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DOI: 10.1113/JP272191
10.1113/JP272191

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Document Version
Peer reviewed version

Citation for published version (Harvard):

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Checked April 2016

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Author Conflict: No competing interests declared

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Adrenaline release evokes hyperpnoea and an increase in ventilatory CO$_2$ sensitivity during hypoglycaemia: a role for the carotid body

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Key words: Adrenaline, Carotid body, Hypoglycaemia

Word count (excluding references and figure legends): 4747

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Table of contents category: Integrative

Key points summary

- Hypoglycaemia is counteracted by release of hormones and an increase in ventilation and CO$_2$ sensitivity to restore blood glucose levels and prevent a fall in blood pH.
- The full counter-regulatory response and an appropriate increase in ventilation is dependent on carotid body stimulation.
• We show that the hypoglycaemia-induced increase in ventilation and CO₂ sensitivity is abolished by preventing adrenaline release or blocking its receptors.

• Physiological levels of adrenaline mimicked the effect of hypoglycaemia on ventilation and CO₂ sensitivity.

• These results suggest that adrenaline, rather than low glucose, is an adequate stimulus for the carotid body mediated changes in ventilation and CO₂ sensitivity during hypoglycaemia to prevent a serious acidosis in poorly controlled diabetes.
Abstract

Hypoglycaemia in vivo induces a counter-regulatory response that involves the release of hormones to restore blood glucose levels. Concomitantly, hypoglycaemia evokes a carotid body mediated hyperpnoea that maintains arterial CO2 levels and prevents respiratory acidosis in the face of increased metabolism. It is unclear whether the carotid body is directly stimulated by low glucose or by a counter-regulatory hormone such as adrenaline. Minute ventilation was recorded during infusion of insulin-induced hypoglycaemia (8-17 mIU kg⁻¹ min⁻¹) in Alfaxan-anaesthetized male Wistar rats. Hypoglycaemia significantly augmented minute ventilation (123±4 to 143±7 ml min⁻¹) and CO2 sensitivity (3.3±0.3 to 4.4±0.4 ml min⁻¹ mmHg⁻¹). These effects were abolished by either β-adrenoreceptor blockade with propranolol or adrenalectomy. In this hypermetabolic, hypoglycaemic state, propranolol stimulated a rise in PaCO₂, suggestive of a ventilation-metabolism mismatch. Infusion of adrenaline (1µg kg⁻¹ min⁻¹) increased minute ventilation (136±4 to 161±5 ml min⁻¹) without altering PaCO₂ or pH and enhanced ventilatory CO2 sensitivity (3.4±0.4 to 5.1±0.8 ml min⁻¹ mmHg⁻¹). These effects were attenuated by either resection of the carotid sinus nerve or propranolol. Physiological concentrations of adrenaline increased the CO2 sensitivity of freshly dissociated carotid body type I cells in vitro. These findings suggest that adrenaline release can account for the ventilatory hyperpnoea observed during hypoglycaemia by an augmented carotid body and whole body ventilatory CO2 sensitivity.

Abbreviations: ABP, arterial blood pressure; Adr, adrenaline; AdrX, adrenalectomy; CB, carotid body; CSNX, carotid sinus nerve section; HC, hypercapnia; HG, hypoglycaemia; Prop, propranolol; fR, respiratory frequency; Ê, minute ventilation; VT, tidal volume
Introduction

In mammals, regulation of arterial blood glucose is an essential process that allows for maintenance of glycolysis and ATP production. The counter-regulatory response to hypoglycaemia (HG) is highly integrated and involves a number of neuro-endocrine responses arising from stimulation of multiple peripheral and central glucose sensors including the gastrointestinal tract, portal mesenteric vein, hypothalamus and hindbrain (Bohland et al., 2014). More recently it has been suggested that, in addition to their more recognised role as hypoxia sensors (Kumar & Prabhakar, 2012) the carotid body (CB) chemoreceptors may also play a key role in mediating the counter-regulatory response to HG in both animals and humans (Alvarez-Buylla & de Alvarez-Buylla, 1988; Koyama et al., 2000; Wehrwein et al., 2010; Limberg et al., 2015; Wehrwein et al., 2015) with corrective reflexes being attenuated by carotid body ablation either surgically or chemically. Although an assumption has been made that the reduction in plasma glucose concentration is directly sensed by the carotid body, this remains controversial and the exact stimulus that excites the CB during HG in vivo remains undetermined.

