The nitric oxide dependence of cutaneous microvascular function to independent and combined hypoxic cold exposure

TITLE

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RUNNING TITLE

NO vasoactivity during hypoxic-cold

ABSTRACT

Hypoxic modulation of nitric oxide (NO) production pathways in the cutaneous microvasculature and its interaction with cold-induced reflex vasoconstriction, independent of local cooling, has yet to be identified. This study assessed the contribution of NO to non-glabrous microvasculature perfusion during hypoxia and whole-body cooling with concomitant inhibition of NO synthase (NOS; via L-NAME) and the nitrite reductase, xanthine oxidase (via allopurinol), two primary sources of NO production. Thirteen volunteers were exposed to independent and combined cooling via water perfused suit (5ºC) and normobaric hypoxia (F_iO_2, 0.109 ± 0.002). Cutaneous vascular conductance (CVC) was assessed across four sites with intradermal microdialysis perfusion of 1) Lactated Ringers solution (control), 2) 20 mmol L-NAME 3) 10 μmol allopurinol, or 4) combined L-NAME/allopurinol. Effects and interactions were assessed via 4-way repeated measures ANOVA. Independently, L-NAME reduced (43%, $p < 0.001$), while allopurinol did not alter CVC ($p = 0.5$). Cooling decreased CVC ($p = 0.001$) and the reduction in CVC was consistent across perfusates (~30%, $p = 0.9$). Hypoxia increased CVC (16%, $p = 0.01$), with this effect abolished by L-NAME infusion ($p = 0.04$). Cold-induced vasoconstriction was blunted by hypoxia, yet importantly hypoxia increased CVC to a similar extent (39% at the Ringer site) irrespective of environmental temperature, thus no interaction was observed between cold and hypoxia ($p = 0.1$). L-NAME restored vasoconstriction during combined cold-hypoxia ($p = 0.01$). This investigation suggests that reflex cold-induced cutaneous vasoconstriction acts independently of NO suppression, while hypoxia-induced cutaneous vasodilatation is dependent on NOS derived NO production.

KEY WORDS

Microdialysis, Vasoconstriction, Vasodilatation, Cold, Hypoxia, Nitric Oxide,

NEW AND NOTEWORTHY

Whole-body cooling when separated from local cooling, elicited cutaneous reflex vasoconstriction via mechanisms independent of nitric oxide removal. Hypoxia elicited cutaneous vasodilatation via mechanisms mediated primarily by nitric oxide synthase, rather than xanthine oxidase mediated nitrite reduction. Cold-induced vasoconstriction was blunted by the opposing effect of hypoxic vasodilatation, while the underpinning mechanisms do not interrelate in the absence of local cooling. Full vasoconstriction was restored with nitric oxide synthase inhibition.

INTRODUCTION

Exposure to acute hypoxia results in net vasodilatation across human non-glabrous skin in thermoneutral conditions (3, 28, 41, 48, 54, 58). This net increase in skin blood flow in hypoxia has also been reported to blunt the degree of cold-induced cutaneous vasoconstriction (46). From a thermoregulatory standpoint, even small impairments in cold-induced cutaneous vasoconstriction with hypoxia can lead to significant increases in heat loss. Indeed, previous studies report higher skin temperature and lower core temperature when cold exposure is combined with hypoxia compared to normoxia (2, 9, 24). However, the exact mechanisms that regulate cutaneous vasodilatation during acute hypoxic exposure and its mechanistic interaction with cold-induced vasoconstriction have yet to be resolved.

Mechanistically, cold-induced cutaneous vasoconstriction can be mediated locally, via the direct cooling of the neuroeffector junction and cutaneous vascular smooth muscle, and systemically, via a sympathetic reflex response to whole-body activation of cold sensitive receptors (23). The role of NO in regulating cutaneous vascular tone to cold stress is well documented, with up to 40% of local-cold mediated vasoconstriction accounted for by the suppression of tonic NO and NO synthase (NOS) activity (20, 22, 61). With regard to reflex sympathetic vasoconstriction in response to whole-body cooling, cutaneous vasoconstriction has been reported to be attenuated following administration of the NOS-independent NO donor, sodium nitroprusside (SNP) (11), and augmented with NOS inhibition via NG-nitro-L-arginine methyl ester (L-NAME) (43). However, since neither study reported whether local skin temperature was fixed at each microdialysis site or was free to fluctuate during whole-body cooling, these findings cannot exclude the possibility that local cooling mechanisms contributed to these effects. Furthermore, both studies pharmacologically elevated cutaneous blood flow, via either SNP or adenosine, throughout cooling procedures which does not

usually occur under real world settings. Fixation of local skin temperature at thermoneutral temperatures (~33°C) during concomitant whole-body cooling without pharmacologically mediated elevations in skin blood flow is important to independently assess the impact of NO on systemic reflex cutaneous vasoconstriction and has yet to be investigated in this context.

The extent to which cutaneous vascular tone is NO mediated in hypoxia, in particular the contribution of NOS-dependent and independent NO synthesis, have yet to be experimentally determined. Although O_2 is required for NOS-derived NO (4), the Michaelis constant (K_M) of the different NOS isoforms for O_2 is not uniform, with endothelial NOS (eNOS) exhibiting the lowest K_M (highest sensitivity) for O_2 (50). Therefore, eNOS has the potential to contribute to vascular tone in hypoxic exposure as empirically documented previously (3, 41, 56). An alternative NOS independent possibility is that NO is transported in the form of S-nitrosothiols (SNOs), yet this has been questioned given the low levels of SNOs in human blood and lack of a detectable arteriovenous SNO gradient (18). Finally, NO can also be synthesized from the one-electron reduction of nitrite (NO_2^-) (35) with this reaction potentiated in acidosis and hypoxia (7, 38). While numerous proteins and enzymes can catalyze NO_2^- reduction to NO (57), xanthine oxidase (XO) is considered a principal NO_2^- reductase in hypoxia (35), and administration of the XO inhibitor, allopurinol, has been reported to thwart the vasodilatory effects of exogenous NO_2^- administration in different vascular beds (6, 17, 32). It has been reported that declines in NO production (55) and tissue perfusion (12) with L-NAME can be offset by increasing circulating NO_2^-, and that XO-derived NO_2^- reduction can elevate NO to a greater extent than achievable by maximally activating NOS during severe ischemia (31). Research is required to assess how independent and combined NOS and XO inhibition impact cutaneous vascular function in hypoxic, cold and combined hypoxic cold conditions.

Using a robust analytical approach, all variances were accounted for using a four-way analysis of variance assessing both main effects and all associated interactions, this study aimed to assess the contribution of NO to skin perfusion in the presence of independent and combined hypoxic and whole-body cold stress with concomitant independent and combined inhibition of NOS and XO. It was hypothesised that: 1) cutaneous vasoconstriction during whole-body cooling in normoxia would be augmented with NOS suppression, with little or no effect from XO suppression; 2) hypoxic cutaneous vasodilatation would be impaired with XO and NOS suppression, with a dominant effect from XO; and that, 3) blunted vasoconstriction would occur during combined whole-body cooling and hypoxia and that this response would be reversed primarily with XO and NOS suppression, with a dominant effect from XO. Addressing a number of aforementioned gaps in the literature, applications of this knowledge might extend to both clinical and occupational settings where hypoxia and cold stress are often incurred in tandem. For example, intensive care where hypothermia is often induced as a means to offset the effects of hypoxia. Furthermore, military and expeditionary settings, where circulatory responses directly compete for thermal balance and adequate oxygenation.

METHODS

Ethical Approval

Ethical approval was granted by the Ethics Approvals Committee at the University of Tsukuba (approval number, 29-24). The research was conducted in accordance with the Declaration of Helsinki, 2013, except for registration in a database. Participants provided written informed consent before participating.

