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

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Key points

- Carotid body dysfunction is recognized as a cause of hypertension in a number of cardio-respiratory 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 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 show CD73 to be a novel regulator of carotid body sensory function and therefore suggest that 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 present study investigated 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 CD73 by α,β-methylene ADP (AOPCP) in the whole CB 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 indicated by reduced elevations in heart rate and exaggerated changes in femoral vascular conductance and mean arterial blood pressure. Thus we identify CD73 as a novel regulator of CB sensory activity. Future investigations are warranted to clarify whether inhibition of CD73 can effectively reduce CB activity in CB-mediated cardiovascular pathology.

Abbreviations   ABP, arterial blood pressure; AOPCP, α,β-methylene ADP; CB, carotid body; CD39, ectonucleoside triphosphate diphosphohydrolase 1; CD73, ecto-5′-nucleotidase; CSN, carotid sinus nerve; ENT, equilibrative nucleotide transporter; f_R, respiratory frequency; FVC, femoral vascular conductance; HR, heart rate; HVR, hypoxic ventilatory response; MABP, mean arterial blood pressure; NBTI, nitrobenzylthioinosine; NO_2^-, nitrite; 8-SPT, 8-(p-sulfophenyl) theophylline; $\dot{V}_E$, minute ventilation; V_t, tidal volume.

Introduction

Chronic carotid body (CB) overactivation has emerged as an important driver of hypertension in a number of cardiovascular related diseases, including heart failure, sleep disordered breathing and diabetes (Ribeiro *et al.* 2013; Schultz *et al.* 2015; Del Rio *et al.* 2016; Prabhakar, 2016). Thus, the 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 suggest that bilateral CB resection decreases sympathetic outflow and improves exercise tolerance in human heart failure 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 ∼50% of patients 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), as well as 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 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, ACh, dopamine, serotonin, adrenaline and adenosine (Nurse, 2010) (McQueen & Ribeiro, 1986; Fitzgerald *et al.* 1999; Zhang *et al.* 2000; Conde *et al.* 2006, 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, 2012; Xu *et al.* 2006) and humans (Tubek *et al.* 2016). Better characterization of the physiological relevance of adenosine and its generation and/or signalling pathways may provide useful information for identifying new potential targets that reduce 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 diphosphohydrolase 1 (CD39) and ecto-5′-nucleotidase (CD73) (Bianchi & Spychala, 2003). The 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, 2008; Xu *et al.* 2006; Livermore & Nurse, 2013) or by directly activating postsynaptic sensory fibres (via A_{2A} receptor stimulation) (Conde *et al.* 2006), with 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 is released directly into the synapse through the bidirectional equilibrative nucleotide transporter (ENT) (Cass *et al.* 1998).

The present study investigated whether pharmacological targeting of either CD73 or ENT effectively reduces CSN discharge frequency under normoxic or hypoxic conditions. Furthermore, we examined whether ventilation, heart rate, blood pressure and vascular conductance responses to hypoxia were modified by targeting CD73 *in vivo*. The data obtained suggest that antagonism of CD73 but not ENT inhibits basal CSN frequency and blunts the response to hypoxia. Inhibition of CD73 also attenuates ventilatory and cardiovascular responses to hypoxia. Thus, we propose that CD73e is a novel modulator of CB chemoafferent outflow.

Methods

Ethical approval

The procedures conducted 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 and were 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, 3 L min^{-1}). 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^{-1} collagenase type II, 0.0025 mg ml^{-1} dispase type I; Sigma-Aldrich, St Louis, MO, USA), at 37°C, for 20–30 min.

Extracellular recordings of chemoafferent activity were made from the cut end of the CSN as described previously (Holmes *et al.* 2014, 2016). The superfusate P_{O_2} was continuously measured using an O_2 electrode (ISO2; World Precision Instruments, Sarasota, FL, USA) and O_2 meter (OXELP; World Precision Instruments). The P_{O_2} and chemoafferent derived voltage were both recorded using a CED micro1401 (Cambridge Electronic Design, Cambridge, UK) and visualized on a personal computer via Spike2, version 7.1 (Cambridge Electronic Design). Chemoafferent voltage signal was sampled at 15 000 Hz and the P_{O_2} at 100 Hz. Single fibres were used for the 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$_2$PO$_4$, 5 Na$_2$SO$_4$, 1.3 MgSO$_4$, 24 NaHCO$_3$, 2.4 CaCl$_2$ and 11 D-glucose at 37°C (pH 7.4). For normoxia/hyperoxia, the superfusate P_{O_2} was maintained throughout at a level that kept spontaneous chemo-discharge between 0.25 and 1.0 Hz (Holmes *et al.* 2014). P_{CO_2} was maintained at 40 mmHg. For hypoxia, the super-fusate P_{O_2} was gradually reduced, at constant P_{CO_2}, until discharge was elevated above 10 Hz. This was repeated in the presence of pharmacological agents used to target adenosinergic signalling pathways: α,β-methylene ADP (AOPCP) (100 μM) (Conde & Monteiro, 2004; Holmes *et al.* 2015); 8-(p-sulfophenyl) theophylline (8-SPT) (300 μM) (Wyatt *et al.* 2007; Holmes *et al.* 2015); and nitrobenzylthioinosine (NBTI) (10 μM) (Conde *et al.* 2012) and again after drug washout.

