on hypercapnic ventilatory sensitivity

Figure 13 shows raw traces demonstrating the response to hypercapnia, the main effect of which was an increased V_t, and slightly increased R_f. HR and FVC decreased and BP slightly increased in this hypercapnic trace compared to control, but mean group data showed no significant cardiovascular effects of hypercapnia (paired t-tests vs control, $P > 0.05$, shown in Table 3). The 3rd trace shows hypercapnia in the presence of adrenaline; V_t is increased as in the 1st hypercapnic response, but R_f increased further. HR and ABP appear increased compared to the 1st response, which may be accounted for by an effect of the adrenaline. Figure 14 A shows the hypercapnic ventilatory response at control, and with adrenaline and propranolol. Adrenaline increased basal ventilation and also increased the response to CO_2. Propranolol alone and with adrenaline reduced this response to below the control. Figure 14 B shows the slopes of the hypercapnic responses i.e. CO_2 sensitivity, demonstrating that there was no significant difference between any of the slopes.

4.3 Group 3

The effect of hypoglycaemia, hyperoxia and propranolol, on ventilation

Figure 15 shows raw traces demonstrating the effect of hypoglycaemia; R_f increased, reflected in the V_t trace. ABP stayed constant, but HR and FBF showed an increase (the 3rd trace shows a hypercapnic response during hypoglycaemia – see Figure 18). However, Table 4 shows that hypoglycaemia had no significant effect on the cardiovascular parameters (paired t-tests vs control, $P > 0.05$). Figure 16 A shows basal ventilation and three levels of insulin-induced hypoglycaemia causing an increased V_E. A one-way ANOVA with post-hoc Bonferroni showed no significant increase in V_E overall or between each pair of blood glucose levels.

Hyperoxia shifted the response down, but V_E still increased at each blood glucose level. Propranolol appeared to prevent the increase, with V_E actually decreasing. Hyperoxia and propranolol in combination showed an additive effect, shifting V_E down and preventing the increase. However, as this data was not paired and each animal had slightly different blood glucose values, linear regression fits were deemed more useful in assessing the relationship between hypoglycaemia and V_E. Slopes for each response were determined and plotted as a bar chart in Figure 16 B. Normoxia (the control response to hypoglycaemia) vs. hyperoxia, and propranolol vs. hyperoxia with propranolol were not significantly different, but all other slopes were significantly different from each other (one-way ANOVA and post-hoc Bonferroni, $P < 0.05$) and all slopes were significantly different from 0 (one-sample t tests, $P < 0.05$).

The effect of hypoglycaemia on P_aCO_2

Figure 17 is a scatter graph showing the association between the increased V_E caused by hyperinsulinaemic hypoglycaemia, and P_aCO_2. Under the normoxic conditions i.e. the control response to hypoglycaemia, V_E increased and P_aCO_2 remained constant. In the presence of propranolol, V_E decreased in spite of the hypoglycaemia (as seen in Figure 16 A), and P_aCO_2 increased. However comparison of the slopes of the fitted linear regression lines showed no significant difference between the two conditions (unpaired t-test, $P > 0.05$), and that neither was significantly different from 0 (one-sample t-tests, $P > 0.05$), potentially due to the large error bars.

The effect of hypoglycaemia on hypercapnic ventilatory sensitivity

The 3rd raw trace of Figure 15 shows an example hypercapnic response in hypoglycaemia, which resembles the trace seen in hypoglycaemia alone, except V_t also increased. Figure 18 A shows the control hypercapnic ventilatory response, and the corresponding responses in hypoglycaemia, with propranolol alone and in combination with hypoglycaemia.

Hypoglycaemia increased V_E basally, and caused an increased response to CO_2. The response with propranolol was not different from control, but the response during hypoglycaemia and propranolol infusion was blunted to below control levels. The slopes of these response lines provided a measure of CO_2 sensitivity, represented by the bar chart in Figure 18 B. The significances shown on the slopes are two-fold: one-way ANOVA with post-hoc Bonferroni showed no significant difference between the control vs hypoglycaemic, control vs propranolol and propranolol vs hypoglycaemia & propranolol CO_2 sensitivities, and significant differences between hypoglycaemic/control vs hypoglycaemic & propranolol sensitivities. The animals were not paired between all groups, however a paired t-test could be carried out between control and hypoglycaemia, revealing a significant difference ($P < 0.05$), and an unpaired t-test between hypoglycaemia and hypoglycaemia with propranolol confirmed significance ($P < 0.05$).

Table 1: Cardiovascular effects of adrenaline and propranolol

Mean ± SEM	Control	Adrenaline $1\mu g.kg^{-1}min^{-1}$	Propranolol	Adrenaline & propranolol
HR (bpm)	449 ± 7.7	448 ± 11.9 (ns)	369 ± 5.4 (*)	374 ± 13.9 (ns)
MABP (mmHg)	123 ± 2.2	132 ± 4.7 (ns)	109 ± 3.8 (*)	120 ± 7.9 (ns)
FVC (U)	0.023 ± 0.003	0.026 ± 0.005 (ns)	0.021 ± 0.002 (ns)	0.026 ± 0.002 (*/ns)

Table 1: Mean group cardiovascular data ± SEM. Adrenaline had no significant effect on any of the mean cardiovascular parameters, although MABP and FVC both increased (paired t-tests vs control, $P > 0.05$). Propranolol alone significantly decreased HR and MABP ($P < 0.05$) but had no significant effect on FVC (paired t-tests vs. control, $P > 0.05$). Adrenaline had no significant effect on HR or MABP during propranolol infusion, but FVC did significantly increase (paired t-tests propranolol vs adrenaline and propranolol, $P < 0.05$). However the FVC seen with adrenaline and propranolol was not significantly different to control (paired t-test adrenaline and propranolol vs control, $P > 0.05$).

Table 2: Cardiovascular effects of hyperoxia

Mean ± SEM	Control	Hyperoxia
HR (bpm)	449 ± 8.7	438 ± 7.2 (*)
MABP (mmHg)	123 ± 2.6	129 ± 2.6 (*)
FVC (U)	0.023 ± 0.003	0.019 ± 0.002 (*)

Table 2: Mean group cardiovascular data ± SEM. Hyperoxia significantly increased MABP, and decreased HR and FVC (paired t-tests vs control, $P < 0.05$).

Table 3: Cardiovascular effects of hypercapnia

Mean ± SEM	Control	Hypercapnia
HR (bpm)	449 ± 8.7	425 ± 9.0 (ns)
MABP (mmHg)	123 ± 2.6	120 ± 3.7 (ns)
FVC (U)	0.023 ± 0.003	0.025 ± 0.003 (ns)

Table 3: Mean group cardiovascular data ± SEM. Hypercapnia had no significant effect on HR, MABP or FVC (paired t-tests vs control, $P > 0.05$).

Table 4: Cardiovascular effects of hypoglycaemia

Mean ± SEM	Control	Hypoglycaemia
HR (bpm)	449 ± 8.7	453 ± 10.5 (ns)
MABP (mmHg)	123 ± 2.6	123 ± 4.5 (ns)
FVC (U)	0.023 ± 0.003	0.024 ± 0.003 (ns)

Table 4: Mean group cardiovascular data ± SEM. Hypoglycaemia had no significant effect on HR, MABP or FVC (paired t-test vs control, $P > 0.05$).

