The combination of theophylline and endothelin receptor antagonism improves exercise performance of rats under simulated high altitude

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The combination of theophylline and endothelin receptor antagonism improves exercise performance of rats under simulated high altitude. J Appl Physiol 113: 1243–1252, 2012. First published August 16, 2012; doi:10.1152/japplphysiol.01622.2011.—Decreased physical performance at high altitude is a well-known consequence of rapid ascent to high altitude. Hypoxic pulmonary vasoconstriction (HPV) potentially limits cardiac output and systemic blood flow, thus preventing successful adaptation to rapid ascent. We hypothesized that pharmacological enhancement of the heart rate with theophylline, combined with reversal of HPV via endothelin blockade, could increase exercise performance at high altitude. Female Sprague-Dawley rats were treated with combinations of 1) theophylline, 2) the endothelin receptor antagonists sitaxsentan/ambrisentan, and/or 3) phosphodiesterase-5 inhibitor sildenafil and exposed to either a simulated high altitude (4,267 m) or 12% oxygen. Exercise capacity, peripheral blood flow, hemodynamics, and pulmonary leak were examined. Combination treatment with theophylline and endothelin blockade, but not with the respective single compounds, significantly prolonged run-to-fatigue time under simulated high altitude. No such efficacy was found when theophylline was combined with sildenafil. Neither theophylline nor sitaxsentan or their combination influenced breathing rates and hemoglobin oxygen saturation. Whereas under hypoxia, theophylline significantly increased muscular blood flow, and sitaxsentan increased tissue oxygenation, the combination improved both parameters but in a reduced manner.

Under hypoxia, the combination treatment but not the single compounds significantly enhanced pulmonary arterial pressure compared with controls (13.1 ± 6.3 vs. 11.9 ± 5.2 mmHg), whereas mean arterial pressure remained unaffected. Pulmonary wet-to-dry weight ratios were unaffected by combination treatment. We conclude that concomitant dosing with a cardiac stimulant and endothelin antagonist can partially reverse loss of physical performance capacity under hypobaric hypoxia, independent from improving blood oxygen saturation.

blood pressure homeostasis; hypoxia; pulmonary vasoconstriction

ACUTE EXPOSURE TO ALTITUDES above 1,500 m decreases human physical performance capacity, and the primary factor contributing to this decrement is reduced oxygen availability due to decreased atmospheric pressure (22). Further complications arise from hypoxic pulmonary vasoconstriction (HPV) and peripheral hypoxic vasoconstriction—the most plausible cause of orthostatic challenge at high altitudes (22, 36, 39, 48). Medications that have shown promise for treating high-altitude-induced performance loss include xanthene derivatives (23), corticoids (21), carbonic anhydrase inhibitors (32), beta adrenergic receptor blockers (49), and perhaps receiving the most attention, pulmonary vasodilators (29, 31, 39, 40).

The circulatory system responds paradoxically to acute high-altitude exposure. In the lung, alveolar hypoxia leads to pulmonary vasoconstriction, whereas systemic hypoxia induces vasodilatation in most other vascular beds in the body (1), making the ascent to high altitude a unique challenge to blood pressure homeostasis (9, 48). Whereas cardiac activity increases in response to exposure to high altitude, probably in response to systemic hypotension, there is evidence that the increased pulmonary vascular resistance resulting from HPV has the potential to limit the amount of blood that flows through the lung and can thus be transported to the periphery (39). On the other hand, endothelin receptor blockers have shown promise to block HPV (19, 39). We hypothesized that the concomitant application of a cardiostimulant drug, such as theophylline, and an endothelin receptor antagonist (ERA) would improve the mammalian physiological response to acute hypobaric hypoxia more effectively than either drug class alone, independent from blood oxygen content. Following our hypothesis, pharmacological support of the favorable adaptive responses to hypoxia—including cardiac stimulation—while simultaneously counteracting HPV, would lead to enhanced blood flow and enhanced oxygen delivery to hypoxic tissue, resulting in improved physical performance under diminished oxygen levels. To test this hypothesis, we treated rats with the cardiac stimulant theophylline, the endothelin-targeting pulmonary vasodilators sitaxsentan or ambrisentan, or a combination of theophylline with either sitaxsentan or ambrisentan; exposed them to acute hypobaric and normobaric hypoxia; and subsequently determined the running time to fatigue, voluntary running volume, hemodynamic and tissue oxygenation parameters, and pulmonary vascular leak. To test whether phosphodiesterase-5 (PDE5) inhibition can achieve the same effect as endothelin blockade, we also tested whether sildenafil, if combined with theophylline, would enhance hypobaric exercise performance of rats.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with Duke University and Colorado State University Institutional Animal Care and Use Committee-approved protocols. Sprague-Dawley rats, aged at 9–12 wk (200–250 g), were used for all experiments. Female rats were used for run-to-fatigue experiments, anesthetized studies, and pulmonary leak experiments. Male rats were used for voluntary running experiments.
Drugs and Dosing Regimen