The single fibre chemoafferent discharge frequency was plotted against the superfusate P_{O_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 P_{O_2} (in mmHg), a is the discharge frequency as the P_{O_2} tends to infinity (offset), b is the discharge frequency when the P_{O_2} is 0 mmHg (minus the offset) and c is the exponential rate constant. In addition, for any given discharge frequency, the corresponding P_{O_2} could be calculated using the inverse function of the exponential decay curve, $x = -(\text{Ln}[(y-a)/b])/c$, where x is the P_{O_2} (in mmHg), y is the single fibre discharge frequency in Hz and a, b and c are constants as above. The P_{O_2} at a frequency of 5 Hz was used to quantify a P_{O_2} shift in the hypoxic response curve. A value of 5 Hz was chosen because it lies on the exponential region of the hypoxic response curve

but is not of a magnitude at which the discharge is probably has started to diminish.

To evaluate chemoafferent responses to mitochondrial inhibition, the rapidly reversible mitochondrial inhibitor nitrite (NO$_2^-$, 10 mM) was used to induce moderate elevations in chemoafferent discharge (Holmes *et al.* 2016). Responses to NO$_2^-$ were measured under control conditions, in the presence of pharmacological agents targeting adenosinergic signalling pathways, as described above, and again after drug washout.

In vivo ventilatory and cardiovascular responses to hypoxia

Adult male Wistar rats (Charles River Laboratories) were initially anaesthetized with 3–4% isoflurane in O_2 at 3–4 L min^{-1} (Merial Animal Health Ltd, Woking, UK). Following cannulation of the right jugular vein, isoflurane was removed and anaesthesia was maintained with I.V. Alfaxan® (Vétoquinol UK Ltd, Buckingham, UK), 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 homeo-thermic heat pad system (Harvard Apparatus, Cambridge, MA, USA).

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, London, UK) allowing variation of inspiratory gases. Animals breathed room air as the normoxic control. For hypoxia, animals inspired hypoxic gas mixture for 2 min 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 minute 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 (Transonic Systems Inc., Ithica, NY, USA) was measured from the left femoral artery. Arterial blood samples were taken from the right femoral artery, utilizing a looped cannula technique to reduce blood loss when sampling pure mixed arterial blood. Drug infusions of AOPCP (160 μg 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).

Statistical analysis

Values are expressed as the mean ± SEM unless otherwise stated. Statistical analysis was performed using (i) a paired two-tailed Student's *t* test or (ii) repeated measures one-way ANOVA with Bonferroni or Dunnett's *post hoc* analysis where appropriate (StatView, version 5; SAS

Institute Inc., Cary, NC, USA; or Prism, version 6; GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

CD73 regulates peripheral chemoreceptor activity in normoxia and hypoxia

Inhibition of CD73 using 100 μM AOPCP (Conde & Monteiro, 2004; Holmes *et al.* 2015) reduced basal single fibre activity by $76 \pm 5\%$ (Fig. 1*A–C*). AOPCP also significantly attenuated the CB chemoafferent response to hypoxia. A characteristic example of a single fibre hypoxic response is shown in Fig. 1(*A* and *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 (Fig. 1*A* and *B*). Mean chemoafferent discharge frequency was significantly depressed over a range of hypoxic superfusate P_{O_2} values from 175 to 100 mmHg (Fig. 1*D*). Grouped paired measurements showed that AOPCP significantly decreased the superfusate P_{O_2} required to elicit a discharge frequency of 5 Hz by a mean value of ~30 mmHg, which is consistent with blunted hypoxic sensitivity (Fig. 1*E*).