Thus, the evidence that long-term, reduced in vitro CB preparations are acutely stimulated by low glucose (Pardal & Lopez-Barneo, 2002; Garcia-Fernandez et al., 2007; Zhang et al., 2007) has been countered by findings that freshly isolated intact CBs and dissociated type I cells lack any inherent low glucose sensitivity (Bin-Jaliah et al., 2004; Conde et al., 2007; Kim et al., 2011; Gallego-Martin et al., 2012; Holmes et al., 2014). In view of this, it has been suggested that the CB-mediated reflex changes observed in HG may be an indirect action in response to a systemically released hormone(s) rather than low glucose per se (Kumar, 2007; Ribeiro et al., 2013; Holmes et al., 2014). HG is known to promote the release of glucagon, cortisol, noradrenaline (NA) and adrenaline (Adr) (Ward et al., 2007; Wehrwein et al., 2010). Of these, NA and Adr are recognised respiratory stimulants; exogenous infusion of NA, Adr or adrenergic agonists increase minute ventilation in humans (Heistad et al., 1972; Butland et al., 1982) and similar findings have been reported in animals (Joels & White, 1968; Folgering et al., 1982; Hauton et al., 2013). This ventilatory response can be attenuated
by Propranolol (Prop), carotid sinus nerve section (CSNX) and hyperoxia, consistent with a role for both β-adrenoreceptors and CB chemoreceptors (Joels & White, 1968; Folgering et al., 1982). Subcutaneous injection of Adr also increases CB cAMP content, an effect attributed to β-adrenoreceptor stimulation (Mir et al., 1983). Additionally, Adr augments CO₂ production as evidenced by increases in the respiratory exchange ratio (RER), the rate of glycogen breakdown and metabolism in skeletal muscle (Watt et al., 2001). These actions of Adr may account for the reflex hyperpnoea previously observed in HG and which has been shown to be critically dependent on an increase in CB chemoafferent activity mediated by an increase in CB CO₂ sensitivity (Bin-Jaliah et al., 2004, 2005). As yet, however, a potential role for Adr in evoking the hyperpnoea and enhanced CB CO₂ sensitivity during HG has not been investigated.

Using whole animal in vivo experimentation we show here that, during HG, Adr released from the adrenal medulla is sufficient to evoke hyperpnoea by increasing ventilatory CO₂ sensitivity. Adr infusion mimics the effect of HG and enhances the CB type I cell response to CO₂. Thus, the importance of CB activation by Adr during HG appears not only to help restore arterial blood glucose levels but also to control PₐCO₂ and pH, thereby preventing respiratory acidosis.
Methods

Ethical approval

The following procedures on animals were carried out in line with the current Home Office (UK) and University of Birmingham guidelines on ethical use of animals. Food and water was available *ad libitum*, until food was withdrawn from 8 hours before experimentation to ensure stable basal blood glucose levels.

Anaesthesia and surgery

Adult male Wistar rats (Charles River Laboratories) were initially anaesthetised with 3-4% isoflurane in O₂ at 3-4 L min⁻¹ (Merial Animal Health Ltd). Following cannulation of the right jugular vein, isoflurane was removed and anaesthesia was maintained with i.v. Alfaxan® (Vétoquinol UK Ltd), at 17-20 mg kg⁻¹ h⁻¹ with 0.1 ml boluses as necessary. Core body temperature was maintained at 37°C with a homeothermic heat pad system.

The trachea was cannulated and a spirometer was attached to measure airflux; respiratory frequency (fᵣ), tidal volume (Vᵢ) and minute ventilation (Vₑ = fᵣ x Vᵢ) were derived from this. Animals breathed room air throughout, except when inspiratory gases were changed to induce hypercapnia (HC; 8% CO₂ for 5 min). The tracheal cannula was connected to a system of rotameters in a gas proportioner frame (CP Instruments Co. Ltd) allowing variation of inspiratory gases. The response to HC was calculated as the mean of the last 2 min of the exposure to HC after ventilation had stabilised.

Arterial Blood Pressure (ABP) was measured from the right brachial artery and heart rate (HR) was derived. Femoral blood flow (FBF; Transonic Systems Inc) was measured from the left femoral artery. Arterial blood samples were taken from the right femoral artery, utilising a looped cannula technique to reduce blood loss when sampling pure mixed arterial blood. Arterial blood was used to monitor blood glucose (0.6 µl;
Accu-Chek®, Aviva) and blood gases (150 µl; Gem® 4000 premier analyser, Instrumentation Laboratory Ltd). Arterial blood gases were measured prior to, and in the final 2 min of, the exposure to HC. Drug infusions were given via the right femoral vein.

For adrenalectomy (AdrX) experiments, a midline incision was made and the kidneys located. The adrenal glands were located bilaterally, vascularly isolated and removed. For CSNX experiments, the carotid artery bifurcations were isolated, the carotid sinus nerves identified and sectioned bilaterally at the junction with the glossopharyngeal nerve. Denervation was confirmed by the absence of a response to hypoxia (as described previously by Bin Jaliah et al 2004, 2005). Following surgery, a 40 min stabilisation period was allowed before commencing the experimental protocols.