Rats were divided randomly into six groups with all dosing done intraperitoneally (ip): 1) vehicle control; 2) theophylline (15–30 mg/kg); 3) ERAs (sitaxsentan at 1 mg/kg or ambrisentan at 0.1 mg/kg); 4) the combination of theophylline and ERA; 5) sildenafil (4.5 mg/kg); and 6) the combination of theophylline and sildenafil. Theophylline was delivered in alkaline saline adjusted to pH 10, whereas sitaxsentan and ambrisentan were injected in a 0.9% NaCl solution (saline) ip. Sildenafil was dissolved in DMSO and coinjected with theophylline or saline (vehicle control).

Drug doses for theophylline, sitaxsentan, and ambrisentan were chosen to be within the range of equivalent doses that have been used previously in humans (14, 24, 26) as follows: 1) sitaxsentan at 1 mg/kg in rats, ~11.3 mg/70 kg in humans; 2) high-dose theophylline at 30 mg/kg in rats, ~338.7 mg/70 kg in humans; low-dose theophylline at 15 mg/kg in rats, ~169.4 mg/70 kg in humans; and 3) ambrisentan at 0.1 mg/kg in rats, ~1.1 mg/70 kg in humans (48a). Sildenafil was dosed within the range of what has been used in rats and humans in the past (4.5 mg/kg in rats, ~51 g/70 kg in humans; recommended dose, 25–100 mg/day) (2, 33, 43). Theophylline and sildenafil were obtained from Sigma (St. Louis, MO). Sitaxsentan and ambrisentan were produced by the Duke University Small Molecule Synthesis Facility (Durham, NC).

Exercise Models

Run-to-fatigue measurements. Female Sprague-Dawley rats were used for the run-to-fatigue experiments because of the high risk of injury associated with male reproductive anatomy on a motorized wheel. To determine a maximal physical performance in rats exposed to simulated altitude, a run-to-fatigue protocol was developed. Rats were habituated to the motorized wheels (Lafayette Instrument, Lafayette, IN) for 10 min/day, starting at 3 m/min for 3 days, followed by 6 m/min for 7 days. This protocol was specifically established to provide the rats with sufficient time to become accustomed to running in the motorized wheels yet to avoid physical training effects. Rats were subjected to an altitude equivalent of 4,267 m (14,000 ft; barometric pressure, ~440 mmHg) in the Duke University hypobaric chamber, creating an equivalent of ~12% oxygen under hypobaric hypoxia. Running time to fatigue was determined by the following protocol: 10 min at 6 m/min, 80 min at 9 m/min, and 30 min at 12 m/min. The experiment was terminated after completion of this sequence to avoid injury associated with forced running over a long time. Preliminary experiments showed that this protocol was sufficient to induce fatigue under simulated high altitude in most of the untreated rats. Fatigue was determined by hind-limb sliding, skidding, and sideways movement or flipping in the wheel and verified by observation for locomotion after being placed on a level surface for 30 s. Fatigued animals showed lack of motivation for voluntary locomotion, often recovering lying on their bellies or on their sides. Any animal that showed signs of injury during the experiment was removed from the wheels immediately. The study protocol is outlined in Fig. 1A.

Two trials were carried out to test the efficacy of theophylline combined with ERAs in improving the exercise capacity of rats running at simulated high altitude. In the first trial, theophylline was combined with sitaxsentan and in the second trial, combined with ambrisentan. In a third trial, theophylline was combined with sildenafil.

