Published in final edited form as:

Free Radic Biol Med. 2013 October ; 63: 264–273. doi:10.1016/j.freeradbiomed.2013.05.024.

Nrf2 Activation: A potential strategy for the prevention of Acute Mountain Sickness

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Abstract

Introduction—Reactive oxygen species (ROS) formed during acute high altitude exposure contributes to cerebral vascular leak and development of acute mountain sickness (AMS). Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) is a transcription factor that regulates expression of greater than 90% of antioxidant genes, but prophylactic treatment with Nrf2 activators has not yet been tested as an AMS therapy. We **hypothesized** that prophylactic activation of the antioxidant genome with Nrf2 activators would attenuate high altitude-induced ROS formation and cerebral vascular leak, and that some drugs currently used to treat AMS symptoms have an additional trait of Nrf2 activation.

Methods—Drugs commonly used to treat AMS were screened with a luciferase reporter cell system for their effectiveness to activate Nrf2, as well as tested for their ability to decrease high altitude cerebral vascular leak *in vivo*. Compounds that showed favorable results for Nrf2 activation from our screen and attenuated high altitude cerebral vascular leak *in vivo* were further tested in brain microvascular endothelial cells (BMEC) to determine if they attenuated hypoxia-induced ROS production and monolayer permeability.

Results—Of 9 drugs tested, with the exception of dexamethasone, only drugs that showed the ability to activate Nrf2 (Protandim, methazolamide, nifedipine, amlodipine, ambrisentan, and sitaxentan) decreased high altitude-induced cerebral vascular leak *in vivo*. *In vitro*, Nrf2 activation in BMEC prior to 24 h hypoxia exposure attenuated hypoxic-induced hydrogen peroxide production and permeability.

Conclusions—Prophylactic Nrf2 activation is effective at reducing brain vascular leak from acute high altitude exposures. Compared to acetazolamide, methazolamide may offer better

protection against AMS. Nifedipine, in addition to its known vasodilatory activities in the lung and protection against high altitude pulmonary edema, may provide protection against brain vascular leak as well.

INTRODUCTION

Acute mountain sickness is a well-described syndrome that affects 60% of unacclimated individuals ascending to altitudes above 8,000 ft (1–4) with the prevalence increasing to 75% of individuals ascending to 12,000 ft (1–4). The most common symptoms of AMS are headache, nausea, and fatigue, (3) however in rare cases high altitude illness can progress to the life threatening conditions of high altitude cerebral (HACE) (3) or pulmonary edema (HAPE) (4). The decreased barometric pressure and subsequent reduction of available oxygen is the primary causal factor of AMS, but the exact mechanism(s) by which hypoxia induces AMS are unclear. Recently, it has been suggested that hypoxia-induces cerebral vascular leak and subsequent astrocyte swelling in the trigeminal areas plays a key role in the development of AMS (1). This has led some investigators to hypothesize that hypoxia triggers increased production of reactive oxygen species (ROS) in the brain, which are subsequently responsible for endothelial cell barrier dysfunction, increased cerebral vascular permeability, and astrocyte swelling (1, 5–8).

An innate defense mechanism of the body towards increased oxidative stress is the activation of nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) transcription factor. Nrf2 is responsible for regulating the gene expression of phase II detoxification enzymes and antioxidant proteins through an enhancer sequence known as the antioxidant responsive element (ARE) (9). Importantly, ARE is a promoter element common to nearly all of the antioxidant enzymes, including peroxiredoxins, thioredoxins, catalase, glutathione peroxidase, and heme oxygenase-1 (9–11). Hence, Nrf2 has been termed “the master regulator” of the ARE-driven cellular defense system against oxidative stress. Nrf2 is held inactive in the cytosolic compartment by its association with binding protein, Keap1 (9–11). In the presence of oxidative stress, Keap1 releases Nrf2, which, now activated, relocates to the nucleus and activates the transcription of ARE-driven genes.

Two distinct mechanisms have been demonstrated to be responsible for the release of Nrf2 from Keap1. The first mechanism involves the oxidation of thiol groups on Keap1, causing it to release Nrf2, (12, 13) thus accounting for the activation of ARE-driven genes by reactive oxygen species (ROS). The second mechanism involves the phosphorylation of Nrf2, which has been proposed to both stabilize Nrf2 and to cause its release from Keap1(10). For example, recently it has been discovered that Nrf2 can be activated by treatment with a variety compounds, including curcumin and quercetin, via a phosphoinositide 3-kinase (PI3K) dependent mechanism (10). Thus, via induction of Nrf2 by a mechanism other than oxidation of Keap1, it may be possible to increase cellular concentrations of antioxidant enzymes prior to incurring altitude-induced oxidative stress thereby attenuating vascular damage incurred from increased cellular concentrations of ROS.

We hypothesized that induction of Nrf2 by Protandim, a known “nonoxidizing” Nrf2 activator (11, 14, 15), prior to and during high altitude exposure would attenuate high altitude induced cerebral vascular leak *in vivo* as well as decrease hypoxia-induced ROS and brain endothelial cell (BMEC) permeability *in vitro*. Secondly, we hypothesized that some drugs currently used for AMS treatment, such as carbonic anhydrase inhibitors and calcium channel blockers, in addition to their primary actions, could induce Nrf2 via a PI3K “nonoxidizing” mechanism and protect the cerebral vasculature against high altitude-induced oxidative stress similar to Protandim.