Figure 9: The effect of adrenaline on ventilation

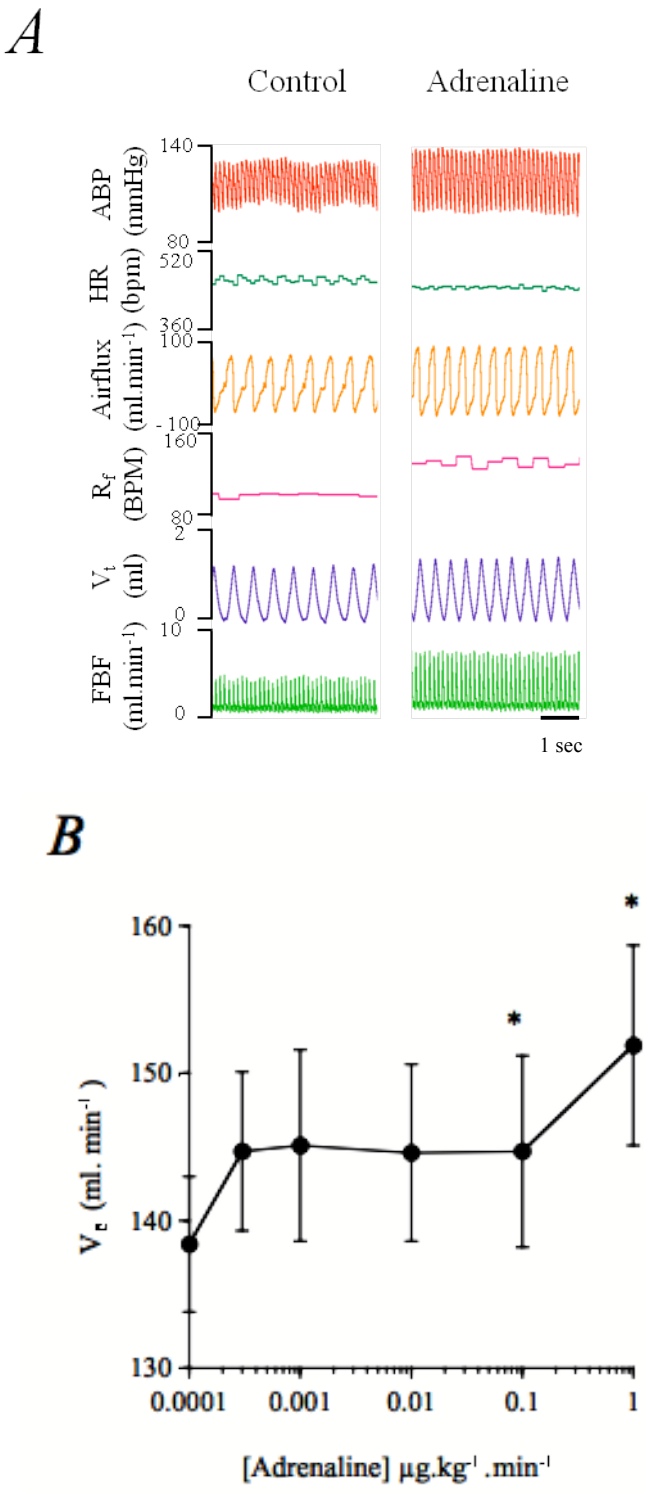

Figure 9 A: Raw traces showing baseline recordings of ABP, HR, airflux, R_f, V_t and FBF, and the effect of $1\mu g.kg^{-1}min^{-1}$ adrenaline upon them. The main effects of this adrenaline dose were increased R_f, V_t, ABP and FBF, and a decreased HR. Mean cardiovascular data is shown in Table 1. B: An adrenaline dose-response curve; adrenaline significantly increased V_E (repeated measures ANOVA, $P < 0.05$) but not between every dose tested, reaching significance at 0.1 and $1\mu g.kg^{-1}min^{-1}$ (post-hoc Bonferroni).

Figure 10: The effect of hyperoxia and propranolol (raw traces)

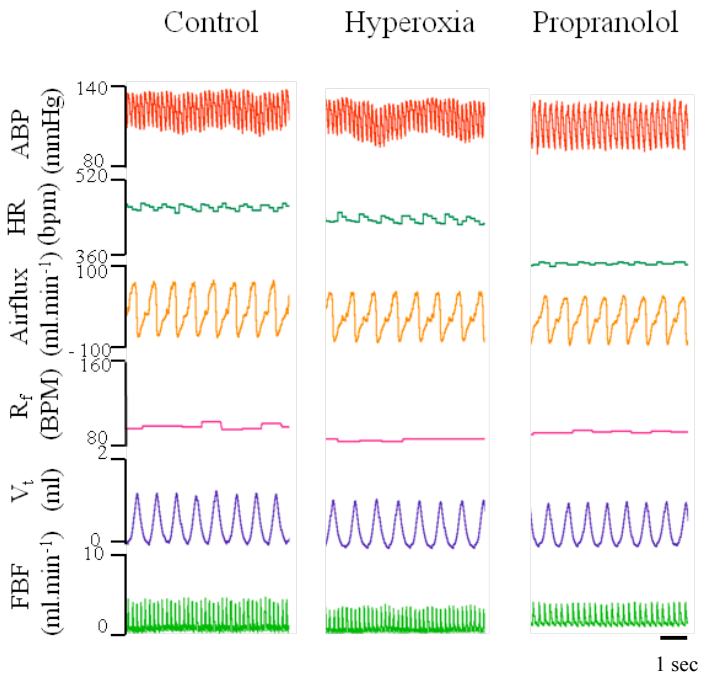

Figure 10: Raw traces showing the effects of hyperoxia and propranolol, compared to control. The main effect of hyperoxia was a decreased R_f. Hyperoxia also significantly decreased HR and FBF. Whilst this trace shows no significant effect on ABP, overall MABP significantly increased, as shown by group mean data in Table 1 (paired t-tests vs control, $P < 0.05$). The major effect of propranolol was to cause a significant decrease in HR (cardiovascular data shown in Table 1), as well as a significantly decreased ABP and a slight but non-significant decrease in FVC (as seen in the FBF trace). R_f slightly decreased compared to control, but not as much as with hyperoxia.

