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**Ecto-5'-nucleotidase (CD73) regulates peripheral chemoreceptor activity and cardiorespiratory responses to hypoxia.**

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**Table of Contents Category: Respiratory****Key Points**

- Carotid body dysfunction is recognised as a cause of hypertension in a number of cardiorespiratory diseases states and has therefore been identified as a potential therapeutic target.
- Purinergic transmission is an important element of the carotid body chemotransduction pathway.
- We show that inhibition of ecto-5'-nucleotidase (CD73) *in vitro* reduces carotid body basal discharge and responses to hypoxia and mitochondrial inhibition.
- Additionally, inhibition of ecto-5'-nucleotidase (CD73) *in vivo* decreased the hypoxic ventilatory response, reduced the hypoxia-induced heart rate elevation and exaggerated the blood pressure decrease in response to hypoxia.
- Our data shows ecto-5'-nucleotidase to be a novel regulator of carotid body sensory function and therefore suggests this enzyme may offer a new target for reducing carotid body activity in selected cardiovascular diseases.

**Abstract**

Augmented sensory neuronal activity from the carotid body (CB) has emerged as a principal cause of hypertension in a number of cardiovascular related pathologies including obstructive sleep apnoea, heart failure and diabetes. Development of new targets and pharmacological treatment strategies aiming to reduce CB sensory activity may thus improve outcomes in these key patient cohorts. The current study tested whether ecto-5'-nucleotidase (CD73), an enzyme that generates adenosine, is functionally important in modifying CB sensory activity and cardiovascular respiratory responses to hypoxia. Inhibition of ecto-5'-nucleotidase by  $\alpha,\beta$ -methylene ADP (AOPCP) in the whole CB

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preparation *in vitro* reduced basal discharge frequency by  $76 \pm 5\%$  and reduced sensory activity throughout graded hypoxia. AOPCP also significantly attenuated elevations in sensory activity evoked by mitochondrial inhibition. These effects were mimicked by antagonism of adenosine receptors with 8-(p-sulfophenyl) theophylline. Infusion of AOPCP *in vivo* significantly decreased the hypoxic ventilatory response ( $\Delta\dot{V}_E$  control  $74 \pm 6\%$ ,  $\Delta\dot{V}_E$  AOPCP  $64 \pm 5\%$ ,  $P < 0.05$ ). AOPCP also modified cardiovascular responses to hypoxia, as evidenced by reduced elevations in heart rate and exaggerated changes in femoral vascular conductance and mean arterial blood pressure. Thus we identify ecto-5'-nucleotidase as a novel regulator of CB sensory activity. Future investigations are warranted to evaluate whether inhibition of ecto-5'-nucleotidase can effectively reduce CB activity in CB-mediated cardiovascular pathology.

### Abbreviations

CB - carotid body. CD73 - ecto-5'-nucleotidase. AOPCP -  $\alpha,\beta$ -methylene ADP. ENT - equilibrative nucleotide transporter. 8-SPT - 8-(p-sulfophenyl) theophylline. NBTI - nitrobenzylthioinosine.  $\text{NO}_2^-$  - nitrite. MABP – mean arterial blood pressure. FVC – femoral vascular conductance. HR – heart rate.  $\dot{V}_E$  – minute ventilation.  $V_t$  – tidal volume.  $f_R$  – respiratory frequency. HVR – hypoxic ventilatory response.

### Introduction

Chronic carotid body (CB) over-activation has emerged as an important driver of hypertension in a number of cardiovascular related diseases including heart failure (HF), sleep disordered breathing (SDB) and diabetes (Ribeiro *et al.*, 2013; Schultz *et al.*, 2015; Del Rio *et al.*, 2016; Prabhakar, 2016). Thus, development of novel treatment strategies that target the CB either pharmacologically or surgically may improve outcomes in these large patient populations. Indeed, some preliminary data suggests that bilateral CB resection decreases sympathetic outflow and improves exercise tolerance in human HF patients (Niewinski *et al.*, 2017). In a small cohort of patients with drug resistant hypertension, unilateral CB resection caused a reduction in ambulatory blood pressure in approximately 50% of those studied (Narkiewicz *et al.*, 2016). However, in view of the potential

safety concerns of bilateral resection, as indicated by a substantial reduction in O<sub>2</sub> saturation during sleep (Niewinski *et al.*, 2017), and the variable efficacy of unilateral resection (Narkiewicz *et al.*, 2016), pharmacological dampening of chemoreceptor activity may still offer a more viable treatment alternative.

Carotid sinus nerve (CSN) discharge frequency, transmitted into the central nervous system (CNS), is the neuronal signal that promotes ventilatory and cardiovascular reflex responses originating from the CB, most notably in response to systemic hypoxia (Kumar & Prabhakar, 2012). Modification of CSN outflow is controlled by a number of important neurotransmitters and neuromodulators including ATP, acetylcholine, dopamine, serotonin, adrenaline and adenosine (Nurse, 2010) (McQueen & Ribeiro, 1986; Fitzgerald *et al.*, 1999; Zhang *et al.*, 2000; Conde *et al.*, 2006; Conde *et al.*, 2012; Hauton *et al.*, 2013; Thompson *et al.*, 2016). Adenosine is an established CB chemostimulant in both animals (Runold *et al.*, 1990; Vandier *et al.*, 1999; Conde *et al.*, 2006; Xu *et al.*, 2006; Conde *et al.*, 2012) and humans (Tubek *et al.*, 2016). Better characterising the physiological relevance of adenosine and its generation and/or signalling pathways may provide useful information for identifying new potential targets for reducing CB chemoafferent activity.