Our data showed that Nrf2 activation by either Protandim or from “off-target” effects of other compounds prior to high altitude or hypoxia exposure decreased cerebral vascular leak *in vivo*. *In vitro*, Nrf2 activation decreased hypoxia-induced endothelial hydrogen peroxide production and permeability.

METHODS

Animals

Male Sprague-Dawley rats (280–350 g and 10–12 weeks of age) were obtained from a commercial vendor (Charles River, Wilmington, MA) and housed in the University of Colorado Anschutz Medical Campus’ Center for Comparative Medicine (elevation = 1,609 m; 5,280 ft). Animals were allowed ad libitum access to food and water and kept on a 14:10 hour day: night cycle. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Colorado Denver Anschutz Medical Campus.

Compound screening for Nrf2 activators

A breast cancer cell line (AREc32) was stably transfected with a luciferase gene attached to the antioxidant response element (ARE, the Nrf2 promoter) and was used to screen pharmaceutical compounds for the ability to induce Nrf2. All compounds were tested in a dose response fashion (1 – 300 µg/ml) in the presence or absence of phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Cell Signaling Technologies, Cat.# 9901) and evaluated with the Nrf2 activator Protandim (11, 14–19). Results are shown in Table 1.

In vivo methods

Drug administration and dosing—All therapeutic compounds, unless otherwise stated, were solubilized in PEG400, administered by I.P. injection, and tested in a dose response fashion of high and low doses in respected groups. Doses were chosen based on data obtained from our luciferase screen. These doses were then compared to the established human doses and translated to the rodent equivalent doses calculated from the formula between humans and rodents based on body surface area (20).

For all high altitude studies, animals were administered drugs starting two days prior to high altitude exposure, day of high altitude exposure, and at 24 hour of exposure. These protocols were determined from initial studies that showed that compared to vehicle control, Protandim decreased cerebral vascular leak after 4 doses (two days of pretreatment, day of

exposure, and at 24 h of exposure; 10 mg/kg, I.P. (Table 1)). This dose of Protandim was chosen because it has previously shown to be effective at activating Nrf2 in rats (16).

High altitude exposure—Animals assigned to the high altitude exposure groups were exposed for 48 h to a simulated high altitude (5,500 m; 18,000 ft; Pb=80 mm Hg) in a specially designed rodent hypobaric chamber facility as previously described (21).

Brain vascular leak determination by Evans blue dye—Brain vascular leak was quantified with Evans blue dye assay as previously described (22). In brief, after 48 h of high altitude exposure, animals were removed from simulated high altitude, anesthetized with ketamine hydrochloride and xylazine hydrochloride (100 mg/kg; 16 mg/kg) and injected via a tail vein with Evan's blue dye (30 mg/kg; solubilized in 0.9% saline; 30 mg/ml). Evans blue dye was allowed to circulate for 30 min. During this time period, animals were kept in specially designed hypoxic cages flushed with 10% oxygen. Afterwards, animals were humanly euthanized with an injection of pentobarbital (100 µg/kg; I.P.) and the vasculature flushed with 10 mL of saline at constant pressure (10 cm). The brain was removed and cut along the corpus callosum into two hemispheres. Both hemispheres were weighed. The left hemisphere was oven dried (5 days at 60°C) for wet-weight to dry-weight ratio. The right hemisphere was placed into 750 µL of 50% trichloroacetic acid, 50% diH₂O solution and homogenized (Fisher Scientific, tissue-miser). The homogenate was centrifuged at 2500 rpm for 10 minutes at room temperature, the supernatant was removed, the protein was re-suspended in 100% ethanol, and centrifuged again. After which, 50 µL of the supernatant was dispensed into a clear bottom 96 well plate (CELLTREAT, 229196) and read at 595 nm (BioTek, Synergy 2) against a standard curve. Data were quantified as mg of Evans blue dye normalized to calculated dry tissue weight.

Chronic catheterization—Chronic indwelling catheters were placed in the jugular vein and carotid artery for determination of blood gases, as previously described (23). In brief, rats were anesthetized via isoflurane with induction at 4% and maintenance of surgical plane between 1.5–3% in 100% O₂. Animals were administered buprenorphine (0.05 mg/kg SQ), Carprofen (5 mg/kg SQ) or Meloxicam (1–2 mg/kg SQ) for pain management post-surgery. The jugular vein and carotid artery were exposed and isolated via blunt dissection, and cannulated with PE-50 catheters. Catheters were flushed with heparinized saline, tied off, tunneled subcutaneously to the dorsal neck region and exteriorized at the back of the neck. Animals were allowed 48 h to recover before any treatments. Animals that demonstrated any signs of infections were excluded from the study.