A similar reduction in basal frequency was observed in the presence of the adenosine receptor antagonist 8-SPT (300 μM) (Wyatt *et al.* 2007; Holmes *et al.* 2015)) (Fig. 2*A* and *B*). 8-SPT decreased chemoafferent activity at all P_{O_2} levels, although its inhibitory effect was enhanced during
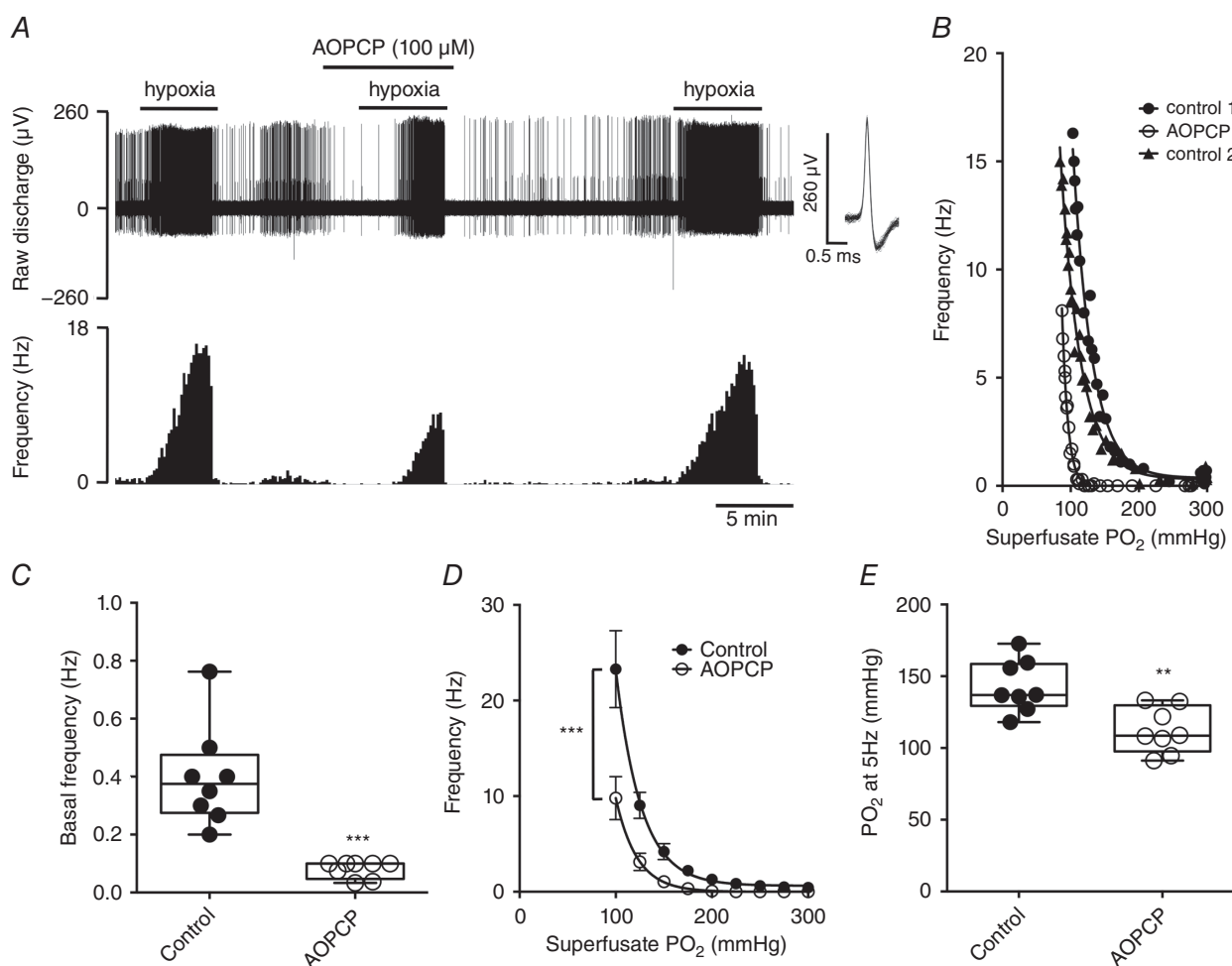

Figure 1. CD73 inhibition reduces CB sensory activity in hypoxia
A, characteristic example of CB chemoafferent response to graded hypoxia (300–100 mmHg P_{O_2}) recorded in the presence and absence of 100 μM AOPCP. Overdrawn action potentials demonstrate single fibre discrimination. *B*, hypoxic response curves for the corresponding fibre recorded in (*A*). *C*, mean basal single fibre frequency $\pm$ AOPCP. *D*, mean frequency $\pm$ AOPCP during graded hypoxia. *E*, P_{O_2} values required to stimulate a discharge frequency of 5 Hz during hypoxia $\pm$ AOPCP. (*C*) and (*E*) **P < 0.01 and ***P < 0.001 AOPCP *vs.* control ($n = 8$ fibres, $N = 5$ CB preparations).

hypoxia (Fig. 2A). In all fibres tested, the addition of 8-SPT reduced CB hypoxic sensitivity, as indicated by evoking a leftward shift in the chemoafferent hypoxic response curve, with a mean superfusate P_{O_2} reduction at 5 Hz measuring ∼40 mmHg (Fig. 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 NBTI (10 μM, 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 (Fig. 2C and D). NBTI did not significantly alter the P_{O_2} required to generate a 5 Hz frequency and produced no change of chemoafferent activity throughout exposure of the CB to graded hypoxia (Fig. 2C and D).

CD73 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 μM AOPCP on

the response to mitochondrial inhibition using NO_2^- is shown in Fig. 3(A). AOPCP almost completely abolished basal frequency and the response to NO_2^- (Fig. 3B). The absolute elevation in chemoafferent activity evoked by NO_2^- (NO_2^- − basal) was significantly decreased in the presence of AOPCP, measuring only ∼0.4% of control (Fig. 3C). 8-SPT (300 μM) also significantly diminished frequency responses to NO_2^- (Fig. 3D and 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 ∼20% of control (Fig. 3D). Thus, AOPCP and 8-SPT are effective in attenuating CB responses to both hypoxia and mitochondrial inhibition.