Participants

A convenience sample of thirteen healthy volunteers, ten males and three females (age, 25 ± 3 yrs; stature, 1.71 ± 0.08 m; body mass, 67.3 ± 9.5 kg; body mass index, 23 ± 3 kg·m^2) were recruited from Tsukuba Japan between July and September 2019. All participants were physically active, non-smoking, East Asian individuals, over 18 years of age. Females were not taking oral contraceptives and were tested between days 1-8 of the menstrual cycle, wherein levels of sex hormones remain low. This control is necessary, as previous research has shown female hormones to alter the mechanisms underlying reflex cutaneous vasoconstriction during cold exposure (49).

Procedure

The study required a single visit to the laboratory comprising five key 20 min experimental phases: an absolute baseline, '**Baseline [Ringers]'**; a post-drug infusion relative baseline, '**Baseline [Drug]'**; independent cold exposure, '**Cold'**; independent hypoxia exposure, '**Hypoxia'**; and combined cold and hypoxia exposure, '**Cold/Hypoxia'** (*Figure 1*).

Participants arrived at 07:00 having abstained from alcohol, strenuous physical activity and caffeine for the previous 12 hrs and food for > 2 hrs prior to arrival. Environmental laboratory conditions were maintained at 26.2 ± 0.8°C and 66.0 ± 3.9% relative humidity for the duration of the trial (52). In order to control whole-body temperature, participants first donned a water perfused suit (Med-Eng, Ottawa, ON, Canada) with a 25% tubing density, leaving the experimental portion of the left forearm uncovered. The suit was connected to a water bath via inline water pump (Variable-Flow Pump, Thermo Fisher Scientific, MS, U.S.), with water circulated at 1.3 L·min^{-1}, regulated at ~35°C during all normothermic phases. Participants then rested in a semi-recumbent position allowing microdialysis fibers to be inserted (detailed below). Participants maintained this position for the remainder of the trial. At the end of the

90 min resolution phase, perfusion of lactated Ringer solution (Composition; Na^+ 130.4, K^+ 4.0, Ca^{++} 2.7, Cl^- 109.4, $Lact.^-$ 27.7 $mmol \cdot L^{-1}$) to all fibers was changed from 10 $\mu L \cdot min^{-1}$ to 2.5 $\mu L \cdot min^{-1}$.

When ready all variables were set to record, initiating the start of the Baseline [Ringers] phase. Skin and core temperatures were measured every 1 s using copper-constant thermocouples and automatically logged (GM10, Yokogawa Electric, Tokyo, Japan). Skin temperature was assessed at six consistent representative locations on the body under the suit. A weighted mean skin temperature (T_{sk}) was generated as follows: $T_{sk} = 0.22\ T_{ch} + 0.21\ T_{ub} + 0.19\ T_{lb} + 0.14\ T_{ab} + 0.14\ T_{th} + 0.11\ T_{cf}$, where the sites are chest, upper back, lower back, abdomen, thigh, and calf, respectively (53). Core temperature was monitored via esophageal probe, self inserted via the nasal passage to a distance equivalent to one-fourth of the participant's height. This length is estimated to be posterior to the lower border of the left atrium (59). In participants who were unable to insert the esophageal probe (n = 3), core temperature was monitored at the sublingual sulcus. Finally, a forearm thermocouple probe was attached near the microdialysis sites as a proxy measure of local skin temperature. Expired respiratory variables were measured via mass spectrometer (ARCO1000, ARCO, Chiba, Japan), calibrated against a standardized gas of known composition (O_2, 15.00 %; CO_2, 5.04 % and N_2, balanced), and connected to the expiratory side of a Hans Rudolf valve attached to a face mask. In addition, heart rate was assessed every 1 s via remote chest transmitter (RS800; Polar, Finland), peripheral oxygen saturation was assessed every 1 s via forehead pulse oximeter (N-595, Nellcor, Hayward, Canada) and blood pressure was assessed every 5 min via automated sphygmomanometer at the right arm positioned at heart level (TM-2580, A&D Ltd, Tokyo, Japan) throughout the trial.

Following a 20 min perfusion of lactated Ringer solution (Fuso Pharmaceutical Industries, Osaka, Japan) for absolute baseline quantification, four perfusates (lactated Ringers, L-NAME, allopurinol, combined L-NAME/allopurinol) were randomly assigned to independent fibers, maintaining 2.5 $\mu L \cdot min^{-1}$, for subsequent experimental comparisons. A standardized 30 min period was then allocated for drugs to saturate each fiber site, followed by a 20 min period for a second post-perfusate baseline (Baseline [Drug] phase), before the participant was sequentially exposed to both independent and combined cold and hypoxia. During cooling phases the bath temperature supplying the suit was regulated at ~5°C, known to reliably reduce mean skin temperature with little reduction in core temperature over an acute period (13). Water circulation was increased to 2.7 $L \cdot min^{-1}$ during cooling and recovery phases. Simulating this set up on a thermal manikin (Andi Thermal Manikin System, Thermetrics, WA, US), the water perfused suit presented an increased cooling capacity of 178 W during cooling phases compared to normothermic phases. During hypoxic phases, inspired air of F_iO_2, = 0.109 ± 0.002 (equivalent to ~5000 m above sea level) was administered via a face mask, connected to a 500 L Douglas bag via low-resistance silicon pipe to the Hans Rudolf three-way valve. During normoxic phases, the valve at the Douglas bag end of the system was left closed and participants breathed ambient sea level air (F_iO_2, ~0.209). The order of exposure to cold and hypoxic phases was counterbalanced between participants. A 30-40 min recovery phase separated independent exposure to cold and hypoxia, allowing either mean skin temperature or arterial oxygen saturation to restore to normothermic normoxic values (T_{sk} within 0.5 °C; S_aO_2 within 3 %) (*Figure 1*).

In addition to dialysate collection (detailed below), a 5 mL venous blood sample was drawn from the antecubital fossa into a tube containing ethylenediaminetetraacetic acid (Nipro, Osaka, Japan) 7 min prior to the end of each phase (*Figure 1*). Blood samples were

immediately centrifuged at 4000 rpm and 4 °C for 10 min. Plasma was then aliquoted into 1 mL microtubes and frozen at -80 °C for subsequent analysis of NO_2^- concentration. At the termination of the combined hypoxic-cold phase, data capture of all variables was stopped, except for laser doppler flow and blood pressure. Perfusion to all fibers was changed from their respective perfusates to 25 mmol SNP (Nacalai Tesque, Kyoto, Japan) in combination with local heating at 44 °C for 30 min in order to estimate maximal skin perfusion (10). Maximal skin perfusion was measured to assess if drugs modulated maximal skin vasodilatory capacity.

Analysis of dialysate and plasma samples was undertaken within one month of sample collection. Concentrations of NO_2^-, which has been reported to sensitively reflect NO activity (27), was quantified by ion chromatography via the Eicom ENO-20 NO_x– analysis system (Eicom, Kyoto, Japan) with on-line reduction of NO_3^- to NO_2^- and post-column Griess diazotization as detailed by Bryan and Grisham (5). Immediately prior to analysis, plasma samples were diluted 1:1 with methanol (Nacalai Tesque, Kyoto, Japan) and centrifuged at 10,000 G and 4 °C for 10 min for deproteinization. Dialysate data from the Ringers fiber alone is reported due to interactions between L-NAME/allopurinol and the carrier solution in the dialysate analysis procedure.