Western blot analyses—Standard western blot technique was used to determine Nrf2 concentration *in vivo* or *in vitro*. Nuclear protein was extracted with NE-PER Kit (Thermo Scientific; #78835) per manufacture's instruction. Nuclear protein was incubated overnight in 4°C with a Nrf2 antibody (Abcam, ab31163) at 1:250 concentrations. Afterwards, a secondary antibody (Vector Labs, PI-1000) was applied and incubated for 1 h at room temperature. The membrane was developed using Thermo Scientific substrate (80196). Quantification was determined by densitometry using ImageJ (Version 1.46j, NHI) and normalized to vehicle control.

Immunostaining microscopy—To determine whether Nrf2 nuclear translocation occurred *in vivo* or *in vitro*, standard immunostaining techniques were used. In brief, five-micron sections of formalin-fixed, paraffin-embedded brain tissues were double stained for the presence of Nrf2 and factor-XIII expression. Antigen retrieval was performed on serial brain sections and then subjected to antibody against Nrf2 (Abcam, 31163) and Factor 8 (Abcam, 778). Slides were incubated with a fluorescent secondary antibody (either Alexa Fluor 488, 1:300 or Alexa Fluor 555, 5 µL/mL, Invitrogen, Carlsbad, CA). As a control for non-specific secondary antibody binding, some slides were simultaneously subjected to the same protocol without the primary antibody. Brain sections or cells were observed with a Nikon Eclipse Ti-E inverted epi-fluorescent microscope (Nikon Instruments, Tokyo Japan). Brightfield, phase contrast, and fluorescent digital deconvolution images were captured to a personal computer with an Andor Clara high resolution CCD camera (Andor Technology plc, Belfast, Northern Ireland) and analyzed using NIS-Elements AR software (Nikon Instruments).

Experimental design—To verify drugs had similar effects *in vivo* as observed in our luciferase reporter cell screen, animals were randomized to two groups: (1) Nrf2 activators and; (2) non-Nrf2 activators. Drug treatments were given once a day for 4 consecutive days in sea level conditions. Nrf2 activators included Protandim, methazolamide, nifedipine, and ambrisentan. Non-Nrf2 activators included acetazolamide, verapamil dexamethasone, theophylline, sildenafil and tadalafil.

High altitude cerebral vascular leak studies were completed by the following protocol. Once animals arrived at University of Colorado Anschutz Medical Campus, they were immediately placed in customized barrels pressurized to sea level conditions to avoid acclimatization to Denver altitude (5,280 ft; 1,609 m; Pb~630 mmHg). All animals remained at sea level conditions for 7 days prior to studies. Animals were divided into three groups; (1) sea level vehicle control; (2) high altitude vehicle control and; (3) high altitude treated. Treatments included: Protandim (10 mg/kg), acetazolamide (4, 10, 20 and 40 mg/kg), methazolamide (1, 4 and 10 mg/kg), nifedipine (4 and 10 mg/kg), verapamil (4 and 10 mg/kg), sildenafil (1 and 5 mg/kg), ambrisentan (0.5 and 2 mg/kg), sitaxentan (1 and 10 mg/kg), dexamethasone (0.1 and 1 mg/kg), and theophylline (30 and 60 mg/kg).

Respiratory rate and blood gas analysis of carbonic anhydrase inhibitors (CAI) were completed by the following protocol. Forty-eight hours after placement of carotid arterial catheter, rats were placed in a custom designed small, rectangular Plexiglas chamber with a portal for the catheters. Respiratory rate was determined and blood samples (arterial catheter) were collected 15 min prior to, at time of CAI administration, and 15, 30, 60, 90 and 120 min post-administration. Acetazolamide (10 mg/kg) or methazolamide (4 mg/kg) was administered I.V. through the jugular catheter. Blood was withdrawn from the carotid catheter into blood gas syringes and immediately analyzed on Radiometer ABL 800 flex machine (Copenhagen).

***In vitro* methods**

Brain vascular endothelial cells—Bovine Brain microvascular endothelial cells (BMEC) were obtained from Cell Applications INC, (B841-25, San Diego, California) and cultured in endothelial cell basal media (Cell Applications, INC B819-500). For all experiments, BMEC were used from passages 4–8, grown to confluence, and cultured under standard cell culture conditions (21% O₂, 'normoxia', 5% CO₂) unless otherwise noted.

Hypoxic incubation—Cell cultures were placed in a special humidified hypoxic chamber for up to 48 h as previously described (24). Use of an antechamber ensured that once cells became hypoxic, they were never reexposed to a normoxic environment. The chamber utilized a positive pressure system and was supplied with a gas mixture of 3% O₂, 5% CO₂, and balance of nitrogen. All relevant permeability tests and preparation for hydrogen peroxide (H₂O₂) production assays were conducted within the hypoxia chamber to avoid introduction of normoxia during data collection.

Nrf2 siRNA transfections—BMEC were transfected with Nrf2 siRNA (Santa Cruz, Cat. # SC-37030) following the manufacture's protocol (XTREMEGENE; Roche, 06 365 787 001). Cells were transfected 4 h prior to drug treatments and drug treatments were incubated for 24 h prior to hypoxic exposure in standard cell culture conditions (21% O₂) then moved into hypoxic conditions (3% O₂) for 24h.