Voluntary Physical Activity Measurements. To determine voluntary physical activity, male Sprague-Dawley rats were given free access to voluntary physical activity wheels (Mini Mitter/Philips Respironics, Bend, OR) for a minimum of 7 days, and activity was recorded with VitalView data acquisition software (Mini Mitter/Philips Respironics). Following acclimation to the activity wheels, rats were matched for baseline (normoxic) voluntary running volume, such that each pharmacological treatment group (or vehicle control group) had the same average normoxic running volume. Rats were then subjected to an altitude equivalent of 4,267 m for 48 h in the Colorado State University hypobaric chamber. Study drugs were injected immediately before ascent to simulated altitude and once again 24 h later while maintaining the 4,267 altitude equivalent. Voluntary physical activity (running distance) was measured continuously during the 48-h hypobaric hypoxic exposure. Two studies were carried out to test the effects of combined dosing with theophylline/sitaxsentan and theophylline/ambrisentan on the voluntary physical activity in rats exposed to simulated high altitude. The outline of the study schedule is given in Fig. 1B.

Hemodynamic Measurements and Breathing Rate

Rats were anesthetized with ketamine/xylazine (80 mg/kg; 8 mg/kg), and body temperature was maintained with a water-circulating heating pad. Animals were placed on their backs, and indwelling catheters were placed in the carotid polyethylene catheter (PE-50) artery for blood pressure measurements as described previously (30). Blood pressures were recorded with an APT300 arterial pressure transducer (Hugo Sachs Elektronik, Germany) interfaced with LabChart software through an ADI bridge amplifier and PowerLab module (ADI Instruments, Australia). A pulse oximetry foot clip was applied to the left hind limb of the animal (MouseOx, Starr Life Sciences, Oakmont, PA) to record arterial hemoglobin oxygen saturation (HbO2) and heart rate. The experimental protocol consisted of 15 min at normoxia (21% oxygen, 79% nitrogen), followed by drug injection and after 15 more minutes, by inspired hypoxia (12% balance nitrogen) for at least 30 more minutes. Breathing rate was measured by counting chest movements over successive time periods: directly before drug injection (minutes 10–15), directly before onset of hypoxia (minutes 25–30), and under hypoxia (minutes 50–55).

Muscular Blood Flow and Tissue Oxygen Partial Pressure

To enable proper probe placement, the skin was removed from the hind limb to expose the surface of the vastus lateralis muscle. For tissue oxygen partial pressure (pO2) measurements, a needle-encased optical pO2 probe (Oxford Optronix, Oxford, UK) was placed in the hind-limb muscle, and data were acquired with LabChart interfaced with the PowerLab module. Acceptability of needle placement of the pO2 needle was tested by subjecting the rat to an initial hypoxic episode of 15 min of breathing a 12% oxygen gas mixture (hypoxia), followed by 10 min of breathing room air before dosing with drugs or controls (Fig. 1D). The measurement was accepted when hypoxia was inducing a change in tissue pO2. A laser Doppler analyzer probe (OxyFlow, Oxford Optronix) was placed perpendicularly to the surface directly onto the muscle to record peripheral blood flow. Flow values are arbitrary [blood perfusion units (BPU)] and are normalized to the baseline at time of injection.

Pulmonary Vascular Leak Measurements

After injection of study drugs, animals were exposed to a simulated altitude of 4,267 m and were either subjected to the run-to-fatigue protocol or remained unchallenged on nonmoving wheels for 120 min. Immediately after return to normobaria, animals were killed, and lungs were removed and weighed on an analytical scale. Lungs were subsequently transferred to an oven and dried at 37°C until a stable weight was achieved. As a separate sea-level control group, wet-to-dry weights were obtained from one set of unexercised animals, which remained under normobaric conditions (n = 10, all groups).

