Increased hemoglobin oxygen affinity protects during acute hypoxia

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ABSTRACT

Acclimatization to hypoxia requires time to complete the adaptation mechanisms that influence oxygen (O$_2$) transport and O$_2$ utilization. Although decreasing hemoglobin (Hb) O$_2$ affinity would favor the release of O$_2$ to the tissues, increasing Hb-O$_2$ affinity would augment arterial O$_2$ saturation during hypoxia. This study was designed to test the hypothesis that pharmacologically increasing the Hb O$_2$ affinity will augment O$_2$ transport during severe hypoxia (10% and 5% inspired O$_2$) compared to normal Hb O$_2$ affinity. Erythrocyte Hb O$_2$ affinity was increased by infusion of 20 mg/kg of 5-hydroxymethyl-2-furfural (5HMF). Control animals received only the vehicle. The effects of increasing Hb O$_2$ affinity were studied in the hamster window chamber model, in terms of systemic and microvascular hemodynamics and partial pressures of O$_2$ (PO$_2$s). Pimonidazole binding to hypoxic areas of mice heart and brain was also studied. 5HMF decreased the P50 (PO$_2$ at which the Hb is 50% saturated with O$_2$) by 12.6 mmHg. During 10% and 5% O$_2$ hypoxia, 5HMF increased arterial SO$_2$ by 35 and 48% from the vehicle group, respectively. During 5% O$_2$ hypoxia, blood pressure and heart rate were 58% and 30% higher for 5HMF compared to the vehicle. In addition, 5HMF preserved microvascular blood flow, whereas blood flow decreased to 40% of baseline in the vehicle group. Consequently, perivascular pO$_2$ was three times higher in the 5HMF group compared to the control group at 5% O$_2$ hypoxia. 5HMF also reduced heart and brain hypoxic areas in mice. Therefore, increased Hb O$_2$ affinity resulted in hemodynamics and oxygenation benefits during severe hypoxia. This acute acclimatization process may have implications in survival during severe environmental hypoxia when logistic constraints prevent chronic acclimatization.

Keywords: Microcirculation, oxygen release, P50, Hb oxygen affinity, critical oxygen supply, high altitude.
INTRODUCTION

Red blood cells (RBCs) contain hemoglobin (Hb) which reversibly binds oxygen (O\textsubscript{2}). The delivery of O\textsubscript{2} to tissues is drastically affected by reductions in a fraction of inspired O\textsubscript{2} (hypoxia), which decreases arterial pO\textsubscript{2} and blood O\textsubscript{2} saturation (SO\textsubscript{2}) (20). Exposure and adaptation to hypoxia (and/or high altitude) decrease Hb O\textsubscript{2} affinity, introducing a right shift to the blood O\textsubscript{2} equilibrium curve (9). The decrease in Hb O\textsubscript{2} affinity during hypoxia has been explained to favor O\textsubscript{2} offload to tissues (9). When exposed to hypoxia, the magnitude of the decrease in Hb O\textsubscript{2} affinity depends on the acid-base status (Bohr Effect and Haldane Effect). Whereas during acclimation to hypoxia, the change in Hb O\textsubscript{2} affinity depends on the total concentration of organic phosphates in the erythrocyte, mainly 2,3-diphosphoglycerate (2,3 DPG) and ATP (10).

Comparison between individuals acutely and chronically exposed to hypoxia showed that when the partial pressure of O\textsubscript{2} in air breathed is reduced by 60% (equivalent to altitudes higher than 5000 m), the arterial SO\textsubscript{2} decreased by more than 30% during acute exposure, whereas in chronically exposed the arterial SO\textsubscript{2} decreased by only 20% (18). Therefore, accelerated adaptation to low inspired O\textsubscript{2} produces severe hypoxemia and eventual death, while the lack of adaptation induces acute mountain sickness (AMS), high altitude cerebral edema (HACE), high-altitude pulmonary edema (HAPE) or death within a few hours (22). Adaptation to hypoxia is the hallmark of survival at high altitude. Human and mammalian natives of high altitudes have adapted to the low inspired O\textsubscript{2} by increasing their Hb O\textsubscript{2} affinity compared to their relatives at sea level, thus enabling higher O\textsubscript{2} uptake, increasing arterial SO\textsubscript{2} and O\textsubscript{2} delivery at lower partial pressure of O\textsubscript{2} in air breathed (32).

The Hb O\textsubscript{2} affinity can be modified with Hb allosteric effectors. We have previously increased Hb O\textsubscript{2} affinity during anemic conditions using 5-hydroxymethyl-2-furfural (5HMF) and found that a moderate increase in O\textsubscript{2} affinity (a decrease in P50 of 6 mmHg) maintained hemodynamic conditions, O\textsubscript{2} delivery, and increased tissue PO\textsubscript{2} (6, 30). 5HMF is a low
molecular weight five carbon-ring aromatic aldehyde with limited toxicological response (21). SHMF is active with high bioavailability, it can be administered orally, intraperitoneally or intravenously, and it has high red cell membrane permeability (2). SHMF binds covalently with intracellular Hb to form a high-affinity Schiff-base Hb adduct in a symmetrical fashion with the N-terminal alpha Valine-1 of Hb, allosterically shifting the Hb O₂ equilibrium curve at relatively low SHMF concentrations (1).

This study investigates the protective effects of artificially increased Hb O₂ affinity during acute hypoxia. We propose that SHMF can be used for acute adaptation and acclimation of personnel exposed to high altitude, accelerating critical acclimation and preventing AMS, HACE, HAPE or hypoxemic death. Hamsters fitted with the window chambers were treated with SHMF (20 mg/kg), and the control group was given only the vehicle solution. We selected the hamster window chamber model because of its capacity to analyze the exchange of O₂ in the microcirculation, accounting for the contributions of RBC and the processes associated with interactions between Hb and O₂. SHMF treatment was administered one hour before the onset of hypoxia (normobaric 10% inspired O₂) followed by severe hypoxia (normobaric 5% inspired O₂). Our experimental model allows us to measure systemic and microvascular hemodynamics and microvascular pO₂ levels to calculate O₂ transport. Validation of the benefit in O₂ transport produced by SHMF during acute hypoxia in the hamster model was confirmed in mice by immunohistochemistry staining for hypoxic zones in brain and heart sections using intraperitoneal pimonidazole. Pimonidazole acts as a probe specific for hypoxic cells, because, at pO₂ lower than 10 mm Hg, it is reduced to a reactive intermediate that binds covalently to molecules containing a thiol group, including proteins, and can be detected by a specific monoclonal antibody. Based on our results, the beneficial effect of SHMF will be most noticeable upon exposure to severe hypoxia where O₂ uptake in the lungs is insufficient to maintain health. The use of SHMF may reduce the need for chronic adaptation and improve performance at limited inspired O₂, clearly, of practical importance for flight crews, military and emergency personal. Moreover, the effects of left shifted Hb O₂ affinity during acute hypoxia is
information useful in understanding the role of Hb O$_2$ affinity and hypoxic hypoxia in O$_2$ transport in the microcirculation.