Immunostaining microscopy—Brain microvascular endothelial cells (BMEC) were seeded (250 cells/chamber) and grown (48 h) on an 8-chamber well slide (Thermo Scientific, 177445). Cells were then fixed in 4% PFA solution for 15 minutes at room temperature, stained for Nrf2 (Abcam, ab31163; 1:100), and counterstained with DAPI as described above under the *in vivo* immunostaining microscopy subheading.

Hydrogen peroxide assay—BMECS were seeded at a density of 5,000 cells per well in a 96-well plate (CELLTREAT, 229196). Hydrogen peroxide production (H₂O₂) was measured as quantitative index of reactive oxygen species (ROS) generation (indirect indicator of the free radical O₂⁻) by an Amplex red assay kit Molecular Probes, (A-22188)] (21). In brief, Amplex red reagent (10-acetyl-3,7-dihydrooxphenoxazine) reacts with hydrogen peroxide in the presence of horseradish peroxidase (HRP) with a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, and has an absorption and fluorescence emission maxima of 571 nm and 585 nm respectively. The rise in H₂O₂ was measured over a 30 min period as an endpoint to the experimental protocol. Hydrogen peroxide production was normalized to the amount of protein per well by the BCA assay kit (Thermo Scientific, 23225). Data are represented as $\mu\text{M min}^{-1} \mu\text{g protein}^{-1}$. Absorption was determined on a Synergy 2 plate reader (BioTek Instruments, Inc; Winooski, VT).

Transendothelial Electrical Resistance—BMEC monolayer permeability was determined using transendothelial electrical resistance (TER) as previously described (25). In brief, cells were seeded 100,000 cells onto 6.5 mm diameter polyethylene terephthalate inserts with a pore size of 0.4 μm (BD Falcon, 351181) 48 hours prior to exposure to any experimental condition. Monolayer permeability was determined by TER after appropriate

cell treatments and 24 h of hypoxic exposure described in experimental design. To verify confluent and consistent monolayers between cell inserts, cells were stained with methylene blue (Sigma-Aldrich, 319112) and viewed under bright field microscope (Nikon Instruments, Tokyo Japan) as a visual conformation of confluent monolayers.

In vitro Experimental Design—Unless otherwise stated, for each BMEC experiment cells were randomly divided into 4 groups: (1) normoxia vehicle control (Nx-CTRL); (2) normoxia-treated; (3) hypoxia (24 h) control (Hx-CTRL) and (4) hypoxia (24 h) treated. As previously described, Nrf2 activators were added 24 h prior to any hypoxic exposures. Cell treatments were; Protandim (100 µg/mL; Life Vantage), methazolamide (125 µg/mL; Sigma Aldrich, M4156), nifedipine (7 µg/mL; Sigma Aldrich, N7643) or ambrisentan (40 µg/mL). In addition, some cells were treated with Nrf2 siRNA (Santa Cruz, sc-37030). In these experiments, siRNA was added 24 h prior to drug treatments (Protandim, methazolamide, or nifedipine). The rationale for 24 h hypoxia exposure for BMEC was to ensure that cells remained transfected with siRNA for the pre-treatment of drugs (24 h in normoxia) and during the 24 h hypoxia exposure. Data was collected from at least three separate cell culture preparations on three separate days (n=9).

Statistical analyses

All *in vivo* and *in vitro* data groups are reported as mean ± S.E.M. Statistical comparisons between *in vivo* groups were analyzed with a multi-factorial (treatment, exposure, dose) analysis of variance (ANOVA). Post hoc analyses were completed with unpaired, two-sided Students *t*-test with a Bonferroni adjustment. Statistical comparisons between *in vitro* groups were determined by ANOVA, and post-hoc analyses were completed with Tukey-Kramer multiple comparison tests. All statistical analyses were completed using the statistical software package JMP (v 5.2 SAS Institute, Cary, NC) on a personal computer. Statistical significance was set at $p = 0.05$.

Results

Compound screening for Nrf2 activators

High altitude illnesses, including Acute Mountain Sickness (AMS) and high altitude pulmonary and cerebral edema (HAPE and HACE), as well as improving general exercise performance at altitude have been effectively treated with a variety of drugs. These include: (1) carbonic anhydrase inhibitors, corticosteroids and methylxanthines to treat AMS symptoms (2–4, 26, 27); (2) calcium channel blockers and phosphodiesterase (PDE) inhibitors, to treat HAPE (2–4, 26, 27); (3) corticosteroids, to treat HACE (2–4, 26, 27) and; (4) endothelin receptor A (ETRA) antagonists to improve general exercise performance while at altitude (28, 29). Thus, we screened eleven compounds spanning these drug classes for potential off-target effects on Nrf2 activation (Table 1). In addition to Protandim, five compounds (methazolamide, nifedipine, amlodipine, ambrisentan, and sitaxentan) demonstrated an ability to induce Nrf2 (Table 1; Figure 1).

