Inhibition of Na^+/K^+-ATPase and K_{IR} channels abolishes hypoxic hyperaemia in resting but not contracting skeletal muscle of humans

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Key points

- Increasing blood flow (hyperaemia) to exercising muscle helps match oxygen delivery and metabolic demand. During exercise in hypoxia, there is a compensatory increase in muscle hyperaemia that maintains oxygen delivery and tissue oxygen consumption.
- Nitric oxide (NO) and prostaglandins (PGs) contribute to around half of the augmented hyperaemia during hypoxic exercise, although the contributors to the remaining response are unknown.
- In the present study, inhibiting NO, PGs, Na^+/K^+-ATPase and inwardly rectifying potassium (K_{IR}) channels did not blunt augmented hyperaemia during hypoxic exercise beyond previous observations with NO/PG block alone. Furthermore, although inhibition of only Na^+/K^+-ATPase and K_{IR} channels abolished hyperaemia during hypoxia at rest, it had no effect on augmented hyperaemia during hypoxic exercise.
- This is the first study in humans to demonstrate that Na^+/K^+-ATPase and K_{IR} channel activation is required for augmented muscle hyperaemia during hypoxia at rest but not during hypoxic exercise, thus providing new insight into vascular control.

Abstract Exercise hyperaemia in hypoxia is augmented relative to the same exercise intensity in normoxia. During moderate-intensity handgrip exercise, endothelium-derived nitric oxide (NO) and vasodilating prostaglandins (PGs) contribute to ∼50% of the augmented forearm blood flow (FBF) response to hypoxic exercise (HypEx), although the mechanism(s) underlying the remaining response are unclear. We hypothesized that combined inhibition of NO, PGs, Na^+/K^+-ATPase and inwardly rectifying potassium (K_{IR}) channels would abolish the augmented hyperaemic response in HypEx. In healthy young adults, FBF responses

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were measured (Doppler ultrasound) and forearm vascular conductance was calculated during 5 min of rhythmic handgrip exercise at 20% maximum voluntary contraction under regional sympathoadrenal inhibition in normoxia and isocapnic HypEx (O_2 saturation $\sim$80%). Compared to control, combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels (L-NMMA + ketorolac + ouabain + $BaCl_2$; Protocol 1; $n = 10$) blunted the compensatory increase in FBF during HypEx by $\sim$50% (29 $\pm$ 6 mL min^{-1} *vs.* 62 $\pm$ 8 mL min^{-1}, respectively, $P < 0.05$). By contrast, ouabain + $BaCl_2$ alone (Protocol 2; $n = 10$) did not affect this augmented hyperaemic response (50 $\pm$ 11 mL min^{-1} *vs.* 60 $\pm$ 13 mL min^{-1}, respectively, $P > 0.05$). However, the blocked condition in both protocols abolished the hyperaemic response to hypoxia at rest ($P < 0.05$). We conclude that activation of Na^+/K^+-ATPase and K_{IR} channels is involved in the hyperaemic response to hypoxia at rest, although it does not contribute to the augmented exercise hyperaemia during hypoxia in humans.

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Introduction

During exercise, acute exposure to systemic hypoxia results in a compensatory increase in skeletal muscle blood flow relative to the same level of exercise under normoxic conditions (Hartley *et al.* 1973; Rowell *et al.* 1986; Wilkins *et al.* 2006, 2008, Casey *et al.* 2009, 2010; Crecelius *et al.* 2011*b*). This augmented hyperaemia compensates for the lower arterial oxygen content in hypoxia to maintain the matching of oxygen delivery with the oxygen demand of the contracting muscle (Koskolou *et al.* 1997; Roach *et al.* 1999; González-Alonso *et al.* 2001; Crecelius *et al.* 2011*b*; Casey & Joyner, 2012). The response appears to be primarily the result of an increase in local vasodilatory factors rather than a decrease in or blunting of vasoconstrictor signals because hypoxia increases sympathetic nervous system activity (Hanada *et al.* 2003; Wilkins *et al.* 2008) but does not affect postjunctional α-adrenergic receptor responsiveness (Dinenno *et al.* 2003; Wilkins *et al.* 2006; Dinenno, 2016). Although numerous studies have attempted to identify the contributing vasodilatory factors, our understanding of the response remains incomplete.

With sympathoadrenal signalling intact, adenosine and circulating epinephrine (and subsequent β-adrenoceptor activation) do not contribute to the augmented vasodilatory response during moderate intensity exercise (Wilkins *et al.* 2008; Casey *et al.* 2009, 2011; Heinonen *et al.* 2010), whereas nitric oxide (NO) has been shown to play a significant role (Casey *et al.* 2010). Our laboratory has investigated the underlying mechanisms of the vascular response to hypoxia at rest and during exercise utilizing regional (forearm) sympathoadrenal blockade aiming to isolate local vasodilator mechanisms without concomitant α- and β-adrenoceptor influences on vascular tone. At rest, we found an interaction between NO and prostaglandins (PGs), with inhibition of these pathways abolishing hypoxic vasodilatation (Markwald *et al.* 2011). However, during

moderate intensity rhythmic handgrip exercise, combined inhibition of NO and PGs only blunts the hypoxic augmentation of blood flow by $\sim$50%, indicating that other vasodilator mechanisms are involved in the response (Crecelius *et al.* 2011*b*; Dinenno, 2016).

Recent investigations in animals and humans have demonstrated a role for Na^+/K^+-ATPase and inwardly-rectifying potassium (K_{IR}) channels in the hyperaemic response to exercise and ischaemia (Edwards *et al.* 1998; Armstrong *et al.* 2007; Crecelius *et al.* 2013*a, b*, 2014; Ahn *et al.* 2017). Whether activation of these pathways contributes to vascular control during hypoxia at rest or in combination with exercise remains unknown. Both Na^+/K^+-ATPase and K_{IR} channels can be stimulated by extracellular K^+ and there is also evidence that hypoxia can (i) increase venous plasma K^+ concentrations (Marshall *et al.* 1993); (ii) stimulate K^+ release from vascular smooth muscle (Park *et al.* 2005), the vascular endothelium or red blood cells (RBCs) (Mo & Ballard, 2005; Aziz *et al.* 2017); and (iii) increase local vasodilator responsiveness to K^+ (Skinner & Costin, 1971). Alternatively, RBCs release ATP in response to haemoglobin deoxygenation, which can subsequently bind to purinergic (P_{2Y}) receptors on the vascular endothelium to stimulate vasodilatation (Ellsworth *et al.* 2009, 2016; Ellsworth & Sprague, 2012). In this context, recent studies in humans have shown that venous plasma ATP concentrations are elevated in the circulation draining hypoxic and active skeletal muscle (González-Alonso *et al.* 2002; Mortensen *et al.* 2011; Kirby *et al.* 2012). Once in circulation, ATP can evoke vasodilatation via stimulation of NO and PG production (Rongen *et al.* 1994; van Ginneken *et al.* 2004; Mortensen *et al.* 2009; Crecelius et al. 2011*a*, 2012) and, importantly, our laboratory has recently demonstrated that $\sim$40–50% of ATP-mediated vasodilatation in the human forearm occurs via activation of K_{IR} channels (Crecelius *et al.* 2012; Hearon *et al.* 2017).

Accordingly, the primary purpose of the present study was to test the hypothesis that combined inhibition of NO, PGs, and Na^+/K^+-ATPase and K_{IR} channel activation would abolish the augmented hyperaemic response during hypoxic exercise in humans. Further, we hypothesized that increased oxygen extraction would compensate for reductions in muscle blood flow (and thus oxygen delivery) to maintain oxygen consumption of both the resting and active skeletal muscle tissue.

Methods

Ethical approval and subjects

The present study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. With approval from the Institutional Review Board at Colorado State University (protocol 14-5392H), a total of 20 young healthy adults participated in the present study after providing their informed, written consent. Protocol 1 (combined inhibition of NO/PG synthesis and Na^+/K^+-ATPase and K_{IR} channel activation) comprised five men and five women; age 23 ± 2 years; weight 70.3 ± 5.2 kg; height 174 ± 3 cm; body mass index 22.9 ± 1.1 kg m^{-2}. Protocol 2 (Na^+/K^+-ATPase and K_{IR} channel inhibition) comprised four men and six women; age 22 ± 2 years; weight 69.3 ± 3.9 kg; height 170 ± 2 cm; body mass index 23.9 ± 0.9 kg m^{-2}. One subject participated in both protocols. All subjects were non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg) and not taking any medications. Studies were performed in the Human Cardiovascular Physiology Laboratory at Colorado State University (altitude: ~1500 m) after a 4 h fast and abstention from caffeine and exercise for 12 h, with subjects in the supine position, with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table. Female subjects were studied during the early follicular phase of their menstrual cycle to minimize potential cardiovascular effects of sex-specific hormones.

Arterial and venous catheterization

For both protocols, a 20-gauge, 7.6 cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anaesthesia (2% lidocaine) for local administration of study drugs and blood sampling. The catheter was connected to a three-port connector and a pressure transducer for mean arterial pressure (MAP) measurement and was continuously flushed at 3 mL h^{-1} with heparinized saline, with the two side ports used for drug infusions (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*). Additionally, in Protocol 1, a 20-gauge, 5.1 cm catheter was inserted in retrograde

fashion into an antecubital vein of the experimental arm for deep venous blood samples (Crecelius *et al.* 2011*b*). Saline was continuously infused through this catheter at a rate of ~2 mL min^{-1} for the duration of the study to keep it patent.

Forearm blood flow and vascular conductance

A 12 MHz linear-array ultrasound probe (Vivid 7; General Electric, Milwaukee, WI, USA) was used to determine brachial artery mean blood velocity (MBV) and diameter proximal to the catheter insertion site as described previously (Crecelius *et al.* 2011*b*; Hearon *et al.* 2017; Richards *et al.* 2017). Foam tape was used to mark the outline of the probe for consistent placement and measurement over the course of the experiments. For blood velocity measurements, the probe insonation angle was maintained at <60° and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analysed via a Multigon 500M TCD (Multigon Industries, Mt. Vernon, NY, USA) spectral analyser from which MBV was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter measurements were made in duplex mode at end-diastole and between contractions (in triplicate) during steady-state conditions (Crecelius *et al.* 2011*b*). FBF was calculated as: $FBF = MBV \times \pi \times$ (brachial artery diameter/2)$^2 \times 60$, where FBF is expressed as mL min^{-1}, MBV as cm s^{-1}, brachial diameter as cm, and 60 was used to convert from mL s^{-1} to mL min^{-1}. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) $\times$ 100, and expressed as mL min^{-1} 100 mmHg^{-1} (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*; Hearon *et al.* 2017; Richards *et al.* 2017). All studies were performed in a cool (20–22°C) temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm haemodynamics.

