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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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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 CO_2 sensitivity is abolished by preventing adrenaline release or blocking its receptors.
- Physiological levels of adrenaline mimicked the effect of hypoglycaemia on ventilation and CO_2 sensitivity.
- These results suggest that adrenaline, rather than low glucose, is an adequate stimulus for the carotid body mediated changes in ventilation and CO_2 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 CO_2 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 CO_2 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 P_aCO_2, 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 P_aCO_2 or pH and enhanced ventilatory CO_2 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 CO_2 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 CO_2 sensitivity.

Abbreviations: ABP, arterial blood pressure; Adr, adrenaline; AdrX, adrenalectomy; CB, carotid body; CSNX, carotid sinus nerve section; HC, hypercapnia; HG, hypoglycaemia; Prop, propranolol; f_R, respiratory frequency; $\dot{V}_E$, minute ventilation; V_T, 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 CO_2 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 CO_2 sensitivity (Bin-Jaliah *et al.*, 2004, 2005). As yet, however, a potential role for Adr in evoking the hyperpnoea and enhanced CB CO_2 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 CO_2 sensitivity. Adr infusion mimics the effect of HG and enhances the CB type I cell response to CO_2. Thus, the importance of CB activation by Adr during HG appears not only to help restore arterial blood glucose levels but also to control P_aCO_2 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 O_2 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 (f_R), tidal volume (V_t) and minute ventilation ($\dot{V}_E = f_R \times V_t$) were derived from this. Animals breathed room air throughout, except when inspiratory gases were changed to induce hypercapnia (HC; 8% CO_2 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

$\dot{V}_E$ 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 CO_2

sensitivity; $\Delta \dot{V}_E$ / mmHg P_aCO_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 Ca^{2+} 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 O_2) 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 $CaCl_2$ (86 μM) and $MgCl_2$ (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% CO_2, 10% O_2, 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% CO_2, 10% O_2, 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 (ΔV_E +20±4ml.min^{-1}, n=12, p<0.05) with no concomitant increase in P_aCO_2, demonstrating a hyperpnoea (Figure 2B). The HG-induced increase in $\dot{V}_E$ was blocked by Prop infusion (ΔV_E -12±3ml.min^{-1}, n=6, p>0.05) and P_aCO_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 $\dot{V}_E$ initiated by HG (ΔV_E -1±2ml.min^{-1}, n=7, p>0.05), but P_aCO_2 was unaffected (see Figure 2C).

Hypoglycaemia-induced changes in CO_2 sensitivity

To examine the impact of HG on respiratory CO_2 sensitivity, the ventilatory response to HC was recorded under normoglycaemic and hypoglycaemic conditions. As expected, HC increased $\dot{V}_E$ in both glycaemic states, but importantly this was exaggerated in HG (Normoglycaemia $\Delta\dot{V}_E$ +66±6 vs HG +82±7ml min^{-1}, n=11, p<0.05, Figure 3A). The ventilatory CO_2 sensitivity (defined as the increase in $\dot{V}_E$ per mmHg increase in P_aCO_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 CO_2 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 CO_2 sensitivity was not different to control, however the HG-mediated augmentation in CO_2 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 $\dot{V}_E$ during exogenous Adr infusion. Adr stimulated a dose-dependent increase in $\dot{V}_E$ (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 $\dot{V}_E$ 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 $\dot{V}_E$ ($\Delta\dot{V}_E$ +25±3ml min^{-1}, n=30, $p<0.05$, Figure 5B). However, in the presence of Prop, the $\dot{V}_E$ response to Adr was diminished (ΔV_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 $\dot{V}_E$ -metabolism mismatch. Following CSNX, P_aCO_2 was significantly elevated compared to control (39±1 vs 47±2mmHg, $p<0.05$). The increase in $\dot{V}_E$ stimulated by Adr was significantly attenuated by CSNX (ΔV_E +6±2ml min^{-1}, n=8, $p<0.05$), but P_aCO_2 did not increase further (Figure 5C).