Data Analysis and Statistics

Run-to-fatigue data were analyzed using Kaplan-Meier plotting and log rank testing of differences among groups. Except for breathing rates, differences in hemodynamic/tissue oxygenation parameters among treatment groups were analyzed from averaged individual...
measurements during 5 min before injection, during 5 min before onset of hypoxia, and between 30 and 40 min postinjection (under hypoxia) using the $t$-test. Changes in hemodynamic parameters compared with the individual baseline were measured using the paired $t$-test throughout. Differences among treatment groups in wet-to-dry ratios (WDR) of lungs were analyzed with the use of the $t$-test. Data are expressed as mean $\pm$ SE unless otherwise indicated. All analyses were done using GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical tests were corrected for multiple comparisons using the false discovery rate method (8). This approach controls the false discovery rate, as opposed to the family error rate, and was applied to all $P$ values within a given endpoint (e.g., all intra- and extracellular parameters).

Fig. 1. Experimental design of (A) run-to-fatigue tests, wet-to-dry weight measurements on rat lungs; (B) voluntary physical activity, (C) hemodynamic studies; and (D) blood flow and tissue oxygen partial pressure ($pO_2$).
intergroup tests for changes in heart rate). The false discovery rate threshold was set at 0.05. For all tests, the raw P values are reported but are described as significant only if they meet this false discovery threshold.

RESULTS

Run to Fatigue

In the theophylline/sitaxsentan trial, the median run-to-fatigue time in the control group was 97 min. No median run-to-fatigue time could be identified in the other treatment groups, because more than one-half of the animals were still running when the experiments were terminated (Fig. 2A). The group treated with the combination of theophylline and sitaxsentan ran significantly longer than the control (log rank test, \( P < 0.005 \)), whereas the groups treated with theophylline or sitaxsentan individually did not (log rank, \( P = 0.0829 \) and 0.1864, respectively). In the theophylline/ambrisentan trial, the median run-to-fatigue time in the control group was 52.5 min, theophylline alone was 113 min [comparison with control (log rank, \( P = 0.083 \)], and ambrisentan alone was 93 min. In the combination group, more than one-half of the animals were still running when the experiment was terminated (Fig. 2B). The only treatment that significantly increased running time was the combination of theophylline and ambrisentan (log rank, \( P < 0.005 \)), whereas treatment with the single drugs did not increase the run-to-fatigue time (log rank theophylline, \( P = 0.0527 \); ambrisentan, \( P = 0.3997 \); Fig. 2, A and B). In the theophylline/sildenafil trial, mean run time in controls was 40 min, theophylline was 115 min, and sildenafil was 79 min, and the mean run-to-fatigue time after combination treatment remained undefined because more than one-half of the animals were still running at the end of the experiment. None of the treatment groups ran longer than controls.

Voluntary physical activity. Acute exposure to simulated altitude decreased voluntary running volume by \( \sim 60-90\% \).
Peripheral Blood Flow, Blood Gases, and Hemodynamics

Peripheral blood flow. Theophylline significantly increased peripheral blood flow during hypoxia compared with control (t-test, \( P = 0.0005 \)). The observed average increase after combination treatment was not significant. Sitaxsentan alone had no effect on blood flow in the hypoxic muscle (Fig. 3A).

Hemoglobin saturation. Average hemoglobin saturation values at the time of injection in all groups were \( 90.0 \pm 18.0 \% \). Hypoxia alone induced the expected significant decrease in HbO2 in all treatment groups to an average of \( 70.4 \pm 3.4 \% \) (Fig. 3B; paired t-test comparing preinjection and hypoxia: \( P < 0.005 \) in all groups). Injection of pH-adjusted saline increased HbO2 in control groups from \( 93.1 \pm 3.7 \%) to 95.6 \pm 2.5 \% \) (paired t-test, \( P = 0.0009 \)). Neither of the single drugs nor their combination altered HbO2 under hypoxia compared with controls.

Tissue \( pO_2 \). Hypoxia decreased hind-muscle \( pO_2 \) in the control group from an average of \( 25.9 \pm 11.1 \) mmHg to \( 15.4 \pm 8.8 \) mmHg. \( pO_2 \) did not decrease significantly in any of the other treatment groups. The observed increase of muscular \( pO_2 \) following combination treatment compared with controls was not significant. Only sitaxsentan treatment led to a tissue \( pO_2 \) that was higher than control treatment (t-test, \( P = 0.0063 \); Fig. 3C). Control treatment with injection of alkaline saline induced an increase in hemoglobin saturation by \( 93.1 \pm 3.7 \) % to \( 95.6 \pm 2.5 \% \) HbO2 (paired t-test, \( P = 0.0009 \)).