Synaptic adenosine may be generated following extracellular breakdown of ATP; a neurotransmitter tonically released from the CB type 1 cell (Piskuric & Nurse, 2013). Conversion of ATP to adenosine requires both membrane bound ectonucleoside triphosphate diphosphohydrolyase 1 (CD39) and ecto-5'-nucleotidase (CD73) (Bianchi & Spychala, 2003). Expression of these two enzymes has been confirmed in extracts of the whole rat CB (Salman *et al.*, 2016). Adenosine causes chemostimulation by either increasing type 1 cell excitability (via A<sub>2A</sub> and/or A<sub>2B</sub> receptors) (Conde *et al.*, 2006; Xu *et al.*, 2006; Conde *et al.*, 2008; Livermore & Nurse, 2013) or by directly activating post-synaptic sensory fibres, (via A<sub>2A</sub> receptor stimulation) (Conde *et al.*, 2006), both mechanisms acting to increase cAMP (Nunes *et al.*, 2014; Holmes *et al.*, 2015). Alternatively, it has been proposed that adenosine may be formed in the type 1 cell and then released directly into the synapse through the bidirectional equilibrative nucleotide transporter (ENT) (Cass *et al.*, 1998).

The current study tested whether pharmacological targeting of either ecto-5'-nucleotidase or ENT effectively reduces CSN discharge frequency in normoxic or hypoxic conditions. Furthermore, we

examined if ventilation, heart rate, blood pressure and vascular conductance responses to hypoxia were modified by targeting ecto-5'-nucleotidase *in vivo*. The data suggests that antagonism of ecto-5'-nucleotidase but not ENT inhibits basal CSN frequency and blunts the response to hypoxia. Inhibition of ecto-5'-nucleotidase also attenuates ventilatory and cardiovascular responses to hypoxia. Thus, we propose ecto-5'-nucleotidase as a novel modulator of CB chemoafferent outflow.

## Methods

### **Ethical approval**

The following procedures on animals were approved and carried out in line with the current Home Office (UK) and University of Birmingham guidelines on ethical use of animals. Adult male Wistar rats (n=32) were used for the study, supplied by Charles River Laboratories, Margate, UK. Animals were housed in individually ventilated cages in the Biomedical Services Unit at the University of Birmingham. Food and water was available *ad libitum*.

### **Extracellular recordings of chemoafferent neurones**

Intact carotid bifurcations containing the CSN and CB were isolated from adult male Wistar rats (100–200 g) under inhalation anaesthesia (2–4% isoflurane in O<sub>2</sub>, 3L min<sup>-1</sup>), death by exsanguination. Following tissue procurement, animals were immediately killed by exsanguination. Connective tissue and surrounding structures were excised and the CSN was sectioned exposing nerve fibres and axons. To facilitate the extracellular neuronal recordings, the whole tissue was partially digested by incubation in enzyme Krebs solution (0.075 mg / ml collagenase type II, 0.0025 mg / ml dispase type I; Sigma Aldrich), at 37°C, for 20–30 minutes.

Extracellular recordings of chemoafferent activity were made from the cut end of the CSN as described previously (Holmes *et al.*, 2014; Holmes *et al.*, 2016). The superfusate PO<sub>2</sub> was continuously measured using an O<sub>2</sub> electrode (ISO2; World Precision Instruments) and O<sub>2</sub> meter

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(OXELP; World Precision Instruments). The PO<sub>2</sub> and chemoafferent derived voltage were both recorded using a CED micro1401 (Cambridge Electronic Design) and visualised on a PC with Spike2 (version 7.1) software (Cambridge Electronic Design). Chemoafferent voltage signal was sampled at 15000Hz and the PO<sub>2</sub> at 100Hz. Single fibres were used for analysis. Electrical activity originating from a single chemoafferent fibre was determined by its unique 'wavemark' signature based on frequency, shape and amplitude.

Throughout experimentation, whole CBs were continuously superfused with a standard bicarbonate buffered Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 5 Na<sub>2</sub>SO<sub>4</sub>, 1.3 MgSO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 2.4 CaCl<sub>2</sub> and 11 D-glucose, 37°C, pH 7.4. For normoxia/hyperoxia the superfusate PO<sub>2</sub> was maintained throughout at a level that kept spontaneous chemodischarge between 0.25 and 1.0Hz (Holmes *et al.*, 2014). PCO<sub>2</sub> was maintained at 40mmHg. For hypoxia, the superfusate PO<sub>2</sub> was gradually reduced, at constant PCO<sub>2</sub>, until discharge was elevated above 10Hz. This was repeated in the presence of pharmacological agents used to target adenosinergic signalling pathways (AOPCP, 100µM (Conde & Monteiro, 2004; Holmes *et al.*, 2015); 8-SPT, 300µM (Wyatt *et al.*, 2007; Holmes *et al.*, 2015) and NBTI, 10µM (Conde *et al.*, 2012)) and again after drug washout.

The single fibre chemoafferent discharge frequency was plotted against the superfusate PO<sub>2</sub>, and fitted to an exponential decay curve with offset,  $y = a + be^{-cx}$ , where,  $y$  is the single fibre discharge frequency in Hz,  $x$  is the superfusate PO<sub>2</sub> in mmHg,  $a$  is the discharge frequency as the PO<sub>2</sub> tends to infinity (offset),  $b$  is the discharge frequency when the PO<sub>2</sub> is 0 mmHg (minus the offset) and  $c$  is the exponential rate constant. In addition, for any given discharge frequency, the corresponding PO<sub>2</sub> could be calculated using the inverse function of the exponential decay curve,  $x = -(\ln((y - a)/b))/c$ , where  $x$  is the PO<sub>2</sub> in mmHg,  $y$  is the single fibre discharge frequency in Hz and  $a$ ,  $b$  and  $c$  are constants as above. The PO<sub>2</sub> at a frequency of 5Hz was used to quantify a PO<sub>2</sub> shift in the hypoxic response curve. A value of 5Hz was chosen as it lies on the exponential region of the hypoxic response curve, but is not of a magnitude at which the discharge is likely to have started to diminish.

To evaluate chemoafferent responses to mitochondrial inhibition, the rapidly reversible mitochondrial inhibitor nitrite (NO<sub>2</sub><sup>-</sup>, 10mM) was used to induce moderate elevations in chemoafferent discharge (Holmes *et al.*, 2016). Responses to NO<sub>2</sub><sup>-</sup> were measured in control conditions, in the presence of

pharmacological agents targeting adenosinergic signalling pathways, as above, and again after drug washout.

