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

Holmes, Andrew; Ray, Clare; Pearson, Selina A; Coney, Andrew; Kumar, Prem

DOI:
10.1113/JP274498

Document Version
Peer reviewed version

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

Andrew P. Holmes[1], Clare J. Ray[2], Selina A. Pearson[3], Andrew M. Coney[2] & Prem Kumar[2]

1. Institute of Cardiovascular Sciences, University of Birmingham, Edgbaston, Birmingham, UK, B15 2TT

2. Institute of Clinical Sciences, University of Birmingham, Edgbaston, Birmingham, UK,

B15 2TT

3. Mouse Pipelines, Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, UK, CB10 1SA

Running Title: Ecto-5'-nucleotidase and carotid body chemosensitivity

Key Words: carotid body, adenosine, ecto-5'-nucleotidase

Corresponding Author: Prem Kumar

Address: Institute of Clinical Sciences, University of Birmingham, Edgbaston, Birmingham, UK, B15 2TT

Email: p.kumar@bham.ac.uk

Tel: 0121 414 6907

This is an Accepted Article that has been peer-reviewed and approved for publication in the The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; doi: 10.1113/JP274498.

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 α,β-methylene ADP (AOPCP) in the whole CB

preparation *in vitro* reduced basal discharge frequency by 76 ± 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±6%, $\Delta\dot{V}_E$ AOPCP 64±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 - α,β-methylene ADP. ENT - equilibrative nucleotide transporter. 8-SPT - 8-(p-sulfophenyl) theophylline. NBTI - nitrobenzylthioinosine. 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_2 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_{2A} and/or A_{2B} 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_{2A} 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_2, 3L min^{-1}), 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 37ºC, 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_2 was continuously measured using an O_2 electrode (ISO2; World Precision Instruments) and O_2 meter

(OXELP; World Precision Instruments). The PO_2 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_2 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_2PO_4, 5 Na_2SO_4, 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$ and 11 D-glucose, 37ºC, pH 7.4. For normoxia/hyperoxia the superfusate PO_2 was maintained throughout at a level that kept spontaneous chemodischarge between 0.25 and 1.0Hz (Holmes *et al.*, 2014). PCO_2 was maintained at 40mmHg. For hypoxia, the superfusate PO_2 was gradually reduced, at constant PCO_2, 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_2, 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_2 in mmHg, a is the discharge frequency as the PO_2 tends to infinity (offset), b is the discharge frequency when the PO_2 is 0 mmHg (minus the offset) and c is the exponential rate constant. In addition, for any given discharge frequency, the corresponding PO_2 could be calculated using the inverse function of the exponential decay curve, $x = -(Ln((y - a)/b))/c$, where x is the PO_2 in mmHg, y is the single fibre discharge frequency in Hz and a, b and c are constants as above. The PO_2 at a frequency of 5Hz was used to quantify a PO_2 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_2^-, 10mM) was used to induce moderate elevations in chemoafferent discharge (Holmes *et al.*, 2016). Responses to NO_2^- 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_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 (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_2. An arterial blood gas sample was taken at each inspired O_2. 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 α,β-methylene ADP (AOPCP; 160μg kg^{-1}, 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 ± 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 μM AOPCP (Conde & Monteiro, 2004; Holmes *et al.*, 2015) reduced basal single fibre activity by 76 ± 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 PO_2 values of 175 to 100mmHg (Figure 1D). Grouped paired measurements showed that AOPCP significantly decreased the superfusate 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 μM (Wyatt *et al.*, 2007; Holmes *et al.*, 2015)) (figure 2A& B). 8-SPT decreased chemoafferent activity at all 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 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; 10μM, 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 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 100μM AOPCP on the response to mitochondrial inhibition using NO_2^- is shown in Figure 3A. AOPCP almost completely abolished basal frequency and the response to NO_2^- (Figure 3B). The absolute elevation in chemoafferent activity evoked by NO_2^- (NO_2^- – basal) was significantly decreased in presence of AOPCP, measuring only approximately 0.4% of control (Figure 3C). 8-SPT (300 μM) also significantly diminished frequency responses to NO_2^- (Figure 3D-E). The rise in discharge frequency elicited by NO_2^- in the presence of 8-SPT was reduced in each fibre studied, with the mean 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±10; AOPCP 195±10 ml 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_aCO_2 (control 45±1; AOPCP 39±1mmHg, $P<0.05$, 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_aCO_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 40mmHg P_aO_2; $\Delta\dot{V}_E$ control 74±6%, $\Delta\dot{V}_E$ AOPCP 64±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±0.0044; AOPCP 0.022±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_aCO_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±12; AOPCP 460±10bpm, n=9) and reduced femoral vascular conductance (control 0.016±0.001; AOPCP 0.012±0.001 ml min^{-1} $mmHg^{-1}$, 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 40mmHg P_aO_2; ΔHR control 15±4%, ΔHR AOPCP 7±3%, n=9), enhanced the hypoxic increase in femoral vascular conductance (FVC) (e.g. at 40mmHg P_aO_2; ΔFVC control 11±5 %, ΔFVC AOPCP 29±5 %, n=8) and exaggerated the fall in MABP (e.g. at 40mmHg P_aO_2; ΔMABP control -19±3%, ΔMABP AOPCP -26±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_2 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 & Spychala, 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_2 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_{2A} and A_{2B} 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_{2A} 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_1 receptors, a functional role is unlikely in our studies given the absence of A_1 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_{2A} 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_2 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_aCO_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_aCO_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 chemorecptor 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.