Microdialysis Instrumentation

Under thermoneutral conditions, four microdialysis fibers were inserted at pre-selected forearm skin sites on the left arm. A 25-gauge needle was aseptically inserted into the dermal layer of the unanaesthetised left dorsal forearm skin. Entry and exit points were separated by ~2.5 cm. A microdialysis fiber made in-house, consisting of a 10 mm regenerated cellulose membrane (0.22 mm outer and 0.20 mm inner diameter, internal volume of 0.31 mm^3)

attached to the inlet and outlet of the polyimide tubes (0.16 mm outer and 0.12 mm inner diameter) was passed through the lumen of the needle, before the needle was withdrawn, leaving the membrane of fiber in the skin. All fibers were placed in a similar manner, separated by >2 cm to avoid any between-site interference of drug administration. Immediately following fiber insertion, participants rested quietly for 90 min to allow local hyperaemia due to insertion trauma to subside. Research shows skin perfusion to return to near normal levels within 60 mins following fiber insertion (1). During this resolution phase, lactated Ringer solution was perfused through all fibers at a rate of 10 $\mu L \cdot min^{-1}$ using a micro-infusion pump (BASi Bee Hive controller and Baby Bee syringe drive; Bioanalytical Systems, West Lafayette, IN, US).

Four selected drugs were randomly assigned to the fibers at the termination of the resolution phase, perfused at a rate of 2.5 $\mu L \cdot min^{-1}$. *1)* Lactated Ringers solution, serving as a control, *2)* 20 mmol solution of L-NAME (molecular weight: 269.69, Nacalai Tesque, Kyoto, Japan), for the inhibition of the NOS pathway of NO production (20, 61) *3)* 10 µmol solution of allopurinol, for the inhibition of XO, a key enzyme catalysing NO_2^- reduction to NO (21, 36, 37). *4)* Combined solution of 20 mmol L-NAME and 10 µmol allopurinol for the impairment of both NO production pathways. In addition to the perfusion of drugs, dialysate from the Ringers fiber alone was collected at a rate of 2.5 $\mu L \cdot min^{-1}$ during the last ten minutes of each experimental phase as indicated in *Fig. 1*. The effluent end of the fiber (opposite to the perfusate end) was connected to a fraction collector (EFC-96FN, Eicom, Kyoto, Japan), passing dialysate (25 µL) into microtubes. The specific time window of collection was offset by 3 mins from experimental phases, accounting the time delay for dialysate to leave the intradermal space and enter the microtube.

To assess cutaneous perfusion at each site, red blood cell flux was measured via integrated laser doppler flowmetry probes with a seven-laser array (Model 413, Perimed, Stockholm, Sweden) and sampled at 32 Hz. Probes were mounted in the centre of thermostatic heating units (Ø 32 mm, Model PF450), clamped at 33 °C and secured to the skin using double sided medical tape directly over the midpoint of each fiber site. Using laser doppler data, cutaneous vascular conductance (CVC) was evaluated as red blood cell flux divided by the closest temporal measurement of mean arterial pressure ([1/3 systolic blood pressure] + [2/3 diastolic blood pressure]), in order to account for changes in perfusion pressure.

Data Analysis

Data across all physiological metrics was downloaded and reduced to 1 min time block averages. For comparisons across environmental conditions, a discrete 5 min time block average was determined for each metric/fiber from the final 5 mins of respective 20 min phases. To directly compare CVC data across independent fiber sites, all recorded values were first normalized against site-specific baseline [Ringers] (normoxic, normothermic, with 2.5 $\mu L \cdot min^{-1}$ Ringers perfusion) and expressed as a percentage, where 100 % equates to baseline [Ringers] perfusion. This was necessary because the range of CVC values observed with vasoconstrictor responses is more comparable with baseline than maximal CVC (23). Data are presented as mean ± SD, while main effects are presented as the difference in estimated marginal means (EMM) with Bonferroni adjusted confidence intervals [95% CI's]. Data from one individual was removed from the CVC data set and two were removed from the $[NO_2^-]$ data set, due to technical difficulties during data collection.

Statistical Analysis

Inferential statistical analysis was conducted using IBM SPSS statistics (version 23, IBM Corp., USA). All data conformed to a normal distribution, assessed via Shapiro-Wilk's test. Baseline and maximal CVC values were first compared using one-way repeated measures analysis of variance (ANOVA, 4 levels) across fibers to ensure consistent microvascular perfusion, irrespective of perfusate administration. Independent samples *t*-tests were also performed to check for sex related differences, revealing no statically significant effects across variables ($p > 0.05$), thus data from males and females were pooled from all subsequent analysis.

A four-way repeated measures ANOVA (2x2x2x2) was performed on CVC data normalized to baseline [Ringers], assessing independent main effects and associated combined interactions between fibers and environmental conditions. Independent factors (main effects) included L-NAME, Allopurinol, Cold and Hypoxia (Organisation of levels found in *Table 1*). Alpha was set a-priori at 0.05. Adding to mechanistic insight, the relative percentage change of each environmental condition from the post infusion 'baseline [drug]' was also determined for each perfusate, calculated as;

$$\text{Relative change (\%)} = \left[\left(\frac{B-A}{A}\right) \times 100\right] \quad \text{(Eq. 1)}$$

Where, *A* is Baseline [Drug], and *B* is for example Cold.

For secondary variables (e.g. skin temperature, $[NO_2^-]$), main effects for cold and hypoxia and their associated interaction were assessed using a two-way repeated measure ANOVA (2x2). Correlations of CVC with mean skin temperature, peripheral oxygen saturation or NO_2^- were investigated using Pearson's correlation test.

Data Interpretation

To better account for all variances and eliminate the need for discretionary post-hoc analysis, and as such multiplicity issues (30), a four-way ANOVA (2x2x2x2) was selected for CVC data over a two-way ANOVA equivalent (4x4). Furthermore, given the clear link between stressors in their independent and combined form, it would be inappropriate to consider combined conditions as strict independent entities (as in the two-way ANOVA), in place of a combination/interaction of independent stressors (as in the four-way ANOAVA). Interactions are defined as per Lloyd and Havenith (33), where the interaction statistic indicates whether the effect of variable A (e.g. thermoneutral vs. cold) is altered with a change in variable B (e.g. Ringers vs L-NAME fiber). Thus, no significant interaction suggests the effect is additive (i.e. the effect of L-NAME is the similar across neutral and cold), while a significant interaction can be hypo- or hyper-additive (antagonistic or synergistic). Where no main effect was observed for a factor, interactions were not explored further.

RESULTS

Environmental Stressors

Both mean skin and forearm temperatures significantly decreased during cooling phases compared to normothermic phases (EMM cold vs. control; mean skin, 4.70 [3.27 to 6.14] ºC, $p < 0.001$; forearm, 1.03 [0.60 to 1.46] ºC, $p < 0.001$), and to a lesser extent during hypoxic compared to normoxic phases (EMM hypoxia vs. control; mean skin, 0.34 [0.17 to 0.50] ºC, $p = 0.001$; forearm, 0.33 [0.13 to 0.53] ºC, $p = 0.003$) (*Figure 2*). Assessed in 10 individuals, esophageal temperature significantly increased during cooling phases compared to normothermic phases (EMM cold vs. control; 0.17 [0.04 to 0.30] ºC, $p = 0.01$), and significantly decreased during hypoxic phases compared to normoxic phases (EMM hypoxia vs. control; 0.09 [0.03 to 0.15] ºC, $p = 0.01$). Peripheral oxygen saturation significantly

decreased during hypoxic phases compared to normoxic phases (EMM hypoxia vs. control; 20 [16 to 24] %, $p < 0.001$). No main effect was seen for cold on peripheral oxygen saturation (EMM cold vs. control; 1 [-1 to 2] %, $p = 0.3$). No interaction was observed between cold and hypoxia exposure for mean skin temperature ($p = 0.2$), forearm temperature ($p = 0.3$), esophageal temperature ($p = 0.7$) or peripheral oxygen saturation ($p = 0.09$). Mean skin temperature ($p = 0.3$), forearm temperature ($p = 0.7$), esophageal temperature ($p = 0.7$) and peripheral oxygen saturation ($p = 0.7$) were successfully restored to baseline [drug] values at the end of the recovery phase.