Figure 11: The effect of hyperoxia and propranolol

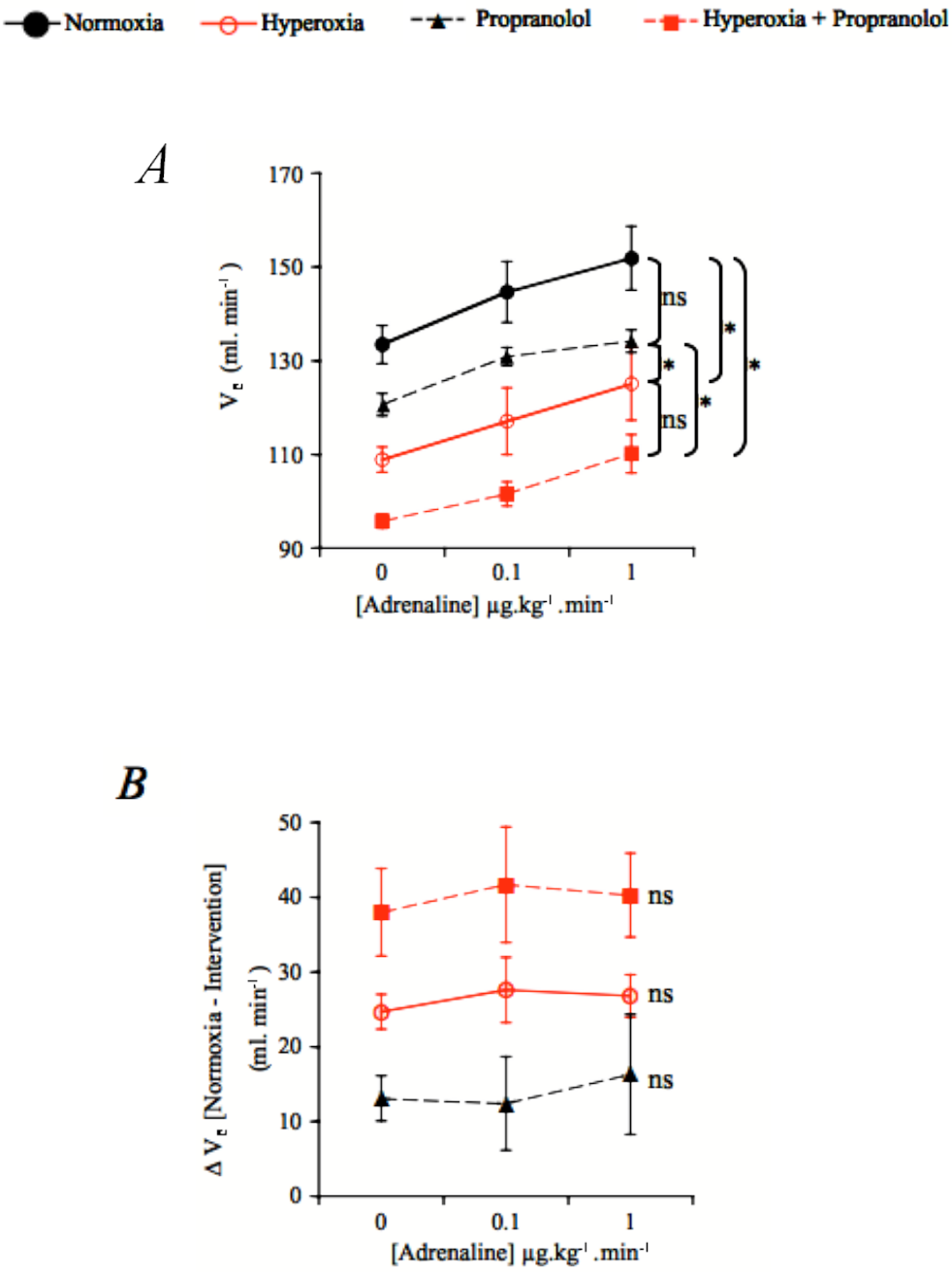

Figure 11: A - The selected adrenaline doses increased V_E (= normoxia). Hyperoxia significantly shifted the response down, but V_E still increased with adrenaline. Propranolol also shifted the response down, but not as far as hyperoxia. V_E still increased with adrenaline at the first dose, however at the top dose, propranolol appears to partly reduce the response, although this didn't reach significance (unpaired t-test, P = 0.0519). The combination of hyperoxia and propranolol was additive (signifcances shown on graph from repeated measures ANOVA & post hoc Bonferroni, comparing means of each line). B: The effect of hyperoxia, propranolol and the combination, shown as the difference from the response at each corresponding adrenaline dose. The hyperoxia and combination differences remained fairly stable at each dose, whereas the propranolol difference slightly, but not significantly, increased at the top dose (one-way ANOVAs & post-hoc Bonferroni, P > 0.05).

Figure 12: The effect of adrenaline on P_aCO_2

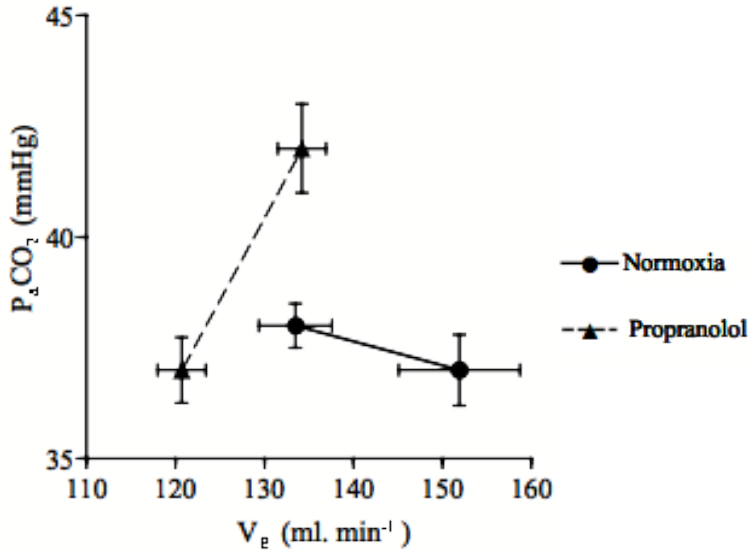

Figure 12: A scatter graph showing the association between the increased V_E caused by adrenaline (only baseline and top dose shown), and the P_aCO_2 levels sampled at the corresponding doses. Under normoxia i.e. the control response to adrenaline, V_E increased and P_aCO_2 remained almost constant, showing just a slight decrease. In the presence of propranolol, adrenaline still increased V_E, but not by as much as in control, as seen in Figure 11 A. However P_aCO_2 increased.

Figure 13: The effect of adrenaline on hypercapnic ventilatory sensitivity (raw traces)

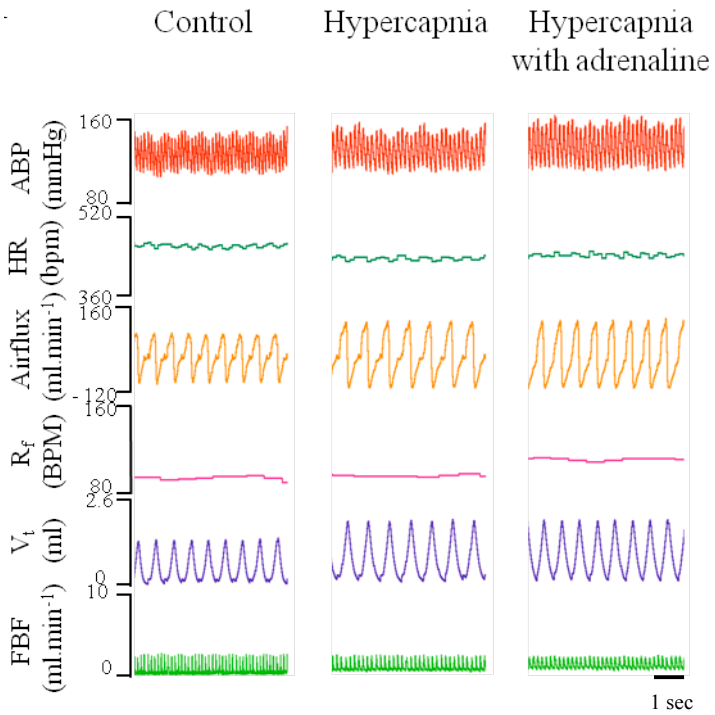

Figure 13: Raw traces showing the response to hypercapnia, the main effect of which was an increased V_t, and slightly increased R_f. HR and FBF also appear slightly decreased in this example, however none of the mean cardiovascular changes in response to hypercapnia were significant (data shown in Table 3, paired t-tests vs control, $P > 0.05$). The 3rd trace shows hypercapnia in the presence of adrenaline; V_t is increased as in the 1st hypercapnic response, but R_f increased further. HR and ABP appear increased compared to the 1st response, which may be accounted for by an effect of the adrenaline.