Data were recorded using PowerLab and Labchart software (ADInstruments Ltd). At the end of the experiment, animals were killed by overdose of Euthatal® (pentobarbital sodium, 200 mg ml⁻¹, Merial Animal Health Ltd) and cervical dislocation.

**In vivo experimental protocols**

A diagrammatic representation of the protocols used in the in vivo experiments below can be seen in Figure 1.

**Group 1: Hyperinsulinaemic hypoglycaemia**

$V_E$ was recorded before and during insulin infusion (n=12; 304±7g). Insulin (Hypurin® Bovine Neutral, CP Pharmaceuticals Ltd) was diluted in Gelofusine® (4% w/v, Dechra Veterinary Products) and infused at 8-17 mIU kg⁻¹ min⁻¹ to induce HG (blood glucose = 3.1±0.1 mmol L⁻¹). Once blood glucose reached the target level, the insulin infusion rate was titrated to keep it at a stable level. The response to HC (to determine CO₂
sensitivity; $\Delta \dot{V}_E/\text{mmHg P}_a\text{CO}_2$) was recorded before and approximately 30 min after the insulin infusion was started. Blood glucose and gas samples were taken periodically throughout each experiment as described above. After recovery from HG, animals were assigned to one of two subgroups; one animal did not fully recover and so was not assigned to a subgroup, whilst an additional two animals were used in the development of the AdrX protocol.

Group 1A: Hyperinsulinaemic hypoglycaemia with propranolol

Prop was infused (0.3 mg kg$^{-1}$ min$^{-1}$; P0884 - Sigma®, n=6; 308±8g) for 10 min until HR stabilised. This dose was shown to significantly antagonise the effects of Adr infusion (see Figure 5C). The response to HC was repeated before and during a further insulin infusion.

Group 1B: Hyperinsulinaemic hypoglycaemia with adrenalectomy

Following AdrX, the response to HC was repeated before and during HG (n=7; 284±8g).

Group 2: Adrenaline infusion

Adr (E4250, Sigma®) was infused at 3 different concentrations (0.1, 1 & 10 µg kg$^{-1}$ min$^{-1}$) to determine a dose that increased $\dot{V}_E$ whilst minimising effects on ABP (see Figure 4). This dose (1 µg kg$^{-1}$ min$^{-1}$) was subsequently used for all other experiments: $\dot{V}_E$ (n=30; 321±7g) and the effect of HC (n=14) were recorded before and during Adr infusion. After recovery from Adr, animals were assigned to one of two subgroups; 8/30 animals were used only for the Adr dose-response experiments to develop the protocol.

Group 2A: Adrenaline infusion with propranolol

Prop was infused and basal $\dot{V}_E$ and the response to HC assessed as in Group 1A. These responses were repeated during combined Prop and Adr infusion (n=14; 333±12g).

Group 2B: Adrenaline infusion with carotid sinus nerve section
Following CSNX, basal $\dot{V}_E$ was recorded and the response to HC was repeated before and during Adr infusion (n=8; 312±7g).

**Carotid body type I cell isolation and Ca²⁺ imaging**

The following procedures comply with the National Institute of Health guide for the care and use of laboratory animals (NIH publications No. 80-23, revised 1996) and were approved by the Wright State University Institutional Laboratory Animal Care and Use Committee.

CBs were isolated from Sprague-Dawley rats (12-19 days old) anaesthetised with isoflurane (4.5% in O₂) and digested in an enzyme solution containing 0.4 mg mL⁻¹ collagenase type I (Worthington Biochemical Corporation) and 0.2 mg mL⁻¹ trypsin type I (Sigma) in DPBS with low CaCl₂ (86 µM) and MgCl₂ (350 µM), for 20 min at 37°C. CBs were mechanically dissociated with forceps and incubated for an additional 7 min. The tissue was centrifuged (115g) for 3 min, supernatant removed and the pellet re-suspended in tissue culture medium (Ham’s F12 (Sigma) supplemented with 10% heat inactivated fetal bovine serum (Biowest). Cells were released by trituration with fire polished silanized Pasteur pipettes (Sigmacote, Sigma). Type I cells were plated on 15 mm round poly-d-lysine coated (0.1 mg mL⁻¹) glass coverslips (Warner Instruments) and incubated at 37°C in 5% CO₂, 10% O₂, for at least two hours before being used. All experiments were performed within 8 hours of isolation.