CD73 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 the result of an increase in tidal volume but not respiratory frequency (Fig. 4A–C). Accordingly, under these normoxic conditions, AOPCP significantly reduced P_{aCO_2} (control 45 ± 1; AOPCP 39 ± 1 mmHg, $P < 0.05$, paired
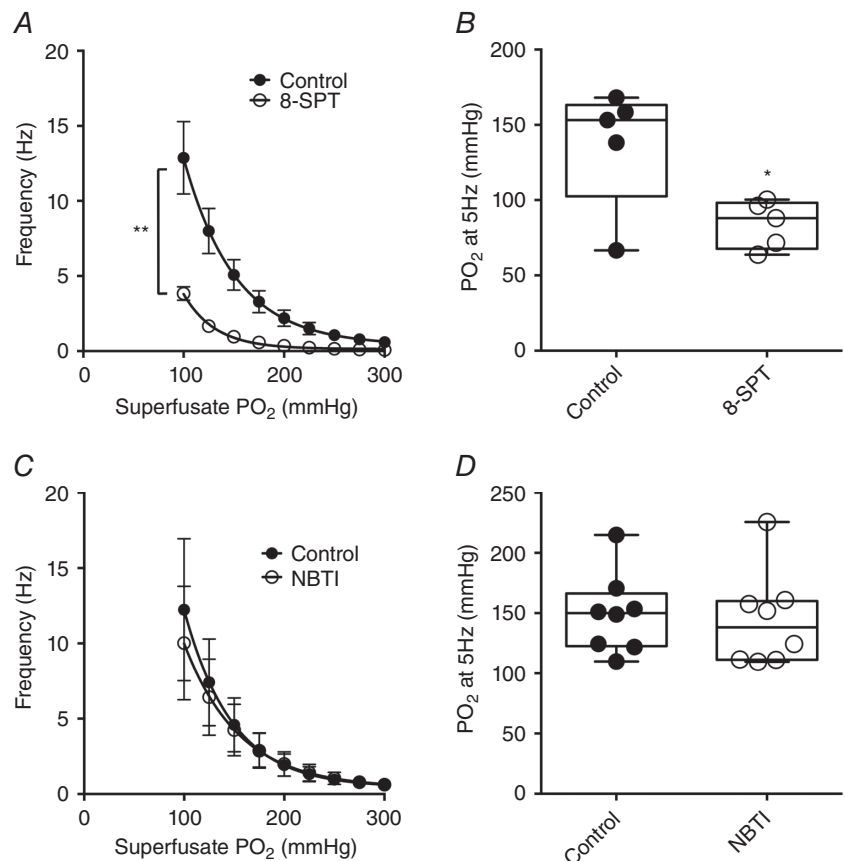

Figure 2. Inhibition of adenosine receptors but not the ENT blunts CB discharge frequency in hypoxia
A, mean single fibre frequency ± 8-SPT (300 μM; adenosine receptor antagonist) during graded hypoxia. B, P_{O_2} values at 5 Hz during hypoxia ± 8-SPT. *$P < 0.05$ and **$P < 0.01$ 8-SPT vs. control ($n = 6$ fibres, $N = 4$ CB preparations). C, mean single fibre frequency ± NBTI (10 μM; ENT antagonist) during graded hypoxia. D, P_{O_2} values at 5 Hz during hypoxia ± NBTI ($n = 8$ fibres, $N = 4$ CB preparations).

two-tailed Student's *t* test, $n = 9$), consistent with hyper-ventilation. Graded hypoxia increased minute ventilation, respiratory frequency and tidal volume (Fig. 4*A–C*) and reduced P_{aCO_2} (Fig. 5*A*) in the control and in the presence of AOPCP. However, AOPCP attenuated the magnitude of the hypoxic ventilatory response (HVR) (e.g. at 40 mmHg P_{aO_2}; $\Delta \dot{V}_E$ control 74 ± 6%, $\Delta \dot{V}_E$ AOPCP 64 ± 5%, $n = 9$) (Fig. 4*F*). This attenuation in HVR was dependent on a reduction in the hypoxia-induced elevation in tidal volume but not respiratory frequency (Fig. 4*D–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 (Fig. 5*A* and *B*). Collectively, these results suggest that, although systemic pharmacological inhibition of CD73 causes a baseline increase in ventilation, it also significantly attenuates the HVR.