Compounds activate Nrf2 via PI3 Kinase

To determine if the off-target effects of Nrf2 activators occurred via a “nonoxidizing”, phosphoinositide 3-kinase (PI3K) dependent mechanism, the AREc32 cells were cotreated with Protandim, methazolamide, nifedipine, amlodipine, ambrisentan, or sitaxentan and the PI3Kinase inhibitor, LY294002. Treatment with LY294002 inhibited Nrf2-induced activation from each of these compounds (Figure 2)

In vivo

Pharmacological activation of Nrf2—To determine *in vivo* whether Nrf2 activator compounds showed evidence of Nrf2 nuclear translocation, we evaluated brain microvasculature by immunohistochemistry microscopy after 4 days of treatment (in sea level conditions) with selected compounds. In addition to vehicle control, as determined by our screen, drugs considered Nrf2 activators were evaluated against drugs within the same drug class but designated as non-Nrf2 activators. Western blot analysis did not reveal increased nuclear Nrf2 protein from whole brain nuclear lysates in any treatment group (data not shown), but immunohistochemistry analysis showed animals treated with Nrf2 activators had increased nuclear Nrf2 concentration in the brain microvasculature, while vehicle control and drugs designated as non-Nrf2 activators did not show Nrf2 translocation (Figure 3).

High altitude-induced Nrf2 activation—Acute high altitude exposure increases the oxidative stress burden (1, 8) while activation of the antioxidant genome via Nrf2 attenuates this stress. To quantify this relationship, we measure nuclear concentration of Nrf2 after 24 and 48 h of hypoxic exposure. Data obtained from Western blot analysis confirmed increased nuclear translocation of Nrf2 at 24 and 48 h of high altitude exposure. This was confirmed further by immunohistochemistry (Figure 4).

Evans blue dye determination of high altitude-induced brain vascular leak—Increased cerebral vascular leak is a primary symptom of AMS and HACE (1, 8). From the ten compounds tested, with the exception of dexamethasone, only compounds that showed an ability to activate Nrf2 attenuated high altitude-induced cerebral vascular leak. Compounds that did not activate Nrf2 had no effect on brain vascular leak (Table 1; Figure 5).

Respiratory rate and blood gases of acetazolamide and methazolamide—Increased arterial oxygenation could account for the differences observed between methazolamide and acetazolamide to decrease high altitude-induced brain vascular leak. Thus, to determine if methazolamide compared to acetazolamide had a more robust carbonic anhydrase inhibitory effect increasing arterial oxygenation, we evaluated the blood gases of rats treated with either acetazolamide (10 mg/kg I.V.) or methazolamide (4mg/kg I.V.) over a 2 h period at ambient pressure. Respiratory rate, PO₂, PCO₂, SaO₂ responses were almost identical with each drug treatment (Figure 6: A,B,C). Whereas in the acetazolamide treated animals after the initial drop in pH, it increased at the 90 and 120 min time points while it remained low in the methazolamide treated animals (Figure 6D).

In vitro

Protandim, methazolamide, nifedipine, and ambrisentan were chosen as representation for Nrf2 activators for further *in vitro* mechanistic evaluation.

Nrf2 activation in bovine brain microvascular endothelial cells (BMEC)—To confirm that Protandim, methazolamide, nifedipine, and ambrisentan induced Nrf2 activation in BMEC, Western blot and immunohistochemistry analysis were done. These results showed that compared to vehicle control, Protandim, methazolamide, nifedipine, and ambrisentan induced Nrf2 activation (Figure 7: A, B, C).

Hypoxia-induced Nrf2 activation *in vitro*—To determine when hypoxia-induced Nrf2 activation naturally occurred via the “injurious pathway”, nuclear translocation of Nrf2 was determined in BMEC exposed to hypoxia (3% O₂; 5% CO₂) for 0, 8, 24, and 48 hours. Our data showed that nuclear protein concentration of Nrf2 was increased at 8, 24, 48 h of hypoxia exposure (Figure 8: A, B).

Hypoxia-induced hydrogen peroxide (H₂O₂) production—We determined H₂O₂ production in BMEC exposed to 24 h of hypoxia. Compared to BMEC incubated in standard cell culture conditions, H₂O₂ was increased at 24 h and was attenuated with Protandim, methazolamide, or nifedipine treatment beginning 24 h prior to hypoxia exposure (Figure 9 A). Similar to permeability studies, these effects were reversed when cells were transfected with siRNA against Nrf2 prior to treatment (Figure 9A).

Hypoxia-induced BMEC monolayer permeability—Endothelial permeability increased in BMEC monolayers at 24 h of hypoxia exposure (Figure 8) and compared to vehicle control, Protandim, methazolamide, nifedipine, and ambrisentan attenuated hypoxia-induced BMEC leak (Figure 9B). These results were reversed when prior to treatment BMEC were transfected with siRNA against Nrf2 (Figure 9B).

Discussion

The main finding of this study was that high altitude-induced cerebral vascular leak in rats could be attenuated by prophylactic activation of the Nrf2 transcription factor. We also found that a subset of therapies (Protandim, methazolamide, nifedipine, ambrisentan, amlodipine, and sitaxentan) from drug classes commonly used to treat high altitude illnesses have the capability to activate Nrf2 as an “off-target” effect via phosphoinositide 3-kinase (PI3K) activation. Our *in vivo* observations were supported by *in vitro* data that showed prophylactic Nrf2 activation in bovine brain microvascular endothelial cells (BMEC) decreased hypoxia-induced hydrogen peroxide production and monolayer permeability. These effects were inhibited with siRNA against Nrf2. These data support the hypothesis that induction of the antioxidant genome prior to ascent to high altitude can protect against high altitude-induced reactive oxygen species (ROS) formation and microvascular permeability in the brain.