Type I cells were loaded with 5 μM FURA-2AM (Invitrogen) in serum free F-12 Ham’s nutrient media for 30 min at room temperature in a humidified chamber at 5% CO₂, 10% O₂, before being transferred to FURA-2AM free media in the same conditions for at least 20 min before imaging. Coverslips were placed in an RC-25F (Warner Instruments) recording chamber (approximate bath volume 500 µL), with gravity fed pre-heated solution being maintained at 34-36°C with an in-line heater (SH-27F, Warner Instruments) with thermistor controlled feedback (TC-344B, Warner...
Instruments), and solution removed with a Rainin-Dynamax RP-1 peristaltic pump at maximum flow rate (8 mL min\(^{-1}\)). Although the chamber is open to ambient air, the gravity-fed, pump-emptied chamber is less than 500µL in volume and perfusate is flowing at ~8mL.min\(^{-1}\) allowing for less mixing with ambient air than might occur at slower flow rates. The PO\(_2\) in the superfusate in the chamber during gassing with 10% O\(_2\) (76 Torr) equilibrated Tyrodes was around 11% O\(_2\) (~85 Torr). Image acquisition was controlled by Metafluor software (Molecular Devices) and cells were visualized using a Nikon TE2000-U inverted microscope with a CFI super fluor x40 oil immersion objective. The FURA-2 loaded cells were excited by 50 ms exposures to 340/380 nm light using a Lambda 10-3 filter wheel (Sutter) and emitted light was recorded at 510 nm using a CoolSnap HQ2 CCD camera (Photometrics) - images were recorded every 5 s.

Cells were continuously perfused with a standard bicarbonate buffered Tyrode solution containing in mM: 117 NaCl, 4.5 KCl, 11 Glucose, 23 NaHCO\(_3\), 2.5 CaCl\(_2\), 1 MgCl\(_2\), equilibrated with 10% O\(_2\), 5% CO\(_2\), pH 7.4 at 37°C. By gassing solutions with 10% O\(_2\) we maintained a more physiological environment for the isolated type I cells. Previous work from multiple groups on isolated type I cells has gassed solutions with 21% O\(_2\), which is extreme hyperoxia. HC was induced by equilibrating extracellular solutions with 15% CO\(_2\), 10% O\(_2\), balance N\(_2\). Type I Cells were exposed to two successive 30 second episodes of HC, with the second being performed in the presence or absence of 10 nM Adr. The change in fluorescence ratio (F\(_{340}/F_{380}\)) from baseline to peak (delta ratio F\(_{340}/F_{380}\)) was measured for each challenge and the percentage change between the first and second exposure calculated. To minimise Adr oxidation to adrenochrome, Adr was prepared in solutions containing 1 mM ascorbic acid– final experimental concentration 125 nM.

**Data analysis**

All data is presented as the mean ± SEM. Statistics were carried out using GraphPad Prism 5 (GraphPad) and graphs were plotted using Deltagraph (Red Rock Software).
Significance (p<0.05) was tested using repeated measures or one-way ANOVA with post-hoc Bonferroni tests, and paired or unpaired t-tests as appropriate.
Results

**Ventilatory and cardiovascular responses to hypoglycaemia**

An example trace illustrating the effect of inducing HG on cardiovascular and respiratory variables is shown in Figure 2A. Reducing blood glucose levels from 6.1±0.3 mM to 3.1±0.1 mM induced an approximately 20% increase in minute ventilation ($\Delta V_E +20\pm4\text{ml.min}^{-1}$, n=12, p<0.05) with no concomitant increase in $P_a\text{CO}_2$, demonstrating a hyperpnoea (Figure 2B). The HG-induced increase in $V_E$ was blocked by Prop infusion ($\Delta V_E -12\pm3\text{ml.min}^{-1}$, n=6, p>0.05) and $P_a\text{CO}_2$ increased in 5 out of 6 rats, although this was not statistically significant (Prop 37±2mmHg vs Prop + HG 41±2mmHg, p=0.06). AdrX blocked the increase in $V_E$ initiated by HG ($\Delta V_E -1\pm2\text{ml.min}^{-1}$, n=7, p>0.05), but $P_a\text{CO}_2$ was unaffected (see Figure 2C).

**Hypoglycaemia-induced changes in CO₂ sensitivity**

To examine the impact of HG on respiratory CO₂ sensitivity, the ventilatory response to HC was recorded under normoglycaemic and hypoglycaemic conditions. As expected, HC increased $V_E$ in both glycaemic states, but importantly this was exaggerated in HG (Normoglycaemia $\Delta V_E +66\pm6$ vs HG $+82\pm7\text{ml min}^{-1}$, n=11, p<0.05, Figure 3A). The ventilatory CO₂ sensitivity (defined as the increase in $V_E$ per mmHg increase in $P_a\text{CO}_2$), was significantly increased by HG (Control 3.3±0.3 vs HG 4.4±0.4ml.min$^{-1}$mmHg$^{-1}$, n=11, p<0.05, Figure 3A). The ventilatory response to HC during Prop infusion was not different to control, however, Prop reduced the CO₂ sensitivity during HG (Prop 3.1±0.2 vs Prop+HG 1.4±0.2ml.min$^{-1}$mmHg$^{-1}$, n=6, p<0.05, Figure 3B). Following AdrX, the response to HC and calculated CO₂ sensitivity was not different to control, however the HG-mediated augmentation in CO₂ sensitivity was abolished (AdrX 4.5±0.9 vs AdrX + HG 3.2±0.9ml.min$^{-1}$mmHg$^{-1}$, n=7, p>0.05, Figure 3C).