CD73 mediates cardiovascular responses to hypoxia

Consistent with the baseline hyperventilation, AOPCP increased heart rate (control 441 ± 12; AOPCP 460 ± 10 beats min^{-1}, $n = 9$) and reduced femoral vascular conductance (control 0.016 ± 0.001; AOPCP 0.012 ± 0.001 ml min^{-1} mmHg^{-1}, $n = 8$) during normoxic
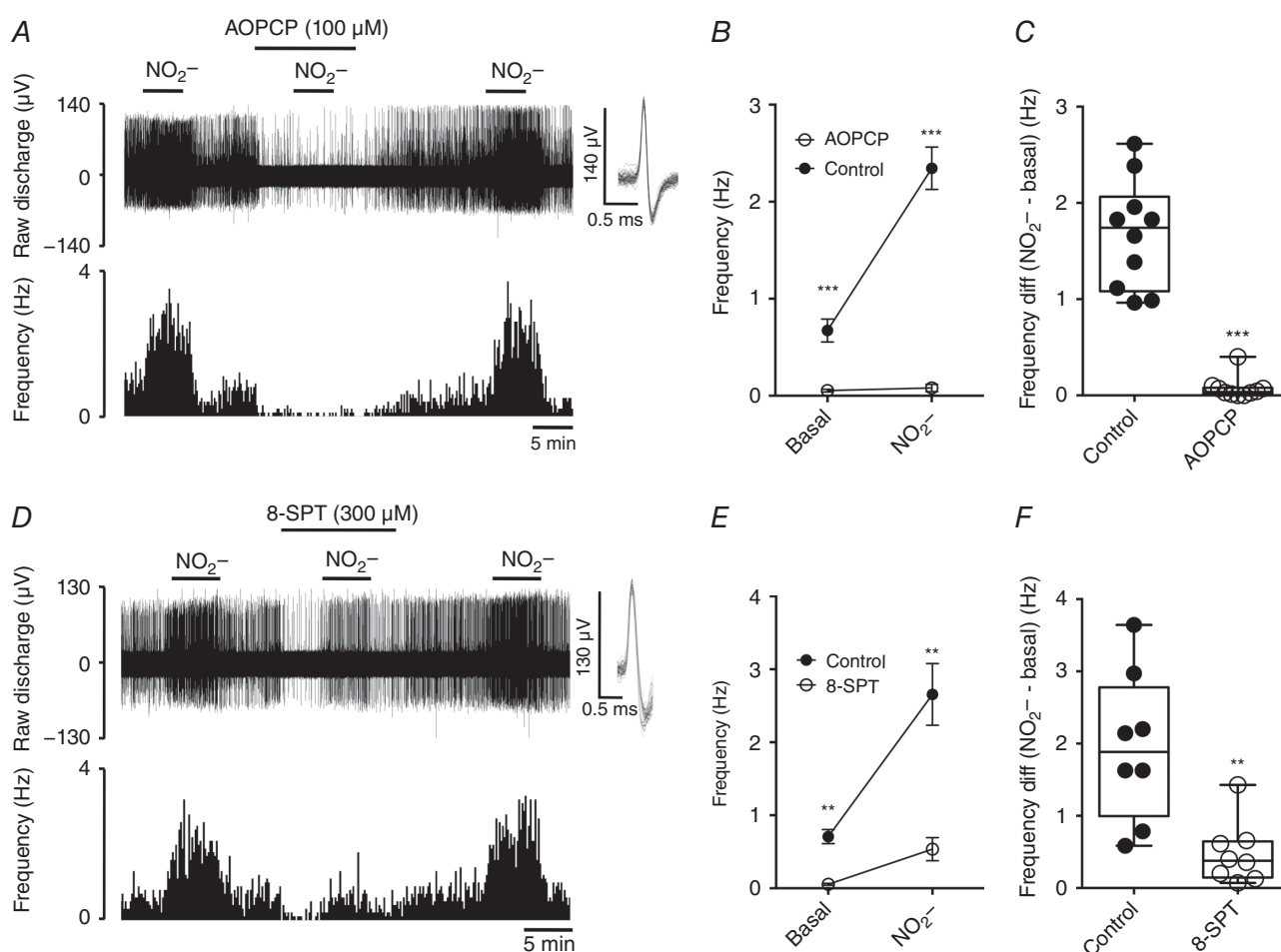

Figure 3. Carotid body stimulation by mitochondrial inhibition is attenuated by antagonism of CD73 and adenosine receptors
A, example recording of the CB sensory response to the mitochondrial inhibitor NO_2^- (10 mM) in the presence and absence of AOPCP (100 μM). Overdrawn action potentials demonstrate 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. ***$P < 0.001$ 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 (300 μM). *E*, mean discharge frequencies recorded under basal conditions and following addition of NO_2^- 8-SPT. *F*, frequency differences (NO_2^- – basal) for each fibre in the presence and absence of 8-SPT. **$P < 0.01$ 8-SPT *vs.* control ($n = 8$ fibres, $N = 5$ CB preparations).

air breathing, although this did not cause an increase in mean arterial blood pressure (MABP) (Fig. 6*A–C*). AOPCP attenuated the hypoxia induced rise in HR (e.g. at 40 mmHg P_{aO_2}; ΔHR control 15 $\pm$ 4%, ΔHR AOPCP 7 $\pm$ 3%, $n = 9$), enhanced the hypoxic increase in femoral vascular conductance (FVC) (e.g. at 40 mmHg P_{aO_2}; ΔFVC control 11 $\pm$ 5 %, ΔFVC AOPCP 29 $\pm$ 5 %, $n = 8$) and exaggerated the fall in MABP (e.g. at 40 mmHg P_{aO_2}; ΔMABP control -19 ± 3%, ΔMABP AOPCP -26 ± 4%, $n = 9$) (Fig. 6*D–F*).