MATERIALS AND METHODS

Hamster preparation. 55 - 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) were fitted with a dorsal window chamber. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee. The hamster window chamber model is widely used for microvascular studies in the unanaesthetized state, and the complete surgical technique is described in detail elsewhere (7, 8). Briefly, the animal was prepared for chamber implantation with a 50 mg/kg ip injection of pentobarbital sodium anesthesia. After hair removal, sutures were used to lift the dorsal skin away from the animal, and one frame of the chamber was positioned on the animal’s back. A chamber consisted of two identical titanium frames with a 15-mm circular window. With the aid of backlighting and a stereomicroscope, one side of the skin fold was removed following the outline of the window until only a thin layer of retractor muscle and the intact subcutaneous skin of the opposing side remained. The intact skin of the other side was exposed to the ambient environment. Animals were allowed 2 days for recovery. Finally, they were anesthetized again, to implant arterial and venous catheters (PE-50) in the carotid artery and jugular vein. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame.

Hamster inclusion criteria. Hamsters were suitable for the experiments if: 1) systemic parameters were within normal range, namely, heart rate (HR) > 340 beat/min, mean arterial blood pressure (MAP) > 80 mmHg, systemic Hct > 45%, and arterial O$_2$ partial pressure (p$_a$O$_2$) > 50 mmHg; and 2) microscopic examination of the tissue in the chamber observed under x650 magnification did not reveal signs of edema or bleeding. Hamsters are a fossorial animal with a low arterial pO$_2$ compared to other rodents; however, the intravascular O$_2$ tension in the
hamster window chamber model is similar to the window chamber model implanted on mice (5).

Test material. Chemicals, including 5HMF, were purchased from Sigma Aldrich Company (St. Louis, MO). Stock solution at 14 mg/mL was prepared, before the experiment, in a degassed (oxygen free) saline (0.9% NaCl) solution. Hypoxyprobe-1 Green Kit (pimonidazole and the corresponden monoclonal antibody conjugated to fluorescein) were purchased form Hypoxyprobe Inc, (Burlington, MA).

Systemic parameters. MAP and heart rate (HR) were recorded continuously (MP 150, Biopac System; Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden). Arterial blood was collected in heparinized glass capillaries (50 µl) and immediately analyzed for pO₂, pCO₂, base excess (BE) and pH (Rapidlab 248, Bayer, Norwood, MA). Arterial Hb saturations were measured on the IL482 CO-Oximeter System (Instrumentation Laboratory, Lexington, MA).

Cardiac Output. Cardiac output (CO) was measured by a modified thermodilution technique (4). Animals instrumented for CO measurements were surgically prepared, and recovered identically to animals studied for microvascular measurements. The complexity of the setup for the thermodilution prohibited positioning them on the microscope. Vascular resistance (VR) was calculated at relation between MAP and CO (VR =MAP/CO). Oxygen delivery (DO₂) was calculated as the product of the total Hb by the O₂ carrying capacity of saturated Hb (1.34 mlO₂/gHb) by the arterial blood O₂ saturation and CO (DO₂ = [RBC_Hb × 1.34 × S_A] × CO).

Blood oxygen equilibrium curve. Oxygen equilibrium curves for Hamster blood were obtained by deoxygenation of O₂-equilibrated samples in a Hemox buffer at 37°C, using a Hemox Analyzer (TCS Scientific Corporation, New Hope, PA). The Hemox buffer pH was adjusted to match the arterial blood pH of the animals using Tris and BisTris buffers. Tris and BisTris buffers...
were prepared by titrating the reagents with HCl before adjusting the pH of the solutions to keep Cl\(^{-}\) ions concentration equal to the buffer at the pH values.

**Microvascular experimental setup.** The animal in the restraining tube with the protruding window chamber was fixed to the microscopic stage for transillumination with the intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were given 20 min to adjust to the tube environment before any measurement. Detailed mappings were made of the chamber vasculature so that the same vessels studied at baseline could be followed throughout the experiment. Observation of the fields was done systematically by displacing the microscopic field of view by a field width in 10-12 successive steps in the lateral direction (relative to the observer). Each step was viewed on the video monitor and was 240 μm long when referred to the tissue. Blood vessels were chosen by a distinctive anatomic landmark to easily and quickly reestablish the same fields and vessels at each observation time point. Six to eight arterioles and venules were selected in each preparation. The tissue image was projected onto a charge-coupled device camera (COHU 4815) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a 40X (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective. The same sites of study were followed throughout the experiment so that comparisons could be made directly to baseline levels.

**Functional Capillary Density (FCD).** Functional capillaries are defined as those capillary with RBC transit of at least a single RBC within a period of 45s. Functional capillaries measurements include 10 successive microscopic fields with a total area of 0.46 mm\(^2\). FCD is defined as the total length of RBC perfused capillaries divided by the area of the microscopic fields (cm\(^{-1}\)).

**Microhemodynamics.** A video image-shearing method was used to measure vessel diameter (D) (13). Changes in arteriolar and venular diameter from baseline were used as indicators of a change in vascular tone. Arteriolar and venular centerline velocities were measured on-line by using the photodiode cross-correlation method (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according
to vessel size to obtain the mean RBC velocity (19). Blood flow (Q) was calculated from the measured values as $Q = \pi \times V (D/2)^2$. This calculation assumes a parabolic velocity profile and has been found to be applicable to tubes of 15 - 80 μm internal diameters and for Hcts in the range of 6 - 60% (19). The same sites of study were followed throughout the experiment so that comparisons could be made directly to baseline levels.