### ***In vivo* ventilatory and cardiovascular responses to hypoxia**

Adult male Wistar rats (Charles River Laboratories) were initially anaesthetised with 3-4% isoflurane in O<sub>2</sub> at 3-4 L min<sup>-1</sup> (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<sup>-1</sup> h<sup>-1</sup> with 0.1 ml boluses as necessary. Core body temperature was maintained at 37°C with a homeothermic heat pad system (Harvard Apparatus).

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. The tracheal cannula was connected to a system of rotameters in a gas proportioner frame (CP Instruments Co. Ltd) allowing variation of inspiratory gases. Animals breathed room air as the normoxic control. For hypoxia, animals inspired hypoxic gas mixture for 2 minute periods over the range of 20 to 8% O<sub>2</sub>. An arterial blood gas sample was taken at each inspired O<sub>2</sub>. The response to hypoxia was calculated as the mean of the last min of the exposure to hypoxia.

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. Drug infusions of  $\alpha,\beta$ -methylene ADP (AOPCP; 160 $\mu$ g kg<sup>-1</sup>, I.V. ) were given via the right femoral vein at a dose known to reduce vasodilation evoked by ATP infusion in similar *in vivo* studies (Skinner & Marshall, 1996).

### **Analysis of data**

Values are expressed as mean  $\pm$  standard error of mean unless otherwise stated. Statistical analysis was performed using i) a paired 2-tailed student's t-test or ii) repeated measures one way Analysis of



Variance (ANOVA) with Bonferroni or Dunnett's post hoc analysis where appropriate (StatView version 5 or GraphPad Prism version 6). Significance was taken as  $p < 0.05$ .

## Results

### **Ecto-5'-nucleotidase regulates peripheral chemoreceptor activity in normoxia and hypoxia.**

Inhibition of ecto-5'-nucleotidase using 100  $\mu\text{M}$  AOPCP (Conde & Monteiro, 2004; Holmes *et al.*, 2015) reduced basal single fibre activity by  $76 \pm 5\%$  (Figure 1A-C). AOPCP also significantly attenuated the CB chemoafferent response to hypoxia. A characteristic example of a single fibre hypoxic response is shown in Figure 1A & B and demonstrates a characteristic leftward shift in the hypoxia response curve caused by AOPCP. The inhibition was rapidly reversible and the original response to hypoxia was almost fully recovered after removal of the agent from the superfusate (Figure 1A & B). Mean chemoafferent discharge frequency was significantly depressed over a range of hypoxic superfusate  $\text{PO}_2$  values of 175 to 100mmHg (Figure 1D). Grouped paired measurements showed that AOPCP significantly decreased the superfusate  $\text{PO}_2$  required to elicit a discharge frequency of 5Hz by a mean value of approximately 30mmHg, consistent with blunted hypoxic sensitivity (Figure 1E).

A similar reduction in basal frequency was observed in the presence of the adenosine receptor antagonist 8-(p-sulfophenyl) theophylline (8-SPT, 300  $\mu\text{M}$  (Wyatt *et al.*, 2007; Holmes *et al.*, 2015)) (figure 2A & B). 8-SPT decreased chemoafferent activity at all  $\text{PO}_2$  levels but its inhibitory effect was enhanced during hypoxia (Figure 2A). In all fibres tested, addition of 8-SPT reduced CB hypoxic sensitivity, as evidenced by evoking a leftward shift in the chemoafferent hypoxic response curve, with a mean superfusate  $\text{PO}_2$  reduction at 5Hz measuring approximately 40mmHg (Figure 2B). To determine whether the neuromodulatory actions of adenosine are dependent on release through the ENT, additional hypoxic responses were performed in the presence of nitrobenzylthioinosine (NBTI; 10 $\mu\text{M}$ , an inhibitor of ENT (Conde *et al.*, 2012)). NBTI did not alter basal single fibre discharge frequency and had no effect on the chemoafferent response to hypoxia (Figure 2C & D). NBTI did not significantly alter the  $\text{PO}_2$  required to generate a 5Hz frequency and produced no change of chemoafferent activity throughout exposure of the CB to graded hypoxia (Figure 2C & D).

**Ecto-5'-nucleotidase and adenosine mediate chemoreceptor responses to mitochondrial inhibitors.**

CB activation by hypoxia is commonly ascribed to be a direct consequence of depletion in mitochondrial energy metabolism (Buckler & Turner, 2013; Holmes *et al.*, 2016). Thus AOPCP and 8-SPT should have similar effects on CB responses to mitochondrial inhibitors. An example trace demonstrating the impact of 100 $\mu$ M AOPCP on the response to mitochondrial inhibition using  $\text{NO}_2^-$  is shown in Figure 3A. AOPCP almost completely abolished basal frequency and the response to  $\text{NO}_2^-$  (Figure 3B). The absolute elevation in chemoafferent activity evoked by  $\text{NO}_2^-$  ( $\text{NO}_2^-$  – basal) was significantly decreased in presence of AOPCP, measuring only approximately 0.4% of control (Figure 3C). 8-SPT (300  $\mu$ M) also significantly diminished frequency responses to  $\text{NO}_2^-$  (Figure 3D-E). The rise in discharge frequency elicited by  $\text{NO}_2^-$  in the presence of 8-SPT was reduced in each fibre studied, with the mean  $\text{NO}_2^-$ -induced frequency elevation in the presence of 8-SPT measuring only approximately 20% of control (Figure 3D). Thus, AOPCP and 8-SPT are effective in attenuating CB responses to both hypoxia and mitochondrial inhibition.