Cutaneous Vascular Conductance

No differences were observed in absolute CVC across fibers at baseline [Ringers] prior to normalisation (Ringers, 0.47 ± 0.24 pu·mmHg^{-1}; L-NAME, 0.61 ± 0.24 pu·mmHg^{-1}; allopurinol, 0.59 ± 0.36 pu·mmHg^{-1}; L-NAME/Allopurinol, 0.53 ± 0.22 pu·mmHg^{-1}; $p = 0.5$) or in heat/SNP induced maximum CVC (Ringers, 4.45 ± 0.52 pu·mmHg^{-1}; L-NAME, 4.68 ± 1.25 pu·mmHg^{-1}; allopurinol, 5.03 ± 1.37 pu·mmHg^{-1}; L-NAME/Allopurinol, 5.01 ± 0.89 pu·mmHg^{-1}; $p = 0.3$). Baseline CVC represented 12 ± 1 % of maximum CVC across fibers. For all subsequent results, CVC data were normalized to baseline [Ringers].

CVC data and statistical findings are presented in *Figure 3*. Independently, L-NAME significantly reduced CVC (EMM; L-NAME vs. control, 43 [28 to 59] %, $p < 0.001$), while no effect was observed for allopurinol (EMM; allopurinol vs. control, 5 [-12 to 22] %, $p = 0.5$). No statistical interaction was observed between allopurinol and any other factor ($p > 0.1$), thus the impact of allopurinol was not explored further. Whole-body cold stress significantly decreased CVC (EMM; cold vs. control, 30 [15 to 44] %, $p = 0.001$). L-NAME antagonistically interacted with cold stress (hypo-additive interaction, $p = 0.01$), in which the

absolute magnitude of cold-induced vasoconstriction was reduced in the presence of L-NAME - i.e. cold-induced vasoconstriction (cold vs control) decreased CVC by an absolute magnitude of 43 % in the absence of L-NAME vs. 17 % in the presence of L-NAME. Yet, when the vasoconstrictive effect of cold was assessed as a percentage change from baseline [drug], the mean difference was the same between the Ringers and L-NAME fiber (relative % change of cold from baseline [drug]; Ringers, -33 % vs. L-NAME, -31 %; $p = 0.9$).

Independently, hypoxia significantly increased CVC (EMM; hypoxia vs. control, 16 [4 to 29] %, $p = 0.01$). L-NAME antagonistically interacted with hypoxic stress (hypo-additive interaction, $p = 0.04$), in which the absolute magnitude of hypoxic-induced vasodilatation was abolished - i.e. hypoxic-induced vasodilation (hypoxia vs. control) increased CVC by an absolute magnitude of 25 % in the absence of L-NAME, vs. 7 % in the presence of L-NAME. The mean difference of hypoxia relative to baseline [drug] also reflected this finding (relative % change of hypoxia from baseline [drug]; +39 % vs. L-NAME, +4 %; $p = 0.003$).

No statistical interaction was observed between cold and hypoxia ($p = 0.1$), while hypoxia blunted cold induced vasoconstriction in a 'additive relative' manner – i.e. in the Ringers fiber, the vasodilatory effect of hypoxia in thermoneutral was 39 %, while hypoxia reduced the vasoconstrictive effect of cold by 39 %. A significant three-way interaction was observed between L-NAME, cold and hypoxia (hypo-additive interaction, $p = 0.01$), in which L-NAME antagonistically abolished the hypoxic effect in cold stress, thus restoring full cold-induced vasoconstriction.

Mean skin temperature was significantly correlated with CVC during the cold phase ($r = 0.72$ $p = 0.01$) and during the combined hypoxia-cold phase ($r = 0.69$, $p = 0.02$) in the Ringers

fiber alone. No correlations were observed between CVC and peripheral oxygen saturation during respective hypoxic phases across fibers ($p > 0.08$).

Plasma and Dialysate [NO_2^-]

Hypoxia significantly increased plasma [NO_2^-] compared to normoxia (EMM; hypoxia vs. control, 19 [10 to 29] nmol·L^{-1}, $p = 0.001$) (*Fig. 4*). No main effect of cold was observed for plasma [NO_2^-] compared normothermic phases (EMM; cold vs. control, 7 [-1 to 15] nmol·L^{-1}, $p = 0.08$). No main effects of cold (EMM; cold vs. control, 5 [-69 to 78] nmol·L^{-1}, $p = 0.8$) or hypoxia (EMM; hypoxia vs. control, 31 [-57 to 120] nmol·L^{-1}, $p = 0.4$) were observed for dialysate [NO_2^-]. No interaction was observed between cold and hypoxia on plasma [NO_2^-] ($p = 0.1$) or dialysate [NO_2^-] ($p = 0.7$). No correlations were observed between CVC responses and assessed [NO_2^-].

Secondary Variables

Cold decreased heart rate and end tidal CO_2 partial pressure, and significantly increased mean arterial pressure compared to normothermic phases (main effects, heart rate, $p = 0.04$; mean arterial pressure, $p = 0.006$; end tidal CO_2 partial pressure, $p = 0.01$) (*Table 2*). Hypoxia significantly increased heart rate and tidal volume, and significantly decreased end tidal O_2 and CO_2 partial pressures compared to normoxic phases (main effects; heart rate, $p = 0.001$; tidal volume, $p = 0.05$; end tidal O_2 partial pressure, $p < 0.001$; end tidal CO_2 partial pressure, $p = 0.02$). No main effect of cold or hypoxia was observed for respiratory rate, minute ventilation or oxygen consumption. No interaction was observed between cold and hypoxia on any secondary variable ($p > 0.1$).

DISCUSSION

409 This study assessed the contribution of NO to cutaneous microvascular perfusion in the
410 presence of independent and combined hypoxic and cold stress by simultaneously inhibiting
411 NOS and XO, both independently and concomitantly. The principle findings of this study
412 were: *1)* whole-body cooling elicited significant cutaneous vasoconstriction via mechanisms
413 independent of NO; *2)* normobaric hypoxia, equivalent to 5000 m above sea level, elicited
414 significant cutaneous vasodilatation mediated primarily by NOS; and *3)* cold-induced
415 vasoconstriction was blunted by the opposing effect of hypoxic-induced vasodilatation, while
416 the mechanisms appear to operate independently of each other. Full cold-induced
417 vasoconstriction is restored with L-NAME administration. Several hypotheses were addressed
418 through this investigation:
419
420 ***Hypothesis 1:*** *Cutaneous vasoconstriction during whole-body cooling in normoxia would be*
421 *augmented with NOS suppression, with little or no effect from XO suppression.* The cold
422 stimulus was effective in inducing cutaneous reflex vasoconstriction across all skin sites (Fig
423 3). This observation is consistent with previous work (19, 26), and suggested to be mediated
424 by sympathetic adrenergic neural mechanisms including the activation of α_2 adrenoceptors
425 and neuropeptide Y receptors (23). Interestingly, the data herein shows a significant
426 antagonistic interaction between cold stress and L-NAME administration, resulting in an
427 attenuation of the cold-induced vasoconstriction effect. It is believed that this interaction is
428 methodological in nature with CVC nearing a lower perfusion limit, whereby the magnitude
429 of cold-induced vasoconstriction is constrained in the presence of significant vasoconstriction
430 with L-NAME administration, thereby inducing 'additive relative' rather than 'additive
431 absolute' effects (33). Indeed, CVC was ~0.25 pu·mmHg^{-1} during cold stress with
432 concomitant L-NAME perfusion. Importantly, when the vasoconstrictive effect of cold was
433 assessed as a relative change from the post infusion baseline [drug] phase, the percentage

decrease was consistent across fibers (-33 and -31 %), suggesting an independence of reflex vasoconstriction from NO mechanisms.