**Responses to adrenaline infusion**

To further explore the acute effect of Adr on respiratory control, we measured $V_E$ during exogenous Adr infusion. Adr stimulated a dose-dependent increase in $V_E$ (Baseline =
136±4, Adr at 0.1 µg kg min⁻¹ = 146±4, 1 µg kg min⁻¹ = 161±5 and 10 µg kg min⁻¹ = 189±9ml min⁻¹, p<0.05) without altering ABP, except at the highest dose (ABP at 1 µg kg min⁻¹ = 129±2 and 10 µg kg min⁻¹ = 146±3mmHg, n=30, p<0.05, Figure 4). Thus, in further experiments Adr was infused at 1 µg kg min⁻¹, to rule out the possibility that any modifications in $V_E$ caused by Adr were an indirect consequence of a change in ABP.

**Carotid body dependent responses evoked by adrenaline infusion**

Figure 5A is an example trace showing the responses evoked by an Adr infusion (1 µg kg min⁻¹). Adr stimulated a hyperpnoea: no change in $P_{aCO_2}$ despite an approximately 20% increase in $V_E$ ($\Delta V_E +25±3ml min^{-1}$, n=30, p<0.05, Figure 5B). However, in the presence of Prop, the $V_E$ response to Adr was diminished ($\Delta V_E +9±4ml min^{-1}$, n=14, p>0.05) and $P_{aCO_2}$ increased significantly (Prop 41±1 vs Prop + Adr 48±4mmHg, p<0.05, Figure 5C), consistent with a $V_E$-metabolism mismatch. Following CSNX, $P_{aCO_2}$ was significantly elevated compared to control (39±1 vs 47±2mmHg, p<0.05). The increase in $V_E$ stimulated by Adr was significantly attenuated by CSNX ($\Delta V_E +6±2ml min^{-1}$, n=8, p<0.05), but $P_{aCO_2}$ did not increase further (Figure 5C).

**Adrenaline-induced changes in CO₂ chemosensitivity**

To investigate a potential role for Adr in modifying ventilatory CO₂ sensitivity, responses to HC were measured under control conditions and during Adr infusion. Adr increased the $V_E$ response to HC (Control $\Delta V_E +59±5$ vs Adr $+75±11$ ml min⁻¹, p<0.05, Figure 6A) and the calculated CO₂ sensitivity (Control 3.4±0.4 vs Adr 5.1±0.8 ml min⁻¹ mmHg⁻¹, n=14, p<0.05, Figure 6A). The Adr-induced increase in CO₂ sensitivity was completely blocked by Prop administration (Prop 3.0±0.4 vs Prop + Adr 2.6±0.5 ml min⁻¹ mmHg⁻¹, n=6, p>0.05, Figure 6B) and by CSNX (CSNX 4.0±0.9 vs CSNX + Adr 3.6±0.6 ml min⁻¹ mmHg⁻¹, n=5, p>0.05, Figure 6C).

**Hypercapnia-induced increases in Type I cell Ca²⁺ levels**

Isolated type I cells were loaded with Fura-2 and their responses to hypercapnic stimuli recorded. Figure 7A is a typical trace showing the control responses to HC. Baseline fluorescence was usually noisy when gassing with 10% rather than 21% O₂ (see (Burlon...
et al., 2009) for examples of quiet baselines at 21%) but 100% of cells recorded from responded rapidly with a rise in the fluorescence ratio when exposed to HC. The second exposure to HC was smaller than the first (see Figure 7A and C). By contrast, Figure 7B shows the effect of Adr (10nM) on the responses to HC. Adr had no effect on baseline activity but potentiated the second response to HC. Figure 7C shows the percentage difference between the second peak response to HC and the first under control conditions (-11±5.8%, n=10) and in the presence of 10nM Adr (+9±8.1%, n=9). The potentiating effect of Adr on HC responses was statistically significant (p<0.05, unpaired t-test).
**Discussion**