Microvascular PO$_2$ distribution. High resolution non-invasive microvascular PO$_2$ measurements were made using phosphorescence quenching microscopy (PQM) (14). PQM is based on the relationship between the decay rate of excited Palladium-mesotetra-(4-carboxyphenyl) porphyrin (Frontier Scientific Porphyrin Products, Logan, UT) bound to albumin and the O$_2$ concentration according to the Stern-Volmer equation (29). The method was used previously in microcirculatory studies to determine pO$_2$ levels in different tissue (14). pO$_2$ measurements by PQM were obtained following these steps for both groups: 1) the probe was injected (intravenous injection of 15 mg/kg at a concentration of 10 mg/ml of the phosphorescence complex 10 min before O$_2$ measurements); 2) the tissue was illuminated (pulsed of light at 420 nm wavelength) to excite the probe into its triplet state; 3) the emitted phosphorescence (680 nm wavelength) was collected and analyzed to yield the phosphorescence lifetime; and 4) the phosphorescence lifetime was converted into O$_2$ concentration, pO$_2$. The phosphorescence lifetimes are concentration independent, which permit extravascular fluid pO$_2$ measurements, although the dye albumin complex that extravasasates is very small. Perivascular PO$_2$ was measured in regions in between functional capillaries.

Microvascular oxygen saturations. Intravascular Hb O$_2$ saturations were calculated using the O$_2$ equilibrium curves measured and the intravascular pO$_2$s measured with the PQM. The O$_2$ equilibrium curves were measured at arterial blood pH as described above.

Oxygen delivery and extraction. The microvascular methodology used in our studies allows a detailed analysis of O$_2$ supply in the tissue. Calculations are made using equation 1 and 2:

$$O_2 \text{ delivery} = [(RBC_{Hb} \times \gamma \times S_A)] \times Q \quad (1)$$
Where, $RBC_{Hb}$ is the total Hb $[\text{g Hb/dl blood}]$, $\gamma$ is the O$_2$ carrying capacity of saturated Hb $[1.34 \text{ mlO}_2/\text{gHb}]$, $S_A$ is the arteriolar blood O$_2$ saturation, $A-V$ indicates the arteriolar/venular differences, and $Q$ is the average microvascular blood flow (arterioles and venules). Intravascular Hb O$_2$ saturations were calculated as described above.

**Experimental groups.** Animals were randomly divided into the following experimental groups before the experiment: 1) **5HMF**, treated with 20 mg/kg of 5HMF in sterile saline (0.9% NaCl) and administered in a single IV infusion of 100 uL; and 2) **Vehicle**, treated with a single IV infusion of sterile saline (0.9% NaCl) solution in a volume of 100 uL. The dose of 20 mg/kg of 5HMF was selected based on previously published studies and various pilot experiments, which appear to be sufficient to reduce P50 by 12 mmHg in hamsters and by 20 mmHg in mice. Additionally, higher doses of 5HMF have not extra effects in P50.

**Acute hypoxia protocol.** The awake animals were placed in a restraining tube with a longitudinal slit from which the protruding window chamber provided a microscopic stage for observation. A plastic tent with an inlet valve connected to the gas tanks was placed in front of the restraining tube. The gas flow rate (0.2 L/min) into the tent was diffused by a cotton filter barrier so that the hamster was not subjected to a direct stream of gas flow. The hamsters were given 20 min to adjust to the experimental environment before baseline (BL) measurements were completed. Immediately after baseline measurements, the hamsters received a bolus IV injection of 5HMF or vehicle. 60 min after treatment, measurements for normoxia (Nor) were completed. Palladium-mesotetra-(4-carboxyphenyl) porphyrin complex was administered 15 min before pO$_2$ measurements. The initial decrease to 10% O$_2$ hypoxia (Hyp10) was induced by normobaric hypoxia (10% O$_2$ balanced N$_2$). The hamsters were given 15 min to adjust to the change in the gas environment before measurements. Severe hypoxia to 5% O$_2$ (Hyp5) was induced by normobaric hypoxia (5% O$_2$ balanced N$_2$), and 15 min were given to adjust to the gas change before measurements. At each time point, systemic parameters, microvascular hemodynamics and PO$_2$ measurements were performed. Blood P50 was
measured at each time point. To prevent animal stress or discomfort, hypoxia was stopped if blood pressure dropped below 40 mmHg, and the animal was excluded from the study.

**Brain and heart hypoxic areas in mice.** 17 - 22 g C57BL/6 mice (Charles River Laboratories, Boston, MA) were used to study brain and heart hypoxic zones. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee. To validate the results obtained in hamsters with 5HMF during hypoxia, we have expanded our study to include immunohistochemistry staining for pimonidazole bound to hypoxic zones in the brain and heart tissues of mice treated with 5HMF during hypoxia. Mice were restrained and exposed to similar hypoxic protocol for the hamsters, as described above. Study mice received a bolus intraperitoneal injection of 5HMF or vehicle. 60 min after treatment, mice received an intraperitoneal (IP) injection of the hypoxic marker Hypoxyprobe-1 (pimonidazole 40 mg/kg) and 5 mg/kg Hoechst 33342 (Invitrogen Corp., Carlsbad, CA) diluted in PBS (total volume, 100 μL) and were exposed to a 10% O₂ hypoxia for 30 min. Then, the mice received a second IP injection of pimonidazole (40 mg/kg) and Hoechst 33342 (5 mg/kg) diluted in PBS (100 μL) and were exposed severe hypoxia at 5% O₂ for an additional 30 min. Mice blood P₅₀ was measured at baseline, 60 min after 5HMF or vehicle treatment, and 15 min after exposing the animals to each hypoxic level form blood samples (20 μL) collected from the tail vein. Finally, mice were euthanized and their brains and hearts were removed. In addition, a Control group of mice, not exposed to hypoxia, was included in the study for comparison. Brain and heart were split sagittally and transverse, respectively. Tissues were fixed by immersion in formalin for 24 hours at room temperature before transfer to 70% (v/v) ethanol. Lastly, tissues were cut into 100-μm thick sections.

Pimonidazole immunohistochemistry. Sections were cleaned and rehydrated according to standard procedures. Monoclonal antibody directed against pimonidazole (included in the Hypoxyprobe-1 green kit) was used for immunohistochemical staining of the brain and heart sections. Fluorescence microscopy was performed using an Olympus BX51WI equipped with a high resolution digital CCD ORCA-285 (Hamamatsu Corp., Hamamatsu city, Japan) illuminated
with a mercury burner and the appropriate fluorescent cubes (XF100-2 and XF02-2, Omega Optical, Brattleboro, VT). Images for pimonidazole antibody-stained areas and Hoechst were prepared using Wasabi Imaging Software (Hamamatsu Corp). The ratio of pixels stained for pimonidazole in each region, to the total cellular area of the image, was calculated. Ten images were analyzed, by sections, and the results were pooled to determine the mean and standard deviation. To indicate the co-localization of pimonidazole and Hoechst in cell, images were superimposed.