Figure 14: The effect of adrenaline on hypercapnic ventilatory sensitivity

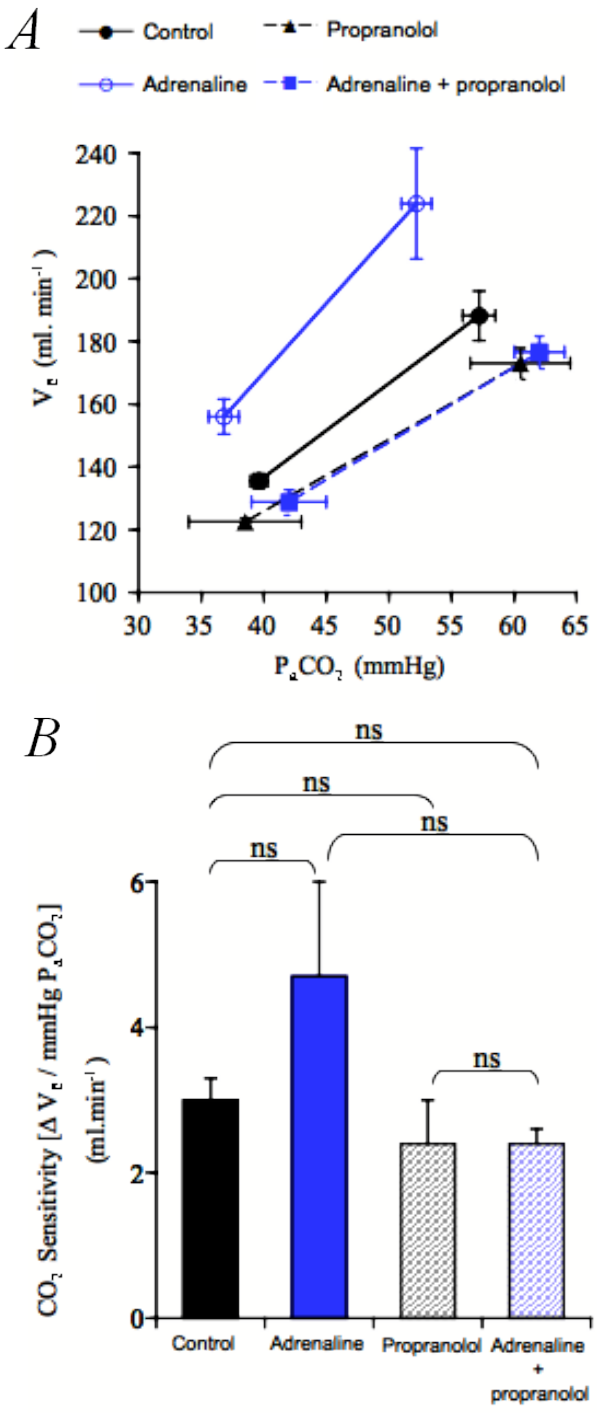

Figure 14: A- The effect of hypercapnia on ventilation under different conditions. Adrenaline increased basal ventilation and also increased the response to CO_2. Propranolol alone and with adrenaline reduced this response to below the control. B: The slopes of the hypercapnic responses (CO_2 sensitivity) shown as a bar chart, demonstrating that the increased response with adrenaline and decreased responses with propranolol (with and without adrenaline) were not significant (one-way ANOVA with post-hoc Bonferroni comparing each group, $P > 0.05$). Propranolol experiments were only carried out in $n = 2$, and so paired and unpaired t-tests were also carried out, also revealing no significance ($P > 0.05$).

Figure 15: The effect of hypoglycaemia, hyperoxia and propranolol, on ventilation (raw traces)

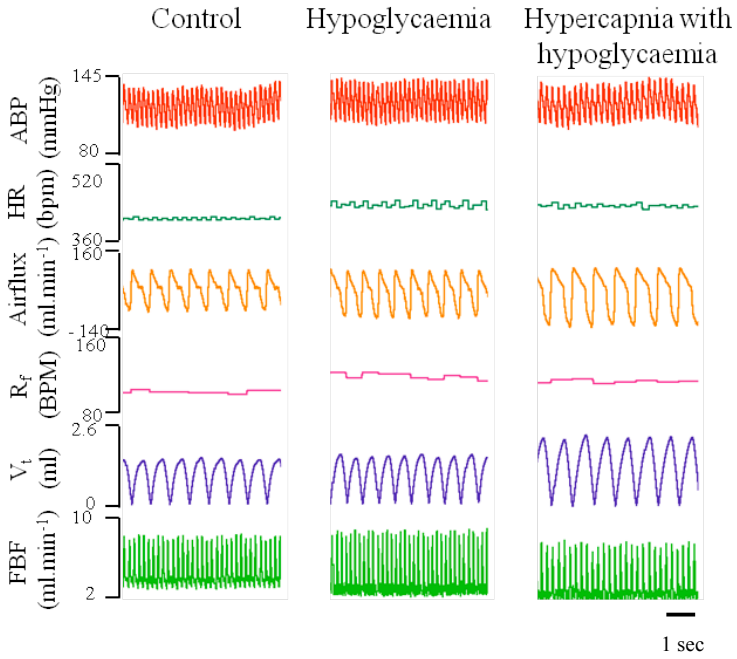

Figure 15: Raw traces showing the effect of hypoglycaemia; R_f increased, reflected in the V_t trace. ABP stayed constant, but HR and FBF showed an increase (3rd raw trace shows a hypercapnic response in hypoglycaemia – see Figure 18). However, as shown in Table 4, hypoglycaemia had no significant effect on the cardiovascular parameters (paired t-tests vs control, $P > 0.05$).

Figure 16: The effect of hypoglycaemia, hyperoxia and propranolol, on ventilation

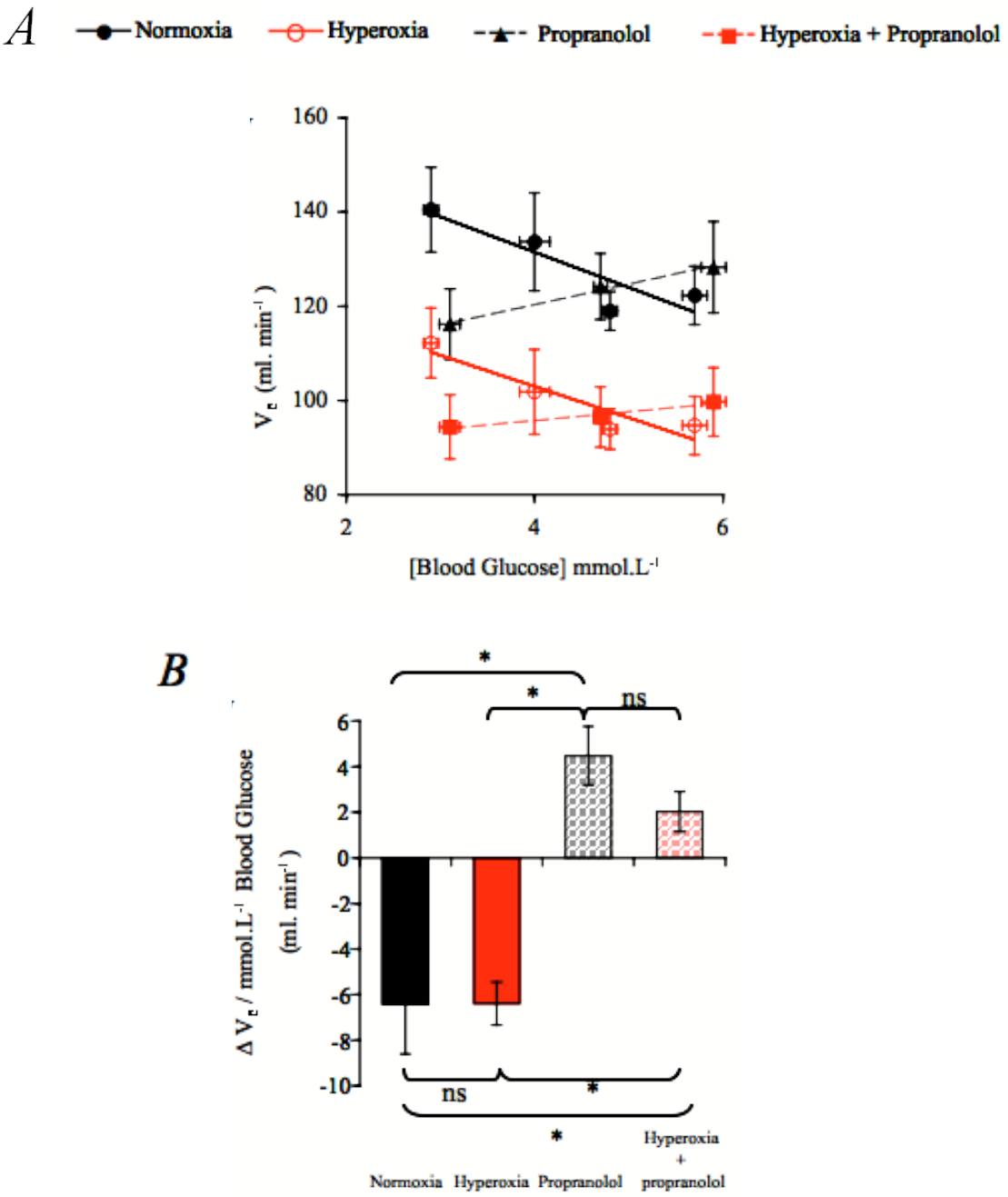

Figure 16: A - Linear regression lines showing basal ventilation and three levels of insulin-induced hypoglycaemia causing an increased V_E. Hyperoxia shifted the response down, but V_E still increased at each blood glucose level. Propranolol appeared to prevent the increase, with V_E actually decreasing. Hyperoxia and propranolol together showed an additive effect, shifting V_E down and preventing the increase. B: Slopes for each linear regression in A plotted as a bar chart. Normoxia (the control response to hypoglycaemia) vs hyperoxia, and propranolol vs hyperoxia with propranolol were not significantly different, but all other slopes were significantly different from one another (one-way ANOVA and post-hoc Bonferroni, $P < 0.05$) and from 0 (one-sample t-tests, $P < 0.05$).