References

Augusto E, Matos M, Sevigny J, El-Tayeb A, Bynoe MS, Muller CE, Cunha RA & Chen JF. (2013). Ecto-5 '-Nucleotidase (CD73)-Mediated Formation of Adenosine Is Critical for the Striatal Adenosine A(2A) Receptor Functions. *J Neurosci* **33,** 11390-11399.

Bianchi V & Spychala J. (2003). Mammalian 5'-nucleotidases. *J Biol Chem* **278,** 46195-46198.

Buckler KJ & Turner PJ. (2013). Oxygen sensitivity of mitochondrial function in rat arterial chemoreceptor cells. *J Physiol* **591,** 3549-3563.

Cass CE, Young JD & Baldwin SA. (1998). Recent advances in the molecular biology of nucleoside transporters of mammalian cells. *Biochem Cell Biol* **76,** 761-770.

Chu S, Xiong W & Parkinson FE. (2014). Effect of ecto-5 '-nucleotidase (eN) in astrocytes on adenosine and inosine formation. *Purinergic Signal* **10,** 603-609.

Conde SV, Gonzalez C, Batuca JR, Monteiro EC & Obeso A. (2008). An antagonistic interaction between A2B adenosine and D2 dopamine receptors modulates the function of rat carotid body chemoreceptor cells. *J Neurochem* **107,** 1369-1381.

Conde SV & Monteiro EC. (2004). Hypoxia induces adenosine release from the rat carotid body. *J Neurochem* **89,** 1148-1156.

Conde SV, Monteiro EC, Rigual R, Obeso A & Gonzalez C. (2012). Hypoxic intensity: a determinant for the contribution of ATP and adenosine to the genesis of carotid body chemosensory activity. *J Appl Physiol* **112,** 2002-2010.

Conde SV, Obeso A, Vicario I, Rigual R, Rocher A & Gonzalez C. (2006). Caffeine inhibition of rat carotid body chemoreceptors is mediated by A2A and A2B adenosine receptors. *J Neurochem* **98,** 616-628.

Del Rio R, Andrade DC, Lucero C, Arias P & Iturriaga R. (2016). Carotid Body Ablation Abrogates Hypertension and Autonomic Alterations Induced by Intermittent Hypoxia in Rats. *Hypertension* **68,** 436-+.

Duchen MR & Biscoe TJ. (1992). Relative mitochondrial membrane potential and [Ca2+]i in type I cells isolated from the rabbit carotid body. *J Physiol* **450,** 33-61.

Fitzgerald RS, Shirahata M & Wang HY. (1999). Acetylcholine release from cat carotid bodies. *Brain Res* **841,** 53-61.

Gauda EB. (2000). Expression and localization of A2a and A1-adenosine receptor genes in the rat carotid body and petrosal ganglia. A2a and A1-adenosine receptor mRNAs in the rat carotid body. *Adv Exp Med Biol* **475,** 549-558.