Recently it has been suggested that high altitude-induced free radical formation may be a culprit in the development of acute mountain sickness (AMS) by causing structural damage

to the blood brain barrier microvascular endothelium, neurons, glia, and induce cell swelling through down regulation of Na^+/K^+ -ATPase pump activities (1). As expected, rats exposed to a simulated high altitude were found to have increased whole brain nuclear Nrf2 concentration. This data indirectly validates that ROS production is increased in simulated high altitude conditions. Importantly, we observed significant Nrf2 activation only at the 48 h time point of hypoxia exposure. These data suggests the high altitude-induced ROS formation reaches a critical level with regard to altering cerebral endothelial cell barrier function prior to intrinsically activating Nrf2. This supports the notion that there is a level of oxidative stress injury prior to Nrf2 activation initiated through the oxidation of thiol groups on Keap1 (10).

In contrast to whole brain Nrf2 activation at 48 h of hypoxia exposure, our data showed pharmacological induction of Nrf2 in sea level conditions appeared to be isolated to the microvasculature. This is in accordance with our data that showed Nrf2 activators attenuated high altitude-induced cerebral endothelial cell barrier dysfunction. However, because high altitude induced ROS species effect many cell types in the brain, it is unclear whether prophylactic Nrf2 activation *only* in the brain microvasculature is enough to attenuate AMS in humans. This is an important issue that will need to be addressed in a clinical study.

Acetazolamide, a carbonic anhydrase inhibitor (CAI), is considered standard of care therapy for acute mountain sickness (AMS) (2, 3, 26, 27). However, our data showed that it was not an Nrf2 activator. Interestingly, another CAI, methazolamide, was a potent Nrf2 activator. Although not as commonly used as acetazolamide, methazolamide is an effective treatment for AMS (3, 30). It should be noted that in our AREc32 reporter cells, methazolamide increased luciferase activity at ~ 40 $\mu\text{g}/\text{ml}$, but a single dose of methazolamide at 4 and 10 mg/kg would achieve plasma concentration of ~3–5 $\mu\text{g}/\text{ml}$ with a plasma half-life of 3–5 h (31). However, CAI are known to be sequestered within red blood cell with a half of ~120 h. Because we did not conduct a pharmacokinetic analysis of plasma or red blood cell concentrations, we don't know exactly what the total plasma concentrations of CAI or the area under the curve after 4 days of CAI, and how this would compare to our in vitro screen. This will need to be completed to better understand if methazolamide has a unique property, other than acting as a carbonic anhydrase inhibitor, capable of inducing Nrf2. Methazolamide was initially designed to diffuse more readily into tissue than acetazolamide, and increased lipophilicity may account for its ability to activate Nrf2 compared to acetazolamide. For a review of the pharmacological differences between these compounds, we refer the reader to reference # (31). However, we can not entirely rule out the possibility that compared to acetazolamide, methazolamide's ability to decrease high altitude-induced cerebral vascular leak is not due to increased respiratory rate and arterial oxygenation. Thus, because of its unique effect to activate the antioxidant genome via Nrf2 activation, we propose that methazolamide may be more effective than acetazolamide at treating AMS symptoms, but this will need to be verified in clinical trials.

Pulmonary vasodilators, including calcium channel blockers (CCB) and phosphodiesterase (PDE) inhibitors, have been effectively used in both prevention and treatment of high altitude pulmonary edema (HAPE) (2–4, 28, 32). Whereas, endothelin receptor antagonists (ETRA) have been investigated to improve exercise capacity at high altitude (28, 29). Of the

pulmonary vasodilators we evaluated for Nrf2 activation, nifedipine, amlodipine, ambrisentan, and, to lesser extent, sitaxentan, were effective from the CCB and ETRA antagonist drug classes. Interestingly, our data did not show those PDE5 inhibitors, sildenafil or tadalafil, or the methylxanthine, theophylline, activated Nrf2.

It is unclear why the calcium channel blockers, nifedipine and amlodipine, activated Nrf2, but not Verapamil. Most likely this is related to the different chemical groups of these compounds. Nifedipine and amlodipine are dihydropyridines (DHP) whereas verapamil is a phenylalkylamine (PAA) (33). In terms of inhibiting the L-type calcium channels, DHPs bind at the N binding site and PAAs bind to the V binding site(33). However, it is unlikely that their binding domain on calcium channels is related to Nrf2 activation, but rather to other factors in the chemical groups such as lipid solubility and cell permeability (34). For a review of the differences between calcium channel blockers, we refer the reader to the manuscript by Araie and Mayama (33). Nifedipine is currently the accepted standard of care for HAPE and has proven to reduce the incidence of HAPE in susceptible individuals if taken prophylactically prior to ascent (2–4). However, in contrast to our data that would suggest prophylactic administration of nifedipine might protect against AMS, a previous study in humans showed nifedipine had no benefit on alleviating symptoms of AMS (35).