Breathing rate. Average respiration rate was \( 41.7 \pm 11.6 \) breaths/min before injection. Breathing rate increased in all treatment groups after onset of hypoxia to \( 56.4 \pm 16.6 \) (paired t-test, \( P < 0.05 \) in all groups). No differences were found among any of the treatment groups for any of the time points (Fig. 3D).

Heart rate. Average preinjection heart rates in all groups were \( 242.2 \pm 27.7 \) beats/min. Theophylline injection increased heart rate significantly from \( 241.8 \pm 25.7 \) to \( 271.0 \pm 33.0 \) beats/min (paired t-test, \( P < 0.01 \)). Under hypoxia, the heart rate decreased in control-treated animals from \( 246.4 \pm 26.1 \) to \( 227.6 \pm 32.0 \) beats/min (paired t-test, \( P < 0.001 \)) and in the sitaxsentan-treated animals from \( 245.6 \pm 30.5 \) to \( 231.7 \pm 33.6 \) beats/min (paired t-test, \( P < 0.001 \)) but remained unaffected in the theophylline- and combination-treated rats (Fig. 4A).

Blood pressures. Average mean arterial blood pressure (MAP) in all groups was \( 79.7 \pm 11.4 \) mmHg preinjection. No changes of MAP were seen following any of the treatments (Fig. 4B). Average pulmonary arterial pressure (PAP) preinjection was \( 13.5 \pm 5.3 \) mmHg, which was slightly increased to \( 14.2 \pm 7.7 \) in all groups after onset of hypoxia; however, this was not significant. No changes were observed in response to any of the treatments, except in the combination group, where hypoxia increased PAP from \( 11.8 \pm 5.4 \) mmHg postinjection to \( 13.1 \pm 6.3 \) mmHg (paired t-test, \( P = 0.0023 \)).

Pulmonary Wet-to-Dry Weight

WDR were \( 4.86 \pm 0.09 \) in normoxic resting rats and \( 4.90 \pm 0.09 \) in hypoxic resting rats. Adding exercise reduced WDR under hypobaric hypoxia (\( 4.80 \pm 0.09 \); t-test, \( P < 0.05 \)). Treatment of exercising rats with theophylline did not change WDR compared with controls (\( 4.77 \pm 0.10 \)). Treatment of exercising rats with sitaxsentan increased WDR compared with exercising controls (\( 4.92 \pm 0.05 \); t-test, \( P < 0.005 \)). The treatment of exercising rats with the drug combination, however, reduced WDR back to control levels (\( 4.77 \pm 0.09 \); comparison with sitaxsentan only; t-test, \( P = 0.0002 \); Fig. 4D).

DISCUSSION

In rats exposed to a simulated, moderately high altitude of 4,267 m or 12% inspired oxygen and treated with theophylline, sitaxsentan, ambrisentan, or the respective combination, the combinational treatments significantly improved maximal exercise performance, whereas the single treatments did not. Sildenafil, when combined with theophylline, did not produce any such synergism. Whereas none of the treatments impacted

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**Fig. 3.** The effects theophylline and sitaxsentan treatment on blood flow, oxygen transport, and tissue oxygenation in anesthetized, hypoxic rats. Asterisks indicate a significantly different physiological response compared with another treatment group or with the respective baseline (*\( P < 0.05 \); **\( P < 0.01 \)). A: increased blood flow during hypoxia compared with control treatment was observed in theophylline-treated animals (t-test, \( P = 0.0005 \)); \( n = 10 \) (control); \( n = 8 \) (theophylline); \( n = 5 \) (sitaxsentan); and \( n = 6 \) (combination). BPU, blood perfusion units. B: hypoxia induced a significant decrease of HbO2 in all groups compared with baseline (paired t-test, \( P < 0.005 \) in all groups). None of the treatments significantly affected hemoglobin saturation under hypoxia compared with controls: \( n = 10 \) (control); \( n = 8 \) (theophylline); and \( n = 9 \) (sitaxsentan and combination). C: inspired hypoxia decreased tissue \( pO_2 \) in the hind-limb muscle of control animals by \( 10 \) mmHg compared with baseline (paired t-test, \( P = 0.0003 \)). Treatment with sitaxsentan reversed the hypoxia-induced change in tissue \( pO_2 \); reflected by a significant difference to control animals under hypoxia (t-test, \( P = 0.0063 \); control: \( n = 9 \); all other groups: \( n = 4 \)); \( n = 9 \) (combination); and \( n = 10 \) (sitaxsentan).
HbO2, breathing rates, or mean MAP, theophylline alone significantly enhanced muscular blood flow under hypoxia, whereas sitaxsentan significantly improved muscular tissue pO2. The combination treatment produced an intermediate effect in both muscular blood flow and tissue oxygenation. Whereas the combination of theophylline and sitaxsentan increased PAP significantly, pulmonary fluid content was unaffected in exercising hypoxic rats after combination treatment. Our results support the initial hypothesis that concomitant dosing with a cardiac stimulant and endothelin blockade can partially reverse loss of physical performance capacity under hypobaric hypoxia.