Figure 17: The effect of hypoglycaemia on P_aCO_2

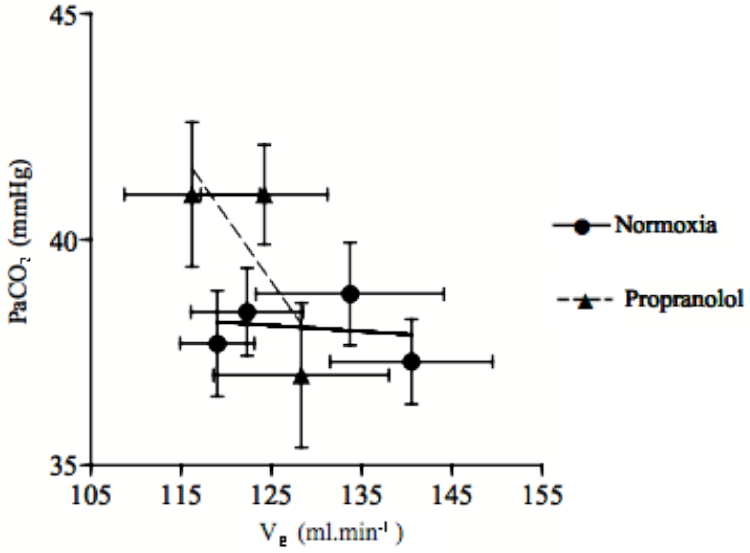

Figure 17: A scatter graph showing the association between the increased V_E caused by hyperinsulinaemic hypoglycaemia, and P_aCO_2. Under the normoxic conditions i.e. the control response to hypoglycaemia, V_E increased and P_aCO_2 remained constant. In the presence of propranolol, V_E decreased in spite of the hypoglycaemia, and P_aCO_2 increased. However, comparison of the slopes of the fitted linear regression lines showed no significant difference between the two conditions (unpaired t-test, $P > 0.05$), and that neither was significantly different from 0 (one-sample t-tests, $P > 0.05$).

Figure 18: The effect of hypoglycaemia on hypercapnic ventilatory sensitivity

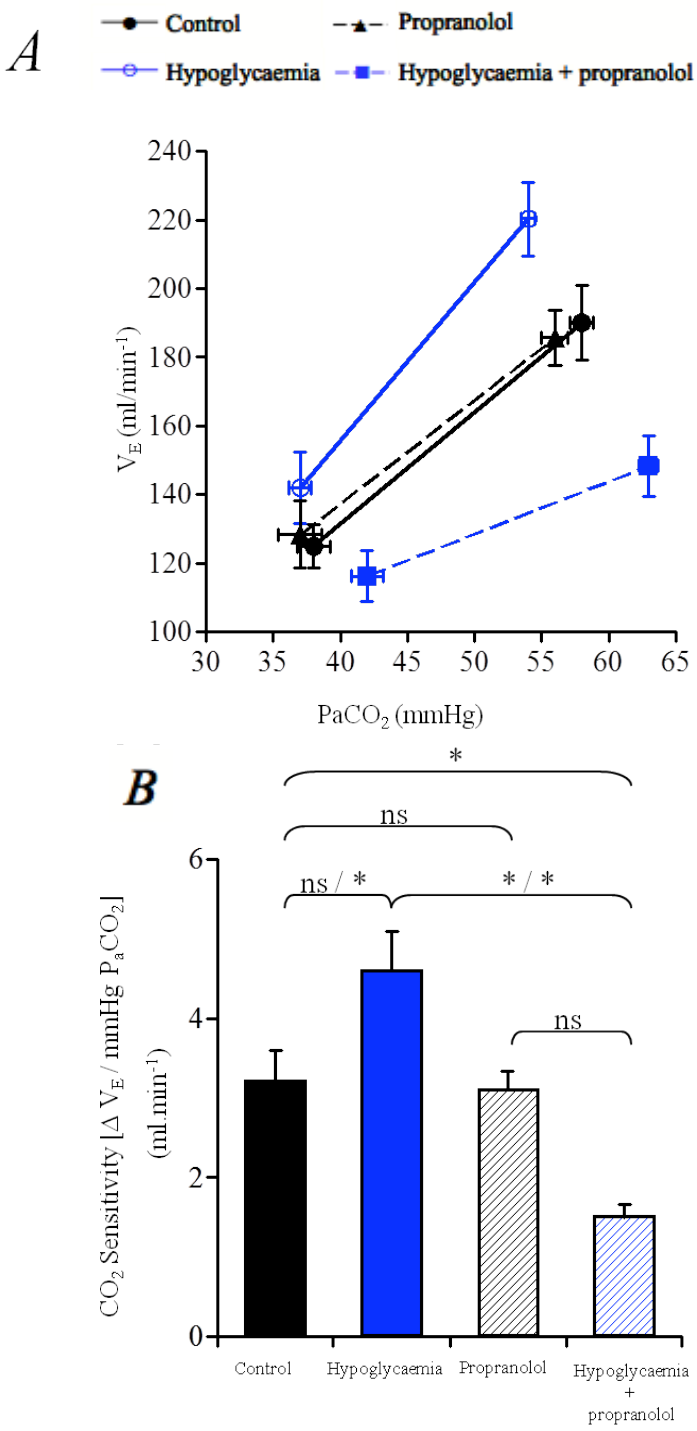

Figure 18: A - The control hypercapnic ventilatory response, and the corresponding responses in hypoglycaemia, with propranolol alone and in combination with hypoglycaemia. Hypoglycaemia increased V_E basally, and also showed an increased response to CO_2. The response with propranolol was not different from control, but the response during hypoglycaemia and propranolol infusion was blunted to below control levels. B: Slopes of each response line in A (CO_2 sensitivity), represented as a bar chart. The significances shown on the slopes are two-fold: one-way ANOVA with post-hoc Bonferroni showed no significant difference between the control vs hypoglycaemic, control vs propranolol and propranolol vs hypoglycaemia with propranolol CO_2 sensitivities, and significant differences between hypoglycaemic/control vs hypoglycaemic with propranolol sensitivities. A paired t-test between control and hypoglycaemia revealed a significant difference ($P < 0.05$), and an unpaired t-test between hypoglycaemia and hypoglycaemia with propranolol confirmed significance ($P < 0.05$).

5. Discussion

5.1 The effect of adrenaline on ventilation and the hypercapnic ventilatory response

Adrenaline caused a significant increase in V_E when administered as an intravenous infusion at 0.1 and 1μg.kg^{-1}min^{-1}, supporting a wealth of data regarding the action of adrenaline to increase respiration (e.g. Whelan and Young, 1953, Young, 1957, Joels and White, 1968, Heistad *et al*, 1972, Maskell *et al*, 2006). For example, Whelan and Young (1953) showed an increased R_f and V_t after intravenous administration of adrenaline at 10-20μg.min^{-1}, which was at its largest during the initial 5 minutes, in line with the infusion length allowed here before respiratory measurements were made. The choice of dose in the present study is justified by the performance of a dose-response curve to observe the effect of a range of doses upon ventilation. The literature reports use of a wide range of doses, although it has been reported that the respiratory response is more readily seen at smaller (μg) doses (Young, 1957), as demonstrated by Linton *et al* (1992), who showed an increased V_E at 1μg.kg^{-1}min^{-1}, but no effect at 0.1 μg.kg^{-1}min^{-1}. No significant effect of adrenaline at the doses used in the present study was seen upon cardiovascular parameters. A slight increase in FVC was seen, which coupled with the lack of effect on blood pressure suggests that the V_E response seen to adrenaline is a direct action on the CB cells rather than an indirect effect via reducing CB blood flow.