Because hypoxia induced vasoconstriction is due, in part, to endothelin, ETRA antagonists have gained interest as a potential therapeutic target towards improving exercise performance at high altitude (28, 29). For example, treatment with the ETRA antagonist, sitaxentan, improved lung diffusion and pulmonary vascular resistance in individuals exposed to acute hypoxia (28, 29). Thus, we evaluated two ETRA antagonists, ambrisentan and sitaxentan, for their ability to induce Nrf2. Our data showed that both compounds activated Nrf2, but ambrisentan induced Nrf2 four times greater than sitaxentan. Interestingly, both compounds reduced high altitude-induced cerebral vascular leak *in vivo* by ~40%. We did not test sitaxentan *in vitro*, but ambrisentan decreased hypoxia-induced H₂O₂ production and permeability in BMEC. There is no reason to believe sitaxentan would not have had a similar effect. Thus, our data would suggest ETRA drugs would have the same ability to protect against high-altitude ROS as nifedipine and may provide a better alternative to nifedipine than the PDE inhibitors, sildenafil and tadalafil.

Interestingly, dexamethasone, a corticosteroid, did not demonstrate an ability to activate Nrf2, yet significantly attenuated cerebral vascular leak. It is well known that dexamethasone is a potent anti-inflammatory and inhibits NF-κB. Both hypoxia and ROS are capable of NF-κB activation (24, 36). Thus, it is plausible that hypoxia-induced ROS can start an inflammatory response via NF-κB activation that would alter brain endothelial cell barrier function properties, which can be attenuated by dexamethasone. However, without additional studies, the pleiotropic nature of dexamethasone makes this connection unclear.

In conclusion, the results of the present study showed prophylactic activation of the transcription factor Nrf2 is a novel therapeutic strategy for the treatment of high altitude-induced cerebral vascular leak. In addition, our data showed that a subset of drugs, (chosen from drug classes typically used to treat high altitude illnesses) had the beneficial “off-target” effect of activating the Nrf2. Protandim, methazolamide, nifedipine, amlodipine,

ambrisentan, and sitaxentan activated Nrf2, decreased high altitude-induced cerebral vascular leak *in vivo*, and hypoxia-induced brain endothelial cell H₂O₂ production and permeability *in vitro*. It is unclear whether these compounds have a unique ability to activate Nrf2, other than a high lipophilicity characteristic that allow for easier diffusion across the cell membrane. This will need to be elucidated in future studies, which we are actively working on. Finally, we propose that when selecting therapies for treatment of high altitude illnesses, the subset of drugs exhibiting the Nrf2 activation trait may provide additional protection and benefit against oxidative stress and AMS than those that do not. However, this will need to be confirmed in clinical trials.

Acknowledgments

Sources of funding.

This study was supported by the Defense Advanced Research Projects Agency (DARPA) Rapid Altitude and Hypoxia Acclimation contract N66001-10-c-2134.

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Figure 1.

Representative line graph of compound-induced luciferase activity in a Nrf2 reporter cell line. Breast cancer cells (MCF7) were stably transfected with a luciferase gene attached to the antioxidant response element (ARE, the Nrf2 promoter) to create the stably transfected AREc32 reporter cell line. AREc32 were treated with compounds listed in Table 1. Methazolamide compared to acetazolamide activated luciferase starting at $\sim 40 \mu\text{g/ml}$ and peaked $\sim 125 - 200 \mu\text{g/ml}$

Figure 2.

AREc32 reporter cells were treated with the PI3K inhibitor LY294002 (20–100 μ M) in the presence of drugs that activated Nrf2. LY294002 blocked Nrf2 activation from: Protandim (30 μ g/mL), methazolamide (120 μ g/mL), nifedipine (7 μ g/mL), amlodipine (4 μ g/mL), or ambrisentan (4 mg/mL).

PI3K inhibition blocks pharmacological induced Nrf2 activation

Figure 3.

Immunohistochemistry overlay of Nrf2 activation in rats treated for 4 days with vehicle control, acetazolamide (40 mg/kg), verapamil (10mg/kg), Protandim (10 mg/kg), methazolamide (4 mg/kg), nifedipine (2 mg/kg), or ambrisentan (1 mg/kg). Only treatment with Protandim, methazolamide, nifedipine, or ambrisentan showed evidence of Nrf2 activation. Green-Factor eight; Red-Nrf2; Blue-DAPI

In vivo Nrf2 activation in Brain Tissue

Figure 4.

A) Western blot analysis of Nrf2 concentration from whole brain nuclear lysates in rats exposed to either normoxia, 24 or 48 h simulated high altitude (17,000 ft). **B)** Immunohistochemistry microscopy analysis of Nrf2 activation in rats exposed to normoxia or 24 or 48 h of hypoxia. Red-Nrf2; Blue-DAPI. White arrows show areas that stained positive for Nrf2. Original magnification 60x * $p = 0.03$ vs. normoxia in vivo time course of high altitude-induced brain Nrf2 activation

Figure 5.

High altitude-induced cerebral vascular leak in rats treated with acetazolamide 10 to 40 mg/kg I.P. and methazolamide 1 to 10 mg/kg. * $p < 0.04$.

Figure 6.