HG has been associated with a CB-mediated increase in ventilation and CO₂ sensitivity (Bin-Jaliah *et al.*, 2004, 2005). We show here for the first time that the *in vivo* augmentation of minute ventilation during HG is prevented by either β-adrenoreceptor blockade or AdrX. This supports the idea that glucose sensing *in vivo* is not a direct effect of low glucose on the CB but rather a consequence of Adr release. These same two interventions also counteracted the HG-induced increase in ventilatory CO₂ sensitivity. Adr infusion mimicked the action of HG by evoking hyperpnoea and by increasing ventilatory CO₂ sensitivity *in vivo*. These responses are dependent on the intact sensory neuronal input from the CB chemoreceptors, as demonstrated by the effect of CSNX. We have also shown that physiological concentrations of Adr increase the CO₂ sensitivity of freshly dissociated CB type I cells. These findings therefore suggest that Adr release during HG is necessary to evoke hyperpnoea and causes an increase in CB and whole body ventilatory CO₂ sensitivity and is consistent with Adr having a major role in the counter-regulatory response to HG.

**A role for adrenaline in mediating hyperpnoea in hypoglycaemia**

The emerging consensus is that the CB is stimulated during HG and contributes to the counter-regulation required to restore a normal plasma glucose concentration (Koyama *et al.*, 2000; Wehrwein *et al.*, 2010; Limberg *et al.*, 2015; Wehrwein *et al.*, 2015). This function is reliant on a heightened chemoafferent input into the NTS that in turn leads to the augmentation of Adr secretion from the adrenal medulla and an increase in hepatic glucose release into the systemic circulation (Alvarez-Buylla & de Alvarez-Buylla, 1988; Alvarez-Buylla *et al.*, 1997). Perhaps as importantly, activation of the CB chemoreceptors during HG is also essential to increase ventilation in a way that precisely matches the concurrent rise in metabolic rate, thereby preventing a rise in PₐCO₂ which if uncorrected could result in a serious systemic acidosis (Bin-Jaliah *et al.*, 2004).
Despite these key homeostatic functions, there remains a debate as to whether the CB responds either directly and rapidly to low glucose, or indirectly to some other blood-borne stimulus released as a consequence of systemic HG. The global counter-regulatory response to HG is multi-faceted and highly integrated and includes sympathetic activation (Fagius et al., 1986), systemic hypokalaemia (Petersen et al., 1982), and an increase in whole body metabolism (Bin-Jaliah et al., 2004). In addition, a number of counter-regulatory endocrine or neuroendocrine factors are released into the systemic circulation including Adr, NA, cortisol and glucagon (Cryer, 1993; Ward et al., 2007).

We have shown that Prop administration and AdrX are both effective in abolishing the HG-induced increase in ventilation. Prop administration during HG caused a rise in $P_aCO_2$, suggestive of a failure to match ventilation to an increased metabolism. Since AdrX also blocked the increase in ventilation during insulin-induced HG, it suggests that the hyperpnoea is not driven by insulin per se. This is in agreement with an earlier study where a euglycaemic-hyperinsulinaemic clamp did not augment ventilation (Bin-Jaliah et al., 2004) however, differs from the paper by (Ribeiro et al., 2013) who concluded the CB response was insulin-triggered. Additionally, there is evidence that insulin can act centrally in the arcuate nucleus of the hypothalamus to stimulate sympathetic nerve activity (Cassaglia et al., 2011). We have no explanation for this difference in results, however, HG may not be the only stimulus for Adr release. Furthermore, exogenous infusion of Adr at levels insufficient to cause ABP changes increased $V_E$ without altering $P_aCO_2$ or pH, indicative of hyperpnoea. Since this latter effect was markedly attenuated by CSNX, the action of Adr appears to be targeted to the CB chemoreceptors. That said, the presence of a small but significant increase in ventilation caused by Adr infusion after CSNX suggests that a small component of this response is CB-independent. Prop administration during Adr infusion abolished the increase in $V_E$ and caused a significant increase in $P_aCO_2$, indicative of ventilation-metabolism mismatch. We have also previously shown that the ventilatory response to HG is absent following CSNX (Bin-Jaliah et al., 2004). Collectively, the data presented
here suggest that Adr released from the adrenal medulla during HG is necessary to stimulate hyperpnoea, via the activation of β-adrenoreceptors and stimulation of the CB.

Abolishing the HG-mediated increase in ventilation with either AdrX or Prop also provides new evidence that the CB is not stimulated directly by low glucose in vivo. This supports previous work where the freshly isolated intact in vitro CB or dissociated type I cells were found to lack any inherent low glucose sensitivity (Bin-Jaliah et al., 2004; Conde et al., 2007; Kim et al., 2011; Gallego-Martin et al., 2012; Holmes et al., 2014). Since our HG is of rapid onset, our data supports the sensing of low glucose centrally (Ibrahim et al., 2003; Burdakov et al., 2005; Fioramonti et al., 2007) to drive sympathetic activation and Adr release from the adrenal medulla into the blood (Levin et al., 2008). The downstream effect of this is CB activation.