Rhythmic handgrip exercise

Maximum voluntary contraction (MVC) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other (Protocol 1: mean 33.8 ± 2.8 kg, range 24.0–50.0 kg; Protocol 2: mean 31.2 ± 2.4 kg, range 22.0–47.0 kg). Rhythmic handgrip exercise during the trials was performed with weight corresponding to 20% MVC (Protocol 1: mean 6.8 ± 0.6 kg, range 4.8–10.0 kg; Protocol 2: mean 6.2 ± 0.5 kg, range 4.4–9.4 kg) attached to a pulley system and lifted 4–5 cm over the pulley at a duty cycle of 1 s contraction/2 s relaxation (20 contractions min^{-1}) using both visual and auditory feedback to ensure the correct timing (Dinenno & Joyner, 2003, 2004). We chose this workload based on recent data suggesting that a

greater potential role for local vasodilators in muscle blood flow regulation during hypoxia at this relative intensity (Wilkins *et al.* 2008) and also to minimize sympathetically-mediated increases in heart rate and mean arterial blood pressure as a result of exercise (Victor & Seals, 1989). Previous studies in our laboratory have determined that MVC is not affected by any of the vasoactive substances administered in the present study, particularly ouabain and $BaCl_2$ (Crecelius *et al.* 2013*a*).

Systemic isocapnic hypoxia

To isolate the effects of systemic isocapnic hypoxia, we used a self-regulating partial rebreathe system developed by Banzett *et al.* (2000) and more recently described by our laboratory (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*; Richards *et al.* 2017). This system allows for constant alveolar fresh air ventilation independent of changes in breathing frequency or tidal volume (Banzett *et al.* 2000; Dinenno *et al.* 2003; Wilkins *et al.* 2008). Using this system, we were able to clamp end-tidal CO_2 levels despite the hypoxia-induced increases in ventilation. The level of oxygen was manipulated by mixing nitrogen with medical air via an anaesthesia gas blender. For the hypoxic trials, inspired oxygen was titrated to achieve arterial oxygen saturations (S_{pO_2}) of ∼80% as assessed via pulse oximetry. For normoxic trials, subjects were placed on the rebreathe system but inspired ambient air. Subjects breathed through a scuba mouthpiece with a nose-clip to prevent nasal breathing. An anaesthesia monitor (Cardiocap/5; Datex-Ohmeda, Louisville, CO, USA) was used to determine heart rate (HR) (3-lead ECG) and expired CO_2 sampled at the mouthpiece. Ventilation was measured via a turbine pneumotachograph (model 17125 UVM; Vacu-Med, Ventura, CA, USA).

Pharmacological infusions

Regional sympathoadrenal inhibition (Protocols 1 and 2). To isolate local vascular control mechanisms, sympathoadrenal influences on forearm vascular tone were blocked using brachial artery catheter administration of phentolamine mesylate (non-selective α-adrenoceptor antagonist; Bedford Laboratories, Bedford, OH, USA) and propranolol hydrochloride (non-selective β-adrenoceptor antagonist; Baxter, Deerfield, IL, USA) as described previously by our laboratory (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*; Richards *et al.* 2014, 2017). A loading dose totalling 1000 μg (200 μg min^{-1} for 5 min) of each drug was infused prior to initiating experimental trials and a maintenance dose (50 μg min^{-1}) was infused throughout the entire study to ensure continuous blockade. The dose of phentolamine used was twice as great as those previously reported to effectively block α-adrenoceptors (Eklund & Kaijser, 1976; Dietz *et al.* 1997; Halliwill *et al.*

1997) and maintains effective α-blockade for several hours (Markwald *et al.* 2011). The dose of propranolol used has been shown to inhibit forearm vasodilatation in response to the non-selective β-adrenoceptor agonist isoproterenol (Johnsson, 1967; Richards *et al.* 2017), as well as reduce vasodilatation in the resting forearm during contralateral isometric handgrip exercise (Eklund & Kaijser, 1976).

Regional NO synthase (NOS) inhibition and cyclo-oxygenase (COX) inhibition (Protocol 1). Intra-arterial administration of N^G-monomethyl-L-arginine (L-NMMA; non-selective NOS inhibitor; Bachem, Bubendorf, Switzerland) was used to inhibit the production of NO. A 5 min loading dose (5 mg min^{-1}, 25 mg total) was infused initially, followed by a maintenance dose (1.25 mg min^{-1}) for the duration of the study to ensure continuous blockade. This dose of L-NMMA has been shown to significantly reduce basal tone and also the vasodilatory effects of ACh (Dietz *et al.* 1994; Eisenach *et al.* 2002; Dinenno & Joyner, 2004), consistent with effective NOS inhibition (Vallance *et al.* 1989). Additionally, intra-arterial administration of ketorolac (non-selective COX inhibitor; trade name Toradol; Hospira, Lake Forest, IL, USA) was used to inhibit the production of PGs (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*). A 10 min loading dose (600 μg min^{-1}, 6 mg total) was infused initially, followed by a maintenance dose (150 μg min^{-1}) for the duration of the study to ensure continuous blockade. This dose of ketorolac is twice that previously demonstrated to reduce forearm blood flow during exercise (Schrage *et al.* 2004) and reduce circulating $PGF_{1\alpha}$ (a stable breakdown product of PGs) at rest and during handgrip exercise (Dinenno & Joyner, 2004).

Regional Na^+/K^+-ATPase and and K_{IR} channel inhibition (Protocols 1 and 2). Intra-arterial administration of ouabain octahydrate (Na^+/K^+-ATPase inhibitor, Sigma-O3125; Sigma-Aldrich, St Louis, MO, USA) was infused at 2.7 nmol min^{-1} in combination with barium chloride ($BaCl_2$; K_{IR} channel inhibitor; 10% w/v BDH3238; EMD Chemicals, Gibbstown, NJ, USA) at 0.9 μmol (dL forearm volume)$^{-1}$ min^{-1} with an absolute dose of 8–10 μmol min^{-1} (Nelson & Quayle, 1995; Edwards *et al.* 1998; Crecelius *et al.* 2012, 2013*a*, 2014, 2015). This approach abolishes vasodilatation to increasing doses of intra-arterial potassium chloride (Crecelius *et al.* 2012). Ouabain and $BaCl_2$ were loaded for 15 and 3 min, respectively, prior to the first trial of the blocked condition and were continued at these same doses for the duration of the trial. In the second trial of the blocked condition, infusion of all inhibitors began 3 min before the start of muscle contractions. Ouabain and $BaCl_2$ were prepared in saline and confirmed sterile

and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use. The forearm volume used for normalization of BaCl$_2$ was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software; Hologic, Inc., Marlborough, MA, USA).

Experimental protocol

A timeline for the overall study and each trial is depicted in Fig. 1. Following catheter placement and experimental set-up in both protocols, resting haemodynamics were determined before and after regional sympathoadrenal inhibition. Normoxic trials consisted of a 3 min baseline period and 5 min of 20% MVC rhythmic handgrip exercise. Hypoxic trials consisted of a 3 min baseline period, 3 min of steady-state hypoxia at ∼80% S_{pO_2} (plus ∼2 min for the normoxia to hypoxia transition; monitored via pulse oximeter) and 5 min of hypoxia plus 20% MVC rhythmic handgrip exercise.

In Protocol 1, we aimed to determine whether combined inhibition of NO, PGs, Na$^+$/K$^+$-ATPase and K$_{IR}$ channels (L-NMMA, ketorolac, ouabain, and BaCl$_2$, respectively) would abolish the augmented vasodilatation and hyperaemia during hypoxic exercise. Thus, subjects performed exercise in normoxia and hypoxia under control (saline) and blocked (aforementioned inhibitors) conditions. In total, four experimental trials each separated by 20 min of rest were performed, and the order of normoxic and hypoxic trials before and after blockade was counterbalanced between subjects. Given the lack of an additional effect of Na$^+$/K$^+$-ATPase and K$_{IR}$ channel inhibition (see Results) compared to our previous findings with NO/PG inhibition alone (Crecelius *et al*. 2011*b*) and the possibility that redundant vasodilator signalling could mask an effect of inhibiting these pathways (Schrage *et al*. 2004; Markwald *et al*. 2011; Joyner & Casey, 2014; Lamb & Murrant, 2015), we performed a follow-up protocol (Protocol 2) to test the contribution of Na$^+$/K$^+$-ATPase and K$_{IR}$ channel activation alone. The experimental approach in Protocol 2 was identical to Protocol 1, with the exception being that only Na$^+$/K$^+$-ATPase and K$_{IR}$ channels were inhibited (ouabain and BaCl$_2$, respectively) in the blocked condition.

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analysed offline with signal-processing software (WinDaq; DATAQ Instruments, Akron, OH, USA). MAP was determined from the brachial artery pressure waveform. FBF, HR, MAP and oxygen saturations (pulse oximetry) represent an average of the last 30 s of the appropriate time period. Minute ventilation and end-tidal

CO$_2$ were determined from an average of the data over the last minute of each time period. The sampling timeframe used for averaging was greater for respiratory variables than hemodynamic variables to ensure an adequate number of sampling points.

In both protocols, blood samples were taken at the end of rest, normoxic exercise, steady-state hypoxia, and hypoxic exercise under control and blocked conditions (Fig. 1). In Protocol 1, brachial artery and deep venous blood samples (∼2 mL) were drawn and immediately (<1 min) analysed with a clinical blood gas analyser (Rapid Point 400 Series Automatic Blood Gas System; Siemens Healthcare Diagnostics, Deerfield, IL, USA) for partial pressures of oxygen and carbon dioxide (P_{O_2} and P_{CO_2}), fraction of oxygenated haemoglobin (FO$_2$Hb), oxygen content and pH. In Protocol 2, only brachial artery blood samples were drawn and analysed as described for Protocol 1. From the blood gas data in Protocol 1, arteriovenous oxygen difference ($a - \dot{V}_{O_2}$) was calculated as the difference between arterial (C_{aO_2}) and venous (C_{vO_2}) oxygen content. Oxygen delivery was calculated as ($C_{aO_2} \times$ FBF $\times$ 0.001) and expressed in mL min^{-1}. Oxygen extraction, reported as a percent, was calculated as $[(C_{aO_2} - C_{vO_2}) \times C_{aO_2}^{-1} \times 100]$. Oxygen consumption across the forearm ($\dot{V}_{O_2}$) was calculated as $[(C_{aO_2} - C_{vO_2}) \times$ FBF $\times$ 0.001] and expressed in mL min^{-1}.