The lack of effect of NOS inhibition on cold-induced cutaneous vasoconstriction differs from both the hypothesis and, at least in part, from the observations by Durand et al. (11) and Shibasaki et al. (43), who reported NO to attenuate cutaneous vasoconstriction during whole-body cooling. Of critical importance however, neither of these previous studies stated whether local skin temperature at the measurement site was explicitly clamped, which is important in order to definitively isolate the independent mechanisms of local vs. reflex vasoconstriction. Indeed, while local temperature of the measurement sites was clamped in the current study, thereby eliminating the effect of local vasoconstrictor mechanisms, skin temperature of the remaining portion of the forearm showed significant reductions during cooling despite freedom from the water perfused suit, as might have been the case to a greater or lesser extent in previous studies. It should be also highlighted that cutaneous vascular conductance during whole-body cooling was largely elevated by pharmacological agents such as SNP and adenosine in the previous studies (11, 43). By contrast, the current protocol did not artificially elevate cutaneous vascular conductance during whole-body cooling. The role of NO on reflex cutaneous vasoconstriction associated with whole-body cooling may be diminished when basal vasoconstrictive tone is strong as reflected by low levels of cutaneous vascular conductance, and this might explain the disparate findings between the present and the above two previous studies. Future study is warranted to elucidate this possibility.

Hypothesis 2: *Hypoxic cutaneous vasodilatation would be impaired with XO and NOS suppression, with a dominant effect from XO.* Previous studies have identified a variety of vascular responses to hypoxic exposure, reporting both increased forearm and muscle blood

459 flow (3, 41, 58), and increased skin perfusion (28, 34, 41, 48). Consistent with the latter, the
460 present study showed net cutaneous vasodilatation with severe hypoxia. In contrast to the
461 hypothesis, no effect was observed for XO inhibition in hypoxia, suggesting that NO_2^-
462 reduction through XO does not play a role in mediating hypoxic cutaneous vasodilatation.
463 This is further supported by the apparent increase in plasma [NO_2^-] and no change in dialysate
464 [NO_2^-] observed with hypoxia herein, thus the apparent absence of systemic and local NO_2^-
465 reduction. On the other hand, L-NAME near-abolished the hypoxia-induced vasodilatation.
466 Unlike *in vitro* studies reporting attenuated NO synthesis from NOS with hypoxia (40, 60),
467 the present study testing human skin *in vivo* lends support to the importance of NOS-derived
468 NO as a central mediator of the cutaneous hypoxic vasodilatory response. Again, this
469 postulate is substantiated by the observation that plasma [NO_2^-] was enhanced following
470 hypoxia exposure. Synthesis of NO through eNOS is activated by the phosphorylation of the
471 serine1177 amino acid residue, a response which is initiated by sheer stress, and various
472 hormones, proteins and kinases (14). For example, heat shock protein 90, which is known to
473 interact with NOS in mediating vasodilatation in human skin (16), may be activated to
474 enhance eNOS activity in hypoxia as was observed in porcine coronary artery (8).
475 Alternatively, hypoxia may activate adenosine receptors, which can cause cutaneous
476 vasodilatation mainly via NOS mechanisms (15), as was demonstrated in human forearm
477 (29). However, the precise molecular mechanisms by which NOS yields NO and regulates
478 cutaneous perfusion under hypoxia remains unclear. Interestingly, when L-NAME was
479 combined with allopurinol, its relative effect was reduced. Given that the concentration of
480 drugs when in their combined form directly equated to their independent form, the reduction
481 of L-NAME effect in this context remains unclear.
482

Hypothesis 3: *Blunted vasoconstriction would occur during combined whole-body cooling and hypoxia and that this response would be impaired primarily with XO and NOS suppression, with a dominant effect from XO.* The vascular response to combined cold and hypoxic stress is unclear with previous literature showing both blunted vasoconstriction (46) or increased vasoconstriction (45), compared to cold stress alone. In agreement with the hypothesis and the findings of Simmons and colleagues (46), hypoxia blunted cold-induced vasoconstriction in the current study, while the interaction between hypoxia and cold was additive, and not statistically significant, i.e. hypoxia increased CVC to a similar extent irrespective of the environmental temperature. L-NAME abolished the vasoactive effect of hypoxia on cold-induced vasoconstriction. Therefore, these results suggest that NOS-dependent cutaneous vasodilatation associated with hypoxia can override some of the sympathetic adrenergic mediated reflex cutaneous vasoconstriction to the cold, yet the mechanisms appear to operate independently and do not interrelate, thus reflecting a new net balance between competing vasoconstrictor and vasodilator drives. Along these lines, Simmons et al. (47) demonstrated that systemic hypoxia has no mechanistic effect on tyramine (α adrenergic- vasoconstrictor) induced cutaneous vasoconstriction. However, Shibasaki et al. (44) demonstrated that the cutaneous vasoconstrictor response to exogenous noradrenaline was blunted at skin sites treated with NO donor SNP compared with a non-NO vasodilator adenosine; they concluded that NO is capable of attenuating cutaneous vasoconstrictor responsiveness to norepinephrine. Yet, it should be noted that in the study by Shibasaki et al., SNP administration increased CVC by 444% compared to baseline, which greatly exceeds the hypoxia-induced cutaneous vasodilatation observed in the present study (39% at the Ringer site). Thus, it appears that hypoxia-induced increases in NO are not sufficient to interfere with adrenergic mediated cutaneous vasoconstriction associated with whole-body cooling. Whether the characteristics of the interaction between cold and hypoxia

alters with the severity/rate of cooling, and/or the severity/type of hypoxia has yet to be determined. Alternatively, given that our results highlight the NO independence of reflex vasoconstriction, while local cold-mediated vasoconstriction and hypoxia vasodilatation are both NO dependent in their action (23), it is possible that hypoxia and cold do not regulate cutaneous vasculature tone through a common mechanism without the presence of local skin cooling.

Considerations

It is recognized that XO is as a dominant catalyzer of NO_2^- reduction to NO and that allopurinol can suppress XO by up to 80% (Faassen *et al.*, 2009). Although XO inhibition has been reported to inhibit vasodilatory responses following NO_2^- administration in animal and cell models (6, 17, 32), oral supplementation with 300 mg allopurinol daily for 4 days did not alter the reduction in systolic blood pressure with $NaNO_2$ infusion in healthy humans (42), in line with the observations of the current study. However, it should be acknowledged that the dose of allopurinol intravenously infused into rats to thwart vasodilatation to NO_2^- administration, 184-368 $\mu mol \cdot kg^{-1}$ (6, 17), was greater than administered in the current study (149 $nmol \cdot kg^{-1}$) and, in spite of the differing methods of allopurinol administration, might have contributed to these interstudy discrepancies. In addition, while XO is highlighted as a dominant NO_2^- reductase many others also exist (van Faassen *et al.*, 2009) and, as such, the extent to which NO derived from NO_2^- reduction was impaired in the current study is unclear. Moreover, since XO is an important source of superoxide (25), it is unclear how XO inhibition impacted redox balance and how this interacted with XO mediated NO_2^- reduction and influenced the results of the current study.

532 The authors also acknowledge that the completion of multiple experimental conditions in a
533 single laboratory session increases the potential risk of carry-over effects between conditions,
534 in particular, persistent sympathoexcitation post hypoxic stress (39). However, it should be
535 noted that this effect is less likely to occur with poikilocapnic hypoxia as used in the current
536 study, compared to isocapnic hypoxia (51). Furthermore, to minimize carryover effects,
537 exposure order to independent cold and hypoxic stress was counterbalanced and separated by
538 a recovery period during which mean skin temperature, peripheral oxygen saturation and
539 CVC were monitored to ensure these returned to a pre-exposure state. It is acknowledged that
540 the slightly lower mean skin temperature and peripheral oxygen saturation observed during
541 combined cold/hypoxia compared to independent phases may have obscured the possibility of
542 a statistical interaction. A second recovery period prior to the combined phase might have
543 resolved this, yet the differences in skin temperature and peripheral oxygen saturation are
544 small (0.5 ºC and 2 % respectively), such that they appear to have minimum physiological
545 impact. Whilst there would have been some benefits of assessing the different experimental
546 conditions during separate laboratory visits, this would have introduced other methodological
547 issues, such as the variance introduced by numerous baseline phases (both [Ringers] and
548 [Drug]) and variable levels of basal NO_2^- across visits, in addition to ethical issues, such as
549 the need to re-insert numerous microdialysis fibers at each visit.
550
551 Finally, the authors recognize that multiple stressors and analytical comparisons within a
552 single research design inflates the risk of incurring type I errors. To best minimize the impact
553 of multiplicity on the results, data was carefully analyzed in relation to a single statistical
554 model (i.e. repeated-measures ANONA), rather than multiple t-test comparisons (30). In this
555 context, the main effect and associated interactions for each stressor were rigorously
556 considered against the variances of the entire data set, and not in their isolated form.