**Adrenaline release in hypoglycaemia augments carotid body and ventilatory CO₂ sensitivity**

Enhanced CB activity in HG is coupled with an elevation in ventilatory CO₂ sensitivity (Bin-Jaliah et al., 2005). After birth, CB chemosensitivity matures rapidly in the first week (Kholawadwala & Donnelly, 1992) and plateaus in the second week (Bamford et al., 1999). Thus, a robust mature response to CO₂ would be expected in our isolated type I cells. Our data shows that Adr increases CB type I cell Ca²⁺ responses to HC in vitro and also augments the ventilatory response to HC in vivo. In both instances, dual application of Adr and HC produced multiplicative responses, signifying stimulus interaction. Thus, exogenous Adr increases the CB and ventilatory sensitivity to CO₂. Additionally, it is unlikely that Adr alters central CO₂ chemoreceptor function directly since peripheral Adr does not cross the blood brain barrier (Axelrod & Tomchick, 1958). However, since the excitability of medullary CO₂ sensitive neurones can be regulated by the input from the peripheral chemoreceptors (Blain et al., 2010; Smith et al., 2015), it is conceivable that Adr indirectly enhances central respiratory CO₂ sensitivity via its action on the CB. Importantly, we also demonstrate that the normal increase in ventilatory CO₂ sensitivity in HG is inhibited by AdrX and Prop. This
therefore indicates that endogenous Adr release in HG enhances the CB and ventilatory CO₂ sensitivity of the whole animal. The possibility that the in vivo CB and ventilatory CO₂ sensitivity is further augmented by a substance released as a consequence of Adr release and β-adrenoreceptor stimulation is also plausible. The mechanism driving Adr release from the adrenal medulla in HG is expected to be an increase in sympathetic nerve activity. Interestingly the ventilatory CO₂ sensitivity was actually reduced in HG during Prop infusion. Whilst the main catecholamine released during HG is Adr, there is also release of NA (Jokiaho et al., 2014). It is possible that, during β-adrenoreceptor blockade with Prop infusion, NA released by HG is acting on inhibitory α₂-adrenoceptors in the CB to reduce CO₂ sensitivity (Kou et al., 1991; Prabhakar & Kou, 1994; Almaraz et al., 1997).

**Effects of different glucose sensors during hypoglycaemia**

Multiple central and peripheral glucose sensors have been identified and play their own significant roles in the response to HG (Donovan & Watts, 2014). An important factor that contributes to the roles played by particular sensors in eliciting the endocrine response is the rate of onset of HG (Bohland et al., 2014). In slow onset HG (60min), peripheral glucose sensors such as the portal mesenteric vein predominate in the sympathoadrenal response and involve a central pathway distinct to central glucose sensors (Jokiaho et al., 2014). In contrast, during rapid onset HG (20min) central glucose sensors mediate the catecholamine release (Bohland et al., 2014). We have reached our HG level within 30min of insulin infusion starting, suggesting central glucose sensors may be most important in causing the release of Adr in our study.

**Potential role for adrenaline in carotid body mediated pathology**

In our experiments we show that Adr stimulates the CB to produce a hyperpnoea during acute HG. Chronic recurrent HG, such as iatrogenic HG is an important clinical issue and is associated with becoming HG unaware and the development of hypoglycaemia-associated autonomic failure (HAAF) (Dunn et al., 2007; Arbelaez et al., 2008). HAAF
is linked to the attenuation of the sympathoadrenal response to HG rather than a change in the action of Adr once released (Moheet et al., 2014). However, chronic CB hyper-excititation is now emerging as an important driving force in establishing the raised arterial blood pressure associated with type II diabetes (Ribeiro et al., 2013) as well as chronic heart failure (Schultz et al., 2013), sleep disordered breathing (Narkiewicz et al., 1998), and spontaneous hypertension (McBryde et al., 2013). As yet little is known of the potential role of Adr in altering CB activity and exacerbating these pathologies. Our data suggest that this mechanism warrants further investigation. In a wider context, the action of Adr to produce a hyperpnoea during raised metabolic states may also implicate a significant role for the CB in matching ventilation to metabolism in other physiological situations such as exercise (Parkes, 2013).