We also have expressed the FBF data in two alternative forms. First, we calculated 'hypoxic augmentation' as the difference between absolute FBF during hypoxic exercise and during normoxic exercise under control (saline), combined NO/PG/Na$^+$/K$^+$-ATPase/K$_{IR}$ channel inhibition (Protocol 1) or Na$^+$/K$^+$-ATPase and K$_{IR}$ channel inhibition alone (Protocol 2) conditions, as described previously (Crecelius *et al*. 2011*b*). We consider that this accurately reflects the underlying physiological responses of the primary research question addressed in the present study. In this context, we are interested in the substances or pathways involved in augmenting FBF during hypoxic compared to normoxic exercise because this difference in blood flow is what drives the maintenance of oxygen delivery during systemic hypoxia when arterial oxygen content is reduced. Second, we calculated the change in FBF from rest under a given physiological (normoxic or hypoxic) and drug condition because there is interest in this means of data expression by investigators in the field. We also performed this calculation with the data obtained in our original study on this topic (Crecelius *et al*. 2011*b*) and have addressed these findings in the Discussion.

Statistical analysis

Data are presented as the mean ± SEM. Differences within and between trials and conditions were determined via two-way repeated measures ANOVA. As a result of large

differences in the magnitude of values of exercise and rest, for comparisons of FBF, FVC, $a - \dot{V}_{O_2}$, oxygen extraction, oxygen delivery and oxygen consumption, two ANOVAs were completed, one including both exercise conditions (normoxic and hypoxic) and one including both rest periods and hypoxia, as described previously (Crecelius *et al.* 2011*b*). For all other variables collected, all time points were included in the ANOVA analysis. Specific hypothesis testing was performed using a two-tailed Student's *t* test for paired data and *post hoc* comparisons were made with Tukey's honestly significant test when appropriate. Significance was set *a priori* at $P < 0.05$.

Results

Effect of regional α- and β-adrenoceptor blockade

Prior to all experimental trials, combined blockade of α- and β-adrenoceptors significantly increased resting FBF (Protocol 1: 44 ± 8 *vs.* 81 ± 12 mL min^{-1}; Protocol 2: 35 ± 4 *vs.* 70 ± 7 mL min^{-1}) and FVC (Protocol 1: 50 ± 10 *vs.* 91 ± 15 mL min^{-1} 100 mmHg^{-1}; Protocol 2: 38 ± 4 *vs.*

74 ± 8 mL min^{-1} 100 mmHg^{-1}) by $\sim 100\%$ ($P < 0.05$). MAP (Protocol 1: 90 ± 2 *vs.* 90 ± 2 mmHg; Protocol 2: 93 ± 2 *vs.* 91 ± 2 mmHg) and HR (Protocol 1: 61 ± 4 *vs.* 59 ± 4 beats min^{-1}; Protocol 2: 67 ± 3 *vs.* 63 ± 3 beats min^{-1}) did not change as a result of local α- and β-receptor blockade. These effects are comparable to our previous observations (Crecelius *et al.* 2011*b*).

Systemic haemodynamic and respiratory responses

Systemic haemodynamic and respiratory responses are presented for each trial and condition in Protocol 1 (Table 1) and Protocol 2 (Table 2). Briefly, the targeted S_{pO_2} of $\sim 80\%$ was achieved under all hypoxic conditions and was similar across trials in both protocols. MAP was increased during normoxic and hypoxic exercise relative to rest and was significantly greater in the blocked *vs.* control condition at all time points in both protocols ($P < 0.05$). As expected, hypoxia at rest and during exercise significantly increased HR and ventilation under all conditions ($P < 0.05$).
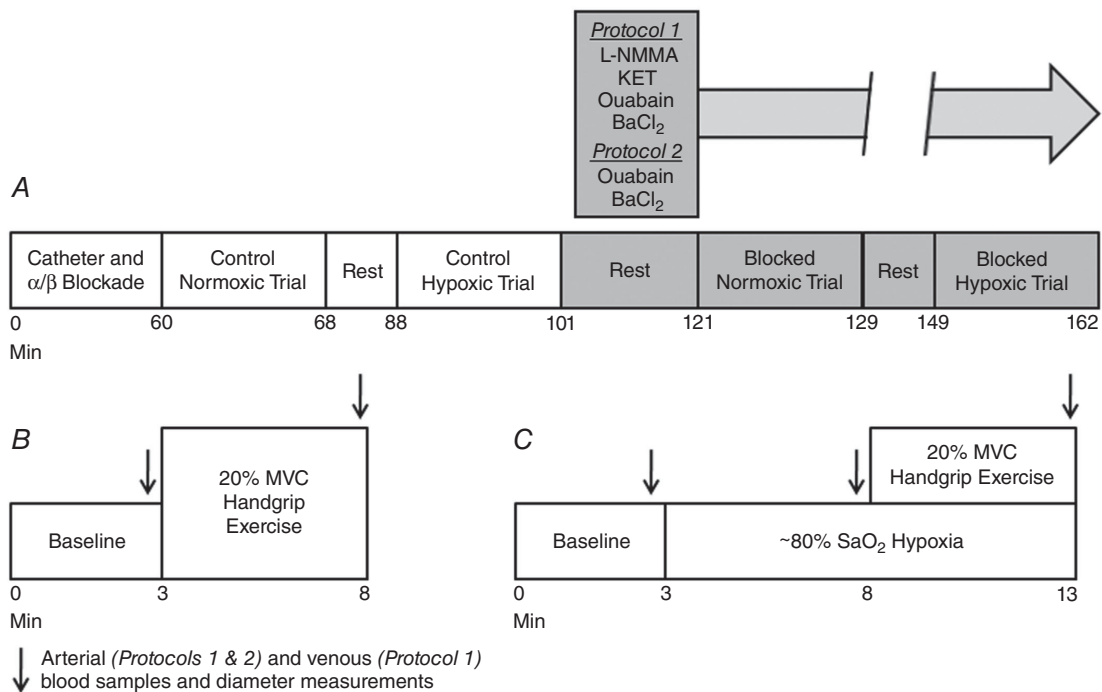

Figure 1. Timeline and experimental protocol
A, overall experimental protocol; after placement of a brachial artery catheter (Protocols 1 and 2) and a deep venous catheter (Protocol 1), phentolamine and propranolol were administered intra-arterially to block α- and β-adrenoceptors, respectively, and to isolate local vascular control mechanisms. Normoxic and hypoxic exercise trials were performed under the control (saline) condition, and then repeated following intra-arterial administration of pharmacological antagonists to inhibit NO/PG synthesis and Na$^+$/K$^+$-ATPase and K$_{IR}$ channel activation (Protocol 1) or only Na$^+$/K$^+$-ATPase and K$_{IR}$ channel activation (Protocol 2). *B*, normoxic trial timeline; baseline measurements were made for 3 min, followed by 5 min of 20% MVC rhythmic handgrip exercise. *C*, hypoxic trial timeline; baseline measurements were made for 3 min and oxygen saturations were then lowered to $\sim 80\%$ within the first 2 min and maintained for the duration of the trial. After 3 min of steady-state hypoxia, subjects performed 5 min of 20% MVC rhythmic handgrip exercise. L-NMMA, N^G-monomethyl-L-arginine; KET, ketorolac; BaCl$_2$, barium chloride.

Table 1. Protocol 1 systemic haemodynamic and ventilatory responses

	Rest 1	Normoxic exercise	Rest 2	Steady-state hypoxia	Hypoxic exercise
Control					
MAP (mmHg)	90 ± 2	$95 \pm 1^\dagger$	91 ± 2	94 ± 2	$97 \pm 2^\dagger$
HR (beats min^{-1})	59 ± 4	$69 \pm 6^\dagger$	59 ± 3	$75 \pm 4^\dagger$	$79 \pm 4^{\dagger\ddagger}$
FVC (mL min^{-1} 100 $mmHg^{-1}$)	91.4 ± 14.9	$357.3 \pm 40.1^\dagger$	91.2 ± 17.1	$117.0 \pm 24.8^\dagger$	$415.9 \pm 44.8^{\dagger\ddagger}$
S_{pO_2} (%)	99 ± 0	98 ± 1	99 ± 0	$82 \pm 2^{\dagger\ddagger}$	$82 \pm 1^{\dagger\ddagger}$
Minute vent. (L min^{-1}; BTPS)	8.4 ± 0.4	10.5 ± 0.6	9.1 ± 0.4	$17.0 \pm 1.4^{\dagger\ddagger}$	$23.1 \pm 2.3^{\dagger\ddagger\#}$
End-tidal CO_2 (mmHg)	40.2 ± 0.7	38.7 ± 0.9	39.1 ± 1.4	38.6 ± 0.9	39.5 ± 1.0
NO, PG, Na^+/K^+-ATPase, K_{IR} channel block					
MAP (mmHg)	$98 \pm 2^*$	$105 \pm 2^{*\dagger}$	$97 \pm 3^*$	$103 \pm 3^{*\dagger}$	$106 \pm 2^{*\dagger}$
HR (beats min^{-1})	57 ± 3	$63 \pm 4^*$	57 ± 3	$75 \pm 3^{\dagger\ddagger}$	$79 \pm 4^{\dagger\ddagger}$
FVC (mL min^{-1} 100 $mmHg^{-1}$)	$41.0 \pm 6.1^*$	$244.4 \pm 31.1^{*\dagger}$	$41.5 \pm 7.4^*$	$36.9 \pm 5.0^*$	$270.3 \pm 31.2^{*\dagger\ddagger}$
S_{pO_2} (%)	99 ± 0	98 ± 1	99 ± 0	$84 \pm 1^{*\dagger\ddagger}$	$82 \pm 1^{\dagger\ddagger}$
Minute vent. (L min^{-1}; BTPS)	9.5 ± 0.7	11.4 ± 0.6	8.8 ± 0.7	$22.1 \pm 1.6^{*\dagger\ddagger}$	$28.8 \pm 2.7^{*\dagger\ddagger\#}$
End-tidal CO_2 (mmHg)	$37.3 \pm 1.3^*$	$36.5 \pm 1.1^*$	38.1 ± 0.8	38.0 ± 0.9	$38.5 \pm 1.0^\ddagger$

BTPS, body temperature and pressure, saturated.
$^*P < 0.05$ *vs.* control; $^\dagger P < 0.05$ *vs.* rest (within trial); $^\ddagger P < 0.05$ *vs.* normoxic exercise; $^\# P < 0.05$ *vs.* hypoxia; all comparisons within protocol; S_{pO_2}, oxygen saturation via pulse oximetry.