557

558 ***Conclusion***

559 This study revealed a clear regulatory role of NO in cutaneous vasodilatation to hypoxic
560 stress, but not cold-induced reflex vasoconstriction. When whole-body cold stress and
561 hypoxia were combined, cold-induced vasoconstriction was partially blunted by the opposing
562 effect of hypoxia-induced vasodilatation, while the two stressors do not appear to share a
563 mechanistic interaction, i.e. hypoxia increased CVC to a similar extent irrespective of the
564 environmental temperature. Uniquely, our data also suggest that NOS-derived NO, rather than
565 NO generated from XO-mediated NO_2^- reduction as conjectured, underpins the hypoxic
566 vasodilatory response. These original observations improve understanding of the mechanisms
567 of hypoxia-induced vasodilatation in the cutaneous vasculature and its interaction with cold-
568 induced vasoconstriction.

569

570 **AUTHOR CONTRIBUTIONS**

571 JA, AL, SB, TN and NF were involved in the conception and design of the research. JA, TF,
572 RM, MT and NF conducted the experiment. JA, AL, SB and NF analyzed the data. JA wrote
573 the manuscript with amendments and suggestions made by all authors.

574

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585

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FIGURE/TABLE CAPTIONS

Fig. 1 Schematic representation of the experimental protocol. NOTE: A single trial in which 13 participants, 10 males and 3 females, visited the laboratory at 07:00 having abstained from alcohol, strenuous physical activity and caffeine for the previous 12 hrs, and food for > 2 hrs prior to arrival. Intradermal microdialysis was used to perfuse drugs across four fibers with simultaneous laser doppler measurement. L-NAME, NG-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; CVC, cutaneous vascular conductance; $T_{sk\ local}$, local skin temperature.

Fig. 2: Mean skin temperature, core temperature and peripheral oxygen saturation in response to independent and combined cold and hypoxia exposure (n = 13). NOTE: Data are mean ± SD with individual data points, ten males and three females. Mean skin temperature; weighted six site measurement. Core temperature; esopageal temperature, (n = 10), eight males and two females. Peripheral oxygen saturation (S_pO_2); forehead pulse oximetry. Cold; water perfused suit circulating water 5ºC. Hypoxia; F_iO_2, 0.109 ± 0.002. Main effects (cold and hypoxia) and interaction (cold x hypoxia) assessed via two-way repeated measures ANOVA ($\alpha = 0.05$).

Fig. 3: Cutaneous vascular conductance in response to independent and combined cold and hypoxia exposure with independent and combined L-NAME and allopurinol administration (n = 12). NOTE: Data normalized and presented against a site-specific pre-infusion baseline (normoxic, normothermic, with 2.5 $\mu L \cdot min^{-1}$ Ringers perfusion). Independent main effects (L-NAME, Allopurinol, Cold, Hypoxia) and combined interactions assessed via four-way repeated measures ANOVA (2x2x2x2). Relative change from baseline [drug] calculated as, Relative change = ([B-A]/A) x100, where, A is Baseline [Drug], and for example B is Cold. Perfusates were administered via microdialysis at 2.5 $\mu L \cdot min^{-1}$. Cold; water perfused suit

circulating water 5ºC. Hypoxia; F_iO_2, 0.109 ± 0.002. Data are mean ± SD with individual data points, nine males and three females.

Fig. 4: Plasma and dialysate nitrite in response to independent and combined cold and hypoxia exposure (n = 11). NOTE: Data are mean ± SD with individual data points, nine males and two females. A 5 mL blood sample was drawn 7 min prior to the end of each phase. Dialysate (25 µL) was collected from the final 10 mins of each corresponding phase during perfusion of Ringers solution at 2.5 $\mu L \cdot min^{-1}$. Plasma Cold; water perfused suit circulating 5ºC water. Hypoxia; F_iO_2, 0.109 ± 0.002. Main effects (cold and hypoxia) and interaction (cold x hypoxia) assessed via two-way repeated measures ANOVA ($\alpha = 0.05$).

Table. 1: Statistical organisation of a four-way repeated measures ANOVA (2x2x2x2), assessing the impact of independent and combined cold and hypoxia stress with concomitant independent and combined L-NAME and allopurinol perfusion. NOTE: 1, indicating no presence of a given stressor (control). 2, indicating active presence of a given stressor.

Table. 2: Physiological responses to independent and combined cold and hypoxia exposure (*n* = 13). NOTE: Data are mean ± SD, ten males and three females. Cold; water perfused suit circulating 5ºC water. Hypoxia; F_iO_2, 0.109 ± 0.002. Main effects (cold and hypoxia) and interaction (cold x hypoxia) assessed via two-way repeated measures ANOVA ($\alpha = 0.05$).

833 Table 1.

Table 1: Statistical organisation of a four-way repeated measures ANOVA (2x2x2x2), assessing the impact of independent and combined cold and hypoxia stress with concomitant independent and combined L-NAME and allopurinol perfusion.

	INDEPENTDENT FACTORS			
	Allopurinol	**L-NAME**	**Hypoxia**	**Cold**
Ringers Fiber – Baseline [Drug]	1	1	1	1
Ringers Fiber – Cold				2
Ringers Fiber – Hypoxia			2	1
Ringers Fiber – Cold/Hypoxia				2
L-NAME Fiber – Baseline [Drug]	1	2	1	1
L-NAME Fiber – Cold				2
L-NAME Fiber – Hypoxia			2	1
L-NAME Fiber – Cold/Hypoxia				2
Allopurinol Fiber – Baseline [Drug]	2	1	1	1
Allopurinol Fiber – Cold				2
Allopurinol Fiber – Hypoxia			2	1
Allopurinol Fiber – Cold/Hypoxia				2
L-NAME/ Allopurinol Fiber – Baseline [Drug]	2	2	1	1
L-NAME/Allopurinol Fiber – Cold				2
L-NAME/Allopurinol Fiber – Hypoxia			2	1
L-NAME/ Allopurinol Fiber – Cold/Hypoxia				2