**Ecto-5'-nucleotidase inhibition reduces hypoxic ventilatory sensitivity**

During normoxic breathing, AOPCP elevated baseline minute ventilation (control  $187 \pm 10$ ; AOPCP  $195 \pm 10$  ml  $\text{min}^{-1}$ ,  $n=9$ ), an effect that was due to an increase in tidal volume but not respiratory frequency (Figure 4A-C). Accordingly, under these normoxic conditions, AOPCP significantly reduced  $P_a\text{CO}_2$  (control  $45 \pm 1$ ; AOPCP  $39 \pm 1$  mmHg,  $P < 0.05$ , paired 2-tailed student's t-test,  $n=9$ ), consistent with hyperventilation. Graded hypoxia increased minute ventilation, respiratory frequency and tidal volume (Figure 4A-C) and reduced  $P_a\text{CO}_2$  (Figure 5A) in control and in the presence of AOPCP. However, AOPCP attenuated the magnitude of the hypoxic ventilatory response (HVR) (e.g. at 40 mmHg  $P_a\text{O}_2$ ;  $\Delta \dot{V}_E$  control  $74 \pm 6\%$ ,  $\Delta \dot{V}_E$  AOPCP  $64 \pm 5\%$ ,  $n=9$ ) (Figure 4F). This attenuation in HVR was dependent on a reduction in the hypoxia-induced elevation in tidal volume but not respiratory frequency (Figure 4D-F). AOPCP also reduced the hypoxic exponential ventilatory rate constant (control  $0.032 \pm 0.0044$ ; AOPCP  $0.022 \pm 0.003$ ,  $n=9$ ,  $P < 0.01$ , paired t-test), signifying a decrease in hypoxic ventilatory sensitivity. Blood gas analysis revealed that the magnitude of the decrease in  $P_a\text{CO}_2$  caused by hypoxia was also attenuated by AOPCP (Figure 5A & B). Collectively

these results suggest that whilst systemic pharmacological inhibition of ecto-5'-nucleotidase causes a baseline increase in ventilation, it also significantly attenuates the HVR.

### **Ecto-5'-nucleotidase mediates cardiovascular responses to hypoxia**

Consistent with the baseline hyperventilation, AOPCP increased heart rate (control  $441 \pm 12$ ; AOPCP  $460 \pm 10$  bpm,  $n=9$ ) and reduced femoral vascular conductance (control  $0.016 \pm 0.001$ ; AOPCP  $0.012 \pm 0.001$  ml min<sup>-1</sup> mmHg<sup>-1</sup>,  $n=8$ ) during normoxic air breathing, but this did not cause an increase in mean arterial blood pressure (MABP) (Figure 6A-C). AOPCP attenuated the hypoxia induced rise in heart rate (HR) (e.g. at 40 mmHg P<sub>a</sub>O<sub>2</sub>;  $\Delta$ HR control  $15 \pm 4\%$ ,  $\Delta$ HR AOPCP  $7 \pm 3\%$ ,  $n=9$ ), enhanced the hypoxic increase in femoral vascular conductance (FVC) (e.g. at 40 mmHg P<sub>a</sub>O<sub>2</sub>;  $\Delta$ FVC control  $11 \pm 5\%$ ,  $\Delta$ FVC AOPCP  $29 \pm 5\%$ ,  $n=8$ ) and exaggerated the fall in MABP (e.g. at 40 mmHg P<sub>a</sub>O<sub>2</sub>;  $\Delta$ MABP control  $-19 \pm 3\%$ ,  $\Delta$ MABP AOPCP  $-26 \pm 4\%$ ,  $n=9$ ) (Figure 6D-F).

### **Discussion**

The present study identifies ecto-5'-nucleotidase (CD73) as a novel functional protein that contributes to CB and whole body cardiorespiratory responses to acute hypoxia. Inhibition of ecto-5'-nucleotidase activity dampens CB sensory neuronal discharge *in vitro*, both under basal conditions and during stimulation by hypoxia. In addition, pharmacological inhibition of ecto-5'-nucleotidase *in vivo* reduces the HVR and attenuates the hypoxia-induced rise in HR. Future investigations could evaluate whether targeting CB ecto-5'-nucleotidase activity may be effective in reducing persistent CB hyperactivity in selected pathologies.

### **Ecto-5'-nucleotidase is a novel regulator of CB sensory discharge in normoxia and hypoxia**

Emerging evidence suggests that a chronic increase in CB sensory output in normoxia and hypoxia contributes significantly to hypertension and cardiac arrhythmia in patients and animal models of SDB, heart failure and diabetes (Ribeiro *et al.*, 2013; Schultz *et al.*, 2015; Del Rio *et al.*, 2016; Prabhakar, 2016). This is due to the CB driving a chronic rise in sympathetic neuronal outflow to the vasculature and heart, thereby leading to persistent vasoconstriction and pro-arrhythmic cardiac

autonomic imbalance (Narkiewicz *et al.*, 1998; Peng *et al.*, 2003; Del Rio *et al.*, 2016; Linz *et al.*, 2016). A number of recent small-scale clinical studies suggest that surgical uni- or bilateral CB ablation may be beneficial in reducing sympathetic outflow and arterial blood pressure in some patient cohorts (Narkiewicz *et al.*, 2016; Niewinski *et al.*, 2017). However, widespread use as a first-line treatment option for CB mediated hypertension may be limited due to the potential safety concerns of complete loss of CB sensory activity, such as exaggerated O<sub>2</sub> desaturation during sleep (Niewinski *et al.*, 2017). This safety concern may be particularly relevant in patients with OSA who rely on CB sensory activity to cause arousal and recovery of airway patency. Furthermore, uni-lateral CB resection seems to confer a relatively short-term (up to 12 months) reduction in blood pressure and is effective in only about 50% of hypertensive patients (Narkiewicz *et al.*, 2016). Thus, identifying new potential drug targets in the CB that can be targeted to reduce, but not abolish chemosensory activity, may offer a safer and more applicable treatment for this form of CB mediated hypertension.