Table 2. Protocol 2 systemic haemodynamic and ventilatory responses

	Rest 1	Normoxic exercise	Rest 2	Steady-state hypoxia	Hypoxic exercise
Control					
MAP (mmHg)	91 ± 2	96 ± 3	91 ± 2	94 ± 3	96 ± 2
HR (beats min^{-1})	63 ± 3	$69 \pm 3^\dagger$	62 ± 2	$78 \pm 2^{\dagger\ddagger}$	$79 \pm 2^{\dagger\ddagger}$
FVC (mL min^{-1} 100 $mmHg^{-1}$)	74.0 ± 7.8	$311.0 \pm 31.5^\dagger$	64.0 ± 6.6	$87.0 \pm 11.3^\dagger$	$370.9 \pm 38.1^{\dagger\ddagger}$
S_{pO_2} (%)	99 ± 0	98 ± 1	98 ± 0	$81 \pm 1^{\dagger\ddagger}$	$79 \pm 0^{\dagger\ddagger}$
Minute vent. (L min^{-1}; BTPS)	8.7 ± 1.0	10.4 ± 0.9	8.7 ± 0.8	$16.2 \pm 2.3^\dagger$	$18.9 \pm 2.4^{\dagger\ddagger}$
End-tidal CO_2 (mmHg)	35.3 ± 1.4	34.7 ± 1.7	35.2 ± 0.9	35.2 ± 1.0	35.8 ± 1.1
Na^+/K^+-ATPase, K_{IR} channel block					
MAP (mmHg)	$98 \pm 3^*$	$105 \pm 3^{*\dagger}$	$98 \pm 2^*$	$102 \pm 3^*$	$106 \pm 3^{*\dagger}$
HR (beats min^{-1})	61 ± 3	$65 \pm 3^*$	62 ± 2	$79 \pm 3^{\dagger\ddagger}$	$79 \pm 2^{\dagger\ddagger}$
FVC (mL min^{-1} 100 $mmHg^{-1}$)	$35.8 \pm 2.1^*$	$215.8 \pm 19.9^{*\dagger}$	$36.6 \pm 2.1^*$	$33.7 \pm 1.8^*$	$259.1 \pm 24.7^{*\dagger\ddagger}$
S_{pO_2} (%)	98 ± 0	98 ± 0	98 ± 0	$80 \pm 1^{\dagger\ddagger}$	$81 \pm 1^{*\dagger\ddagger}$
Minute vent. (L min^{-1}; BTPS)	9.3 ± 0.4	10.6 ± 0.9	9.5 ± 0.8	$21.5 \pm 3.3^{*\dagger\ddagger}$	$23.4 \pm 2.9^{*\dagger\ddagger}$
End-tidal CO_2 (mmHg)	$34.0 \pm 1.1^*$	$33.2 \pm 1.3^*$	35.2 ± 0.7	34.5 ± 1.2	34.8 ± 1.0

BTPS, body temperature and pressure, saturated.
$^*P < 0.05$ *vs.* control; $^\dagger P < 0.05$ *vs.* rest (within trial); $^\ddagger P < 0.05$ *vs.* normoxic exercise; all comparisons within protocol; S_{pO_2}, oxygen saturation via pulse oximetry.

Forearm blood flow and vascular conductance responses

Absolute values of FBF for each trial in both protocols are presented in Fig. 2 and all main effects are similar when the data are presented as FVC (Tables 1 and 2). In Protocol 1 (Fig. 2A), resting FBF was not different between normoxic (Rest 1) and hypoxic (Rest 2) trials. As expected, FBF during control hypoxic exercise was significantly augmented compared to normoxia (401 ± 40 *vs.* 339 ± 37 mL min^{-1}; $P < 0.05$). Combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels significantly reduced resting FBF and, furthermore, significantly reduced steady-state FBF during normoxic exercise (256 ± 31 mL min^{-1} *vs.* 339 ± 37 mL min^{-1}; $P < 0.05$). Blockade of these pathways abolished the FBF response to hypoxia at rest (Fig. 3) and also reduced steady-state FBF during hypoxic exercise (285 ± 32 *vs.* 401 ± 40 mL min^{-1}; $P < 0.05$). The augmentation of FBF during hypoxic exercise was reduced by ∼50% during blockade conditions (ΔFBF: control $= 62 \pm 8$ *vs.* blockade $= 29 \pm 6$ mL min^{-1}; $P < 0.05$) (Fig. 4), although FBF was still greater than normoxic exercise ($P < 0.05$) (Fig. 2A). The change in FBF from rest to steady-state exercise was reduced by ∼20% under both normoxic and hypoxic conditions (Fig. 5B).

Furthermore, the change in FBF was greater during hypoxic compared to normoxic exercise under both control and blockade conditions (Fig. 5*B*).

In Protocol 2 (Fig. 2*B*), resting FBF was not different between normoxic (Rest 1) and hypoxic (Rest 2) trials. As expected, exercise during hypoxia under control conditions significantly augmented FBF compared to normoxia (358 ± 39 *vs.* 297 ± 29 mL min^{-1}; $P < 0.05$). Inhibition of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels significantly reduced resting FBF and, furthermore, significantly reduced steady-state FBF during normoxic exercise (224 ± 19 mL min^{-1} *vs.* 297 ± 29 mL min^{-1}; $P < 0.05$). Blockade of these pathways abolished the FBF response to hypoxia at rest (Fig. 3) and also reduced steady-state FBF during hypoxic exercise (273 ± 32 *vs.* 358 ± 39 mL min^{-1}; $P < 0.05$). By contrast to Protocol 1, the augmentation of FBF during hypoxic exercise was not significantly altered by blockade of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels (ΔFBF: control $= 60 \pm 13$ *vs.* blockade $= 50 \pm 11$ mL min^{-1}; $P = 0.45$) (Fig. 4). The change in FBF from rest to steady-state exercise was reduced by ~20% under both normoxic and hypoxic conditions (Fig. 5*C*). Furthermore, the change in FBF was greater during hypoxic compared to normoxic exercise under both control and blockade conditions (Fig. 5*C*).

Blood gases and forearm oxygen delivery, extraction and consumption

Blood gas data for Protocols 1 and 2 are presented in Tables 3 and 4, respectively. As expected, hypoxia reduced arterial oxygen saturation (S_{aO_2}), arterial partial pressure
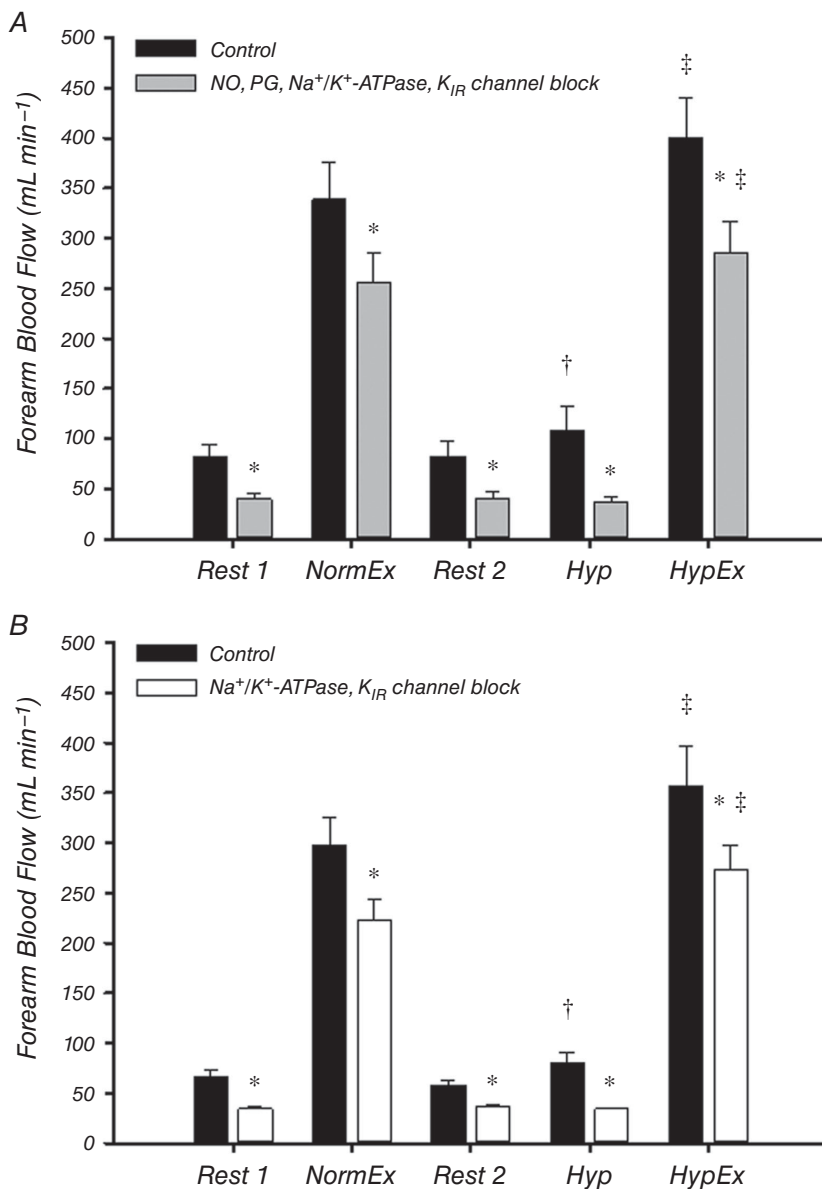

Figure 2. Forearm haemodynamics at rest, during normoxic exercise, hypoxia and hypoxic exercise

Forearm blood flow across all trials in Protocol 1 (*A*) and Protocol 2 (*B*). *A*, combined inhibition of NO, PGs, Na$^+$/K$^+$-ATPase and K$_{IR}$ channels significantly reduced FBF (and FVC) (Table 1) at all time points. Under control conditions, hypoxic exercise (HypEx) FBF was significantly greater than normoxic exercise (NormEx) FBF and this augmentation remained in the blocked condition. FBF during hypoxia (Hyp) under resting conditions was significantly greater than rest (Rest 2) under control conditions but not after blockade. *B*, with combined inhibition of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels, the trends in the data are not different from that observed in Protocol 1 (*A*). *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* Rest 2; ‡$P < 0.05$ *vs.* normoxic exercise (within condition).

of oxygen (P_{aO_2}) and C_{aO_2} compared to normoxia in both protocols ($P < 0.05$). Under control and blocked conditions, there was no significant difference in arterial partial pressure of CO_2 (P_{aCO_2}) between normoxic and hypoxic trials. In Protocol 1, both normoxic and hypoxic exercise significantly reduced venous pH (pH_v), venous P_{O_2} (P_{vO_2}) and C_{vO_2} compared to rest ($P < 0.05$). Venous catheters were not placed in Protocol 2.