Theoretically, two main physiological strategies exist to improve oxygen transport from the lung to peripheral organs: improving blood oxygen loading or increasing blood flow (36). The increased breathing rate in response to hypoxia (also observed in this study) and the increased hematocrit in response to long-term hypoxic exposure are examples of the first strategy (35). Regarding the second mechanism of increased blood flow, it is known that (hypobaric) hypoxia itself increases the heart rate (36); however, in light of the potentially limiting effect of HPV on blood flow through the lung, it can be debated whether pharmaceutical targeting of HPV can improve exercise capacity at altitude (17, 37–39). As part of the criticism, it has been reasoned that a primary effect of theophylline, enhancing human performance at both sea level (28) and under hypobaric hypoxia (23). Treatment with theophylline alone has increased heart rates in anesthetized rats in our trials, and whereas hypoxia reduced heart rate in anesthetized control rats, increased heart rates in anesthetized rats in our trials, and consequently, both adenosine receptor antagonism and PDE3 inhibition were present in our experiments. Caffeine, structurally similar to theophylline, has shown efficacy in enhancing human performance at both sea level (28) and under hypobaric hypoxia (23). Treatment with theophylline alone has increased heart rates in anesthetized rats in our trials, and whereas hypoxia reduced heart rate in anesthetized control rats, theophylline- and combination-treated rats, heart rates in the theophylline group remained elevated under hypoxia (Fig. 4A). Although muscular blood flow was enhanced significantly when animals were treated with theophylline alone, this increase did not lead to a significant improvement of tissue oxygenation (Fig. 3, A and C). One possible explanation for our observation is that theophylline monotherapy may result in increased blood flow through the muscle compared with controls, but parts of this flow bypasses the capillary beds by being shunted to the postcapillary, venous compartment.
ERAs

Sitaxsentan and ambrisentan are both subtype A-specific antagonists of circulating endothelin that were originally designed to treat pulmonary arterial hypertension in human patients (11). Sitaxsentan has been shown to reduce HPV and to improve exercise capacity in humans at high altitude (39). Sitaxsentan has never been approved by the U.S. Food and Drug Administration (FDA) in the United States following concerns of liver toxicity and has recently been taken off the market in Europe (42). Ambrisentan has a more benign safety profile than sitaxsentan and is currently approved by the FDA for human use (24, 34, 42). The observation that endothelin blockade via sitaxsentan alone was sufficient to reverse tissue hypoxia (Fig. 3C), yet did not augment tissue blood flow, might be explained by its direct dilating effect on peripheral arterioles and by the reduced demand for cardiac output in the anesthetized animal.