In this study we provide the first functional evidence that ecto-5'-nucleotidase mediates CB sensory discharge frequency in hypoxia. Ecto-5'-nucleotidase catalyses the formation of adenosine from AMP, following initial breakdown of ATP and ADP by CD39 (Bianchi & Sychala, 2003). In the CB, a significant synaptic ATP concentration is due to the tonic vesicular neurosecretion from the type 1 cell (Zhang & Nurse, 2004) and also through ATP release from type 2 cells via pannexin-1 channels (Murali & Nurse, 2016). The attenuation of sensory discharge by inhibiting ecto-5'-nucleotidase is therefore most likely due to a reduction in adenosine formation. Indeed, we show that antagonism of adenosine receptors reduced normoxic and hypoxic CB sensory activity by an amount that was similar to inhibition of the ecto-5'-nucleotidase. Furthermore, antagonism of ecto-5'-nucleotidase and adenosine receptors both decrease the PO<sub>2</sub> threshold required for CB activation during hypoxia, consistent with a comparable reduction in CB hypoxic sensitivity.

Adenosine generated from ecto-5'-nucleotidase has the potential to modulate CB hypoxic sensitivity through activation of A<sub>2A</sub> and A<sub>2B</sub> receptors on the type 1 cell (Conde *et al.*, 2006; Xu *et al.*, 2006; Conde *et al.*, 2008; Livermore & Nurse, 2013) or by stimulating the nerve ending upon binding to post synaptic A<sub>2A</sub> receptors (Conde *et al.*, 2006). Both of these mechanisms are probable given that ecto-5'-nucleotidase will increase extracellular adenosine in the type 1 cell-chemoafferent synapse, thus having access to both pre-and post-synaptic receptors. Despite 8-SPT potentially also targeting

A<sub>1</sub> receptors, a functional role is unlikely in our studies given the absence of A<sub>1</sub> mRNA expression in the rat CB (Gauda, 2000; Kobayashi *et al.*, 2000).

Importantly, we also demonstrate that pharmacological antagonism of ecto-5'-nucleotidase reduces chemoafferent activity in hypoxia without completely abolishing it. Thus, effective targeting of ecto-5'-nucleotidase would still allow for transmission of a definitive hypoxic neuronal signal into the CNS, albeit significantly reduced. Demonstration of significant ecto-5'-nucleotidase mRNA expression in the rat CB has been confirmed (Salman *et al.*, 2016). However, the precise localisation of ecto-5'-nucleotidase and ectonucleoside triphosphate diphosphohydrolyase 1 (CD39) remains to be defined. Given the known co-localisation between ecto-5'-nucleotidase and A<sub>2A</sub> receptors in striatal neurones in the CNS (Augusto *et al.*, 2013), we suggest that ecto-5'-nucleotidase in the CB is likely located similarly i.e. on type 1 cells and nerve endings.

In contrast, inhibitory targeting of the ENT had no effect on the normoxic or hypoxic discharge frequency suggesting that insufficient quantities of adenosine are released through ENT in normoxia and hypoxia in order to impact directly on hypoxic neuronal discharge. This is consistent with earlier observations demonstrating that extracellular adenosine recovery in normoxia was independent on ENT activity (Conde & Monteiro, 2004; Conde *et al.*, 2012).

A direct up-regulation of ecto-5'-nucleotidase activity in the CB in chronic or chronic intermittent hypoxia remains to be verified. However, recent studies suggest that ATP and 5-HT can both augment ATP release from type 2 cells thereby amplifying synaptic ATP concentration (Murali & Nurse, 2016; Murali *et al.*, 2017). Our data suggests that increased ecto-5'-nucleotidase activity driven by this increased availability of ATP would act to augment CB O<sub>2</sub> sensitivity, and this may contribute to exaggerated chemoafferent responses observed in CH or CIH.

Our data also shows that inhibition of ecto-5'-nucleotidase and adenosine receptors also attenuates sensory discharge frequency in response to mitochondrial inhibition. This is important not only because a run-down in mitochondrial energy metabolism is considered a necessary step in coupling hypoxia with type 1 cell stimulation (Duchen & Biscoe, 1992; Buckler & Turner, 2013; Holmes *et al.*,

2016), but also in view of the evidence that chronic impairment of mitochondrial function is associated with the increase in CB sensory activity in animal models of OSA (Peng *et al.*, 2003). Thus, our data supports the idea that functional inhibition of ecto-5'-nucleotidase inhibits basal and hypoxic discharge frequency and responses to stimuli relevant to CB pathology.

### **Ecto-5'-nucleotidase reduces HVR and modifies cardiovascular responses to hypoxia**

Intravenous infusion of AOPCP to inhibit ecto-5'-nucleotidase *in vivo* caused an inhibition of HVR (Figure 4) and evoked a smaller reduction in  $P_a\text{CO}_2$  during hypoxia (Figure 5), suggestive of a less marked hyperventilation. We also calculated that the hypoxic ventilatory exponential rate constant is depressed in the presence of AOPCP. These observations are in agreement with our *in vitro* data and suggest that AOPCP effectively blunts CB hypoxic sensitivity *in vivo*. Previous work has also show that 8-SPT (a compound that does not cross the blood brain barrier) also inhibits the acute phase of the HVR in rats (Lee *et al.*, 2005), an action that our data implies is due to adenosine being important in establishing the CB sensitivity to hypoxia (Figure 2).

AOPCP also reduced the HR response to hypoxia and exaggerated rise in FVC and fall in MABP. These data are consistent with a reduced elevation in sympathetic outflow to the heart and vasculature in hypoxia caused by ecto-5'-nucleotidase inhibition. In view of the positive correlation between CB chemodischarge and sympathetic outflow during hypoxic stimulation, the reduction in sympathetic activity is likely to be due to reduced CB stimulation. In addition, this could also be due to a lower level of hyperventilation and lung stretch receptor activation, again a consequence of blunted CB hypoxic chemodischarge and respiratory drive. If similar findings are validated in humans then pharmacological inhibition of CB ecto-5'-nucleotidase may offer a novel and important means of reducing cardiovascular sympathetic outflow in CB related pathology.