Data analysis. Results are presented as mean ± standard deviation. The Grubbs' method was used to assess closeness for all measured parameters values at baseline. Data within each group was analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, post hoc analyses were performed with the Dunn's multiple comparison test. Data comparison between groups was analyzed using two way analysis of variance (Two-way ANOVA test). When appropriate, post-test analyses were performed with the Bonferroni posttests comparison. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. The same vessels and capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more robust statistics. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if P<0.05.

RESULTS

Twenty-four hamsters entered the study; fourteen were used for microvascular studies and ten for CO measurements. Fourteen mice were used to study pimonidazole binding to brain and heart hypoxic tissue. All animals tolerated the entire hypoxia protocol. Animals were randomly assigned to the experimental groups, microvascular studies: 5HMF (n = 7; 66 ± 7 g) and Vehicle (n = 7; 68 ± 5 g); CO studies: 5HMF (n = 5; 69 ± 6 g) and Vehicle (n = 5; 70 ± 7 g); mice pimonidazole binding: 5HMF (n = 5; 18 ± 2 g), Vehicle (n = 5; 19 ± 3 g) and Control (no
exposed to hypoxia, n = 4; 19 ± 2 g). All animals passed the Grubbs' test, ensuring that all the measured values at baseline were within a similar population (P<0.05). Similarities between groups at baseline for hamsters and mice were statistically verified between groups (P>0.30).

*Oxygen equilibrium curves.* Blood O₂ equilibrium curves are presented in Figure 1 for normoxia, hypoxia 10% O₂ and hypoxia 5% of O₂. Treatment with 5HMF decreased blood P50 from 32.2 ± 1.2 mmHg at baseline to 19.6 ± 1.8 mmHg within 60 min. The decrease in blood P50 produced by 5HMF was maintained through the entire protocol, and the P50 of 5HMF animals was 20.3 ± 1.9 mmHg during the 10% hypoxia and 20.8 ± 2.4 mmHg during the 5% hypoxia, respectively. The P50 of the animals treated with the vehicle was not different from baseline at normoxia, 32.6 ± 1.4 mmHg; however, during 10% and 5% O₂ hypoxia, their P50 increased to 34.1 ± 2.2 and to 35.4 ± 2.0 mmHg, respectively.

*Blood chemistry and gas parameters.* Blood gas parameters are presented in Table 1. 5HMF did not produce any significant change in blood gas parameter during normoxia. The multiple samples for blood gases and O₂ affinity determination produced a small and not significant decrease in Hct and Hb in both groups. 10% O₂ hypoxia decreased arterial pO₂ in both groups compared to baseline. Arterial pCO₂ also decreased compared to baseline during 10% O₂ hypoxia. The arterial CO₂ tension decreased due to hyperventilation, which should produce an increase in blood pH. However, arterial pH decreased in both groups during hypoxia. Thus, the acid-base status changed towards a mixed respiratory alkalosis and metabolic acidosis, the latter dominating and decreasing blood pH. 5% O₂ hypoxia further decreased arterial pO₂ in both groups, although 5HMF had higher arterial pO₂ compared to the vehicle. During 5% O₂ hypoxia, the arterial blood pH further decreased, triggering reflex response to metabolic acidosis that decreased arterial pCO₂ producing a more profound respiratory alkalosis, as indicated by the blood biochemical analysis.

*Blood pressure and heart rate.* MAP and HR are presented in Figure 2A and 2B. Treatment with 5HMF did not affect MAP or HR compared to baseline. No difference in MAP and HR was
measured between groups at normoxia. MAP and HR were not significantly changed during
10% O2 hypoxia in either group. 5% O2 hypoxia decreased significant MAP in both groups
compared to baseline and normoxia. During 5% O2 hypoxia, the vehicle treated animals showed
a significant decrease in MAP compared to 10% O2 hypoxia and to the 5HMF treated animals.
At 5% O2 hypoxia, the 5MHF treated animals significantly increased their HR compared to
baseline, normoxia and to the vehicle treated animals. The vehicle treated animals had lower
HR compared to 10% O2 hypoxia.

Arterial oxygen saturation. Arterial SO2 are presented in Figure 2C. The 5HMF group showed
an increase in arterial SO2 (P<0.10) compared to baseline and to the vehicle group during
normoxia. 10% O2 hypoxia decreased arterial SO2 in both groups compared to baseline and to
normoxia, although the 5HMS group had significantly higher arterial SO2 compared to the
vehicle group. Similarly, at 5% O2 hypoxia, arterial SO2 in both groups decreased compared to
baseline, normoxia and 10% hypoxia. During 5% O2 hypoxia, the 5HMS group had significantly
higher arterial SO2 compared to the vehicle group.

Cardiac output, vascular resistance and systemic oxygen delivery. CO, VR and DO2 are
presented in Figure 3A, 3B and 3C. CO, VR and DO2 at normoxia did not change after treatment
with 5HMF compared to baseline, in addition, 5HMF and vehicle treated animals were not
difference from each other at normoxia. 5HMF and vehicle groups were not different form
baseline or normoxia at 10% O2 hypoxia. CO was not different between 5HMF and vehicle
treated animals at 10% O2 hypoxia; however, at 5% O2 hypoxia, CO was significantly higher in
the 5HMF group compared to the vehicle group. At 5% O2 hypoxia the 5HMF treated animals
increased CO compared to baseline and normoxia, whereas the vehicle treated animals
decreased their CO compared to baseline, normoxia and 10% O2 hypoxia. The changes in MAP
and CO induced changes in VR in both groups especially at 5% O2 hypoxia. The 5HMF group
systematically decreased in VR as the hypoxia increased, thus at 5% O2 hypoxia VR was lower
compared to baseline, normoxia and 10% O2 hypoxia, whereas in the vehicle group VR
decreased only significantly compared to baseline and normoxia. The increase in CO and the
preservation of arterial $O_2$ saturation in the 5HMF group preserved the calculated $DO_2$ as hypoxia increased, even at 5% $O_2$ hypoxia. In the vehicle group, $DO_2$ decreased proportionally to the hypoxia.