Hauton D, Holmes A, Ziff O & Kumar P. (2013). The impact of acute and chronic catecholamines on respiratory responses to hypoxic stress in the rat. *Pflugers Arch* **465,** 209-219.

Holmes AP, Nunes AR, Cann MJ & Kumar P. (2015). Ecto-5 '-Nucleotidase, Adenosine and Transmembrane Adenylyl Cyclase Signalling Regulate Basal Carotid Body Chemoafferent Outflow and Establish the Sensitivity to Hypercapnia. In *Arterial Chemoreceptors in Physiology and Pathophysiology*, ed. Peers C, Kumar P, Wyatt CN, Gauda E, Nurse CA & Prabhakar N, pp. 279-289. Springer-Verlag Berlin, Berlin.

Holmes AP, Turner PJ, Buckler KJ & Kumar P. (2016). Moderate inhibition of mitochondrial function augments carotid body hypoxic sensitivity. *Pflugers Arch* **468,** 143-155.

Holmes AP, Turner PJ, Carter P, Leadbeater W, Ray CJ, Hauton D, Buckler KJ & Kumar P. (2014). Glycogen metabolism protects against metabolic insult to preserve carotid body function during glucose deprivation. *J Physiol* **592,** 4493-4506.

Kobayashi S, Conforti L & Millhorn DE. (2000). Gene expression and function of adenosine A(2A) receptor in the rat carotid body. *Am J Physiol Lung Cell Mol Physiol* **279,** L273-282.

Kumar P & Prabhakar NR. (2012). Peripheral Chemoreceptors: Function and Plasticity of the Carotid Body. *Comprehensive Physiology***,** 141–219.

Lee SD, Nakano H & Farkas GA. (2005). Adenosinergic modulation of ventilation in obese zucker rats. *Obes Res* **13,** 545-555.

Linz D, Linz B, Hohl M & Bohm M. (2016). Atrial arrhythmogenesis in obstructive sleep apnea: Therapeutic implications. *Sleep Med Rev* **26,** 87-94.

Livermore S & Nurse CA. (2013). Enhanced adenosine A2b receptor signaling facilitates stimulus-induced catecholamine secretion in chronically hypoxic carotid body type I cells. *Am J Physiol Cell Physiol* **305,** C739-750.

McQueen DS & Ribeiro JA. (1986). Pharmacological characterization of the receptor involved in chemoexcitation induced by adenosine. *Br J Pharmacol* **88,** 615-620.

Montandon G, Kinkead R & Bairam A. (2007). Disruption of adenosinergic modulation of ventilation at rest and during hypercapnia by neonatal caffeine in young rats: role of adenosine A(1) and A(2A) receptors. *Am J Physiol-Regul Integr Comp Physiol* **292,** R1621-R1631.

Murali S & Nurse CA. (2016). Purinergic signalling mediates bidirectional crosstalk between chemoreceptor typeI and glial-like typeII cells of the rat carotid body. *J Physiol-London* **594,** 391-406.

Murali S, Zhang M & Nurse CA. (2017). Evidence that 5-HT stimulates intracellular Ca2+ signalling and activates pannexin-1 currents in type II cells of the rat carotid body. *J Physiol*.

Narkiewicz K, Ratcliffe LE, Hart EC, Briant LJ, Chrostowska M, Wolf J, Szyndler A, Hering D, Abdala AP, Manghat N, Burchell AE, Durant C, Lobo MD, Sobotka PA, Patel NK, Leiter JC, Engelman ZJ, Nightingale AK & Paton JF. (2016). Unilateral Carotid Body Resection in Resistant Hypertension: A Safety and Feasibility Trial. *JACC Basic to translational science* **1,** 313-324.

Narkiewicz K, van de Borne PJH, Montano N, Dyken ME, Phillips BG & Somers VK. (1998). Contribution of tonic chemoreflex activation to sympathetic activity and blood pressure in patients with obstructive sleep apnea. *Circulation* **97,** 943-945.

Niewinski P, Janczak D, Rucinski A, Tubek S, Engelman ZJ, Piesiak P, Jazwiec P, Banasiak W, Fudim M, Sobotka PA, Javaheri S, Hart EC, Paton JF & Ponikowski P. (2017). Carotid body resection for sympathetic modulation in systolic heart failure: results from first-in-man study. *European journal of heart failure* **19,** 391-400.