That said, AOPCP also evoked a significant hyperventilation under normoxic conditions, as evidenced by a significant decrease in  $P_a\text{CO}_2$ . It also provoked a rise in baseline HR and reduced FVC in normoxia. Ecto-5'-nucleotidase is known to be expressed in brain regions where it regulates adenosine generation (Chu *et al.*, 2014). Furthermore, A1 receptor activation depresses central respiratory drive (Montandon *et al.*, 2007). Although speculative we therefore suggest that

hyperventilation in normoxic conditions is a consequence of a reduction in adenosine concentration in central respiratory brain regions following inhibition of cerebral ecto-5'-nucleotidase. This data raises the intriguing possibility that ecto-5'-nucleotidase may also have an important functional role in mediating central respiratory drive in normoxic conditions. These findings also suggest that selective targeting of the CB ecto-5' nucleotidase or development of nucleotidase inhibitors that do not cross the blood brain barrier could be necessary to selectively reduce CB sensory activity.

### Limitations

The present studies were performed in rodents and validation of a functional role for CB ecto-5' nucleotidase is necessary in humans. Our *in vivo* experiments were performed specifically using Alfaxan, an anaesthetic that improves preservation of cardiovascular reflexes. However, we cannot rule out that there would be some suppression of chemoreceptor function, although we speculate that if this is the case then our estimates of reduction in HVR caused by ecto-5' nucleotidase may be an underestimate. Furthermore, our experiments used a pharmacological approach to probe the importance of ecto-5'-nucleotidase in CB function and HVR. We targeted ecto-5'-nucleotidase with AOPCP as this is known to be selective for membrane-bound ecto-5'-nucleotidase and has been used as such, previously, to distinguish the activities of membrane bound and cytosolic nucleotidases (Sala-Newby *et al.*, 2003). However, to validate our findings it would be interesting to perform similar experiments in mice deficient in CD73, ideally restricted to CB or tyrosine hydroxylase positive cells. Finally, in view of the present findings evaluation of expression levels of CB ecto-5' nucleotidase and contribution to CB sensory activity in diseases such as OSA, heart failure and diabetes is warranted.

### Conclusions

Ecto-5'-nucleotidase (CD73) is a novel mediator of CB chemoafferent activity. Inhibition of ecto-5'-nucleotidase reduces basal CB sensory neuronal activity and attenuates responses to hypoxia. Inhibition of ecto-5'-nucleotidase *in vivo* blunts HVR and modifies cardiovascular changes in hypoxia, including reduced HR elevation. Future studies will be important in order to assess whether ecto-5'-nucleotidase may be a feasible target for reducing CB activity and sympathetic outflow in CB related cardiovascular pathology.

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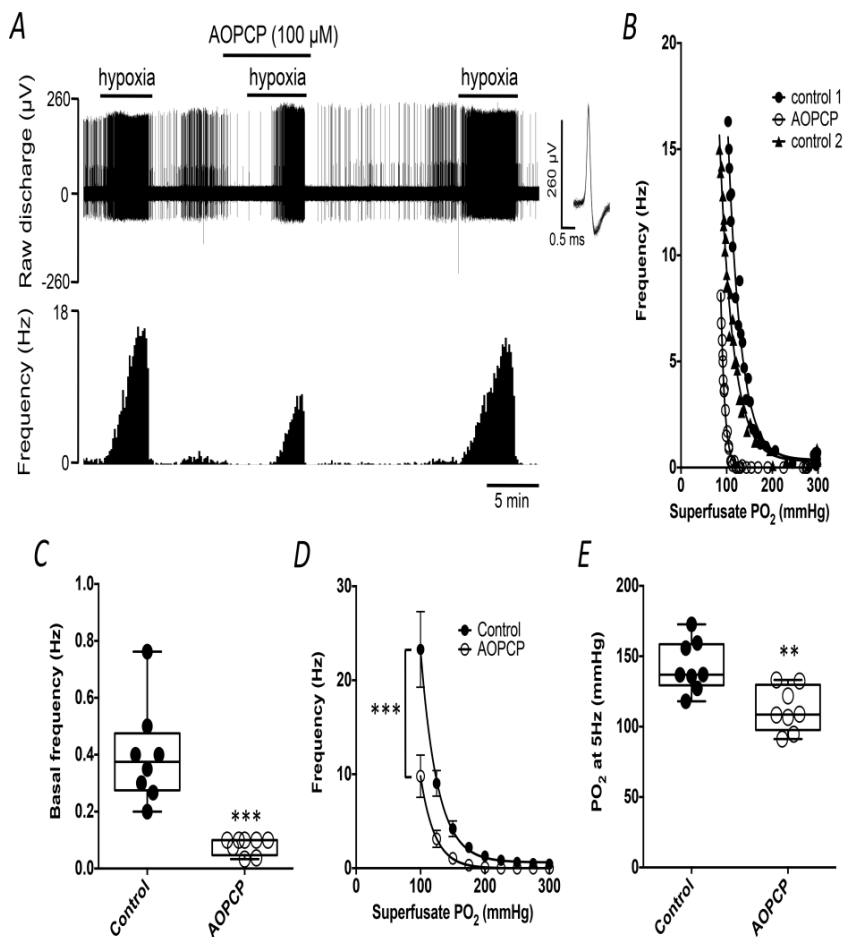
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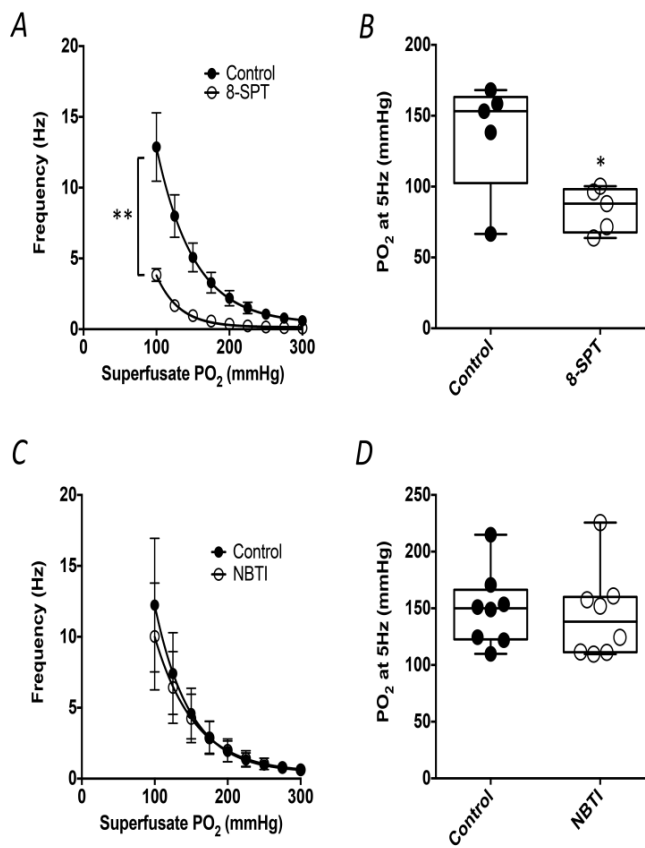
**Figure Legends****Figure 1. Ecto-5' nucleotidase inhibition reduces CB sensory activity in hypoxia**