**Microhemodynamics.** Microvascular changes: diameter, RBC velocity and blood flow in arterioles (range 42 - 74 $\mu$m) and venules (range 41 - 78 $\mu$m) were measured at each time point. Microvascular diameter and blood flow relative to baseline are presented in **Figures 4** of arterioles (panel A) and venules (panel B), and absolute values are presented in the figure legend. Arteriolar and venular diameters were not affected by 5HMF treatment. 10% $O_2$ hypoxia produced an increase in arteriolar and venular diameters in both groups compared to baseline and to normoxia. 5% $O_2$ hypoxia produced arteriolar and venular vasodilation only in the 5HMF group compared to baseline and normoxia, and the arteriolar and venular diameters of the vehicle group were not different from baseline or normoxia. 5HMF group showed significant arteriolar and venular vasodilation compared to the vehicle group. Arteriolar and venular RBC velocities decreased at 5% $O_2$ hypoxia in both groups compared to baseline, although the decrease in arteriolar and venular RBC velocity was significantly more drastic in the vehicle group compared to the 5HMF group. Arteriolar and venular blood flows relative to baseline are presented in **Figure 4**, panel C (arterioles) and D (venules), and absolute values are presented in the figure legend. Arteriolar and venular blood flows were not affected by 5HMF treatment during normoxia. 10% $O_2$ hypoxia increased arteriolar blood flow in the 5HMF group compared to the vehicle group. No changes in venular blood flow compared to baseline, normoxia, or between groups were measured at 10% $O_2$ hypoxia. Analogous to the changes in microvascular diameters, the 5HMF group preserved higher arteriolar and venular blood flow compared to vehicle group during the 5% $O_2$ hypoxia. The blood flows in the vehicle group during the 5% $O_2$ hypoxia were also significantly decreased in arterioles and venules compared to baseline, normoxia, and 10% $O_2$ hypoxia.

**Functional Capillary Density.** FCD at baseline was 114 ± 7 cm$^{-1}$ for 5HMF and 109 ± 9 cm$^{-1}$ for the vehicle, respectively. 5HMF treatment did not affect FCD (5HMF: 108 ± 10 cm$^{-1}$; vehicle:
During 10% O$_2$ hypoxia, the FCD was not different from baseline or normoxia (5HMF: 104 ± 8 cm$^{-1}$; vehicle: 97 ± 10 cm$^{-1}$) in both groups. 5% O$_2$ hypoxia produced a significant decrease in FCD (5HMF: 78 ± 6 cm$^{-1}$; vehicle: 42 ± 10 cm$^{-1}$) compared to baseline and normoxia in both groups. As a consequence of the hemodynamic changes produced by 5% O$_2$ hypoxia in the vehicle group, the vehicle group was significantly lower FCD compared to the 5HMF group.

Microvascular Oxygen Distribution. Intravascular and perivascular (tissue) O$_2$ tensions are presented in Figure 5. During normoxia, 5HMF treatment did not affect arteriolar, venular or perivascular pO$_2$ compared to the vehicle group. Additionally, 5HMF treatment at normoxia appears not to have a significant effect compared to previous measurements in O$_2$ tension for the specie and the window model preparation. According to previous studies during normoxia, the perivascular P$_O_2$ for the hamster window model is 20.9 ± 2.9 mmHg, and intravascular P$_O_2$ in arterioles and venules were 46.2 ± 4.8 and 27.0 ± 3.6 mmHg, respectively (3). 10% O$_2$ hypoxia decreased arteriolar, venular and tissue P$_O_2$ in both groups. The 5HMF group had significantly higher venular and tissue P$_O_2$ compared to the vehicle group (P<0.10). 5% O$_2$ hypoxia further decreased arteriolar, venular and tissue P$_O_2$ in both groups compared to normoxia and 10% O$_2$ hypoxia, although the vehicle group was significantly reduced compared to the 5HMF group. Perivascular P$_O_2$ decreased with the hypoxic level, although 5HMF partially prevented the drastic decrease measured in the vehicle group. Arteriolar O$_2$ saturation in the 5HMF group were 89.1 ± 5.2% during normoxia, 65.0 ± 4.6% during 10% O$_2$ hypoxia and 26.6 ± 3.5 during 5% O$_2$ hypoxia, while the arteriolar O$_2$ saturation in the vehicle group were 74.1 ± 5.0% during normoxia, 32.2 ± 4.0% during 10% O$_2$ hypoxia and 6.4 ± 1.6 during 5% O$_2$ hypoxia. Therefore, 5HMF increased two and fourfold, in arteriolar SO$_2$ relative to vehicle treated animals at 10% and 5% O$_2$ hypoxia.

Calculated Oxygen transport. Figure 6 shows the analysis of arterial and arteriolar O$_2$ transport, and microvascular O$_2$ extraction. Arterial O$_2$ supply decreased with the degree of hypoxia in both groups. The vehicle group had significantly lower arterial O$_2$ supply compared to the
5HMF group at 10% and 5% O₂ hypoxia, respectively. Arteriolar O₂ supply also decreased with the degree of hypoxia. The vehicle groups had lower arteriolar O₂ supply compared to 5HMF group during hypoxia. Microvascular (arteriolar – venular) extraction reflects the O₂ consumed by the tissue, and it remained stable at 10% O₂ hypoxia compared to normoxia, but at 5% O₂ hypoxia the vehicle group had significantly lower microvascular extraction compared to normoxia, 10% O₂ hypoxia, and to the 5HMF group. Therefore, at 5% O₂ hypoxia the tissue of the vehicle group was not able to extract sufficient O₂ from the O₂ transported by the blood.

Pimonidazole binding to mice brain and heart hypoxic tissues. To expand and validate the results obtained using the hamster window model, C57BL/6 mice were treated with 5HMF or vehicle and exposed to identical hypoxic protocol. Treatment with 20 mg/kg of 5HMF decreased mice blood P50 from 41.5 ± 2.0 mmHg at baseline to 23.8 ± 2.0 mmHg within 60 min, and the change in P50 was maintained through the entire protocol (10% O₂ hypoxia: 22.5 ± 1.7 mmHg and 5% O₂ hypoxia: 22.0 ± 1.5 mmHg). The P50 of the animals treated with the vehicle was not different from baseline (41.0 ± 2.3 mmHg), at normoxia, however it decreased at 10% and 5% O₂ hypoxia to 39.7 ± 1.9 and 39.2 ± 2.3 mmHg, respectively. Pimonidazole was injected to detect hypoxic areas in the brain and heart tissues. The degree of hypoxia was assessed from binary masked chromogenic images from different areas of the heart and brain. Pimonidazole staining was viewed covering large areas of the heart and brain in mice exposed to hypoxia, compared to the control group (Figure 7, A and E). A group wise comparison revealed highly significant differences, the vehicle mice demonstrated an approximately fourfold and double higher degree of pimonidazole reactivity than the 5HMF mice in the heart and brain, respectively. Superimposed images (Figure 7, B-D and F-H) indicate clear co-localization of pimonidazole (green) and Hoechst (blue). Very few areas seemed to be hypoxic in control mice that were not exposed to hypoxia (Figure 7, D and H). In 5HMF mice (Figure 7, B and F) some cells stained positive for hypoxia, these cells were scattered and non-uniformly distributed. In the Vehicle mice (Figure 7, B and F), the number of positive cells was visibly higher, and cells were often grouped in multicellular foci. Moreover, in 5HMF mice, the intensely labeled cells often were surrounded by areas of low-intensity intercellular and intracellular staining.
DISCUSSION