Hyperoxia was used to isolate any CB-mediated input to the response to adrenaline, and was seen to reduce the offset in the ventilatory response to adrenaline. However hyperoxia did not block the action of adrenaline (as observed by Joels and White, 1968, and Heistad *et al*, 1972), as V_E still increased during adrenaline infusion. Propranolol significantly decreased HR, as well as MABP, demonstrating its widely reported role as a β-adrenoreceptor antagonist. The dose used was the same as that used by Maskell *et al* (2006). The cardiovascular data recorded during concurrent adrenaline and propranolol infusion was inconclusive. HR was still reduced

compared to control and not significantly different to propranolol alone, however MABP increased and FVC increased significantly. This either suggests that the β block was incomplete and so the β action of adrenaline to cause vasodilatation was still able to occur, or that an α action of adrenaline was responsible. For example adrenaline could have acted via α receptors to increase MABP (by vasoconstriction), activating a baroreceptor reflex resulting in decreased sympathetic output to muscle. This would cause vasodilatation and therefore an increased FVC. Another possibility is that an α-mediated metabolic action of adrenaline resulted in release of some by-product which was responsible for the cardiovascular changes. Propranolol showed a similar effect to hyperoxia on V_E, except at 1 $\mu g.kg^{-1}min^{-1}$ adrenaline where the V_E response appeared slightly (but not significantly) reduced.

Taken together, these data suggest that the action of adrenaline on V_E is CB independent, or that O_2 and adrenaline act via independent pathways within CB. This latter possibility is supported by the fact that the response with hyperoxia and propranolol in combination was additive rather than multiplicative, suggesting no interaction between pathways. Previous studies have supported this idea of independent transduction pathways within the CB, for example Zhang *et al* (2007) showed that the *in vitro* response to hypoglycaemia and hypoxia/hypercapnia were additive, and Garcia-Fernandez *et al* (2007) showed that a metabolic inhibitor blocked the response to hypoxia but not to low glucose.

It must also be considered whether the hyperoxia applied here was sufficient to ablate CB function, as Kumar *et al* (2007) reported that a tonic *in vivo* firing could still be seen at PO_2s in excess of 400mmHg, whilst Biscoe *et al* (1970) reported in vivo single afferent fibre firing up to 600mmHg. Therefore it is likely that the > 300 mmHg P_aO_2 utilised here did not fully ablate CB function, explaining why hyperoxia did not completely prevent the response to adrenaline. This is supported by Heistad *et al* (1972) who described, in man, that 100% O_2

could block the increased ventilation seen in response to an i.v. infusion of a β-agonist. Additionally they showed that propranolol could also block this ventilatory response, thus further supporting a β- and CB-mediated increase in V_E.

Although we were unable to measure metabolic rate in our study, we found that the increased ventilation in response to adrenaline was independent of P_aCO_2 thus suggesting that the increased V_E seen was an appropriate hyperpnoea in response to increased metabolic rate, rather than a hyperventilation, as observed by Bin Jaliah et al (2004) and Maskell *et al* (2006). Whelan and Young (1953) reported a fall in alveolar CO_2 and increased O_2 consumption during adrenaline infusion, which they described as calorigenic i.e. metabolic, although they also suggested the increased respiration may have been independent of the increased metabolic rate.

It is known that increased metabolism can be induced by adrenaline via both adrenoreceptor groups. α receptors activate downstream pathways inducing liver and adipose tissue glycogenolysis and gluconeogenesis, inhibition of insulin release and stimulation of glucagon release from the pancreas. Stimulation of β receptors (via increased cAMP levels) can also lead to increased glucagon release and lipolysis (Rang and Dale, 2006). Hence metabolic effects of adrenaline can be primarily attributed to α-action, whilst the respiratory actions are suggested to be via β receptors, as demonstrated by Heistad *et al* (1972) and Maskell *et al* (2006). Therefore, in the current study, adrenaline should have increased both V_E and metabolism, but the primary effect of propranolol would have been upon V_E. However the α-actions of adrenaline to increase metabolism would have been unaffected (Rang and Dale, 2006, Yeo and Sawdon, 2007), as supported by the fact that blood glucose still increased in the presence of propranolol (although this was not significant, potentially due to the higher baseline levels seen with propranolol alone). This would explain the increased P_aCO_2 seen during β-block. This rise in P_aCO_2 would be expected to stimulate central chemoreceptors, and

this increased central chemoreceptor drive is the most likely explanation for the increased V_E which was still seen during adrenaline infusion despite the presence of propranolol. These data, coupled with the lack of cardiovascular effects of adrenaline i.e. no α-mediated increase in ABP and no β-mediated increase in HR, but just a slight increase in FVC, suggests that different tissues can have different sensitivities to adrenaline, possibly via different adrenoreceptor subtypes.

Adrenaline showed a trend to increase CO_2 sensitivity in the present experiment, increasing the hypercapnic ventilatory response, which could subsequently be reduced by propranolol. Maskell *et al* (2006) have also shown that adrenaline (at a higher dose of $10\mu g.kg^{-1}min^{-1}$) increases CO_2 sensitivity, and that this was abolished by CSNX, hence it was CB-mediated. They also showed the increased chemosensitivity was blocked by infusion of propranolol ($0.3mg.kg^{-1}min^{-1}$, as used here), supporting the present data, and showing that the effect was β-mediated. Along with the present data showing no change in P_aCO_2 with the increased V_E associated with adrenaline infusion, and that this hyperpnoea could be prevented by propranolol, the trend for an increased CO_2 sensitivity supports the hypothesis by Maskell *et al* (2006); that adrenaline may be one of the blood-borne factors that mediate hyperpnoea by an action on CO_2 chemosensitivity.

5.2 The effect of hyperinsulinaemic hypoglycaemia on ventilation

A decreased blood glucose caused an increased V_E, with a linear regression slope significantly different to zero. This has been shown previously by Bin Jaliah *et al* (2004, 2005) and Ward *et al* (2007). Bin Jaliah *et al* (2004) isolated the role of the CB, showing reduced basal V_E and no increased V_E in response to hypoglycaemia in CSNX animals, supporting a role for the CB in mediating the increased V_E seen.

It has previously been shown that hypoglycaemia is associated with an increased release of counter-regulatory hormones including adrenaline (Vollmer *et al*, 1997, Koyama *et al*, 2000, 2001, Ward *et al*, 2007, Yeo and Sawdon, 2007, Wehrwein *et al*, 2010). Both the increased V_E and counter-regulatory hormone release has been shown to be reduced by CBR (Koyama *et al*, 2000, 2001) or application of hyperoxia (Wehrwein *et al*, 2010), suggesting a CB-dependency of the effect. This mechanism explains the need for an increased glucose infusion to clamp plasma glucose during hyperinsulinaemia, in absence of CB input (Koyama *et al*, 2000, 2001, Wehrwein *et al*, 2010). These findings are consistent with the results of the present study, where hyperoxia reduced the ventilatory response to hypoglycaemia. However, hyperoxia did not block the response, with a constant difference between normoxic and hyperoxic V_E at each blood glucose resulting in a slope that was offset, but not different from control (normoxia). However propranolol was able to abolish the increase in V_E that was seen in hypoglycaemia. This suggests that the rise in V_E associated with hypoglycaemia is β-dependent. Taken in combination with the hyperoxia data, this suggests that the increased V_E in hypoglycaemia is either a CB independent mechanism, or that an O_2-independent pathway exists within the CB for sensing hypoglycaemia (supported by Garcia-Fernandez *et al*, 2007 and Zhang *et al*, 2007, as discussed above). The fact that the effect of hyperoxia and propranolol together appeared additive (slope not significantly different from propranolol alone), supports this idea of independent pathways within the CB.