Discussion

The present study identifies CD73 as a novel functional protein that contributes to CB and whole body cardio-respiratory responses to acute hypoxia. Inhibition of CD73 activity dampens CB sensory neuronal discharge *in vitro*, both under basal conditions and during stimulation by hypoxia. In addition, pharmacological inhibition of CD73 *in vivo* reduces the HVR and attenuates the hypoxia-induced rise in HR. Future investigations could
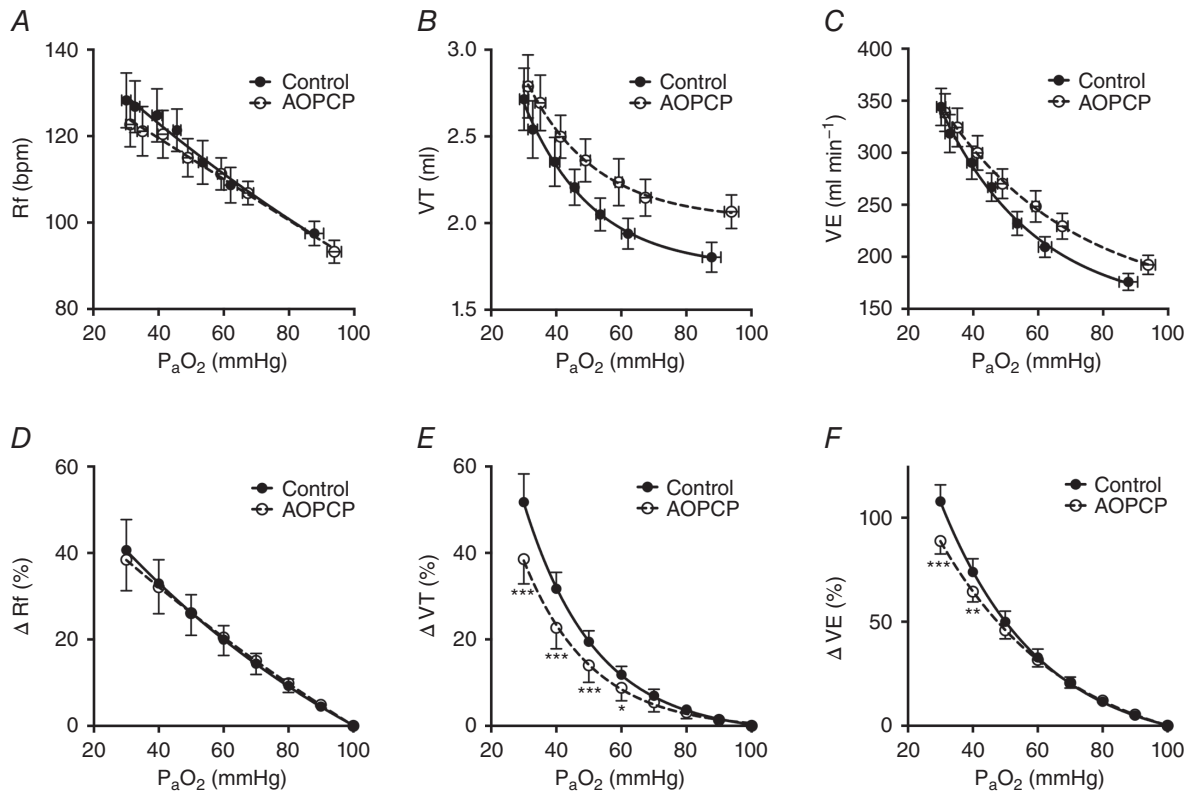
Figure 4. Ventilatory responses to hypoxia are reduced by antagonism of CD73
A–C, f_R, V_t and $\dot{V}_E$ at baseline and in response to graded hypoxia $\pm$ AOPCP (160 μg.kg^{-1}, I.V.). *D* and *E*, percentage changes in f_R, V_t and $\dot{V}_E$ at different calculated P_{aO_2} compared to 100 mmHg baseline for control and during AOPCP infusion. (*D*) to (*F*) *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ AOPCP *vs.* control ($N = 9$).
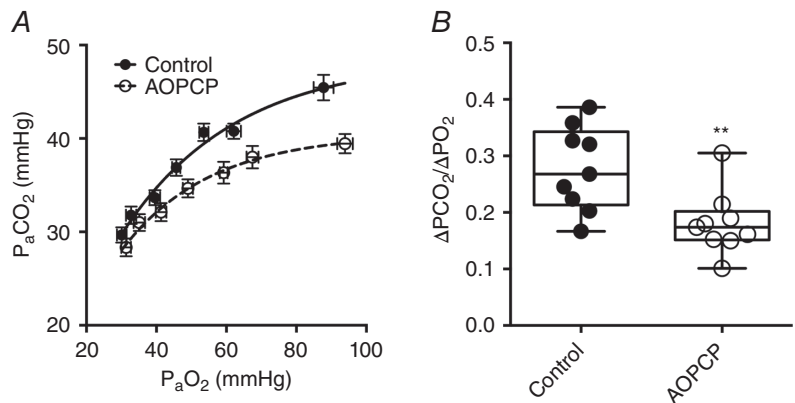
Figure 5. Hypoxia induced fall in P_{aCO_2} is blunted by inhibition of CD73
A, absolute changes in P_{aCO_2} during graded hypoxia in the presence and absence of AOPCP. *B*, calculated reduction in P_{aCO_2} per mmHg fall in P_{aO_2}, in the presence and absence of AOPCP. **$P < 0.01$ ($N = 9$).

evaluate whether targeting CB CD73 activity may be effective in reducing persistent CB hyperactivity in selected pathologies.