**A** Characteristic example of CB chemoafferent response to graded hypoxia (300–100mmHg PO<sub>2</sub>) recorded in the presence and absence of 100μM AOPCP. Overdrawn action potentials demonstrate the single fibre discrimination. **B** Hypoxic response curves for the corresponding fibre recorded in **A**. **C** Mean basal single fibre frequency ± AOPCP. **D** Mean frequency ± AOPCP during graded hypoxia. **E** PO<sub>2</sub> values required to stimulate a discharge frequency of 5Hz during hypoxia ±AOPCP. For C-E \*\* and \*\*\* denotes P<0.01 and P<0.001 AOPCP vs control, n=8 fibres, N=5 CB preparations.



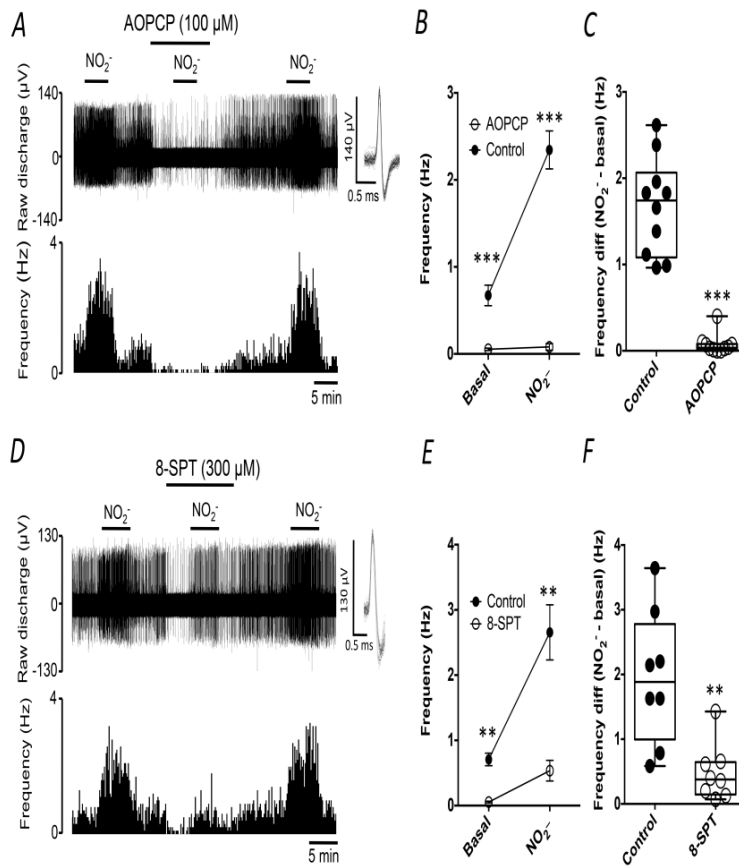
**Figure 2. Inhibition of adenosine receptors but not the equilibrative nucleotide transporter (ENT) blunts CB discharge frequency in hypoxia.**

**A** Mean single fibre frequency  $\pm$  8-SPT (300 $\mu$ M; adenosine receptor antagonist) during graded hypoxia. **B** PO<sub>2</sub> values at 5Hz during hypoxia  $\pm$  8-SPT. \* and \*\* denotes P<0.05 and P<0.01 8-SPT vs control n=6 fibres, N=4 CB preparations. **C** Mean single fibre frequency  $\pm$  NBTI (10 $\mu$ M; ENT antagonist) during graded hypoxia. **D** PO<sub>2</sub> values at 5Hz during hypoxia  $\pm$  NBTI. n=8 fibres, N=4 CB preparations.



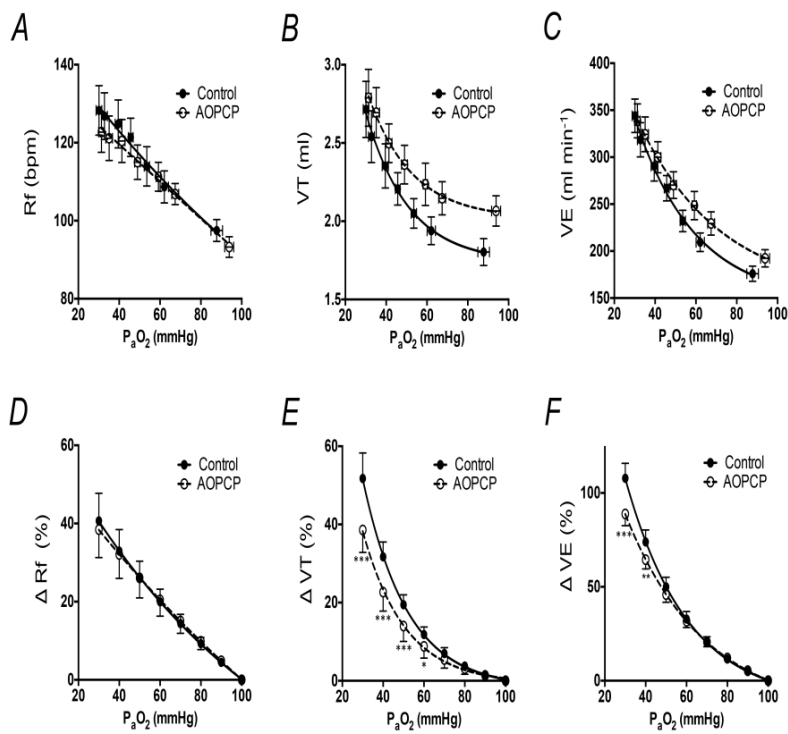
**Figure 3. Carotid body stimulation by mitochondrial inhibition is attenuated by antagonism of ecto-5'-nucleotidase and adenosine receptors.**