Adrenaline-induced changes in CO_2 chemosensitivity

To investigate a potential role for Adr in modifying ventilatory CO_2 sensitivity, responses to HC were measured under control conditions and during Adr infusion. Adr increased the $\dot{V}_E$ response to HC (Control ΔV_E +59±5 vs Adr +75±11 ml min^{-1}, $p<0.05$, Figure 6A) and the calculated CO_2 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 CO_2 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 Ca^{2+} 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% O_2 (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 CO_2 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 CO_2 sensitivity. Adr infusion mimicked the action of HG by evoking hyperpnoea and by increasing ventilatory CO_2 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 CO_2 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 CO_2 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 P_aCO_2 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 $\dot{V}_E$ 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 $\dot{V}_E$ 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 CO_2 sensitivity

Enhanced CB activity in HG is coupled with an elevation in ventilatory CO_2 sensitivity (Bin-Jaliah *et al.*, 2005). After birth, CB chemosensitivity matures rapidly in the first week (Kholwadwala & Donnelly, 1992) and plateaus in the second week (Bamford *et al.*, 1999). Thus, a robust mature response to CO_2 would be expected in our isolated type I cells. Our data shows that Adr increases CB type I cell Ca^{2+} 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 CO_2. Additionally, it is unlikely that Adr alters central CO_2 chemoreceptor function directly since peripheral Adr does not