CD73 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 sleep disordered breathing, heart failure and diabetes (Ribeiro *et al.* 2013; Schultz *et al.* 2015; Del Rio *et al.* 2016; Prabhakar, 2016). This is a result of 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 because of 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 obstructive sleep apnoea who rely on CB sensory activity to cause arousal and recovery of airway patency. Furthermore, uni-lateral CB resection appears to confer a relatively short-term (up to 12 months) reduction in blood pressure and is effective in only ∼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 the present study, we provide the first functional evidence indicating that CD73 mediates the CB sensory discharge frequency in hypoxia. CD73 catalyses the formation of adenosine from AMP, following the initial breakdown of ATP and ADP by CD39 (Bianchi & Spychala, 2003). In the CB, a significant synaptic ATP concentration is a result of the tonic vesicular neuro-secretion 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 CD73 is therefore most probably the result of a reduction in adenosine formation.
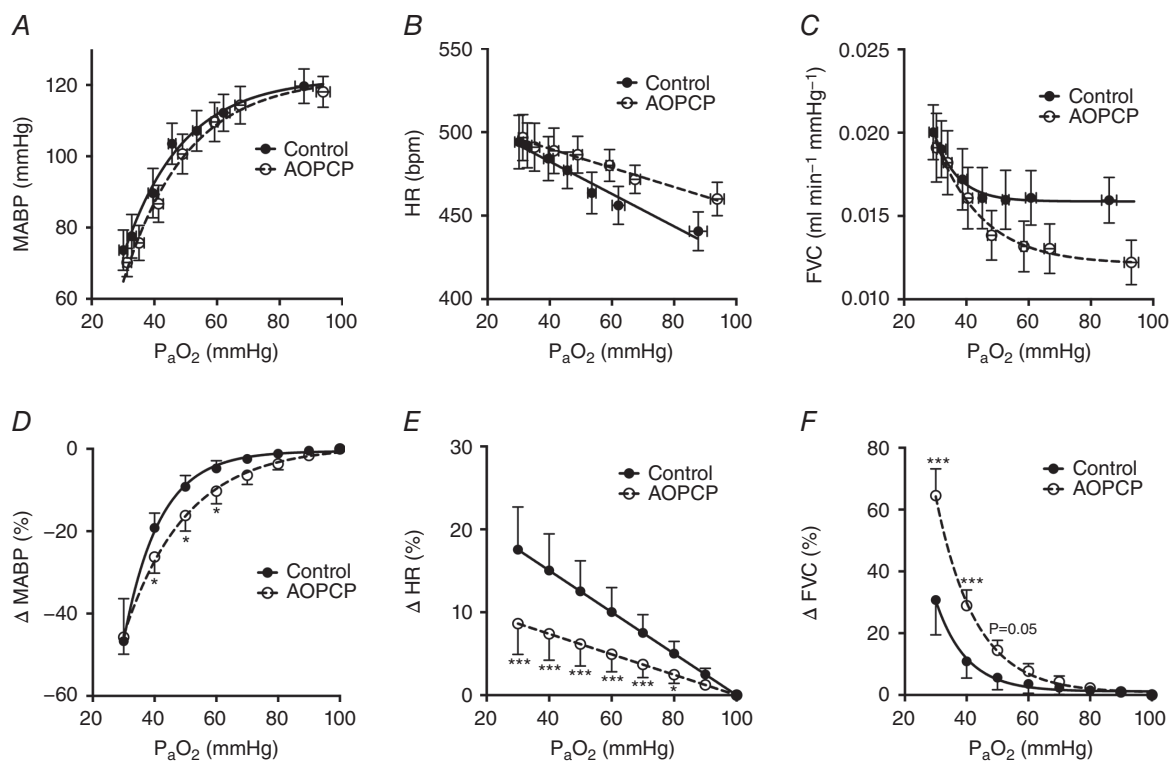

Figure 6. Cardiovascular responses to hypoxia are modified by inhibition of CD73
A–C, MABP, HR and FVC during air breathing and in response to graded hypoxia ± AOPCP (160 μg kg^{-1}, i.v.). *D–E*, percentage changes in MABP, HR and FVC at different calculated P_{aO_2} compared to 100 mmHg baseline for control and AOPCP infusion. (*D*) to (*F*) *$P < 0.05$ and ***$P < 0.001$ AOPCP *vs.* control [$N = 9$ (MABP and HR), $N = 8$ FVC].