In Protocol 1, oxygen delivery was significantly reduced at all time points under the blocked condition compared to control ($P < 0.05$) (Fig. 6A) primarily as a result of the decrease in FBF because C_{aO_2} was unaffected by the blockade (Table 3). Oxygen extraction was significantly greater during the hypoxic *vs.* normoxic exercise trial under the control condition and was also greater at all time points under the blocked condition compared to control

Figure 3. Effect of blockade on hypoxia-induced hyperaemia

The effect of hypoxia on forearm blood flow at rest was calculated for each condition as the difference in absolute FBF between hypoxia (Hyp) and rest (Rest 2). In Protocol 1, combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels abolished the hypoxia-induced increase in FBF at rest. In Protocol 2, combined inhibition of Na^+/K^+-ATPase and K_{IR} channels also abolished the hypoxia-induced increase in FBF at rest. *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* zero.
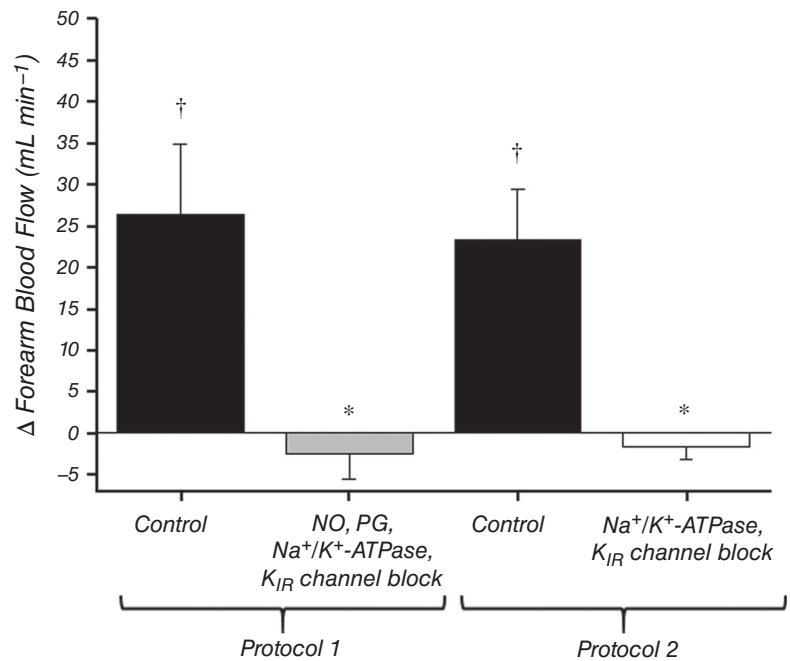

Figure 4. Effect of blockade on augmented hyperaemia during hypoxic exercise

Hypoxic augmentation of forearm blood flow during exercise was calculated for each condition as the difference in absolute FBF between hypoxic exercise (HypEx) and normoxic exercise (NormEx). In Protocol 1, combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels blunted the hypoxic augmentation by ~50%. In Protocol 2, combined inhibition of Na^+/K^+-ATPase and K_{IR} channels did not significantly affect hypoxic augmentation. *$P < 0.05$ *vs.* control; †$P < 0.05$ *vs.* zero.
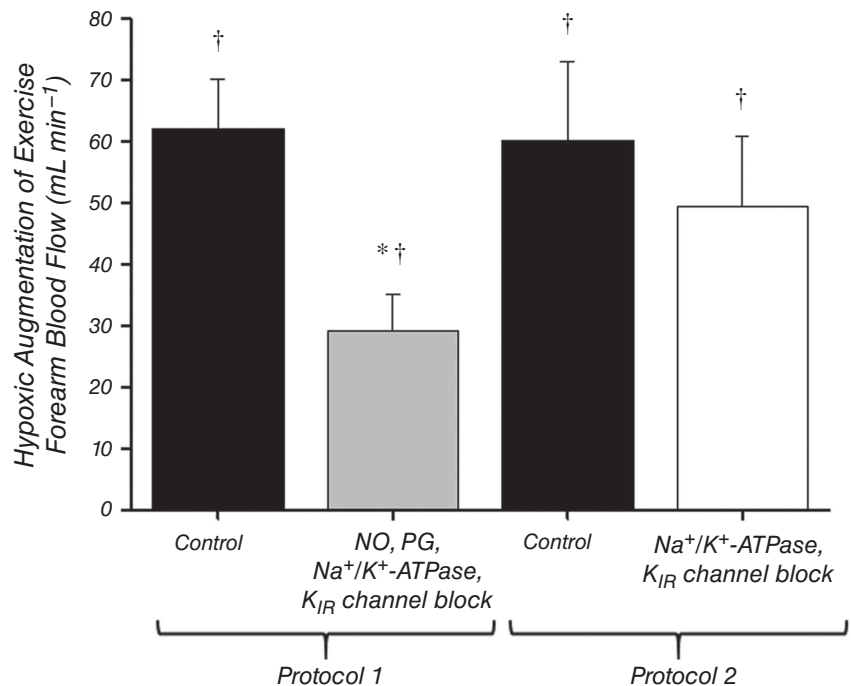
($P < 0.05$) (Fig. 6B). Forearm oxygen consumption ($\dot{V}_{O_2}$) was not significantly different at any time point under blocked *vs.* control conditions ($P = 0.08$–0.86) (Fig. 6C).

Discussion

The present study provides several primary new findings. First, under conditions of forearm sympathoadrenal blockade to isolate local vasodilatory mechanisms, combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels blunts the augmented skeletal muscle hyperaemia during hypoxic exercise $\sim 50\%$ (Protocol 1) (Figs 2A and 4). These data are almost identical to our recent findings showing that inhibition of NO and PGs reduces the response to a similar extent (Crecelius *et al.* 2011b). As a result of concerns regarding redundant pathways masking
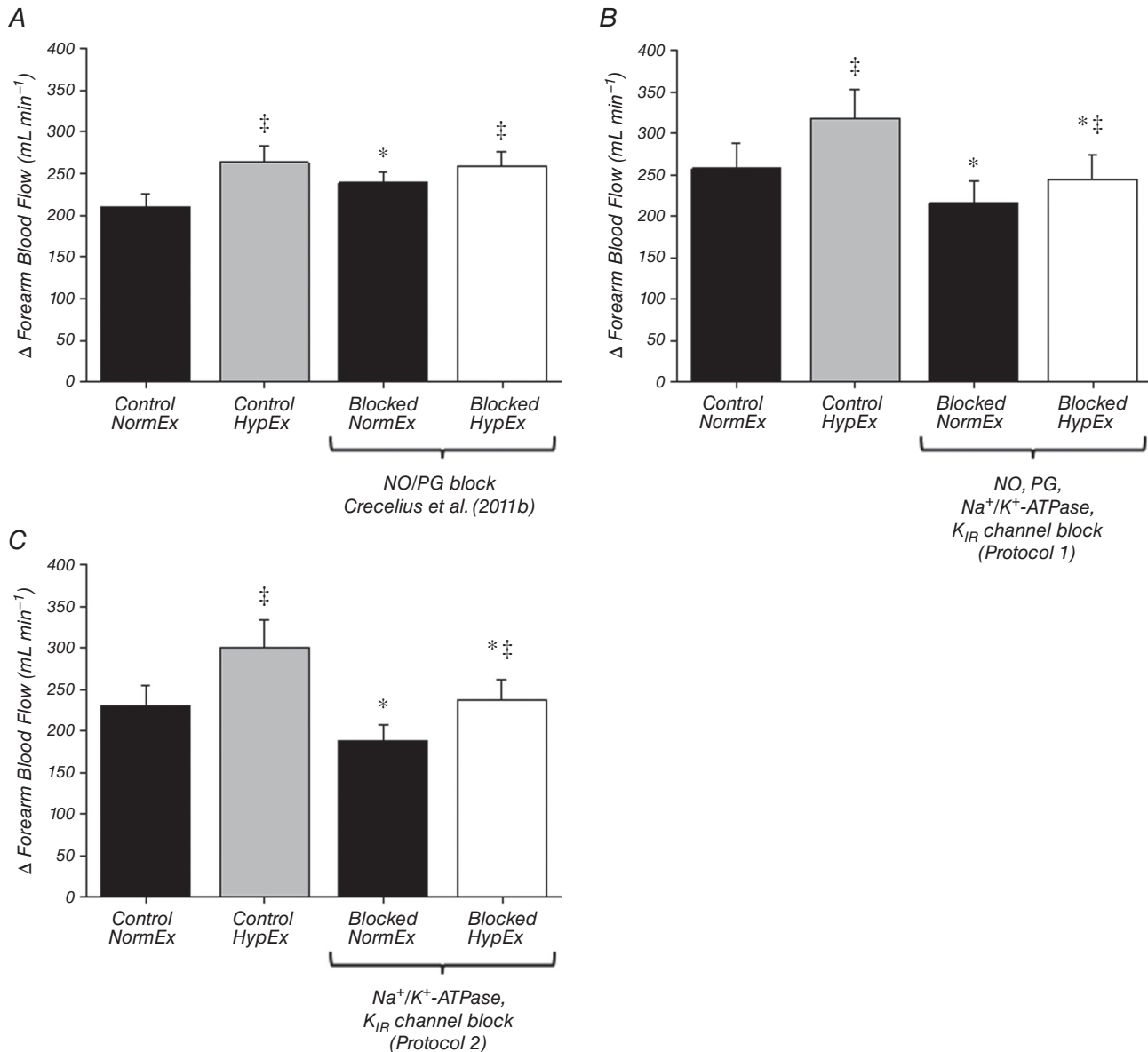

Figure 5. Effect of blockade on change in forearm blood flow from rest to steady-state exercise in normoxia and hypoxia

The absolute change in forearm blood flow from rest to exercise at 20% MVC in normoxia and hypoxia in each condition was calculated using data from our previous study (*A*) (Crecelius *et al.* 2011b) and also for Protocol 1 (*B*) and Protocol 2 (*C*) of the present study. The increase in FBF during hypoxic exercise was not reduced by combined NO/PG blockade (*A*). Combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels significantly reduced the change in FBF from rest to exercise in both normoxia and hypoxia by $\sim 20\%$ (*B*), as did inhibition of Na^+/K^+-ATPase and K_{IR} channels alone (*C*). *$P < 0.05$ *vs.* control (within normoxic/hypoxic condition); ‡$P < 0.05$ *vs.* normoxic exercise (within drug condition).