The principal finding of the study is that 5HMF increased Hb $O_2$ affinity, prevented hemodynamic disturbance produced by severe hypoxia, and partially maintained microvascular oxygenation, compared to animals with normal Hb $O_2$ affinity. The decrease in the fraction of inspired $O_2$ decreased arterial $SO_2$ and blood $O_2$ content. In animals with normal Hb $O_2$ affinity, hypoxia produced systemic and microvascular hemodynamic changes that further limited tissue oxygenation. 5HMF increased Hb $O_2$ affinity, arterial $SO_2$ and $O_2$ content, and partially prevented the hemodynamic disturbances induced by severe hypoxia by preserving MAP, CO and microvascular blood flow. The increase in Hb $O_2$ affinity during hypoxia partially preserved systemic $O_2$ delivery (Figure 3C). These results suggest that the hemodynamic adjustments to maintain blood flow and $O_2$ supply during hypoxia are limited by arterial and arteriolar $SO_2$. Increased Hb $O_2$ affinity during normoxia had minor effects on perivascular $pO_2$, although it maintained tissue $O_2$ extraction by generating the appropriate $pO_2$ gradients to extract sufficient $O_2$. Thus, 5HMF treatment increased Hb $O_2$ affinity, provided an advantage for blood $O_2$ loading, and increased arterial $SO_2$ during severe hypoxia, preserving blood pressure and heart rate, compared to vehicle treated animals, and supported microvascular blood flow and oxygenation. These changes consequently increased microvascular $O_2$ delivery, maintained $O_2$ extraction and tissue $pO_2$ compared to animals with normal Hb $O_2$ affinity during severe hypoxia. Additionally, these results appeared not to be specie specific, as staining for hypoxic tissues in heart and brain of mice suggest that the increased Hb $O_2$ affinity after 5HMF treatment reduced hypoxic areas in mice in similar hypoxia protocol.

5HMF acute adaptation to extreme hypoxic environments is very significant since chronic physiological adaptation requires time, as several systems (i.e., cardiovascular, pulmonary, endocrine) gradually adjust. Our results suggest that 5HMF may prevent tissue anoxia during severe hypoxia and reduce the incidence of AMS, HACE and HAPE. 5HMF has also been
reported to prevent sickling and hemorrhological complications induced by hypoxia in sickle mice (1). 5HMF protective effects in sickle cell disease and during severe hypoxia are associated with an increase in Hb O2 affinity and the maintenance of central hemodynamics. While cardiac function is limited by myocardial O2 delivery (12), the overall cardiac dysfunction observed in animals with normal Hb O2 affinity during severe hypoxia restricted the maximum hydraulic cardiac power (MAP x HR) to 50% of the hydraulic cardiac power in the 5HMF treated animals. The preservation of cardiac function induced by 5HMF increased CO and systemic O2 delivery to vital organs, including the heart and brain. Moreover, 5HMF effects in O2 delivery to vital organs are derived from the increase in Hb O2 affinity, as confirmed by pimonidazole hypoxic staining heart and brain tissues in mice. Although no neurological function was measured in this study, during 5% O2 hypoxia, 5HMF animals were alert, whereas control animals were lethargic. These results suggest that 5HMF can improve survivability at 5% O2 (38 mmHg) without a hypoxic adaptation process at an O2 partial pressure lower than the highest place on earth (Mount Everest, 43 mmHg, 5.6% O2).

A decrease of 50% in the fraction of inspired O2 is mostly compensated for through vasodilation and increased blood flow, although as the inspired O2 reaches a critical limit, the changes in vascular tone and resistance become ineffective to maintain O2 delivery. 5HMF increased Hb O2 affinity allowed the physiological response to hypoxia to be extended to 5% inspired O2, where vasodilation and the increase in blood flow favored oxygenation. At 5% O2 hypoxia, vascular resistance in hamsters treated with 5HMF decreased to 58% of baseline, whereas the resistance in vehicle treated hamsters was 70% of baseline. Increasing blood O2 content during hypoxia by increased Hb O2 affinity also has positive systemic hemodynamic effects. Hamsters treated with 5HMF had an 87% higher CO compared to vehicle treated hamsters. Similar advantage was previously described in studies by Turek et al. as enhanced O2 pulmonary uptake (27, 28). Their results showed that an increase in Hb O2 affinity provides an advantage for tissue O2 delivery under severe hypoxemia, which increased CO and redistributed blood flow to vital organs (28). Although FCD decreased in both groups with the reduction of inspired O2, the increased Hb O2 affinity preserved FCD above 50%, suggesting
redistribution of blood flow for 5HMF treated animals. The arteriolar vasoconstriction in the vehicle treated hamsters during severe hypoxia, with a consequential effect on perfusion, can be attributed to the autoregulatory process that attempts to maintain O₂ supply to vital organs, while compromising peripheral tissues (17). 5HMF treatment mediated a rapid adaptive process that maintained microvascular function and partially preserved oxygenation during severe hypoxia.

The hamster chamber model includes muscle and connective tissue with stable O₂ consumption; therefore changes of O₂ supply determine tissue oxidative state. Comparing our published intravascular and perivascular pO₂ for the hamster chamber model (3), 5HMF did not affect intravascular pO₂S and SO₂S for arterioles and venules, however perivascular (tissue) pO₂ appeared to decrease in 5HMF treated animals (P < 0.10). Whereas during 10% and 5% hypoxia, the 5HMF group arteriolar SO₂ was two and fourfold in the arteriolar SO₂ of the vehicle group, respectively. At 10% O₂ hypoxia, venules SO₂, which reflects O₂ extraction, increased fourfold for the 5HMF compared to control group, and at 5% O₂ hypoxia, the difference in venular SO₂ was sixfold. Although these changes in SO₂ appear dramatic, they reflect the range in the Hb O₂ equilibrium curve where each group of animals manages their O₂ exchange (Figure 2). During 10% and 5% O₂ hypoxia, the 5HMF group maneuvers O₂ within the steepest section of the Hb O₂ equilibrium curve, while the vehicle group operates at lower section of their Hb O₂ equilibrium curve. In the present study, hypoxia increased the P50 from 32.6 to 34.1 and to 35.4 mmHg at 10% and 5% O₂ in the hamsters. This appears to result from an augmented metabolic acidosis, whereas the P50 in mice decreased from 41.0 to 39.7 and 39.2 at 10% and 5% O₂, suggesting a respiratory alkalosis. Hypoxia may induce different ventilation changes in both species; however, 5HMF’s protective effect during hypoxia appears to be specific to increasing Hb O₂ affinity and is independent of the animal specie.