As mentioned above, given the reports that *in vivo* CB afferent firing can still be seen at P_aO_2s of up to 400-600mmHg (Kumar, 2007, Biscoe *et al* 1970), it is likely that the level of hyperoxia used here did not fully ablate CB function, offering further explanation as to why a V_E response to hypoglycaemia was still seen and supporting a CB-dependent mechanism.

This is further supported by the evidence for a CB role in glucosensing (see section 1.4), and it therefore seems unlikely that these data represent a CB-independent ventilatory response to hypoglycaemia.

The data therefore disagrees with the results of *in vitro* studies that suggest the CB responds to low glucose directly (Pardal and Lopez-Barneo, 2002, Lopez-Barneo, 2003, Garcia-Fernandez *et al*, 2007, Zhang *et al*, 2007). In addition the present data contradicts the speculations from *in vivo* studies such as Koyama *et al* (2000, 2001) and Wehrwein *et al* (2010), which suggest, but provide no direct evidence for, low glucose acting directly on the CB. It also offers *in vivo* support for those *in vitro* studies that showed no effect of low glucose on chemoafferent discharge; despite showing an *in vivo* increase in V_E during hypoglycaemia, which was ablated by CSNX (Bin Jaliah *et al*, 2004, 2005). Based on this data, Bin Jaliah *et al* (2004, 2005) suggested a potential role for one of the associated counter-regulatory hormones as a blood-borne mediator sensed by the CB, a possibility that was also recognised by Ward *et al* (2007). Given the present data showing that propranolol was able to block the V_E response to hypoglycaemia, this blood-borne mediator may be adrenaline.

The relationship seen between hypoglycaemia and P_aCO_2 suggests that the increased V_E seen was a hyperpnoea rather than a hyperventilation, as P_aCO_2 remained constant. This suggests that the hyperinsulinaemic hypoglycaemia caused an increased metabolism, which is consistent with previous findings by Bin Jaliah *et al* (2004, 2005). In the present study, P_aCO_2 fell slightly, as was also seen in response to adrenaline. However, this fall does not appear large enough to be explained by a hyperventilation. For example, a fall in P_aCO_2 of around 5mmHg was observed in response to a 20% increase in V_E generated in response to graded hypoxia (i.e. a hyperventilation) (Marshall and Metcalfe, 1988), whereas we only observed a fall of around 1mmHg in response to a hypoglycaemia- (and presumably adrenaline-)

mediated 15% increase in ventilation. This is in line with data reported by Forster and Pan (1994), where P_aCO_2 only changes by 1-2mmHg in human exercise (a situation of increased metabolism).

In the presence of propranolol, both hypoglycaemia and adrenaline did not induce as much of an increased V_E, and P_aCO_2 increased. This suggests that propranolol prevented the increase in V_E that is usually matched to the increased metabolism i.e. it occurs via a β-mediated mechanism, whilst metabolism was still able to increase via the action of adrenaline at α receptors, as well as via the other associated counter-regulatory hormones in hypoglycaemia. However, the data presented did not show statistically significant results, possibly due to the high variability associated with the V_E data.

The effect of hyperinsulinaemic hypoglycaemia on hypercapnic ventilatory sensitivity

Bin Jaliah *et al* (2005) showed an increased CO_2 chemosensitivity during hypoglycaemia which was reduced by ~75% after CSNX, presenting a possible CB-mediated mechanism by which P_aCO_2 could remain constant during increased metabolism. Increasing the sensitivity of the chemoreceptors for CO_2 would lead to an increased V_E, facilitating matching to the increased CO_2 production associated with increased metabolism. This was demonstrated in the present study, where the ventilatory response to hypercapnia was increased during hypoglycaemia, showing a significantly increased CO_2 sensitivity. Bin Jaliah *et al* (2005) also showed that a fall in glucose concentration decreased CO_2 sensitivity in an *in vitro* CB preparation, suggesting that the *in vivo* increase in CO_2 sensitivity and hyperpnoea was not via a direct action of low glucose. This was supported by the present finding that propranolol was able to block, and even significantly reduce, the increased CO_2 sensitivity associated with hypoglycaemia, suggesting a β-mediated mechanism i.e. adrenaline.

5.3 Comparison of the responses to adrenaline and hypoglycaemia

The response to application of exogenous adrenaline and hyperinsulinaemic hypoglycaemia are similar; both causing an increase in V_E of similar magnitude (~20ml.min^{-1}). Both responses were affected similarly by application of hyperoxia, i.e. V_E was reduced, but still showed an increase with increasing stimulus intensity. This may be explained by the fact that the CB was not completely switched off by the hyperoxia, or, based on the assumption that CB activity was reduced, suggests that the responses were either independent of the CB or sensed via O_2 independent pathways within the CB. Propranolol had differing effects, causing a small but non-significant decrease in the V_E response to 1μg.kg^{-1}min^{-1} adrenaline, but causing a complete attenuation of the increased V_E caused by hypoglycaemia, with V_E even decreasing. The application of hyperoxia and propranolol in combination had an additive effect in both experiments, supporting the idea of independent pathways within the CB for different stimuli i.e. O_2 and adrenaline/low glucose.

The maintenance of P_aCO_2 during the increased V_E caused by both adrenaline and hypoglycaemia, suggested that metabolic rate had increased and the observed increase in V_E was a hyperpnoea as opposed to a hyperventilation. In the presence of propranolol in both experimental groups, P_aCO_2 showed a tendency to rise, although this did not reach statistical significance in either group. These data suggest that metabolism rises via an α-receptor mechanism, whilst matching V_E to this metabolism is β-dependent.

Finally, CO_2 sensitivity was increased during infusion of adrenaline and significantly during insulin-induced hypoglycaemia, which would provide a mechanism for the ability to maintain isocapnia in the face of increased metabolism.

Based on these findings and the knowledge that adrenaline is a major counter-regulatory hormone released in response to hypoglycaemia (Vollmer *et al*, 1997, Koyama *et al*, 2000, 2001, Yeo and Sawdon, 2007, Ward *et al*, 2007, Wehrwein *et al*, 2010), it would not be unreasonable to conclude that the hyperinsulinaemic hypoglycaemia had caused endogenous release of adrenaline, and that this adrenaline caused the respiratory responses observed via β-receptors, as the response was reduced by propranolol. When taken alongside previous studies already discussed, which have suggested the CB does not respond to low glucose directly (e.g. Almaraz *et al*, 1984, Conde *et al*, 2007, Fitzgerald *et al*, 2009) but to some other blood borne mediator (Bin Jaliah *et al*, 2004, 2005) and that adrenaline can increase V_E and CO_2 sensitivity via CB-dependent mechanisms (Heistad *et al*, 1972, Maskell *et al*, 2006), it may be suggested that this data demonstrates a β-receptor and CB-dependent role for adrenaline in situations of increased metabolism.

A physiological example of such a situation is exercise. The CBs have been shown to play a neuroendocrine role during exercise, as demonstrated by Koyama *et al* (2001). They showed that CBR dogs had reduced levels of glucagon and noradrenaline released during exercise compared to sham animals, and a mismatch between glucose use and production, thus also linking back to the proposed role in glucoregulation.

An immediate matching of V_E to metabolism is seen in exercise, maintaining isocapnia or slight hypocapnia, as well as normoxia and normal arterial pH. Although peripheral chemoreceptors are not thought to be the major mediators of the hypercapnic response, they do give a rapid response to increased PCO_2, increasing V_E. The timing of this response therefore fits with the almost immediate increase in V_E seen during exercise. Based on the present experiment and literature, this fast response to increased CO_2 from increased metabolism may be mediated by an increased CB CO_2 chemosensitivity, which is potentially mediated by adrenaline, either directly or indirectly via increased sympathetic activity.

Further implicating the CB in exercise hyperpnoea is the evidence that the response is depressed in CBR subjects compared those with intact carotid bodies (Honda, 1985, Wasserman *et al*, 1975).

The ventilatory response to exercise is in three phases: phase I shows the rapid increase in V_E that occurs at the start of the exercise, or even in anticipation before exercise begins, phase II; an exponential increase in V_E during dynamic exercise, and phase III; a steady-state of V_E.