Nunes AR, Holmes AP, Conde SV, Gauda EB & Monteiro EC. (2014). Revisiting cAMP signaling in the carotid body. *Frontiers in physiology* **5,** 406.

Nurse CA. (2010). Neurotransmitter and neuromodulatory mechanisms at peripheral arterial chemoreceptors. *Exp Physiol* **95,** 657-667.

Peng YJ, Overholt JL, Kline D, Kumar GK & Prabhakar NR. (2003). Induction of sensory long-term facilitation in the carotid body by intermittent hypoxia: implications for recurrent apneas. *Proc Natl Acad Sci U S A* **100,** 10073-10078.

Piskuric NA & Nurse CA. (2013). Expanding role of ATP as a versatile messenger at carotid and aortic body chemoreceptors. *J Physiol* **591,** 415-422.

Prabhakar NR. (2016). Carotid body chemoreflex: a driver of autonomic abnormalities in sleep apnoea. *Exp Physiol* **101,** 975-985.

Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteiro EC & Conde SV. (2013). Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. *Diabetes* **62,** 2905-2916.

Runold M, Cherniack NS & Prabhakar NR. (1990). Effect of adenosine on isolated and superfused cat carotid body activity. *Neurosci Lett* **113,** 111-114.

Sala-Newby GB, Freeman NVE, Curto MA & Newby AC. (2003). Metabolic and functional consequences of cytosolic 5 '-nucleotidase-IA overexpression in neonatal rat cardiomyocytes. *Am J Physiol-Heart Circul Physiol* **285,** H991-H998.

Salman S, Vollmer C & Nurse CA. (2016). Characterization of Ectonucleotidase Expression in the Rat Carotid Body: Potential Regulation by Hypoxia? *The FASEB Journal* **30,** 983.982.

Schultz HD, Marcus NJ & Del Rio R. (2015). Mechanisms of carotid body chemoreflex dysfunction during heart failure. *Exp Physiol* **100,** 124-129.

Skinner MR & Marshall JM. (1996). Studies on the roles of ATP, adenosine and nitric oxide in mediating muscle vasodilatation induced in the rat by acute systemic hypoxia. *J Physiol* **495 (Pt 2),** 553-560.

Thompson EL, Ray CJ, Holmes AP, Pye RL, Wyatt CN, Coney AM & Kumar P. (2016). Adrenaline release evokes hyperpnoea and an increase in ventilatory CO2 sensitivity during hypoglycaemia: a role for the carotid body. *The Journal of physiology* **594,** 4439-4452.

Tubek S, Niewinski P, Reczuch K, Janczak D, Rucinski A, Paleczny B, Engelman ZJ, Banasiak W, Paton JF & Ponikowski P. (2016). Effects of selective carotid body stimulation with adenosine in conscious humans. *J Physiol*.

Vandier C, Conway AF, Landauer RC & Kumar P. (1999). Presynaptic action of adenosine on a 4-aminopyridine-sensitive current in the rat carotid body. *J Physiol* **515 (Pt 2),** 419-429.

Wyatt CN, Mustard KJ, Pearson SA, Dallas ML, Atkinson L, Kumar P, Peers C, Hardie DG & Evans AM. (2007). AMP-activated protein kinase mediates carotid body excitation by hypoxia. *J Biol Chem* **282,** 8092-8098.

Xu F, Xu J, Tse FW & Tse A. (2006). Adenosine stimulates depolarization and rise in cytoplasmic [Ca2+] in type I cells of rat carotid bodies. *Am J Physiol Cell Physiol* **290,** C1592-1598.

Zhang M & Nurse CA. (2004). CO2/pH chemosensory signaling in co-cultures of rat carotid body receptors and petrosal neurons: role of ATP and ACh. *J Neurophysiol* **92,** 3433-3445.

Zhang M, Zhong H, Vollmer C & Nurse CA. (2000). Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. *J Physiol* **525 Pt 1,** 143-158.

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_2) 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_2 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.