Experimental evidence in isolated canine muscle and humans supports that the rate at which O₂ diffuses from the erythrocyte to the tissue determines maximal O₂ uptake (11). The O₂ diffusion can be divided in the O₂ conductance within the tissue and the release from the
erythrocyte Hb to the tissue (11). For a given O\textsubscript{2} transported by the blood (flow x O\textsubscript{2} content), the O\textsubscript{2} extracted by tissue is determined by the pO\textsubscript{2} gradient between the blood and the tissue. Therefore, as the O\textsubscript{2} conductance is held constant, Hb-O\textsubscript{2} affinity regulates the rate at which the O\textsubscript{2} is released. Therefore, changes in Hb-O\textsubscript{2} affinity affect the radial pO\textsubscript{2} gradient between blood vessels and perivascular tissues required to satisfy the flux of O\textsubscript{2} leaving the blood vessel to maintain the O\textsubscript{2} consumed by perivascular tissues (26). Previous results obtained by Stein and Ellsworth suggest that an increase in Hb O\textsubscript{2} affinity did not affect the RBC supply rate, a measurement of convective O\textsubscript{2} transport, between animals with high O\textsubscript{2} affinity and controls during hypoxia (24). Their first study concludes that the amount of O\textsubscript{2} lost across the capillary network, which reflects the O\textsubscript{2} extraction during a hypoxia (10% inspired O\textsubscript{2}), was not affected by Hb O\textsubscript{2} affinity (24). Their results are consistent with the present results, where the high-affinity and control groups at normoxia and moderate hypoxia had similar arteriolar – venular O\textsubscript{2} extraction (Figure 5) even though arteriolar supply was higher for the group with increased Hb O\textsubscript{2} affinity (24). Stein and Ellsworth also found that blood pressure and arteriolar blood flow were higher in animals with increased Hb O\textsubscript{2} affinity during hypoxia (24). In a subsequent paper, Stein and Ellsworth found a beneficial effect of an increase in O\textsubscript{2} affinity during anemia (40% reduction in systemic Hct) and hypoxia (10% inspired O\textsubscript{2}) (23). The practicality and limited toxicity of the acute treatment with 5HMF (20 mg/kg) provides significant benefits compared to sodium cyanate (0.2 mg/kg five times per 2 weeks) used by Stein and Ellsworth, which creates similar changes in Hb O\textsubscript{2}.

The present study was carried out to determine the systemic and microvascular hemodynamic changes induced by acute modifications in Hb O\textsubscript{2} affinity during severe and extreme hypoxia. The principal hemodynamic change identified in the study was the preservation of central and peripheral hemodynamics in the animals with higher Hb O\textsubscript{2} affinity by partially preserving O\textsubscript{2} delivery. A reduced fraction of inspired O\textsubscript{2} limits the amount of O\textsubscript{2} that can be bound to the Hb in pulmonary circulation, therefore by simply increasing Hb O\textsubscript{2} affinity the O\textsubscript{2} transported to the tissues is increased. Vascular resistance was not affected by the increase in Hb O\textsubscript{2} affinity during normoxia. 10% O\textsubscript{2} hypoxia decreased vascular resistance
equally, independently of Hb O\textsubscript{2} affinity, while at 5% O\textsubscript{2} hypoxia the group with increased Hb O\textsubscript{2} affinity showed lower vascular resistance compared to unmodified Hb O\textsubscript{2} affinity. The vascular resistance in the vehicle group decreased to 67% of baseline during 5% O\textsubscript{2} hypoxia, whereas in the animals with increased Hb O\textsubscript{2} affinity the vascular resistance decreased to 60% of baseline. Previous studies had shown similar effects in vascular resistance by increasing P50 at any given level of arterial O\textsubscript{2} content, consistent with the concept that O\textsubscript{2} transport is regulated in specific vascular beds (16).

**Limitation of the study.** Hamsters are fossorial animals, which explains their low arterial PO\textsubscript{2} at normoxia, since their respiratory exchange is adapted to their burrow environment (25). Other fossorial rodents withstand conditions of extreme hypoxia, however hamsters are sensitive to hypoxia and hypercapnia (25, 31). Hamsters and humans have different ventilation regulation during hypoxia; hamsters increase their ventilation during hypoxia primarily by increasing frequency with minor changes in tidal volume, which results in minimal activation of pulmonary stretch receptors (31). Additionally, hamster’s vasodilatation during hypoxia might be limited for humans, since hypoxic hypocapnic in humans produces central inhibition of the peripheral chemoreflex and induces peripheral vasoconstriction (15). Small animals have a high HR, this high HR reduces the fraction of time spent in diastole, thus shortens the time for subendocardial perfusion. Therefore, hamster and mice critical fraction of inspired O\textsubscript{2} level may be higher than other larger mammalian with slower HR. Hamster’s high baseline HR limits the increase in CO during hypoxia; therefore their limited CO response to hypoxia appears to produce metabolic acidosis. Therefore, the early phase of human acclimatizing to high altitude is different from the hamster, since it includes respiratory alkalosis producing a small increase in Hb O\textsubscript{2} affinity, which is later offset by a decrease by 2,3 DPG (33). The benefits of 5HMF in humans may be limited by hypocapnia which decreases P50. The results of the current study are limited to hypoxic hypoxia, it does not account for the changes in barometric pressure and air dryness that occur at high altitude. The extent of hypoxia used in our study was selected to determine the effects of increased Hb O\textsubscript{2} affinity in the early acclimatization process to hypoxia, before 2,3 DPG and erythropoietin affect O\textsubscript{2} transport.
CONCLUSION

This study indicates that 5HMF increases tolerance to normobaric severe hypoxia without an adaptation process. The potent effects on Hb O\textsubscript{2} affinity, low molecular weight, high bioavailability and low toxicity of 5HMF make it an attractive candidate drug for further detailed studies as a treatment for acute adaptation to extreme hypoxic environments. 5HMF protective mechanisms appear closely associated to the increase of Hb O\textsubscript{2} affinity, extending physiological hemodynamics responses and changing microvascular O\textsubscript{2} transport. This suggests that O\textsubscript{2} content rather than pO\textsubscript{2} is the critical determinant of O\textsubscript{2} supply during hypoxia. Further studies may be necessary to better understand how the protective role of S-HMF in response to severe hypoxia affect O\textsubscript{2} transport from the blood to the mitochondria.