**A** Example recording of the CB sensory response to the mitochondrial inhibitor nitrite ( $\text{NO}_2^-$ ; 10mM) in the presence and absence of AOPCP (100 $\mu\text{M}$ ). Overdrawn action potentials demonstrate single fibre discrimination. **B** Mean discharge frequencies recorded under basal conditions and following addition of  $\text{NO}_2^-$ ,  $\pm$ AOPCP. **C** Frequency differences ( $\text{NO}_2^-$  – basal) for each fibre in the presence and absence of AOPCP. \*\*\* denotes  $P < 0.001$  AOPCP vs control,  $n = 10$  fibres,  $N = 5$  CB preparations. **D** Recording of the CB sensory response to  $\text{NO}_2^-$  in the presence and absence of 8-SPT (300 $\mu\text{M}$ ). **E** Mean discharge frequencies recorded under basal conditions and following addition of  $\text{NO}_2^-$ ,  $\pm$  8-SPT. **F** Frequency differences ( $\text{NO}_2^-$  – basal) for each fibre in the presence and absence of 8-SPT. \*\*denotes  $P < 0.01$  8-SPT vs control,  $n = 8$  fibres,  $N = 5$  CB preparations.



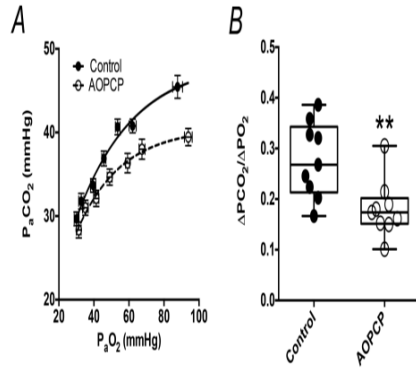
**Figure 4. Ventilatory responses to hypoxia are reduced by antagonism of ecto-5'-nucleotidase.**

**A-C** Respiratory frequency ( $f_R$ ), tidal volume ( $V_T$ ) and minute ventilation ( $\dot{V}_E$ ) at baseline and in response to graded hypoxia,  $\pm$ AOPCP ( $160\mu\text{g}\cdot\text{kg}^{-1}$ , I.V.). **D-E** % changes in  $f_R$ ,  $V_T$  and  $\dot{V}_E$  at different calculated  $P_a\text{O}_2$ , compared to 100mmHg baseline, for control and during AOPCP infusion. For D-F \*, \*\* and \*\*\* denotes  $P<0.05$ ,  $P<0.01$  and  $P<0.001$  AOPCP vs control,  $N=9$ .

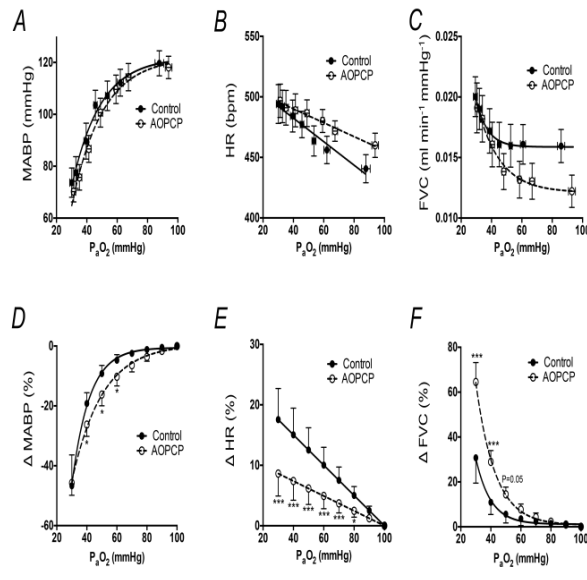




**Figure 5. Hypoxia induced fall in  $P_a\text{CO}_2$  is blunted by inhibition of ecto-5'-nucleotidase. A** Absolute changes in  $P_a\text{CO}_2$  during graded hypoxia in the presence and absence of AOPCP. **B** Calculated reduction in  $P_a\text{CO}_2$  per mmHg fall in  $P_a\text{O}_2$ , in the presence and absence of AOPCP. \*\* denotes,  $P < 0.01$ ,  $N = 9$ .



**Figure 6. Cardiovascular responses to hypoxia are modified by inhibition of ecto-5'-nucleotidase. A-C** Mean arterial blood pressure (MABP), heart rate (HR) and femoral vascular conductance (FVC) during air breathing and in response to graded hypoxia,  $\pm$ AOPCP ( $160\mu\text{g kg}^{-1}$ , I.V.). **D-E** % changes in MABP, HR and FVC at different calculated  $P_a\text{O}_2$ , compared to 100mmHg baseline, for control and AOPCP infusion. For D-F \*and \*\*\* denotes  $P < 0.05$ , and  $P < 0.001$  AOPCP vs control,  $N = 9$  (MABP and HR),  $N = 8$  FVC.



**Competing Interests**

The authors declare no competing interests.

**Author Contributions**

Experimental concepts and design were devised by PK, APH, CJR & AMC. Data was collected by APH, CJR, SAP & AMC. APH, CJR, AMC & PK interpreted and analysed data. Original drafting of the manuscript was performed by APH and was edited by all authors.

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