cross the blood brain barrier (Axelrod & Tomchick, 1958). However, since the excitability of medullary CO_2 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 CO_2 sensitivity via its action on the CB. Importantly, we also demonstrate that the normal increase in ventilatory CO_2 sensitivity in HG is inhibited by AdrX and Prop. This

therefore indicates that endogenous Adr release in HG enhances the CB and ventilatory CO_2 sensitivity of the whole animal. The possibility that the *in vivo* CB and ventilatory CO_2 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 CO_2 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 α_2-adrenoceptors in the CB to reduce CO_2 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-excitation 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 CO_2 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 P_aCO_2.

Figure Legends

Figure 1: Diagram illustrating the *in vivo* experimental protocols. **A** Hyperinsulinaemic hypoglycaemia (HG) induced under control conditions and after either adrenalecotomy (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 (f_R) and Tidal Volume (V_T)) 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 (f_R) and Tidal Volume (V_T)) 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_aCO_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_aCO_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 1st and 2nd HC challenge for control (n=10) and 10 nM Adr (n=9). * denotes $p< 0.05$, unpaired t-test.

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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.

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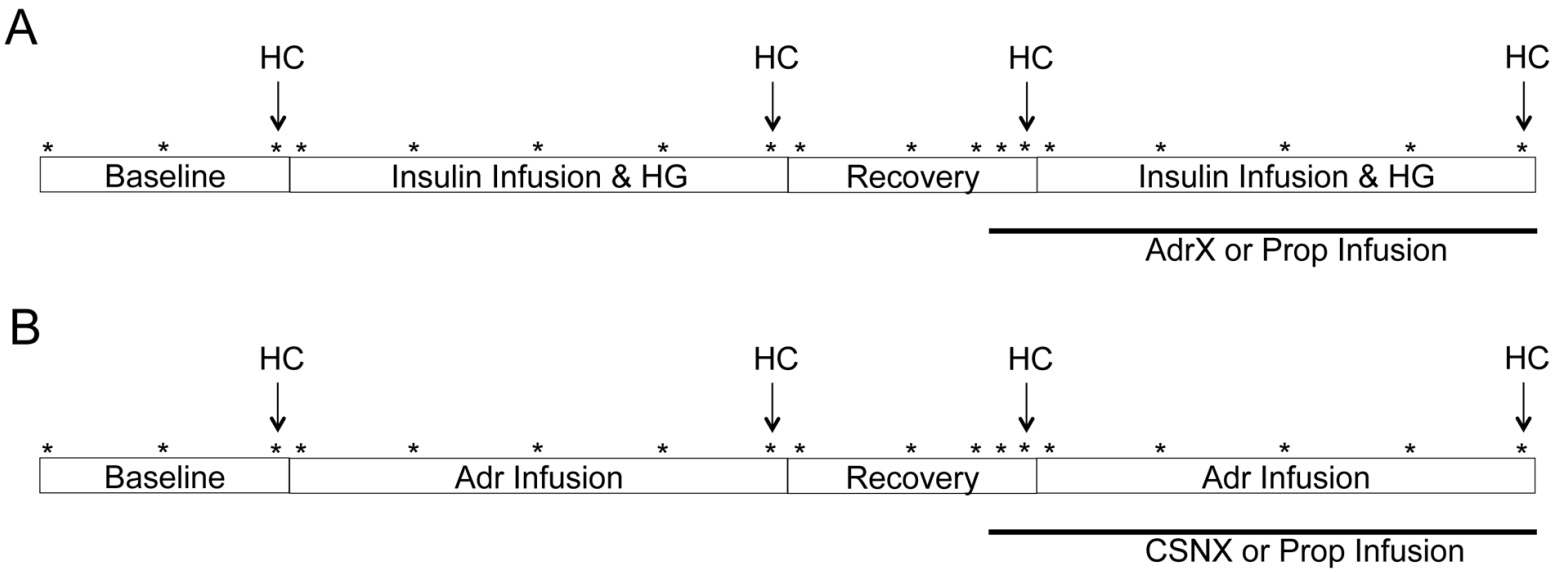