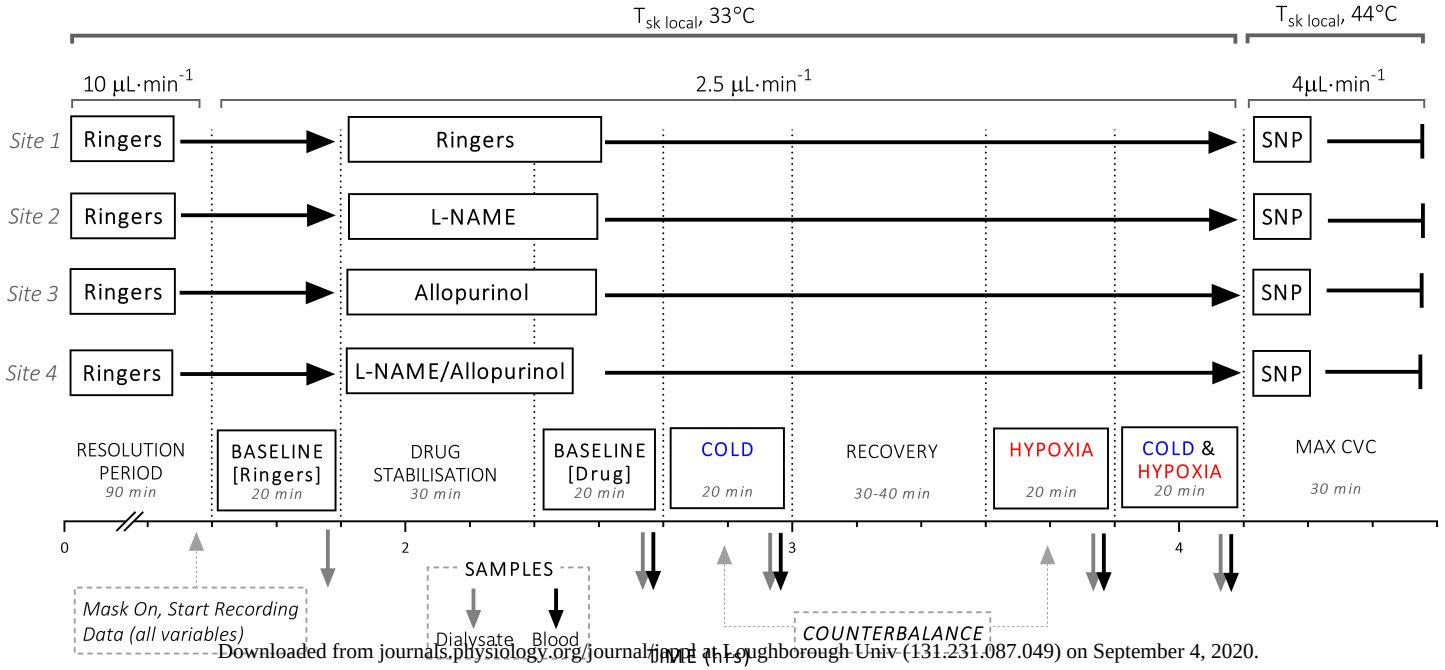
834

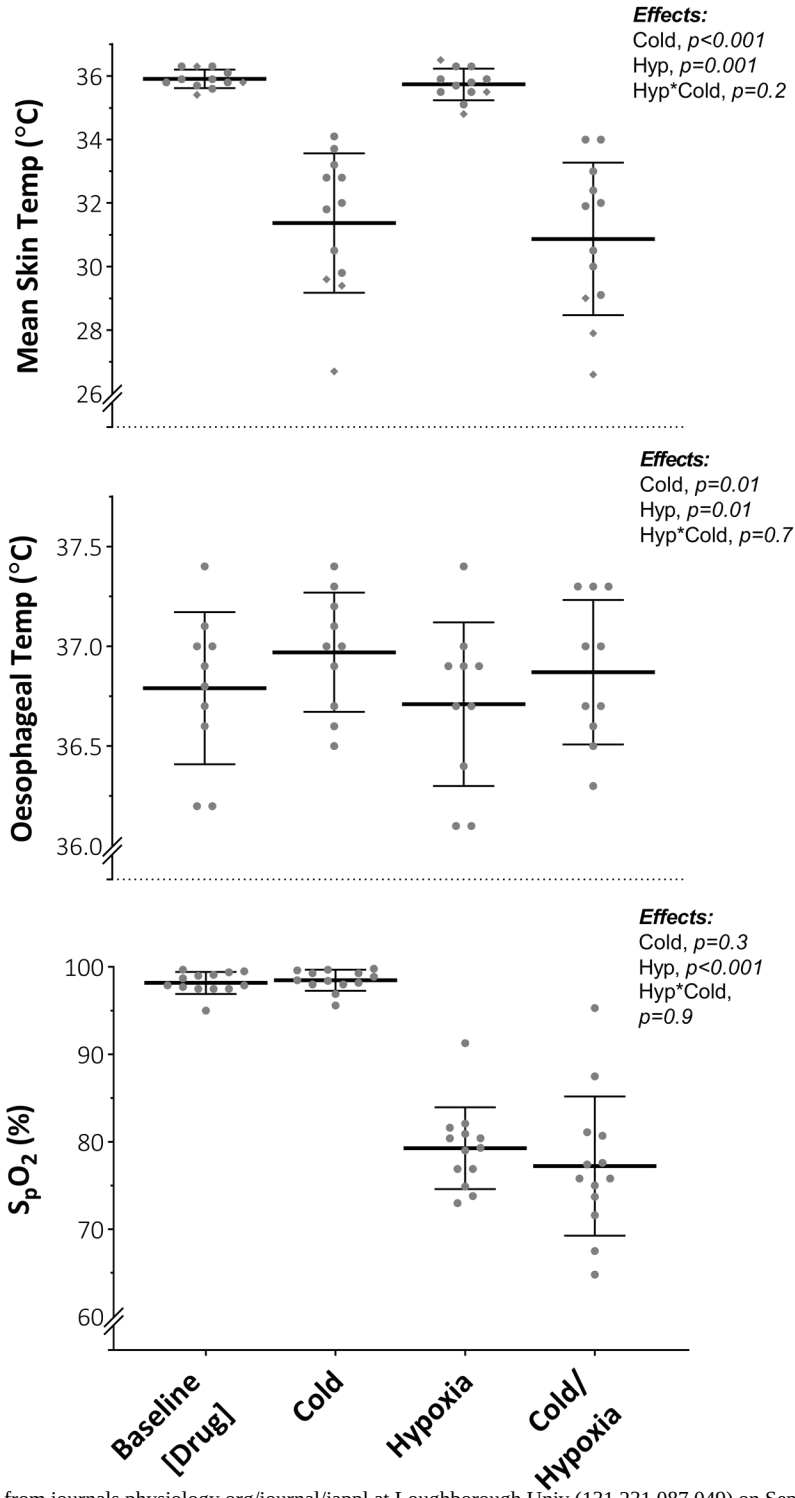
835 Table 2.

Table 2: Physiological responses to independent and combined cold and hypoxia exposure (n = 13).

	Baseline [Drug]	Cold	Hypoxia	Cold/ Hypoxia	Main Effects
Heart Rate (beats·min^{-1})	66 ± 8	61 ± 9	72 ± 6	71 ± 8	*Cold, Hyp*
Mean Arterial Pressure (mm Hg)	80 ± 9	85 ± 9	79 ± 9	85 ± 10	*Cold*
Respiratory Rate (breaths·min^{-1})	19 ± 3	18 ± 3	17 ± 3	18 ± 3	
Tidal Volume (L)	0.63 ± 0.07	0.61 ± 0.12	0.68 ± 0.12	0.67 ± 0.15	*Hyp*
Minute Ventilation (L·min^{-1})	11.3 ± 2.2	10.5 ± 2.2	10.7 ± 1.9	11.8 ± 2.7	
O_2 Consumption (mL·min^{-1})	299.6 ± 44.1	294.6 ± 49.6	290.5 ± 96.7	291.9 ± 124.1	
End Tidal O_2 partial pressure (mm Hg)	109.6 ± 15.5	111.6 ± 12.7	57.9 ± 22.8	62.1 ± 34.0	*Hyp*
End Tidal CO_2 partial pressure (mm Hg)	35.6 ± 2.2	34.6 ± 2.6	33.9 ± 2.9	32.9 ± 3.9	*Cold, Hyp*