Table 3. Protocol 1 blood gases

	Rest 1	Normoxic exercise	Rest 2	Steady-state hypoxia	Hypoxic exercise
Control					
pH$_a$	7.40 ± 0.01	7.40 ± 0.01	7.42 ± 0.01	7.42 ± 0.01	7.41 ± 0.01
pH$_v$	7.39 ± 0.01	7.30 ± 0.01†	7.40 ± 0.01	7.41 ± 0.01‡	7.31 ± 0.01$^{\dagger\#}$
P_{aCO_2} (mmHg)	37.2 ± 1.1	37.2 ± 1.0	35.4 ± 1.5	35.9 ± 1.1	36.0 ± 1.2
P_{vCO_2} (mmHg)	40.6 ± 0.8	54.1 ± 2.5†	38.8 ± 1.6	37.8 ± 1.5‡	52.7 ± 2.7$^{\dagger\#}$
[Hb]$_a$ (g dL^{-1})	15.0 ± 0.4	15.1 ± 0.4	15.0 ± 0.4	14.9 ± 0.4	14.9 ± 0.4
S_{aO_2} (%)	95.2 ± 0.1	95.7 ± 0.2	95.7 ± 0.3	81.8 ± 1.4†‡	81.6 ± 1.4†‡
S_{vO_2} (%)	82.9 ± 3.1	46.4 ± 2.5†	78.7 ± 1.6	71.9 ± 1.1†	35.3 ± 2.3$^{\dagger\#}$
P_{aO_2} (mmHg)	80.0 ± 1.0	83.2 ± 1.6	83.3 ± 2.2	46.5 ± 1.6†‡	47.8 ± 2.0†‡
PvO$_2$ (mmHg)	53.2 ± 5.2	29.3 ± 0.8†	44.7 ± 2.2	38.6 ± 1.0	23.9 ± 0.5$^{\dagger\#}$
C_{aO_2} (mL L^{-1})	199 ± 5	200 ± 5	200 ± 6	167 ± 5†‡	168 ± 7†‡
C_{vO_2} (mL L^{-1})	167 ± 7	95 ± 7†	163 ± 7	148 ± 5‡	72 ± 6$^{\dagger\ddagger\#}$
Extraction (%)	17 ± 3	53 ± 3†	20 ± 2	13 ± 2‡	58 ± 3$^{\dagger\#}$
NO, PG, Na$^+$/K$^+$-ATPase, K$_{IR}$ channel block					
pH$_a$	7.43 ± 0.01*	7.42 ± 0.01*	7.42 ± 0.01	7.43 ± 0.01	7.42 ± 0.01
pH$_v$	7.36 ± 0.01*	7.28 ± 0.02†	7.36 ± 0.01*	7.36 ± 0.01*‡	7.29 ± 0.02$^{\dagger\#}$
P_{aCO_2} (mmHg)	33.1 ± 1.7*	32.6 ± 1.2*	35.1 ± 1.0	34.3 ± 1.1	34.9 ± 1.1
P_{vCO_2} (mmHg)	40.6 ± 1.2	58.1 ± 5.0†	41.1 ± 2.0	41.8 ± 1.5‡	55.7 ± 3.9$^{\dagger\#}$
[Hb]$_a$ (g dL^{-1})	15.0 ± 0.4	14.8 ± 0.4*	15.0 ± 0.4	14.8 ± 0.4†	14.8 ± 0.4
S_{aO_2} (%)	95.8 ± 0.3	96.4 ± 0.2	95.7 ± 0.3	82.6 ± 1.3*†‡	82.8 ± 1.1*†‡
S_{vO_2} (%)	65.6 ± 3.9*	33.6 ± 2.7*†	65.7 ± 8.7	46.7 ± 4.5*†	26.1 ± 3.0*$^{\dagger\#}$
P_{aO_2} (mmHg)	83.8 ± 2.7*	87.3 ± 1.1*	82.8 ± 2.0	47.0 ± 1.6†‡	47.8 ± 1.4†‡
P_{vO_2} (mmHg)	36.7 ± 2.3	21.4 ± 1.6†	39.3 ± 5.1	26.7 ± 1.6†	18.3 ± 1.3†
C_{aO_2} (mL L^{-1})	200 ± 5	201 ± 5	198 ± 6	168 ± 6†‡	170 ± 6†‡
C_{vO_2} (mL L^{-1})	126 ± 9*	60 ± 9*†	124 ± 17*	84 ± 8*†‡	47 ± 8*$^{\dagger\#}$
Extraction (%)	38 ± 4*	71 ± 4*†	37 ± 9*	50 ± 5*†‡	73 ± 4*$^{\dagger\#}$

*$P < 0.05$ *vs.* control; $^\dagger P < 0.05$ *vs.* rest (within trial); $^\ddagger P < 0.05$ *vs.* normoxic exercise; $^\# P < 0.05$ *vs.* hypoxia; all comparisons within protocol.

Table 4. Protocol 2 blood gases

	Rest 1	Normoxic exercise	Rest 2	Steady-state hypoxia	Hypoxic exercise
Control					
pH$_a$	7.45 ± 0.01	7.44 ± 0.01	7.44 ± 0.01	7.45 ± 0.01	7.43 ± 0.01
P_{aCO_2} (mmHg)	31.2 ± 1.5	32.0 ± 1.7	32.7 ± 1.0	32.1 ± 1.1	32.0 ± 1
[Hb]$_a$ (g dL^{-1})	14.7 ± 0.4	14.7 ± 0.4	14.5 ± 0.4	14.5 ± 0.4	14.4 ± 0.3
S_{aO_2} (%)	96.0 ± 0.5	96.3 ± 0.3	96.1 ± 0.3	79.6 ± 0.8†‡	81.6 ± 0.7†‡
P_{aO_2} (mmHg)	85.9 ± 3.0	88.4 ± 2.8	84.5 ± 1.5	43.1 ± 0.7†‡	46.0 ± 0.6†‡
C_{aO_2} (mL L^{-1})	197 ± 5	197 ± 5	195 ± 5	159 ± 5†‡	164 ± 5†‡
Na$^+$/K$^+$-ATPase, K$_{IR}$ channel block					
pH$_a$	7.45 ± 0.01	7.44 ± 0.01	7.44 ± 0.01	7.45 ± 0.01	7.44 ± 0.01
P_{aCO_2} (mmHg)	29.7 ± 1.0*	29.2 ± 1.1*	31.7 ± 1.1	30.0 ± 1.2*	31.2 ± 1.1
[Hb]$_a$ (g dL^{-1})	14.9 ± 0.4	14.4 ± 0.4†	14.9 ± 0.4*	14.8 ± 0.4	15.0 ± 0.4*
S_{aO_2} (%)	96.4 ± 0.2	96.2 ± 0.2	96.1 ± 0.2	80.5 ± 0.9†‡	80.4 ± 0.7†‡
P_{aO_2} (mmHg)	87.0 ± 2.1	86.9 ± 2.1	84.3 ± 1.7	43.9 ± 0.8†‡	44.4 ± 0.9†‡
C_{aO_2} (mL L^{-1})	201 ± 6	194 ± 6	200 ± 6	165 ± 6†‡	168 ± 5†‡

*$P < 0.05$ *vs.* control; $^\dagger P < 0.05$ *vs.* rest (within trial); $^\ddagger P < 0.05$ *vs.* normoxic exercise; all comparisons within protocol.

an effect of inhibiting Na$^+$/K$^+$-ATPase and K$_{IR}$ channels during concomitant NO and PG inhibition, we performed additional experiments with Na$^+$/K$^+$-ATPase and K$_{IR}$ channel inhibition alone (Protocol 2). Thus, our second primary finding is that activation of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels does not contribute to the augmented skeletal muscle hyperaemia during hypoxic exercise (Figs 2*B* and 4) and thus FBF remains elevated compared to normoxic exercise. Third, despite the lack of contribution of Na$^+$/K$^+$-ATPase and K$_{IR}$ channel activation to

hypoxic augmentation during exercise, these hyperpolarizing pathways are an essential component of the hyperaemic response to hypoxia at rest (Fig. 3). Finally, the decline in blood flow and therefore oxygen delivery in all experimental trials following combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels does not significantly impact oxygen consumption as a result of significant increases in oxygen extraction (Fig. 6).