Respiratory and blood gas analysis of rats treated with acetazolamide (10 mg/kg) or methazolamide (4 mg/kg). **A)** respiratory rate; **B)** blood oxygen saturation; **C)** blood PO₂ and PCO₂, and; **D)** blood pH values. * $p < 0.05$ vs. acetazolamide.

Figure 7.

Analysis of Nrf2 nuclear concentration in Bovine Brain Microvascular Endothelial (BMEC) cells treated for 24 h with Protandim (125 $\mu\text{g/mL}$), methazolamide (100 $\mu\text{g/mL}$), nifedipine (7.5 $\mu\text{g/mL}$), or ambrisentan (40 $\mu\text{g/mL}$). **A)** Original western blot showing both nuclear and cytosolic concentration of Nrf2. **B)** Representative Western blot showing only nuclear fraction spliced from “A” and corresponding densitometry. **C)** Immunohistochemistry microscopy analysis of Nrf2 nuclear translocation in BMECs. Original magnification 60x * $p < 0.001$ vs. CTRL; ** $p = 0.045$ vs. CTRL.

Drug-induced Nrf2 activation in BMECs in normoxic environment.

Figure 8.

A) Western blot analysis of hypoxia-induced Nrf2 activation in Bovine Brain Microvascular Endothelial cells (BMEC) exposed to 3% oxygen for 0, 8, 24, and 48 hours. **B)**

Immunohistochemistry microscopy analysis of Nrf2 activation in BMECs exposed to 3% oxygen for 0, 8, 24 and 48 hours. Original magnification 60X. White arrow show regions that stained positive for Nrf2. * p < 0.001.

In vitro time course of hypoxia-induced Nrf2 activation.

A. Hydrogen Peroxide Production **B. TER** **C. Nrf2 siRNA** **Figure 9.**

A) Hydrogen peroxide production in Bovine Brain Microvascular Endothelial Cells (BMECs) treated with Protandim (125 µg/mL), methazolamide (100 µg/mL), nifedipine (7.5 µg/mL), or ambrisentan (40 µg/mL) in the presence or absence of siRNA against Nrf2. **B)** Hypoxia-induced leak in BMECs exposed to 3% oxygen and treated with either Protandim (125 µg/mL), methazolamide (100 µg/mL), nifedipine (7.5 µg/mL), or ambrisentan (40 µg/mL) in the presence or absence of siRNA against Nrf2. **C)** Representative Western blot from BMEC nuclear lysates showing Nrf2 knockdown. ** p<0.001 vs. Nx CTRL; * p<0.05 vs. siRNA against Nrf2.

siRNA Experiments

Table 1

Compounds with ability to activate Nrf2 *in vitro* and *in vivo*.

AGENT	DOSE (<i>In vitro</i>) screening µg/ml	MAXIMAL Nrf2 FOLD INDUCTION In AREc32 cell line (max response)	DOSE (<i>In vivo</i> , LP) mg/kg		% DECREASE CEREBRAL VASCULAR LEAK Numbers in () signify increased leak.		DRUG CLASS
			Low	High	Low	High	
PROTANDIM	0-50	20 (30 µg/ml)	10*	10*	30±15	62±5 [#]	Nrf2 ACT.
ACETAZOLAMIDE	0-200	<2	4	10	(10)±15	5±12	CAI
METHAZOLAMIDE	0-200	17 (150 µg/ml)	4	10	45±15*	52±9**	CAI
NIFEDIPINE	0-14	10 (7 µg/ml)	4	10	20±14	50±15*	CCB
VERAPAMIL	0-14	<2	4	10	5 ±10	(10) ±6	CCB
AMLODIPINE	0-14	8 (6 µg/ml)	NA	NA	NA	NA	CCB
SILDENAFIL	0-50	<2	1	5	(17)±25	(23)±25	PDE-5 IN.
TADALAFIL	0-50	<2	NA	NA	NA	NA	PDE-5 IN.
AMBRISANTAN	0-10	13 (2 µg/ml)	0.5	2	(15)±20	32±5*	ETRA
SITAXENTAN	0-10	3 (4 µg/ml)	1	10	36 ± 13*	13±9	ETRA
DEXAMETHASONE	0-10	<2	0.1	1	70±19**	75±27*	CORT.
THEOPHYLLINE	0-10	<2	30	60	+18±6	(2)±15	METHXYN

Gray rows - signify drugs that showed ability to induce Nrf2 activation.

Protandim - *rats were all treated with 10 mg/kg. Low dose is considered to be rats treated day of altitude exposure and at 24 h (2 doses) and high Dose is considered to be rats treated two days prior to altitude exposure, day of and at 24 h (4 doses). [#]protandim p=0.001 vs vehicle control.

Methazolamide - *Low dose p= 0.051 and **high dose p=0.019 vs. vehicle control

Nifedipine - *high dose p=0.04 vs. vehicle control

Ambrisentan - *high dose p= 0.044 vs. vehicle control

Sitaxentan - *low dose p=0.045 vs. vehicle control

Dexamethasone - *p=0.03 vs. vehicle control

CAI – Carbonic anhydrase inhibitor

CCB – Calcium channel blocker

PDE – Phosphodiesterase inhibitor

ETRA- Endothelin receptor A antagonist

Cort. – Corticosteroid

Methxyn – Methylxanthine