Run to Fatigue and Voluntary Running

Our most important observation was that the combination of theophylline and endothelin blockade consistently enhanced run-to-fatigue time at altitude compared with the single compounds. These results, together with the absence of alterations to respiratory rates or blood oxygenation levels in any of the groups, support our initial hypothesis that it is possible to counteract tissue hypoxia and performance loss under hypobaric hypoxia by pharmacologically improving blood flow rate, accomplished by countering endothelin-mediated vasoconstriction and accelerating heart rate (37). The absence of a significant increase in PAP in control animals after onset of hypoxia (Fig. 4C) and the absence of increased pulmonary fluid content after onset of exercise and theophylline treatment (Fig. 4D) illustrate that HPV was only weakly expressed in our model and might not be limiting to blood flow through the lung under these conditions. It is therefore possible that the observed beneficial effect of ERAs is due to extrapulmonary mechanisms. It is known that the vasoconstrictive activity of endothelin-1 affects not only the pulmonary but also all other peripheral vasculature (12). Importantly, endothelin blockade was only effective in our exercise models when combined with theophylline. The observation that combining sildenafil (as opposed to ambrisentan or sitaxsentan) with theophylline did not lead to any additional benefit over the single compounds argues for the importance of targeting endothelin to achieve the observed synergism. The voluntary running data suggest that both theophylline alone and combined with endothelin receptor blockade may have the potential to increase adaptation to high altitude over a period of ~1 day; however, further experimentation needs to be conducted to definitively conclude this.

Pulmonary WDR

Heavy exercise in conjunction with acute exposure to hypoxia at altitude has the potential to increase pulmonary leak, as both exercise and hypoxia increase heart rate, leading to increased hydrostatic pressure on the pulmonary vasculature (16). The fact that exercise reduced, rather than increased, vascular fluid content is therefore surprising. However, the redistribution of blood flow in response to high altitude and exercise in rats is different from the human situation, at least in part, due to differences in size and posture. We also initially expected that treatment with theophylline of rats undergoing the run-to-fatigue protocol in hypobaric hypoxia would further augment pulmonary extracellular fluid content reflected by an increase of lung weight WDR. The observation that theophylline alone did not increase vascular leak might be explained by the known vasodilatory effects of theophylline and its reported ability to reduce HPV (5). Sitaxsentan, however, had the opposite effect, increasing the fraction of blood present in the pulmonary vasculature, which is reflected by the increased lung weight WDR after sitaxsentan treatment (Fig. 4D). The overall ratios obtained in our study are similar to those found in other rodent studies (41). Whereas the highest increase of ratios in our study were from 4.8 in controls to 5.0 in the sitaxsentan-treated group, representing an increase of 4%, other studies in rats, including pulmonary edema and reperfusion injury models, demonstrate increases of >66% compared with controls (41). Even the significant increase in WDR after sitaxsentan treatment, therefore, is unlikely to represent a pathophysiological state. Specifically, the observation that PAP increased after combination treatment with theophylline and that sitaxsentan is not reflected by increases in WDR indicates that neither of the treatments sufficiently increases the pressure burden on the pulmonary vasculature enough to cause significant extravasation of fluid. The absence of any aggravating effect of simulated altitude, with and without exercise, on the pulmonary fluid content of the rats and the lack of a reducing effect of sitaxsentan on WDR support the conclusion that at least parts of the beneficial effect of endothelin blockade might be extrapulmonary in nature.

Heart rates measured in anesthetized rats were ~30% lower than values reported in the literature (50). A further significant reduction of heart rates occurred after onset of hypoxia in control- and sitaxsentan-treated animals. This discrepancy is probably due to the effect of ketamine anesthesia. Whereas conscious mammals, including humans and rats, react to inspired hypoxia with an increase in heart rate, hypoxia—in conjunction with ketamine anesthesia—generally appears to reverse this reaction (13, 36, 47).

Blood Pressures

The overall baseline values found for MAP are in accordance with what has been reported elsewhere (13). It has been shown that inspired hypoxia leads to a drop in MAP, which was present but nonsignificant in our study. Theophylline-induced hypotension was nonsignificant in our study (45). Overall, PAP values are, however, lower than those reported before in male-anesthetized and awake Sprague-Dawley rats of similar age and weight (~19 mmHg). Furthermore, the increase in PAP after onset of hypoxia was smaller than in other studies in rats, including pulmonary edema and reperfusion injury models, demonstrate increases of >66% compared with controls (41). The absence of any aggravating effect of simulated altitude, with and without exercise, on the pulmonary fluid content of the rats and the lack of a reducing effect of sitaxsentan on WDR support the conclusion that at least parts of the beneficial effect of endothelin blockade might be extrapulmonary in nature.
gender of the animals, as it has been reported that female mammals show weaker vasoconstrictive and ventilatory responses to hypoxia (25, 46, 51). It is plausible that all changes to PAP, which were observed in this study, are due to increased cardiac output following the treatment.