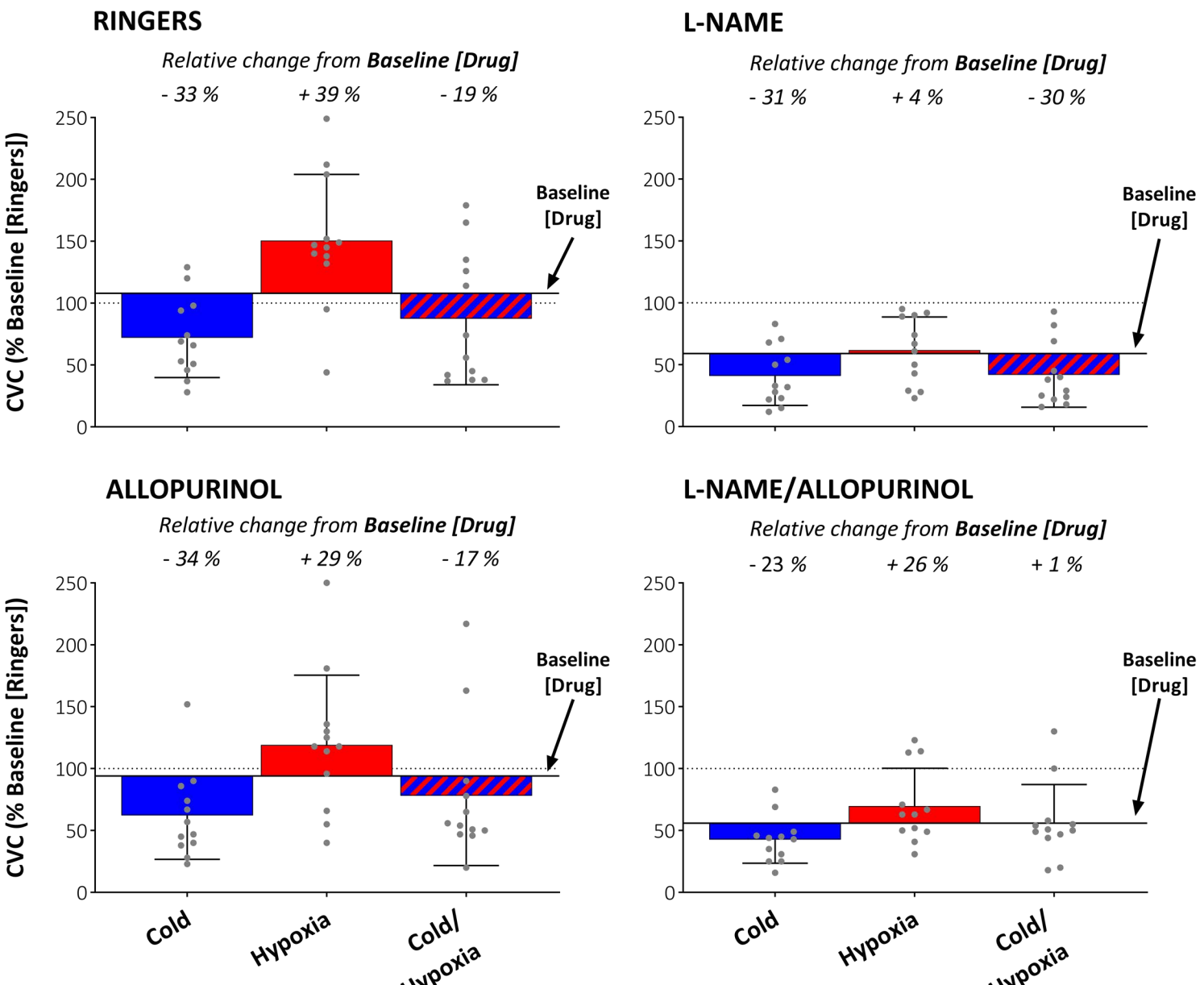
836

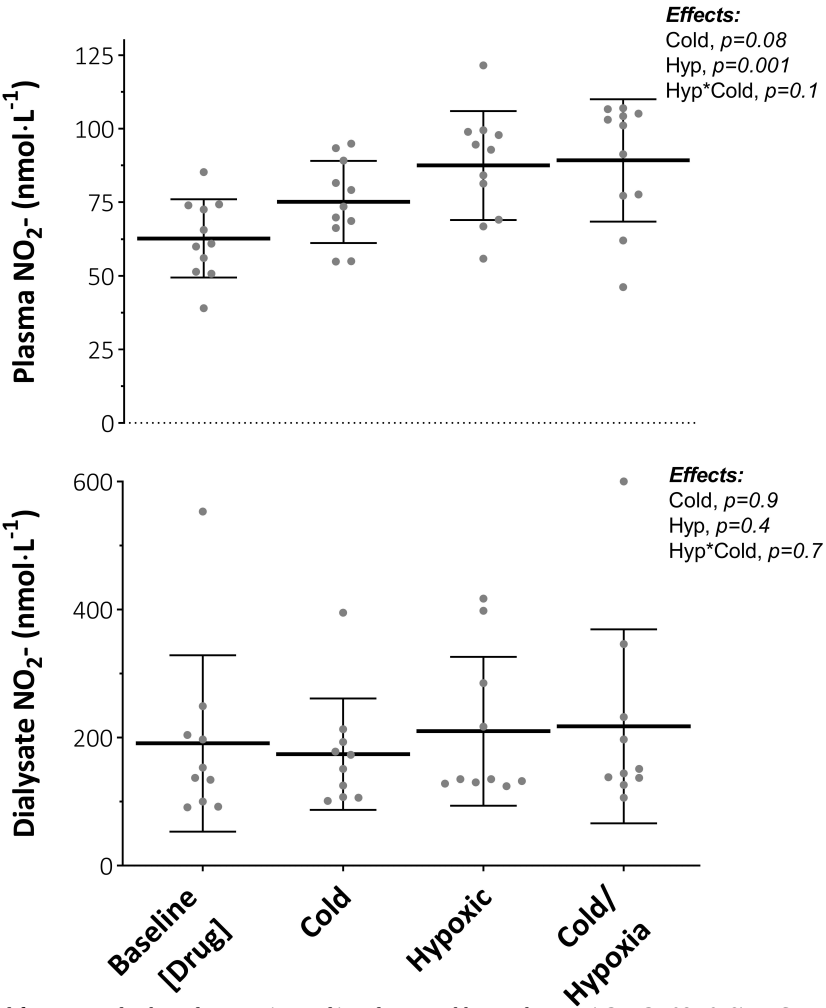




MAIN EFFECTS	
	L-NAME p < 0.001
	Allopurinol p = 0.5
	Cold p = 0.001
	Hypoxia p = 0.01

INTERACTIONS		
L-NAME*Allopurinol p = 0.1	Allopurinol*Hypoxia p = 0.6	L-NAME*Allopurinol*Cold*Hypoxia p = 0.3
Cold*Hypoxia p = 0.1	L-NAME*Allopurinol*Cold p = 0.6	
L-NAME*Cold p = 0.01	L-NAME*Allopurinol*Hypoxia p = 0.07	*Assessed via 4-Way ANOVA (α = 0.05)*
L-NAME*Hypoxia p = 0.04	L-NAME*Cold*Hypoxia p = 0.01	
Allopurinol*Cold p = 0.1	Allopurinol*Cold*Hypoxia p = 0.3	





	INDEPENTDENT FACTORS			
	Allopurinol	**L-NAME**	**Hypoxia**	**Cold**
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Ringers Fiber – Cold				2
Ringers Fiber – Hypoxia			2	1
Ringers Fiber – Cold/Hypoxia				2
L-NAME Fiber – Baseline [Drug]	1	2	1	1
L-NAME Fiber – Cold				2
L-NAME Fiber – Hypoxia			2	1
L-NAME Fiber – Cold/Hypoxia				2
Allopurinol Fiber – Baseline [Drug]	2	1	1	1
Allopurinol Fiber – Cold				2
Allopurinol Fiber – Hypoxia			2	1
Allopurinol Fiber – Cold/Hypoxia				2
L-NAME/ Allopurinol Fiber – Baseline [Drug]	2	2	1	1
L-NAME/Allopurinol Fiber – Cold				2
L-NAME/Allopurinol Fiber – Hypoxia			2	1
L-NAME/ Allopurinol Fiber – Cold/Hypoxia				2

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