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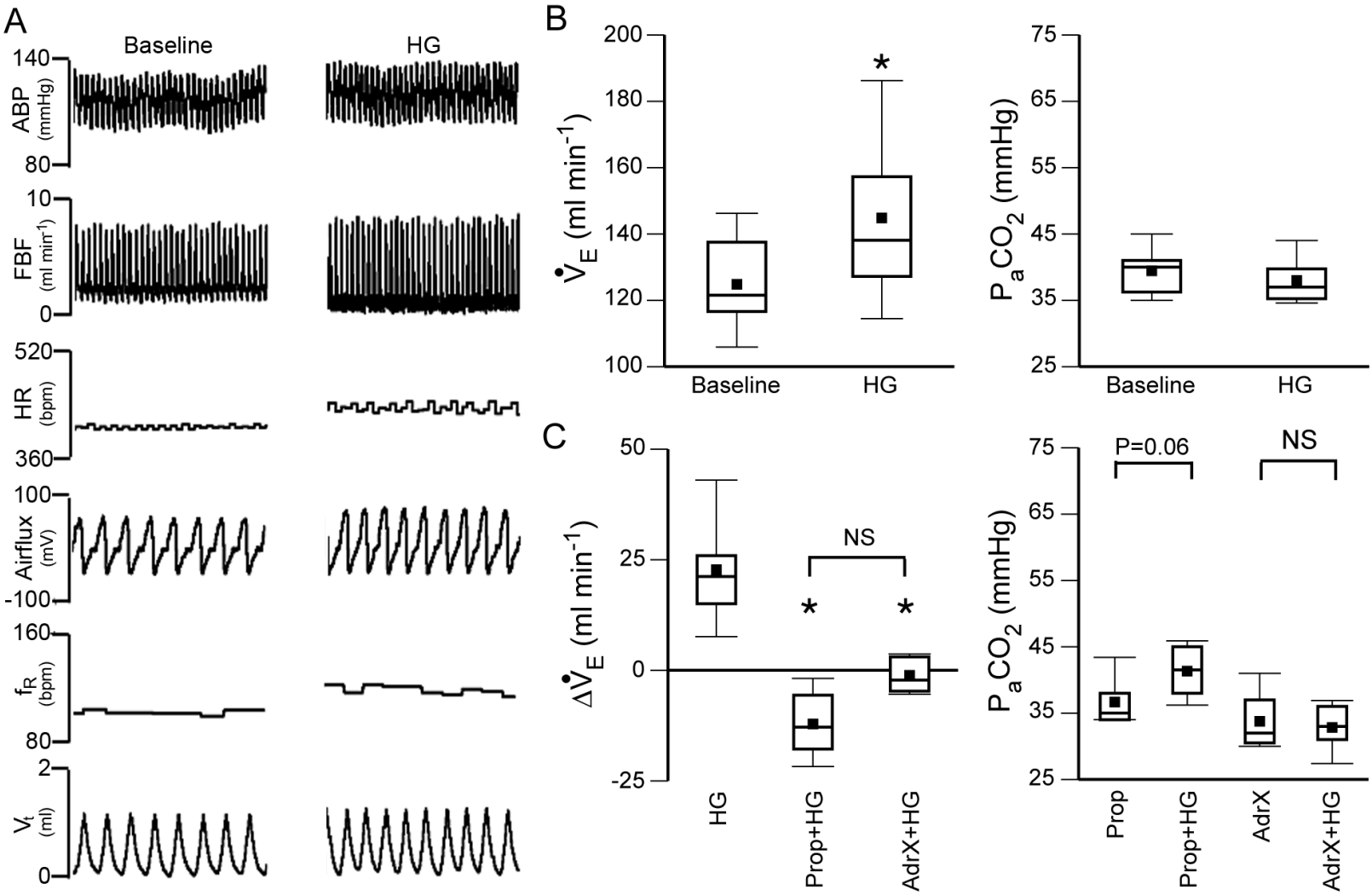
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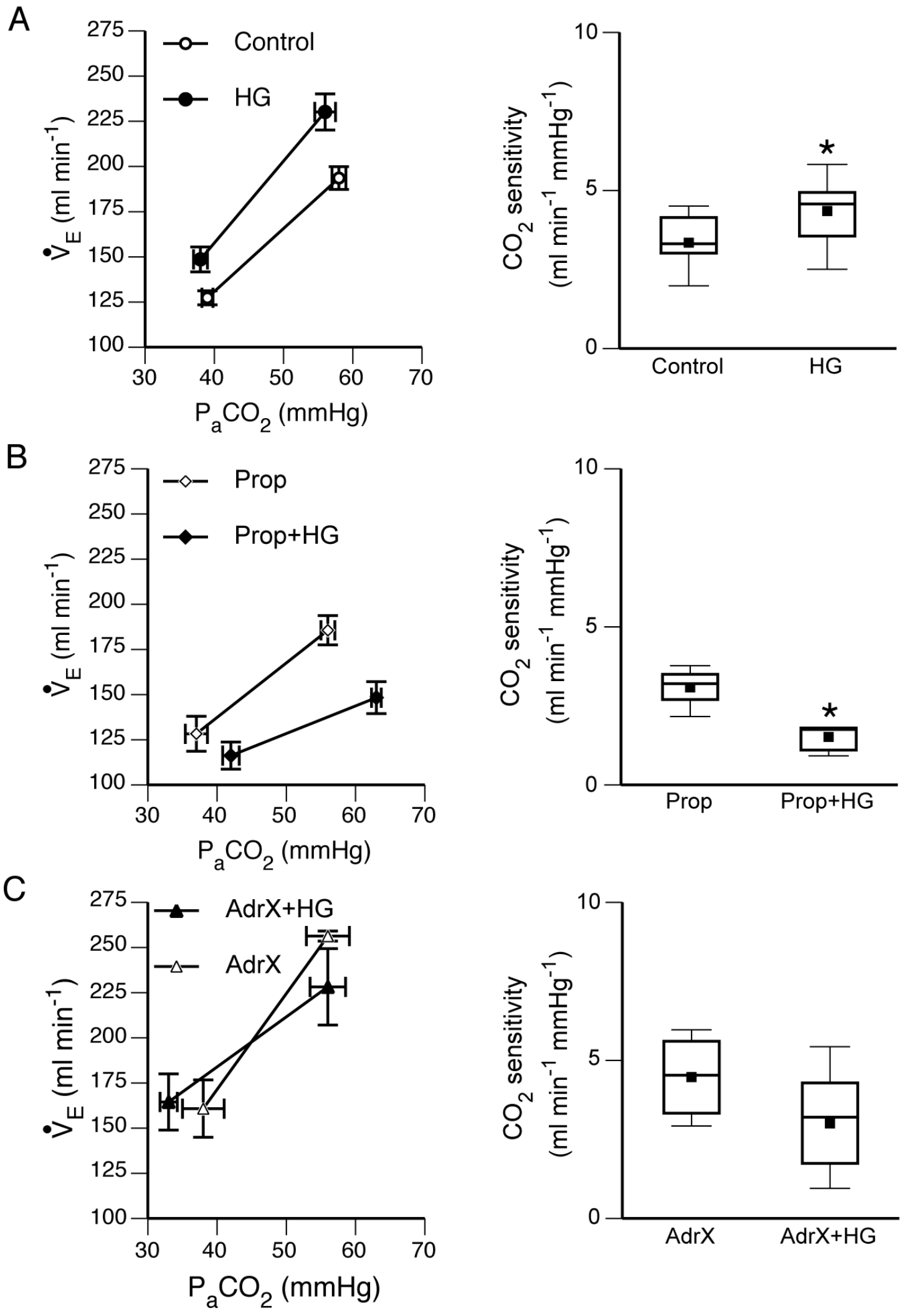
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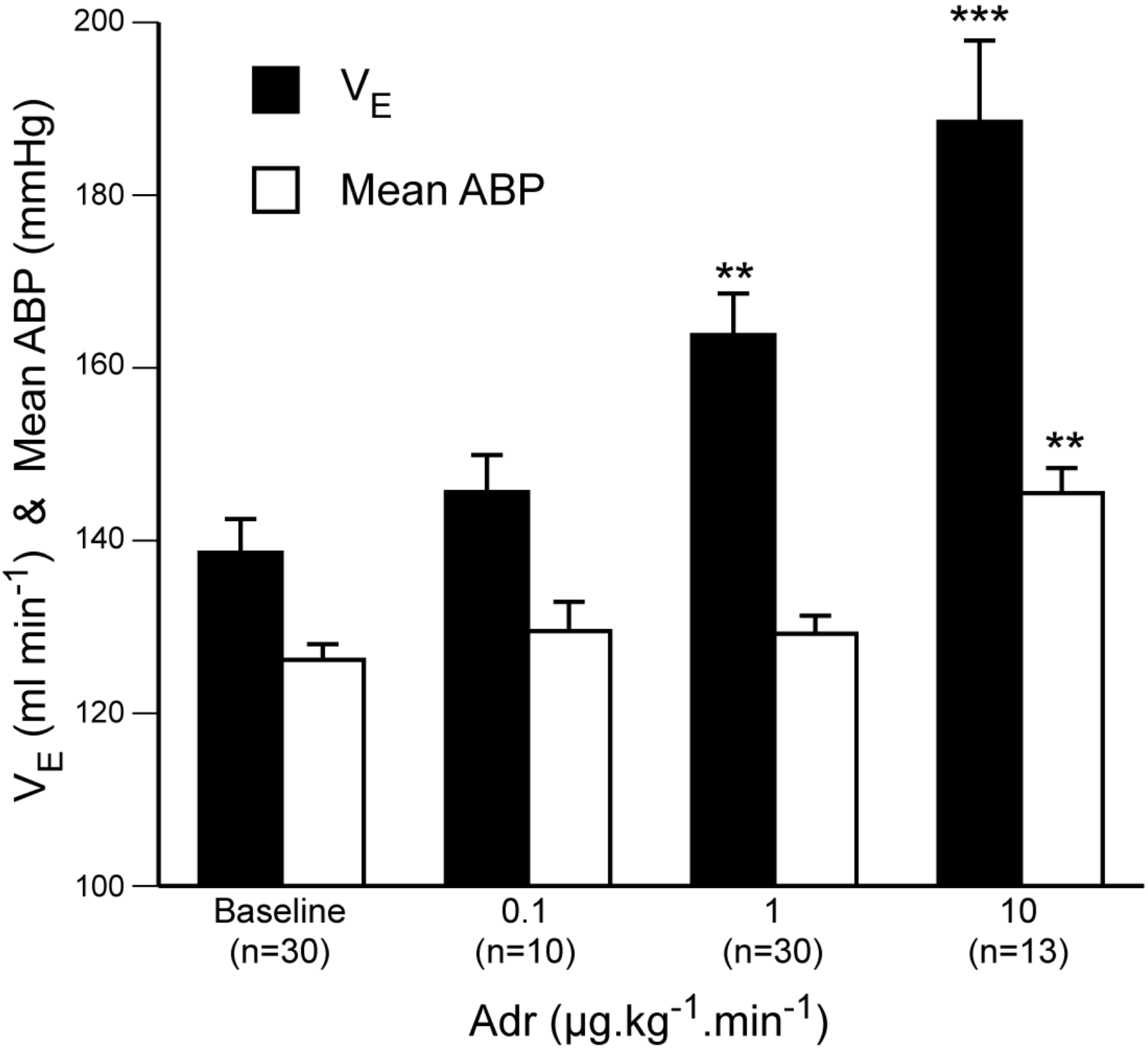
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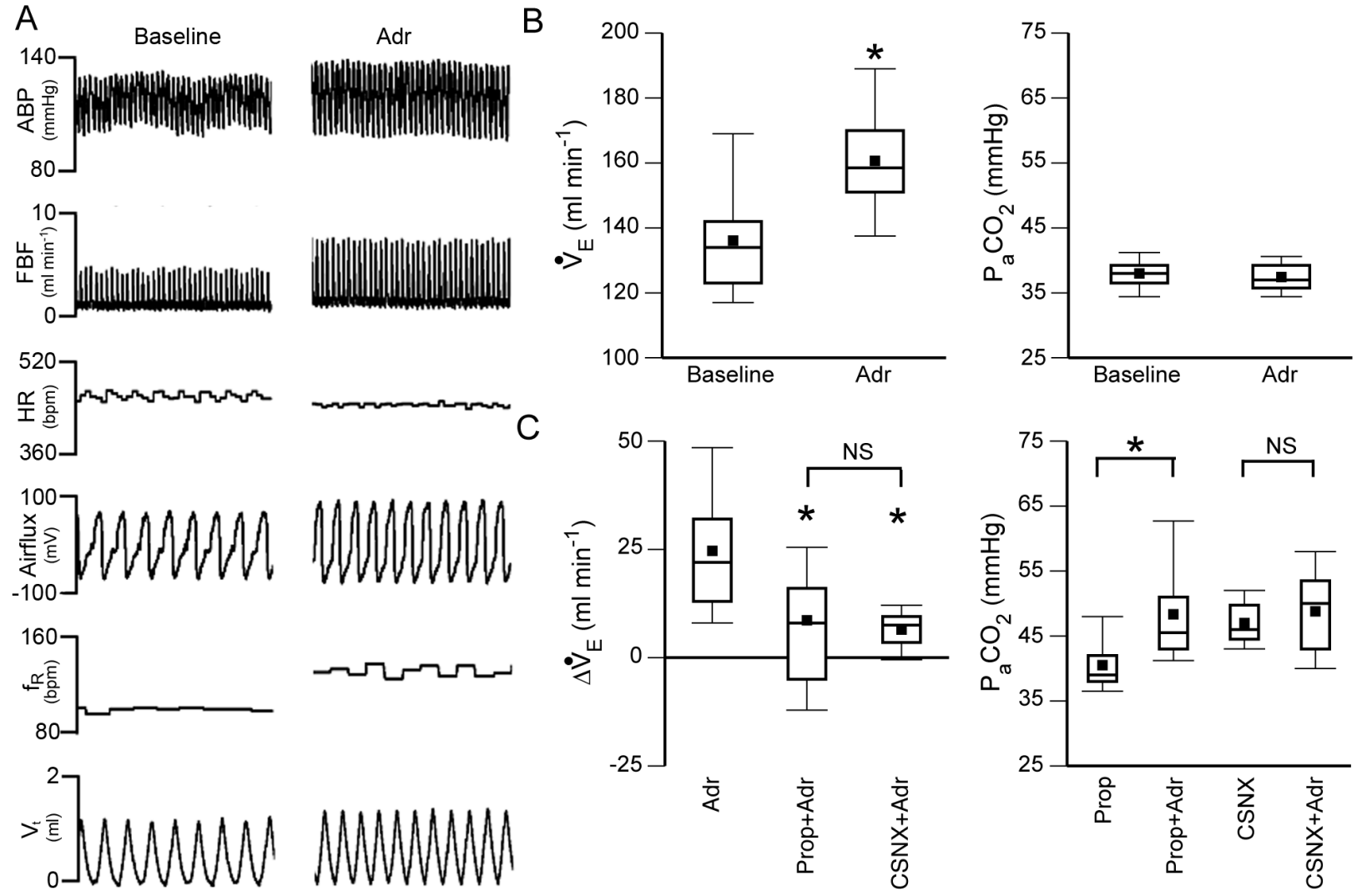
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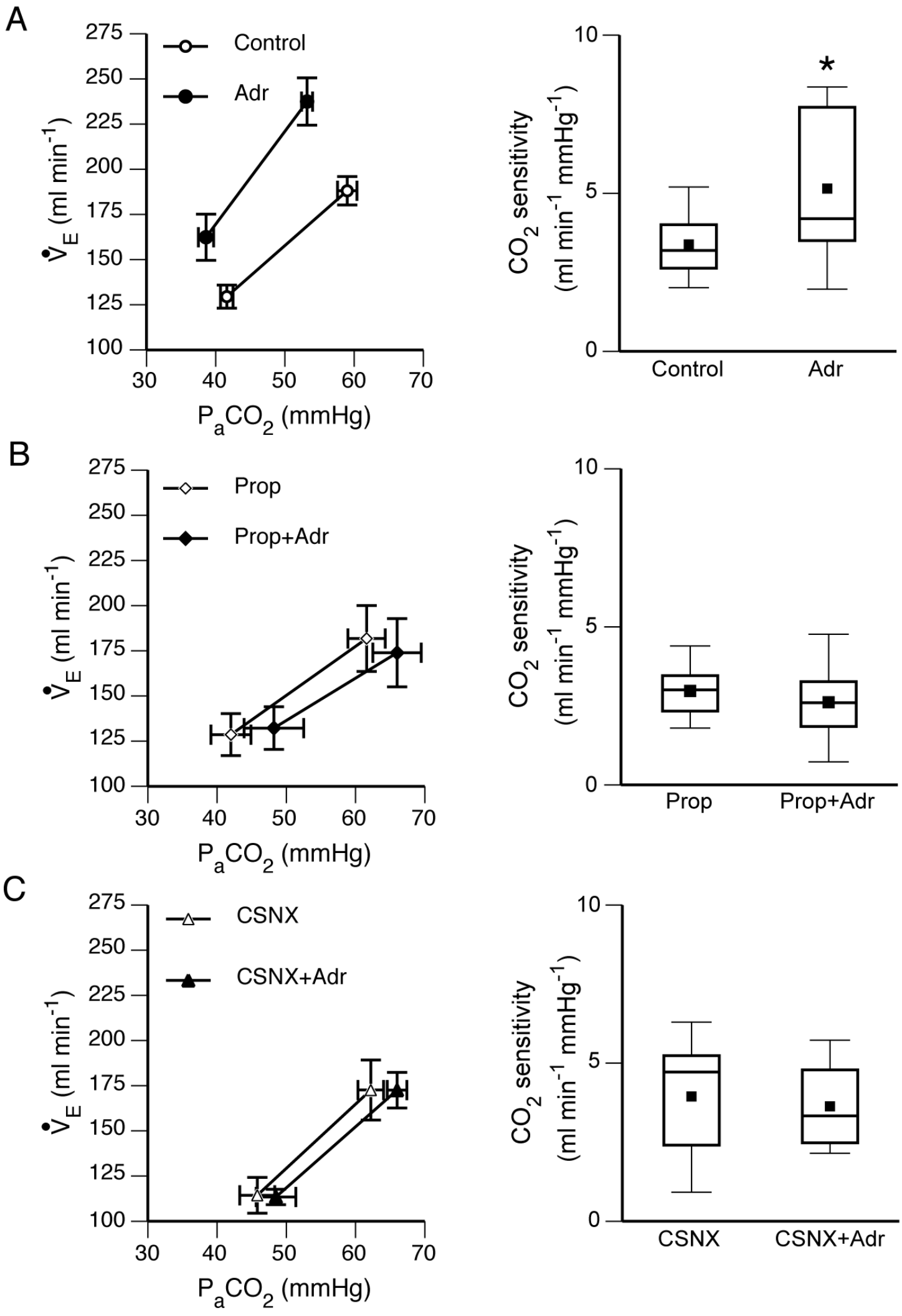
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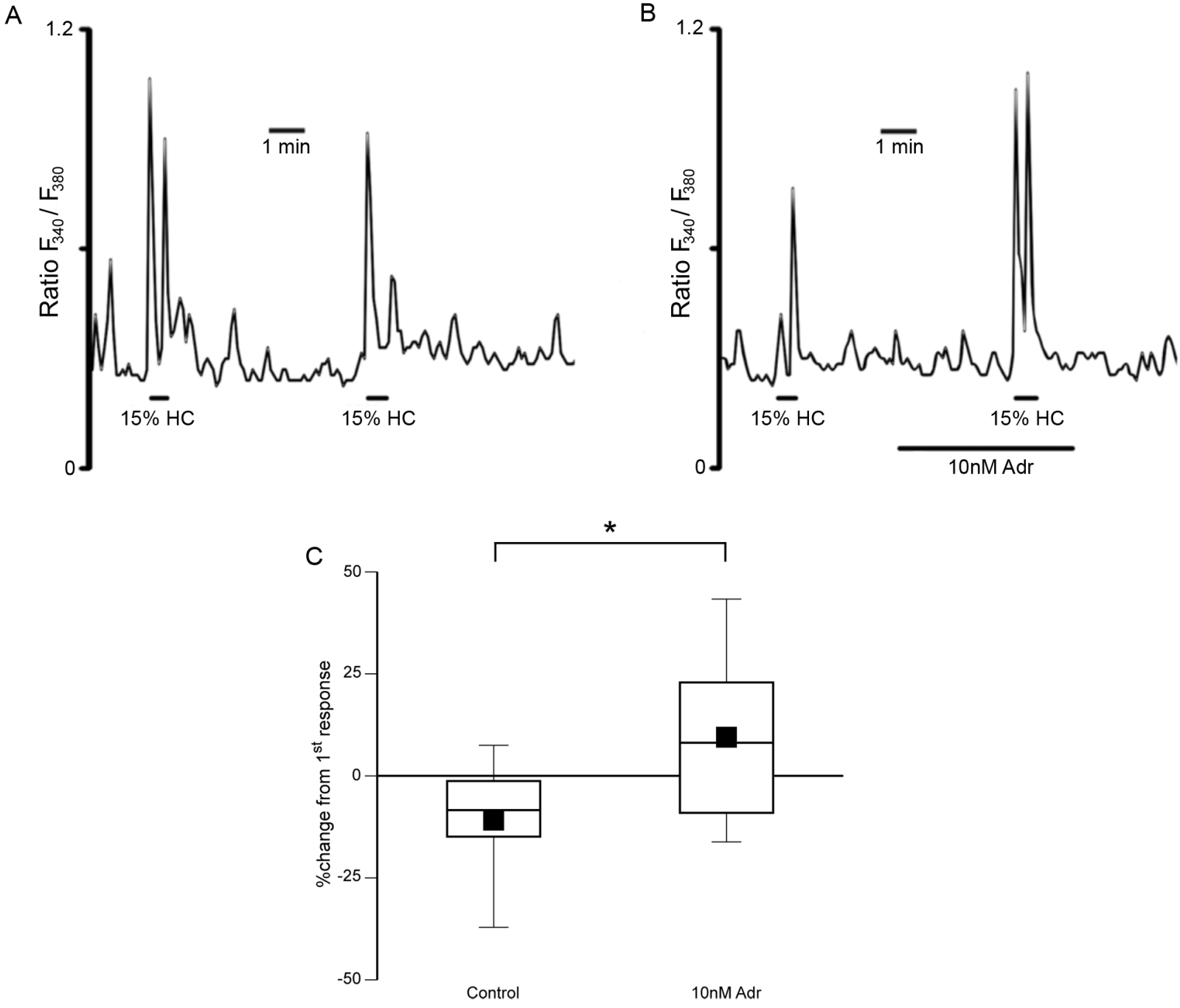
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