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 CD73. Furthermore, antagonism of CD73 and adenosine receptors decreases the P_{O_2} threshold required for CB activation during hypoxia, which is consistent with a comparable reduction in CB hypoxic sensitivity.

Adenosine generated from CD73 has the potential to modulate CB hypoxic sensitivity via activation of A_{2A} and A_{2B} receptors on the type 1 cell (Conde *et al.* 2006, 2008; Xu *et al.* 2006; Livermore & Nurse, 2013) or by stimulating the nerve ending upon binding to postsynaptic A_{2A} receptors (Conde *et al.* 2006). Both of these mechanisms are probable given that CD73 will increase extracellular adenosine in the type 1 cell-chemoafferent synapse, thus having access to both pre- and postsynaptic receptors. Despite 8-SPT potentially also targeting A_1 receptors, a functional role is improbable 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 CD73 reduces chemoafferent activity in hypoxia without completely abolishing it. Thus, effective targeting of CD73 would still allow for transmission of a definitive hypoxic neuronal signal into the CNS, albeit it being significantly reduced. Demonstration of significant CD73 mRNA expression in the rat CB has been confirmed (Salman *et al.* 2016). However, the precise localization of CD73 and CD39 remains to be defined. Given the known co-localization between CD73 and A_{2A} receptors in striatal neurones in the CNS (Augusto *et al.* 2013), we suggest that CD73 in the CB is probably located similarly (i.e. on type 1 cells and nerve endings).

By 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 to impact directly on hypoxic neuronal discharge. This is consistent with earlier observations demonstrating that extracellular adenosine recovery in normoxia was independent of ENT activity (Conde & Monteiro, 2004; Conde *et al.* 2012).

A direct up-regulation of CD73 activity in the CB in chronic or chronic intermittent hypoxia remains to be confirmed. However, recent studies suggest that ATP and 5-HT can both augment ATP release from type 2 cells, thereby amplifying the synaptic ATP concentration (Murali & Nurse, 2016; Murali *et al.* 2017). Our data suggests that increased CD73 activity driven by this increased availability of ATP would act to augment CB O_2 sensitivity, which may contribute to the exaggerated chemoafferent responses observed in chronic hypoxia or chronic intermittent hypoxia.

Our data also show that inhibition of CD73 and adenosine receptors attenuates sensory discharge frequency in response to mitochondrial inhibition. This is important not only because a run-down in mitochondrial energy metabolism is considered to be 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 obstructive sleep apnoea (Peng *et al.* 2003). Thus, our data support the idea that functional inhibition of CD73 inhibits basal and hypoxic discharge frequency and responses to stimuli relevant to CB pathology.

CD73 reduces HVR and modifies cardiovascular responses to hypoxia

Intravenous infusion of AOPCP to inhibit CD73 *in vivo* caused an inhibition of HVR (Fig. 4) and evoked a smaller reduction in P_{aCO_2} during hypoxia (Fig. 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 a result of adenosine being important in establishing the CB sensitivity to hypoxia (Fig. 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 CD73 inhibition. In view of the positive correlation between CB chemodischarge and sympathetic outflow during hypoxic stimulation, the reduction in sympathetic activity is probably a result of reduced CB stimulation. In addition, this could also be the result of a lower level of hyperventilation and lung stretch receptor activation, which again is a consequence of blunted CB hypoxic chemodischarge and respiratory drive. If similar findings are validated in humans, then pharmacological inhibition of CB CD73 may offer a novel and important means of reducing cardiovascular sympathetic outflow in CB related pathology.

Nevertheless, AOPCP also evoked a significant hyperventilation under normoxic conditions, as indicated by a significant decrease in P_{aCO_2}. It also provoked a rise in baseline HR and reduced FVC in normoxia. CD73 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 under normoxic conditions is a consequence of a reduction in adenosine

concentration in the central respiratory brain regions following the inhibition of cerebral CD73. Such data raise the intriguing possibility that CD73 may also have an important functional role in mediating central respiratory drive under normoxic conditions. These findings also suggest that selective targeting of the CB CD73 or the 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 the validation of a functional role for CB CD73 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 a reduction in HVR caused by CD73 may be an underestimate. Furthermore, our experiments used a pharmacological approach to probe the importance of CD73 in CB function and HVR. We targeted CD73 with AOPCP because this is known to be selective for membrane-bound CD73 and has been used as such previously to distinguish the activities of membrane bound and cytosolic nucleotidases (Sala-Newby *et al.* 2003). However, to confirm 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, an evaluation of the expression levels of CB CD73 and the contribution to CB sensory activity in diseases such as obstructive sleep apnoea, heart failure and diabetes is warranted.

Conclusions

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

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

PK, APH, CJR and AMC devised the experimental concepts and were responsible for the study design. APH, CJR, SAP and AMC collected data. APH, CJR, AMC and PK interpreted and analysed data. APH carried out the original drafting of the manuscript, which was edited by all of the authors. All authors approve of the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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