It is recognised that the major cardiovascular and respiratory controller in exercise is a central feed-forward mechanism. Afferent reflexes then provide additional input e.g. respiratory muscle metaboreceptors, locomotor muscle afferents and the CBs (for review see Dempsey, 2012). It has been proposed that the CBs are involved in phases II and III, maintaining P_aCO_2 and preventing hypoxia, providing around a 20% proportion of the ventilatory drive in phase III (Ward, 1994, Whipp, 1994). However, Dempsey and Smith (1994) have shown in various animal models that the CB inhibits respiratory motor output during heavy exercise, and Forster and Pan (1994) ascribed the CB a role in 'fine tuning' ventilation during submaximal exercise, but no major role in exercise hyperpnoea.

5.4 A possible role for potassium

It has long been shown that adrenaline can cause a decrease in plasma K^+ levels (D'Silva, 1934), although some have reported an increase in K^+ associated with adrenaline infusion (Linton *et al*, 1992). It has been observed that this plasma K^+ lowering action of adrenaline is via a β_2-receptor dependent action on the Na^+/K^+ ATPase pump, moving K^+ into cells (Clausen, 1983). This is particularly important in exercise, when K^+ leaks from cells and the adrenaline released in association with exercise acts to counter this hyperkalaemia (Lancet editorial, 1983, Clausen, 1983). Insulin can have the same effect as adrenaline on K^+, as observed by Bin Jaliah *et al* (2004), who reported a decrease in arterial K^+ levels during a

hyperinsulinaemic clamp protocol. However this is via a non-β dependent mechanism (Minaker and Rowe, 1982).

In relation to the current experiment, adrenaline infusion would have driven K^+ into cells, causing hyperpolarisation of all cells, including CB type I cells. This would increase the threshold for excitation of the CB and may explain why the dose-response of adrenaline against V_E was complex. In the presence of propranolol, any direct β effect of adrenaline on the CB to increase V_E would be lost. Additionally, the action of adrenaline on the Na^+/K^+ ATPase acting via β_2 receptors would also be blocked, thus unmasking any effect of K^+. This is supported by the observation that β-blockers can cause hyperkalaemia, especially during exercise when adrenaline levels are increased (Clausen, 1983). Band and Linton (1986) showed that an infusion of KCl produced hyperkalaemia in cats similar to that seen during exercise, resulting in a significantly increased carotid body chemoafferent firing. The K^+ levels increased the level and amplitude of oscillations in CB afferent firing which aligned with respiration and have previously been suggested to play a role in the control of breathing during exercise (Band *et al*, 1978, 1980, Kumar *et al*, 1988). It was therefore suggested that K^+ may underlie this role (Band and Linton, 1986, Nye, 1994). This may explain why adrenaline still caused an increase in V_E during propranolol infusion.

Insulin also drives K^+ plasma levels down in a dose-dependent manner, via the Na^+/K^+ ATPase, but via a non-β mediated mechanism (Minaker and Rowe, 1982). This means that the hyperinsulinamic hypoglycaemia would have also been accompanied by a hypokalaemia, which would then be further accentuated by the release of counter-regulatory adrenaline. Hence the increased V_E seen was a balance between the effect of increased adrenaline and low K^+. When propranolol was given in hypoglycaemia, the effect of adrenaline on K^+ would be removed; however the β-independent effect of insulin would still be present, meaning that

plasma K^+ levels would continue to decrease. In this situation, propranolol is presumably blocking the adrenaline drive to V_E, and insulin is reducing the K^+ drive (unlike the situation with adrenaline and propranolol). This may therefore offer an explanation as to why V_E was seen to decrease with propranolol during hypoglycaemia.

5.5 Methodological limitations

Limitations are recognised within the experiments carried out. For instance a control hyperinsulinaemic euglycaemic clamp was not carried out to control for effect of the high insulin infusion itself. However this has been carried out previously by co-workers (Bin Jaliah *et al*, 2004, 2005) who showed no direct effect of insulin on the CB.

A steady P_aCO_2 during an increased V_E was taken to indicate a hyperpnoea in the presence of increased metabolism. However, use of plethysmography or a spirometer attached to a sealed system would have allowed for evaluation of O_2 consumption or CO_2 production, which are indicators of metabolic activity.

Total V_E was used to here to represent the respiratory response to adrenaline, hypoglycaemia and hypercapnia; however there was no determination of physiological dead space. The amount of gas that was available for gas exchange, the alveolar V_E (V_A) would have been smaller than the data reported. We did not record this and therefore have had to assume V_A to be a constant fraction of total V_E.

Hyperoxia was used in this investigation to 'chemically denervate' the CB. However it has been shown that even at a P_aO_2 of 400-600mmHg, some *in vivo* basal firing remains (Kumar, 2007, Biscoe *et al*, 1970) and if CO_2 is not controlled for, there will still be CB activity (see section 1.2 - hypercapnia). Therefore it must be accepted that the hyperoxia used here

probably reduced but did not ablate CB activity. This may explain why hyperoxia did not prevent the V_E increases seen in response to adrenaline and hypoglycaemia, supporting a role for the CB in mediating the observed responses.

6. Future work

Based on the findings of the current study, several pieces of future work could be carried out to help consolidate the conclusions and address the ideas raised by the data:

Measurement of plasma adrenaline from the blood samples taken during the hyperinsulinaemic hypoglycaemic ramp should be carried out in order to confirm the release of adrenaline and compare the concentration seen to the dose given exogenously in the first set of experiments. Koyama *et al* (2000) reported adrenaline concentrations of around 300pg.ml^{-1} in response to hypoglycaemia, much lower than the exogenous dose of 1μg.kg^{-1}min^{-1} administered here. It may also be prudent to repeat the adrenaline dose-response protocol, taking blood samples at each dose to measure the concentration of adrenaline in the blood, e.g. using HPLC as performed by Maskell *et al* (2006). The exogenous dose given would have been subject to possible uptake mechanisms and metabolism, and so does not represent the circulating levels.

The experiments which used hyperoxia to isolate the role of the CB could be repeated using CSNX or CBR, surgically removing CB input and allowing comparison with the current results. This would determine whether the level of hyperoxia used was ablating all of the CB input and therefore if the increased V_E and CO_2 sensitivity seen during exogenous adrenaline infusion and hyperinsulinaemic hypoglycaemia are CB-dependent mechanisms.

The experiments could also be repeated using a β-agonist such as isoprenaline, in order to reconcile the inconclusive cardiovascular data seen here with adrenaline and propranolol.

Considering the potential role of K^+ in the increased V_E seen with adrenaline and hypercapnia, an improvement for future experiments would be to have the ability to measure K^+ levels. This can often be determined from the sample taken for blood gas analysis, and so would not require further blood sampling or an increased protocol time.

As it is known that the CB possesses functional plasticity, changes in chemoreceptor sensitivity may occur in response to conditions with exposure to chronic/intermittent hypoxia such as obstructive sleep apnoea. These diseases may be associated with the co-morbidity of diabetes, presenting an opportunity for the possible alteration of the glucoregulatory response. Investigation of such conditions in terms of CB function may offer new therapeutic targets.

7. Conclusion

This study provides evidence for a role of adrenaline, a counter-regulatory hormone released in response to hypoglycaemia, in the hyperpnoea of hypermetabolism. The data generated thus supports a body of literature that suggests that the ventilatory response to hypoglycaemia is not mediated by a direct action of low glucose on the CB, but the action of an associated blood-borne mediator.

The observation that CO_2 sensitivity was augmented during adrenaline infusion and hypoglycaemia, offers a mechanism by which V_E could be matched to metabolism in the absence of a change in P_aCO_2. Reduction of the increased V_E, CO_2 sensitivity and hyperpnoea by the β-antagonist propranolol confirms the β-dependence of the mechanism. Further experiments are required to confirm whether adrenaline acts via the CB, in an O_2 independent manner or whether the level of hyperoxia used was able to fully isolate the role of the CB.

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