HbO2 and Breathing Rates

As expected, reduced FiO2 induced a decrease of HbO2 and an increase in breathing rates. In support of our initial hypothesis, no increases in hemoglobin saturation or breathing rates were found after any of the treatments, indicating that the performance-enhancing effect of the combination treatment was not due to increased oxygen content of the blood. The small increase of HbO2 observed after ip injection of control treatment (alkaline saline) might be caused by the Bohr effect, which would favor release of oxygen from hemoglobin under acidic conditions and retention during alkalosis.

Rationale for Modified Experimental Protocol for Tissue pO2 and Blood Flow Measurements

The protocol that was used to measure tissue oxygenation and muscular blood flow, which has been done on the same set of animals, was slightly different from the one used for the other hemodynamic data, as outlined in Fig. 1. To account for the known sensitivity of the pO2 needle-probe measurements to differences in sensor placement, a pretreatment hypoxic episode was introduced into this cohort, which served to verify that the needle was sensitive to tissue pO2 changes in response to inspired hypoxia. As a result of this calibration, three out of 24 measurements were removed from analysis, where due to poor needle placement, the initial hypoxic episode did not cause any change in tissue pO2. A comparison of the relative margin of changes in tissue pO2 during the post-treatment hypoxic cycle with those changes during initial hypoxia in the same animals confirmed the findings with absolute pO2 values in each treatment group, supporting the validity of the measurements (data not shown).

Tissue pO2

Interestingly, the onset of inspired hypoxia caused a significant drop in tissue pO2 in control animals, whereas each of the treatments appeared to prevent such a drop. Only treatment with sitaxsentan led to a significantly increased pO2 under hypoxia compared with control treatment. It is plausible that both increased blood flow, as observed with theophylline treatment, and vasodilatation of pulmonary and peripheral arterioles via endothelin blockade can independently contribute to improved oxygen delivery to the capillary bed. The particular efficacy of sitaxsentan could be due to the special situation of the anesthetized animal, where due to reduced requirements toward homeostasis of blood pressure and blood flow in the resting state, pulmonary and other peripheral arteriolar vasodilatation could be sufficient to allow improved oxygen transport to tissues.

Muscular Blood Flow

It is important for understanding the muscular blood flow data to recognize that laser doppler values (arbitrary BPU) have no dimension and should always be read in relation to a baseline value; therefore, all values have been normalized to the measurement at the time of injection. Because theophylline itself appears to be able to counteract pulmonary vasoconstriction in rats (5), it is possible that a blood flow-enhancing effect through the lung could be caused by this drug alone. Interestingly, the addition of sitaxsentan appeared to partially blunt the flow-enhancing effect of theophylline alone in the muscle. This observation can be explained by vasodilatory effects of endothelin receptor blockade in peripheral tissues. Endothelin blockade may cause vasodilatation of arterioles, thus reducing shunting and diverting more blood to the capillary beds. This would lead to improved delivery of oxygen and nutrients to the parenchymal tissues but at the same time, reduce overall blood flow velocity through the respective organ. It is known that the vasoconstrictive activity of endothelin-1 affects not only the pulmonary but also other peripheral vasculature (12); however, little is known about the influence of sitaxsentan or ambrisentan on muscular vascular resistance and oxygen transport.

Potential Synergism Between Theophylline and Endothelin Receptor Blockers

The combination of theophylline and sitaxsentan did improve tissue oxygenation and tissue blood flow to a lesser extent than the respective single drugs. The synergism observed in our exercise trials could therefore have arisen from the ability of endothelin blockers to reduce shunting by dilating arterioles and to redirect theophylline-enhanced blood flow toward the tissue capillary systems, resulting in improved oxygen delivery to the peripheral capillary beds.

The combination of pharmaceutical-driven cardiac stimulation and vasodilatation is a novel, physiological concept in treating high-altitude-induced decreases in physical performance and potentially other altitude-related illnesses. Our data suggest that cardiac stimulants, such as xanthene derivatives, might be used for this purpose and may have greater therapeutic potential when delivered in combination with endothelin receptor blockers.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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