Na^+/K^+-ATPase and K_{IR} channels in vasodilatation and hyperaemia during hypoxia at rest

Under similar experimental conditions, we have demonstrated previously that combined blockade of NO and PGs decreases resting forearm blood flow and abolishes the vasodilator response to hypoxia in quiescent skeletal muscle (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*). Consistent with this, combined NO, PG, Na^+/K^+-ATPase

and K_{IR} channel blockade in Protocol 1 of the present study also abolished the response. Interestingly, blockade of only Na^+/K^+-ATPase and K_{IR} channels in Protocol 2 both decreased resting blood flow and abolished the hyperaemic response to hypoxia in quiescent skeletal muscle as well (Fig. 3). Although determining the exact mechanisms by which these disparate pathways contribute to hypoxic vasodilatation at rest is beyond the scope of the present study, this merits a brief discussion. First, any effect of our pharmacological inhibitors is not simply the result of reducing forearm blood flow at rest (i.e. vasoconstriction) because studies have shown that infusion of phenylephrine (α_1-adrenergic agonist) to reduce blood flow prior to systemic hypoxia does not reduce the dilatory response (Blitzer *et al.* 1996). Second, this could suggest that NO and PGs and Na^+/K^+-ATPase and K_{IR} channels operate in a redundant fashion. However, if these pathways were operating in a redundant manner, we would expect
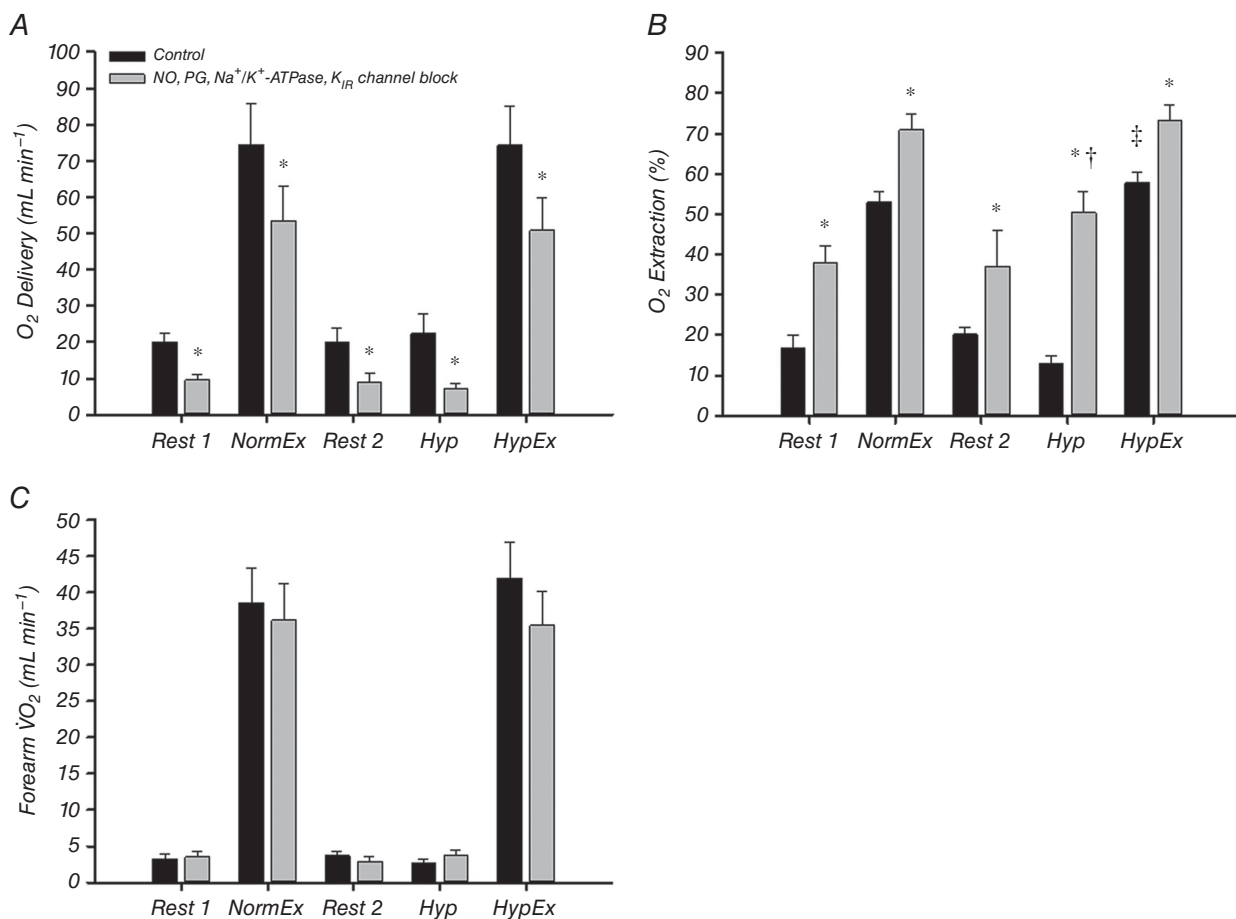

Figure 6. Forearm oxygen delivery, extraction and consumption before and after combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels
Combined inhibition of NO, PGs, Na^+/K^+-ATPase and K_{IR} channels significantly attenuated oxygen delivery in all trials (*A*). Subsequently, oxygen extraction was significantly increased after blockade in all trials (*B*) and thus oxygen consumption (forearm $\dot{V}_{O_2}$) was maintained after blockade in all trials (*C*). All values for NormEx and HypEx under both conditions are significantly greater than rest and hypoxia ($P < 0.05$ within condition). *$P < 0.05$ *vs.* control condition; †$P < 0.05$ *vs.* rest (within trial and condition); ‡$P < 0.05$ *vs.* normoxic exercise (within condition).

that NO/PG inhibition alone, or Na$^+$/K$^+$-ATPase and K$_{IR}$ channel inhibition alone, would not impact hypoxic vasodilatation and that the response would be abolished only when all four pathways are inhibited (Markwald *et al.* 2011; Lamb & Murrant, 2015). Finally, it is possible that multiple pathways are involved in the regulation of vascular tone during hypoxia and perhaps, depending on the study, inhibition of a given pathway may significantly reduce or almost abolish the response. In this context, adenosine receptor antagonism (∼85%: Leuenberger *et al.* 1999), NO inhibition alone (∼60%: Blitzer *et al.* 1996; ∼75%: Casey *et al.* 2010) or in combination with PG inhibition (∼85–90%: Markwald *et al.* 2011; ∼90%: Crecelius *et al.* 2011*b*) and cytochrome P-450 inhibition (∼50%: Spilk *et al.* 2013), have all been demonstrated to significantly reduce forearm vasodilatation during hypoxia. We propose that during systemic hypoxia when S_{aO_2} is reduced to ∼80%, inhibiting the normal vasodilator and hyperaemic response (typically by the order of ∼20–30%) results in a fairly small absolute change in forearm blood flow (Fig. 2) and oxygen delivery (Fig. 6*A*). Given that blood flow and oxygen demand in resting skeletal muscle is low, pharmacological inhibition of a dilatory signal or pathway may not evoke much of an 'error signal' to initiate a redundant response.

Na$^+$/K$^+$-ATPase and K$_{IR}$ channels in vasodilatation and hyperaemia during hypoxic exercise

The primary purpose of the present study was to determine whether Na$^+$/K$^+$-ATPase and K$_{IR}$ channels are involved in the augmented hyperaemia during hypoxic exercise in humans. In Protocol 1, we combined Na$^+$/K$^+$-ATPase and K$_{IR}$ channel inhibition with the same NO/PG blockade approach that decreased the compensatory increase in blood flow during hypoxic exercise by ∼50% in our previous study (Crecelius *et al.* 2011*b*). Although this approach significantly reduced steady-state hyperaemia during exercise in normoxia and hypoxia (Fig. 2), which is most probably primarily a result of K$_{IR}$ channel inhibition (Crecelius *et al.* 2014), it did not result in a further decrease in the augmented hyperaemia during hypoxic exercise beyond ∼50% (Fig. 4). Given that exercise hyperaemia is regulated by many redundant factors (Joyner & Wilkins, 2007) and to test that this lack of an additional effect was not influenced by NO/PG inhibition, we repeated the experiment with combined ouabain and BaCl$_2$ infusion alone (Protocol 2). Consistent with our observations in Protocol 1, inhibition of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels alone significantly reduced exercise hyperaemia in normoxia and hypoxia by ∼25%, although it did not impact the augmented hyperaemia during hypoxic exercise (Figs 2 and 4). Collectively, these data indicate that activation of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels does not contribute to the augmented skeletal muscle hyperaemia during hypoxic exercise in humans (Fig. 4).

Possible mechanisms of the unexplained augmented exercise hyperaemia during hypoxia

Our data clearly indicate that distinct mechanisms beyond the synthesis of the endothelial autocoids NO and PGs and activation of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels must be involved in the augmented hyperaemic response during hypoxic exercise. Although there are many potential explanations, it is of interest that skeletal muscle blood flow responses are more closely associated with reductions in C_{aO_2} than P_{O_2} (Roach *et al.* 1999; González-Alonso *et al.* 2001). Given that changes in C_{aO_2} are directly related to reductions in the oxygenation of haemoglobin, RBCs may be implicated as a potential sensor of the hypoxic stimulus and modulator of vascular tone, blood flow and oxygen delivery through the release of vasodilatory molecules such as ATP (González-Alonso *et al.* 2002; Ellsworth *et al.* 2009, 2016; Dufour *et al.* 2010; Ellsworth & Sprague, 2012; Dinenno, 2016). Circulating ATP can then bind to purinergic P$_{2Y}$ receptors on the endothelium and evoke vasodilatation, in part through the activation of small- and intermediate-conductance calcium-activated potassium channels (SK$_{Ca}$ and IK$_{Ca}$, respectively) (Winter & Dora, 2007) and subsequent hyperpolarization that conducts along and between the endothelium and vascular smooth muscle through gap junctions (Bagher & Segal, 2011; Garland *et al.* 2011; Straub *et al.* 2014; Dora, 2017). Unfortunately, we were unable to inhibit P$_{2Y}$ receptors, SK$_{Ca}$/IK$_{Ca}$ channels or gap junctions in the present study as a result of specific inhibitors being unavailable for use in humans. As such, the vasodilatory response to intravascular ATP that persists following combined inhibition of NO, PGs, Na$^+$/K$^+$-ATPase and K$_{IR}$ channels (∼40% based on Hearon *et al.* 2017) may have contributed to the remaining augmented hyperaemia during hypoxic exercise. RBCs can also act as an endothelium-independent source of NO through the formation of *S*-nitrosohemoglobin in the lung (Jia *et al.* 1996; Stamler *et al.* 1997), hypoxia-induced increases in the conversion of nitrite to NO via the nitrite reductase activity of deoxyhaemoglobin, as well as synthesis via RBC eNOS (Kleinbongard *et al.* 2006; Halpin & Spence, 2010; Cortese-Krott *et al.* 2012; Wood *et al.* 2013), all of which would be unaffected by NOS inhibition in the present study.

Beyond the RBC, other pathways could potentially be involved in the remaining hypoxic response during exercise. For example, adenosine has been shown to contribute to the vasodilatory response to hypoxia alone in both humans and experimental animals (Bryan & Marshall, 1999; Leuenberger *et al.* 1999; Ray *et al.* 2002), as well as to exercise hyperaemia (Marshall, 2007). However,

an obligatory role of adenosine in the augmented hyperaemic response to hypoxic exercise in humans has not been demonstrated (Casey *et al.* 2009; Heinonen *et al.* 2010). It is important to note that these findings may be influenced by experimental limitations associated with the use of adenosine receptor antagonists such as aminophylline in humans, which include non-selective targeting of multiple adenosine receptor subtypes, incomplete inhibition of adenosine-mediated dilatation (∼50% inhibition of the vasodilatory response to intra-brachial adenosine infusion (Kirby *et al.* 2010)) and non-selective inhibition of phosphodiesterases, which can increase resting muscle blood flow (Rådegran & Hellsten, 2000; Casey *et al.* 2009; Lomas & Zaccolo, 2014; Dinenno, 2016). Furthermore, given the reduction in steady-state exercise hyperaemia with Na^+/K^+-ATPase and K_{IR} channel inhibition, it is possible that adenosine could play a role under these conditions of reduced tissue perfusion.