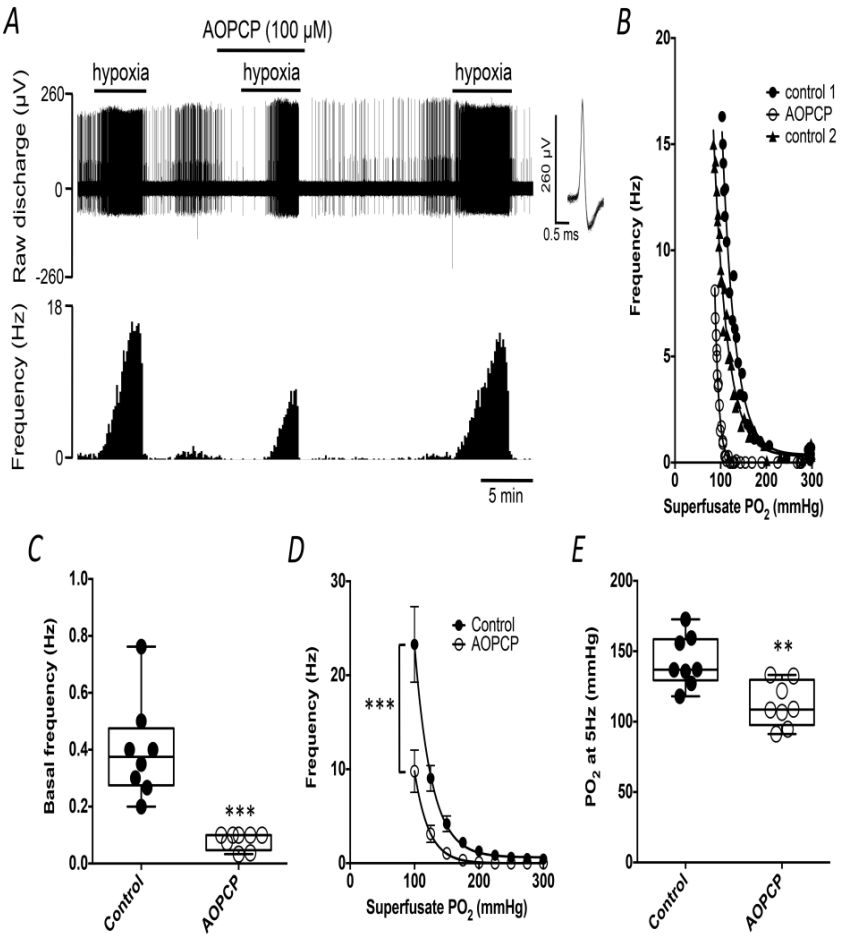


Figure 2. Inhibition of adenosine receptors but not the equilibrative nucleotide transporter (ENT) blunts CB discharge frequency in hypoxia.

A Mean single fibre frequency ± 8-SPT (300μM**;** adenosine receptor antagonist) during graded hypoxia. **B** PO_2 values at 5Hz during hypoxia± 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 ± NBTI (10μM**;** ENT antagonist) during graded hypoxia. **D** PO_2 values at 5Hz during hypoxia ± NBTI. n=8 fibres, N=4 CB preparations.