**Concluding remarks**

In conclusion, this study provides evidence for a role of Adr, a counter-regulatory hormone released in response to HG, in provoking the hyperpnoea of hypermetabolism. The data generated thus supports a body of literature suggesting that the ventilatory response to HG is not mediated by a direct action of low glucose on the CB, but by the action of an associated blood-borne mediator, namely Adr. The observation that CO₂ sensitivity is elevated during Adr infusion and HG, and is blocked by the β-antagonist Prop and by AdrX, offers a mechanism by which ventilation can be matched to metabolism in the absence of a change in P₅CO₂.
**Figure Legends**

**Figure 1:** Diagram illustrating the *in vivo* experimental protocols. **A** Hyperinsulinaemic hypoglycaemia (HG) induced under control conditions and after either adrenalectomy (AdrX) or during propranolol (Prop) infusion. **B** Adrenaline (Adr) infusion under control conditions and after either carotid sinus nerve section (CSNX) or during propranolol (Prop) infusion. HC denotes when responses to hypercapnia were tested, * denotes when arterial blood samples were taken.

**Figure 2:** Ventilatory responses to hypoglycaemia (HG) are blocked by adrenalectomy (AdrX) and by propranolol (Prop). **A** Representative trace (5 sec) showing respiratory (Airflux, Respiratory Frequency (fR) and Tidal Volume (Vt)) and cardiovascular data (Arterial Blood Pressure (ABP) and Femoral Blood Flow (FBF)) at baseline and during HG. **B** Minute ventilation ($\dot{V}_E$) at baseline and in response to HG (n=12) with corresponding $P_aCO_2$. **C** Ventilatory response ($\Delta \dot{V}_E$) to HG before and after Prop (n=6) or AdrX (n=7) with corresponding $P_aCO_2$. * denotes p< 0.05, unpaired t-test.

**Figure 3:** Hypoglycaemia-induced changes in CO$_2$ sensitivity are blocked by both propranolol (Prop) and adrenalectomy (AdrX). **A** HG (n=11) increases CO$_2$ sensitivity which is blocked by **(B)** Prop (n=6) and by **(C)** AdrX (n=7). * denotes p< 0.05, paired t-test.

**Figure 4:** Ventilatory and cardiovascular responses to graded adrenaline (Adr) infusion. Minute ventilation ($\dot{V}_E$) and arterial blood pressure (ABP) were recorded at baseline and during infusion of Adr at 0.1, 1 and 10 µg kg min$^{-1}$. Adr significantly increased $\dot{V}_E$ in a dose-dependent manner, whilst a significant change in ABP was only seen at the top dose (n=30). *, **, *** denotes p<0.05, <0.01, <0.001 respectively one-way ANOVA.
**Figure 5:** Ventilatory responses to adrenaline (Adr) are blocked by propranolol (Prop) and by carotid sinus nerve section (CSNX). A Representative trace (5sec) showing respiratory (Airflux, Respiratory Frequency (fR) and Tidal Volume (VT)) and cardiovascular data (Arterial Blood Pressure (ABP) and Femoral Blood Flow (FBF)) at baseline and during Adr infusion. B (i) Minute ventilation ($\dot{V}_E$) in response to Adr infusion with corresponding P$_a$CO$_2$ (n=30). C Ventilatory response ($\Delta\dot{V}_E$) to Adr before and after Prop (n=14) or CSNX (n=8) with corresponding P$_a$CO$_2$. * denotes p< 0.05, unpaired t-test.

**Figure 6:** Adrenaline (Adr)-induced changes in CO$_2$ sensitivity are blocked by both propranolol (Prop) and carotid sinus nerve section (CSNX). A Adr increases CO$_2$ sensitivity which is blocked by Prop (B) and by CSNX (C). * denotes p< 0.05, paired t-test.

**Figure 7:** Carotid body type I cell Ca$^{2+}$ responses to hypercapnia (HC) are augmented by adrenaline (Adr). A Representative trace of paired control responses to HC (15% CO$_2$). B Representative trace of paired responses to HC before and during 10 nM Adr administration. C Bar chart comparing mean percentage change (±SEM) between 1$^{st}$ and 2$^{nd}$ HC challenge for control (n=10) and 10 nM Adr (n=9). * denotes p< 0.05, unpaired t-test.
References


Additional Information

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

All animal experiments were performed at the University of Birmingham. ELT, CJR, APH, AMC & PK designed the experiments. ELT, CJR & AMC performed the experiments. ELT, CJR, APH, AMC & PK analysed the data and wrote the manuscript. Isolated cell experiments were performed at Wright State University. RP & CNW designed, performed and analysed these experiments. All authors have read, commented on and approved the final version submitted for publication.

Funding

Funding of the BBSRC for ELT during her MRes degree programme is gratefully acknowledged.
A

Baseline | Insulin Infusion & HG | Recovery | Insulin Infusion & HG

HC

AdrX or Prop Infusion

B

Baseline | Adr Infusion | Recovery | Adr Infusion

HC

CSNX or Prop Infusion