Cytochrome P450 metabolites of arachidonic acid are another group of vasoactive molecules that could contribute to the augmented hyperaemic response to hypoxic exercise. These metabolites are comprised of two groups, hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs), with HETEs generally evoking vasoconstriction through inhibition of K_{Ca} channels and activation of PKC in the vascular smooth muscle and EETs generally evoking vasodilatation through activation of K_{Ca} channels (Clifford & Hellsten, 2004; Miyata & Roman, 2005; Félétou & Vanhoutte, 2009; Spector, 2009). 20-HETE is the primary vasoconstricting HETE that has been studied in the context of vascular control, whereas there are multiple EET isoforms and it is less clear which of these contribute to regulation of vascular tone (Félétou & Vanhoutte, 2009; Spector, 2009). The production and release of 20-HETE is oxygen-dependent, such that declines in P_{O_2} decrease [20-HETE]; thus, the contribution of 20-HETE to hypoxic vasodilatation has been proposed to result from less vasoconstrictor signalling through this pathway (Frisbee *et al.* 2001, 2002; Jackson, 2016). By contrast, an increase in EETs in hypoxia would contribute to vasodilatation and, although studies in isolated rat arteries suggest that EETs are not involved in hypoxic vasodilatation (Frisbee *et al.* 2001, 2002), Spilk *et al.* (2013) found that regional infusion of fluconazole (an inhibitor of cytochrome P450 2C9) in the human forearm significantly attenuates hypoxia-induced dilatation. There is also evidence in humans that EETs can compensate for the loss of NO at rest and during exercise in humans, thus indicating potential cross-talk between these two pathways (Hillig *et al.* 2003; Ozkor & Quyyumi, 2011; Ozkor *et al.* 2011). Additionally, given that both vasodilating PGs and EETs are derived from the metabolism of arachidonic acid, it is important to consider that COX inhibition (as in Protocol 1 of the present study) could conceivably shunt arachidonic

acid metabolism through the cytochrome P450 pathway, thereby resulting in a compensatory increase in EETs. Thus, it is possible that reduced 20-HETE and/or increased EET production contributed to the remaining augmented exercise hyperaemia during hypoxia in the present study.

Finally, activation of other K^+ channels could be involved in the response. In isolated vessels and *in situ* preparations, ATP-sensitive K^+ (K_{ATP}) channels have been shown to be involved in the vasodilatory response to hypoxia (Gasser *et al.* 1993; Marshall *et al.* 1993; Frisbee *et al.* 2002; Ngo *et al.* 2010), although other studies have shown they are not involved (Smani *et al.* 2002; Lynch *et al.* 2006). Similarly, studies in experimental animals suggest that K_{ATP} channels mediate arteriolar vasodilatation during muscle contractions, (Murrant & Sarelius, 2002; Dua *et al.* 2009), yet studies in humans do not support this (Keller *et al.* 2004; Schrage *et al.* 2006). K_{Ca} channels have also been shown to be activated by hypoxia (Gebremedhin *et al.* 1994; Frisbee *et al.* 2002) and can be stimulated by factors that were not blocked in the present study including EETs and ATP (Gebremedhin *et al.* 1992; Campbell *et al.* 1996; Zou *et al.* 1996; Eckman *et al.* 1998; Hayabuchi *et al.* 1998; Miura & Gutterman, 1998; Fissltⁿhaler *et al.* 1999; Earley *et al.* 2005; Huang *et al.* 2005; Weston *et al.* 2005; Winter & Dora, 2007). However, confirmation of these findings is not possible as a result of the lack of specific inhibitors available for use in humans. Future studies will be needed to determine whether these channels or other mechanisms contribute significantly to the augmented hyperaemic response during hypoxic exercise.

Experimental considerations and limitations

A key issue for studies on blood flow and vascular control in response to pharmacological or physiological perturbations is the proper quantification of the vasomotor responses. With respect to the research question addressed in the present study, the augmented hyperaemia observed during hypoxic exercise occurs as a means to maintain oxygen delivery for a given oxygen demand. Many studies have demonstrated this, including our own (Koskolou *et al.* 1997; Roach *et al.* 1999; González-Alonso *et al.* 2001; Crecelius *et al.* 2011*b*; Casey & Joyner, 2012) (Fig. 6*A*). Thus, when attempting to understand the signalling mechanisms underlying this response, we consider that quantifying the 'hypoxic augmentation of exercise forearm blood flow' is most appropriate because this difference in blood flow is what drives oxygen delivery to be maintained during systemic hypoxia when arterial oxygen content is reduced. In an effort to be comprehensive, we have also presented the data as a change from the respective baseline condition from both our previous study on this topic (Crecelius *et al.* 2011*b*) and the present study (Fig. 5). When this analysis is performed, the collective data indicate that (i) NO and PGs do not

play a role in the change in forearm blood flow from rest to exercise in either normoxia or hypoxia (Fig. 5*A*) (Crecelius *et al.* 2011*b*); (ii) Na$^+$/K$^+$-ATPase and K$_{IR}$ channels contribute significantly to this change from rest to exercise in both normoxia and hypoxia (Fig. 5*B* and *C*); and (iii) additional vasodilator mechanisms are involved in this change from rest to exercise in hypoxia (Fig. 5*B* and *C*).

Given the specific research question of the present study as it relates to blood flow and oxygen delivery during hypoxic exercise, we do not consider that quantifying the change in blood flow from rest to exercise (within a given normoxia/hypoxia or drug condition) is most reflective of the underlying physiology. For example, despite the appearance of a 'normal' blood flow response from rest to exercise in our prior study with combined NO/PG inhibition (Fig. 5*A*), muscle blood flow and oxygen delivery (Crecelius *et al.* 2011*b*) were clearly reduced during hypoxic exercise compared with normoxic exercise by inhibiting these autocoids, strongly implicating their role in blood flow regulation under hypoxic conditions. In the present study, inhibition of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels (in both protocols) reduced steady-state blood flow during both normoxic and hypoxic exercise (Fig. 2). However, blood flow during hypoxic exercise was greater than normoxic exercise within the blocked condition and, as such, the 'hypoxic augmentation' was largely unaffected (Fig. 4). Thus, we consider this to indicate that Na$^+$/K$^+$-ATPase and K$_{IR}$ channels are not involved in the augmented hyperaemia during hypoxic exercise in humans. However, in an effort to be comprehensive, we present these data because there is interest in this means of data expression by investigators in the field.

One potential limitation of the present study is that we did not test the individual effects of ouabain and BaCl$_2$ and are therefore unable to determine the respective contributions of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels to the hyperaemic response to hypoxia at rest. However, in previous studies, we have found little role for Na$^+$/K$^+$-ATPase activation in the vascular responses to intra-arterial ATP infusion or exercise, whereas activation of K$_{IR}$ channels does contribute significantly (Crecelius *et al.* 2012, 2014). Thus, it is possible that K$_{IR}$ channels were the primary contributor to the hypoxic vasodilatory response at rest. We also did not directly test the efficacy of the pharmacological inhibitors used in the present study, although our laboratory and others have demonstrated these doses are effective in humans (see Methods). Furthermore, in the present study, the combined infusion of ouabain and BaCl$_2$ significantly attenuated blood flow at rest and during exercise in both normoxia and hypoxia in both protocols (Figs 2 and 3). Despite this effect, the augmented hyperaemia during hypoxic exercise was not different from NO/PG block alone in Protocol 1 and unaffected in Protocol 2 (Fig. 4).

Finally, the addition of ouabain and BaCl$_2$ in both protocols significantly increased MAP ($\sim$6–10 mmHg) and decreased FBF under all experimental conditions (Fig. 2 and Tables 1 and 2); in contrast, the combined NO/PG blockade from our previous investigation had no effect on MAP and only decreased FBF at rest and during hypoxic exercise (Crecelius *et al.* 2011*b*). Despite these differences, this probably does not influence our primary conclusion regarding the role of the Na$^+$/K$^+$-ATPase and K$_{IR}$ channels in hypoxic augmentation of blood flow during exercise for two key reasons. First, all experiments were performed during local sympathoadrenal blockade, which would minimize any impact of baroreflex-mediated changes in sympathetic outflow on forearm vascular tone. Second, the blockade-induced change in MAP and FBF during exercise was the same between protocols and between normoxic/hypoxic conditions within each protocol; however, the effect on forearm haemodynamics during hypoxic exercise was different. Specifically, hypoxic augmentation of exercise hyperaemia was blunted only when NO and PGs were included in the blockade conditions, which supports our previous findings (Crecelius *et al.* 2011*b*).

Conclusions

Skeletal muscle blood flow is augmented during hypoxia to maintain oxygen delivery to the tissue. In combination with our previous work (Markwald *et al.* 2011; Crecelius *et al.* 2011*b*), the results from the present study indicate that combined inhibition of NO and PGs, or inhibition of Na$^+$/K$^+$-ATPase and K$_{IR}$ channels, abolishes the hyperaemic response to systemic isocapnic hypoxia at rest. How these pathways are interrelated under these conditions is presently unknown. By contrast, NO and PGs appear to mediate $\sim$50% of the augmented hyperaemia during hypoxic exercise, whereas the present study provides the first experimental evidence that Na$^+$/K$^+$-ATPase and K$_{IR}$ channels do not contribute to this response. The factors or signalling pathways underlying the remaining augmented hyperaemia during hypoxic exercise remain to be determined.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

All experiments were performed in the Human Cardiovascular Physiology Laboratory, Colorado State University, Fort Collins, CO, USA. MLR and FAD contributed to the conception and design of the experiments, collection, analysis and interpretation of the data, as well as the writing of the manuscript. ARC contributed to the conception and design of the experiments, collection, analysis and interpretation of the data, and provided critical revision of the manuscript. GJL and DGL contributed to the experimental design, provided invasive methodology for data collection, and contributed to the critical revision of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

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