ACKNOWLEDGMENTS

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### Table 1. Blood gas parameters

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FIGURE LEGENDS

**Figure 1.** Changes in blood O$_2$ equilibrium curves during normoxia, 10% O$_2$ hypoxia and 5% O$_2$ hypoxia. Each panel includes arterial (square), arteriolar (red circle) and venular (blue triangle) O$_2$ saturation for each group. Legends indicate baseline (BL), normoxia (Nor), 10% O$_2$ hypoxia (Hyp10) and 5% O$_2$ hypoxia (Hyp5). Severe hypoxia was induced by normobaric hypoxia with 10% O$_2$ balanced N$_2$, and after characterization the animals were exposed to extreme hypoxia using 5% O$_2$ balanced N$_2$. Reduction of the fraction of inspired O$_2$ limits the amount of O$_2$ bound to the Hb in pulmonary circulation, therefore increasing Hb O$_2$ affinity augments the O$_2$ transported by the blood to the tissues. 5HMF increased the blood O$_2$ saturation at lower pO$_2$.

**Figure 2.** Changes in A. mean arterial pressure (MAP), B. heart rate and C. arterial oxygen saturation (arterial SO$_2$) during the acute hypoxia protocol. Legends indicate baseline (BL), normoxia (Nor), 10% O$_2$ hypoxia (Hyp10) and 5% O$_2$ hypoxia (Hyp5). †, P < 0.05 relative to baseline; ‡, P<0.05 compared to normoxia; §, P<0.05 compared to 10% O$_2$ hypoxia.

**Figure 3.** Changes in A. cardiac output (CO), B. vascular resistance (VR) and C. systemic oxygen delivery (DO$_2$) during the acute hypoxia protocol. Legends indicate baseline (BL), normoxia (Nor), 10% O$_2$ hypoxia (Hyp10) and 5% O$_2$ hypoxia (Hyp5). †, P < 0.05 relative to baseline; ‡, P<0.05 compared to normoxia; §, P<0.05 compared to 10% O$_2$ hypoxia.

**Figure 4.** Relative changes to baseline in arteriolar and venular hemodynamics during the acute hypoxia protocol. Broken line represents baseline level. Legends indicate baseline (BL), normoxia (Nor), 10% O$_2$ hypoxia (Hyp10) and 5% O$_2$ hypoxia (Hyp5). Panel A: arteriolar diameter, Panel B: venular diameter, Panel C: arteriolar blood flow, Panel D: venular blood flows. Diameters at baseline in each animal group were as follows: 5HMF (arterioles (A): 64.5 ± 7.7 μm, n = 42; venules (V): 69.5 ± 8.9 μm, n = 49) and Vehicle (A: 62.4 ± 6.8 μm, n = 44; V: 67.8 ± 9.2 μm, n = 47). n = number of vessels studied. RBC velocities at baseline in each animal group were as follows: 5HMF (A: 4.6 ± 0.6 mm/s; V: 1.7 ± 0.5 mm/s) and Vehicle (A: 4.5 ± 0.8
Blood flows at baseline in each animal group were as follows: 5HMF (A: 14.4 ± 4.3 nl/s; V: 6.7 ± 2.3 nl/s) and Vehicle (A: 14.6 ± 4.2 nl/s; V: 6.9 ± 2.5 nl/s). †, P < 0.05 relative to baseline; ‡, P<0.05 compared to normoxia; §, P<0.05 compared to 10% O₂ hypoxia.

**Figure 5.** Intravascular and extravascular (tissue) partial pressure of oxygen during the acute hypoxia protocol. Legends indicate baseline (BL), normoxia (Nor), 10% O₂ hypoxia (Hyp10) and 5% O₂ hypoxia (Hyp5). †, P<0.05 compared to normoxia; §, P<0.05 compared to 10% O₂ hypoxia. Dotted lines represent baseline PO₂s (arterioles: 46.2 mmHg; venules: 30.7 mmHg; tissue: 20.9 mmHg (3)).

**Figure 6.** Arterial oxygen delivery and extraction during the acute hypoxia protocol. Legends indicate baseline (BL), normoxia (Nor), 10% O₂ hypoxia (Hyp10) and 5% O₂ hypoxia (Hyp5). ‡, P<0.05 compared to normoxia; §, P<0.05 compared to 10% O₂ hypoxia. Oxygen transport is not directly measurable; however, it can be calculated using the measured parameters. The delivery and extraction were calculated by averaging arterioles and venules of each animal.

**Figure 7.** Brain and heart pimonidazole binding to hypoxic areas in mice. C57BL/6 mice treated with 5HMF or vehicle were exposed to identical hypoxic protocol as hamster. Pimonidazole preferentially binds to hypoxic cells, so detection of pimonidazole adducts using monoclonal antibodies can serve as a method for measuring tissue hypoxia. Pimonidazole stained areas in the heart (panels A-D) and brain (panels E-H) sections after hypoxia. Superimposed images (hearts, B-D and brains F-H) indicate clear co-localization of pimonidazole (green) and Hoechst (blue). Control animals received pimonidazole, but were not exposed to hypoxia. †, P<0.05 compared to control.
REFERENCES


function, and energy metabolism after a trek to Mt. Everest Base Camp. FASEB J 25: 792-796, 2011.


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**Table 1**
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Fig 2. Mean arterial pressure (MAP), heart rate and arterial oxygen saturation (arterial SO₂)
Fig 3. Cardiac output (CO), vascular resistance (VR) and oxygen delivery (DO₂)

Figure 3
Fig 4. Relative changes to baseline in arteriolar and venular hemodynamics

**A. Arterioles**

- **5HMF**
- **Vehicle**

**B. Venules**

**C. Blood flow, relative to BL**

**D. Time point**

Figure 4
Fig 5. Intravascular and extravascular (tissue) partial pressure of oxygen

Figure 5
Fig 6. Systemic oxygen delivery and microvascular oxygen delivery and extraction.

Systemic

Microcirculation

5HMF
Vehicle

Arterial \( \text{DO}_2 \)

Arteriolar \( \text{DO}_2 \)

Artioliar- venular \( \text{VO}_2 \)

Fraction of inspired \( \text{O}_2 \)

Systemic Oxygen delivery, mlo2.min\(^{-1}\)

Microcirculation \( \text{O}_2 \) transport, mlo2.min\(^{-1}\)

Figure 6
Fig 7. Mice brain and heart pimonidazole binding to hypoxic areas.