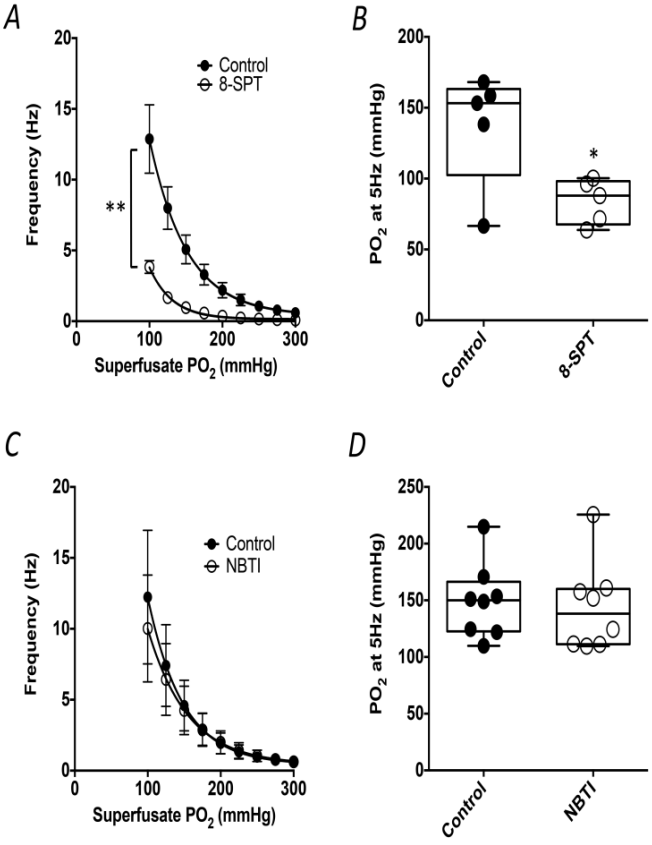


Figure 3. Carotid body stimulation by mitochondrial inhibition is attenuated by antagonism of ecto-5'-nucleotidase and adenosine receptors.

A Example recording of the CB sensory response to the mitochondrial inhibitor nitrite (NO_2^-; 10mM) in the presence and absence of AOPCP (100μM). Overdrawn action potentials demonstrate single fibre discrimination. **B** Mean discharge frequencies recorded under basal conditions and following addition of NO_2^-, ±AOPCP. **C** Frequency differences (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 NO_2^- in the presence and absence of 8-SPT (300μM). **E** Mean discharge frequencies recorded under basal conditions and following addition of NO_2^-, ± 8-SPT. **E** Frequency differences (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.

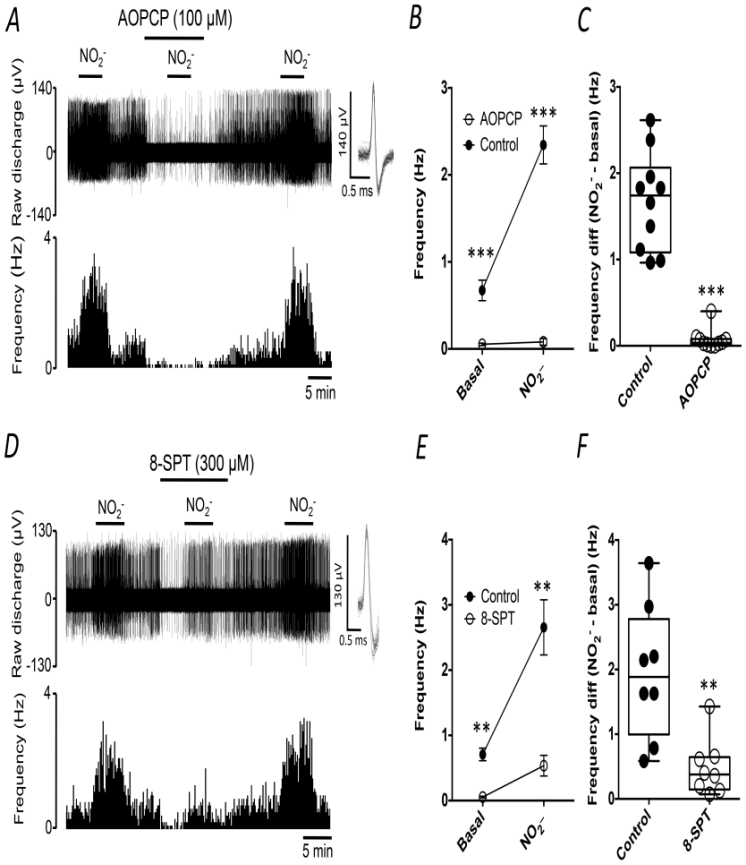


Figure 4. Ventilatory responses to hypoxia are reduced by antagonism of ecto-5'-nucleotidase. **A-C** Respiratory frequency (f_R), tidal volume (V_t) and minute ventilation ($\dot{V}_E$) at baseline and in response to graded hypoxia, ±AOPCP (160µg.kg^{-1}, I.V.). **D-E** % changes in f_R, V_t and $\dot{V}_E$ at different calculated P_aO_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.

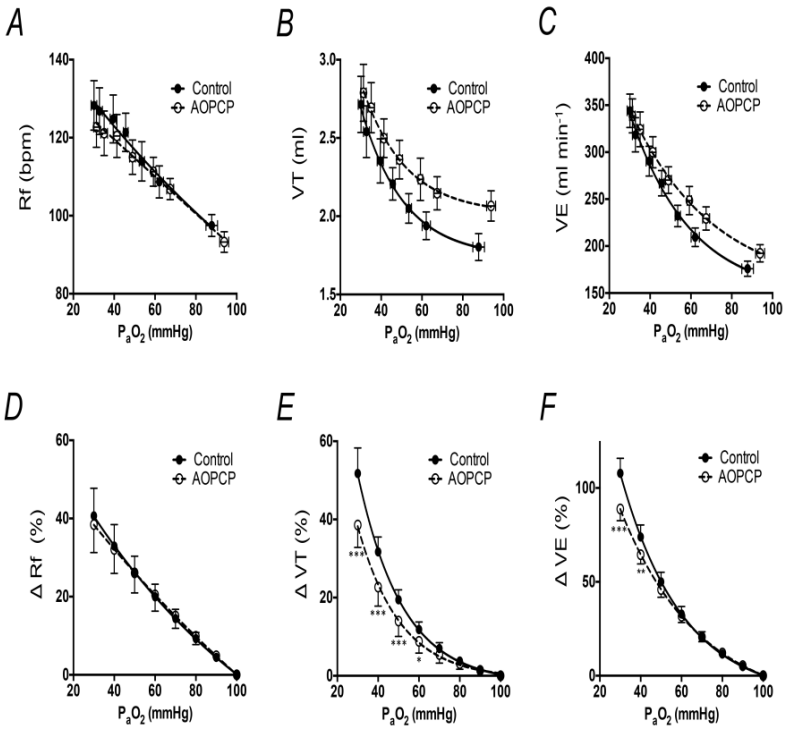


Figure 5. Hypoxia induced fall in P_aCO_2 is blunted by inhibition of ecto-5'-nucleotidase. A Absolute changes in P_aCO_2 during graded hypoxia in the presence and absence of AOPCP. **B** Calculated reduction in P_aCO_2 per mmHg fall in P_aO_2, in the presence and absence of AOPCP. ** denotes, P<0.01, N=9.

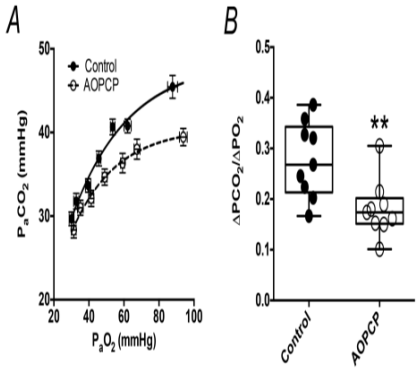
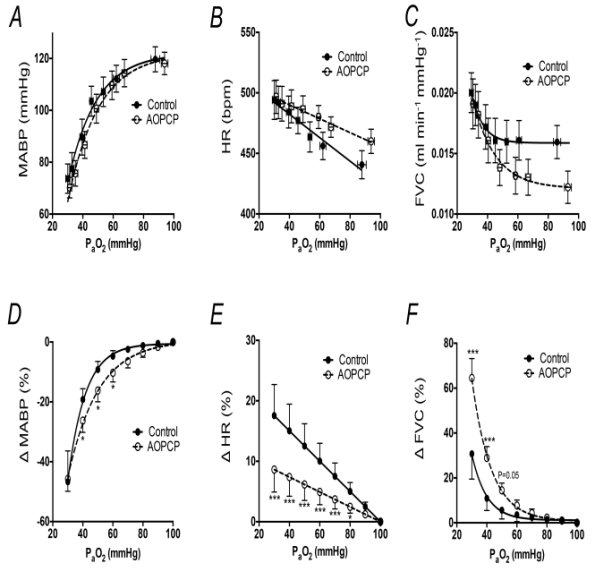


Figure 6. Cardiovascular responses to hypoxia are modified by inhibition of ecto-5'-nucleotidase. **A-C** Mean arterial blood pressure (MABP), heart rate (HR) and femoral vascular conductance (FVC) during air breathing and in response to graded hypoxia, ±AOPCP (160μg kg^{-1}, I.V.). **D-E** % changes in MABP, HR and FVC at different calculated P_aO_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.

Funding

This work was supported financially by the College of Medical and Dental Sciences, University of Birmingham, UK. APH was supported by the A.E. Hills Scholarship awarded by the Postgraduate School, University of Birmingham, UK.