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Original Contribution

Nrf2 activation: A potential strategy for the prevention of acute mountain sickness

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ABSTRACT

Reactive oxygen species (ROS) formed during acute high altitude exposure contribute 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. Drugs commonly used to treat AMS were screened with a luciferase reporter cell system for their effectiveness to activate Nrf2, as well as being 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 (BMECs) to determine if they attenuated hypoxia-induced ROS production and monolayer permeability. Of nine 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 BMECs before 24 h hypoxia exposure attenuated hypoxic-induced hydrogen peroxide production and permeability. 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.

Acute mountain sickness (AMS) is a well-described syndrome that affects 60% of unacclimated individuals ascending to altitudes above 8000 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 are the primary causal factors of AMS, but the exact mechanism(s) by which hypoxia induces AMS is unclear. Recently, it has been suggested that hypoxia-induced cerebral vascular leak and subsequent astrocyte swelling in the trigeminal areas play 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 against increased oxidative stress is the activation of the 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, the 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-

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driven cellular defense system against oxidative stress. Nrf2 is held inactive in the cytosolic compartment by its association with the 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 ROS. The second mechanism involves the phosphorylation of Nrf2, which has been proposed to both stabilize Nrf2 and 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 before 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], before and during high altitude exposure would attenuate high-altitude-induced cerebral vascular leak in vivo as well as decreasing hypoxia-induced ROS and brain microvascular endothelial cell (BMEC) permeability in vitro. Secondarily, 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 either by Protandim or from "off-target" effects of other compounds before 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, USA) and housed in the University of Colorado Anschutz Medical Campus's Center for Comparative Medicine (elevation 1609 m; 5280 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 the University of Colorado at 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 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 the PI3K inhibitor LY294002 (Cell Signaling Technologies, Cat. No. 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 intraperitoneal (ip) injection, and tested in a dose–response fashion of high and low doses in the respective 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

Table 1
Compounds with ability to activate Nrf2 in vitro and in vivo.

Agent	Dose (in vitro screening, μg/ml)	Maximal Nrf2 fold induction in AREc32 cells (max response)	Dose (in vivo, ip, mg/kg)		% Decrease in cerebral vascular leak[a]		Drug class
			Low	High	Low	High	
Protandim	**0–50**	**20 (30 μg/ml)**	**10**[b]	**10**[b]	**30 ± 15**	**62 ± 5**[c]	**Nrf2 act.**
Acetazolamide	0–200	<2	4	10	(10) ± 15	5 ± 12	CAI
Methazolamide	**0–200**	**17 (150 μg/ml)**	**4**	**10**	**45 ± 15**[d]	**52 ± 9**[e]	**CAI**
Nifedipine	**0–14**	**10 (7 μg/ml)**	**4**	**10**	**20 ± 14**	**50 ± 15**[f]	**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	PDE5 in.
Tadalafil	0–50	<2	NA	NA	NA	NA	PDE5 in.
Ambrisentan	**0–10**	**13 (2 μg/ml)**	**0.5**	**2**	**(15) ± 20**	**32 ± 5**[g]	**ETRA**
Sitaxentan	**0–10**	**3 (4 μg/ml)**	**1**	**10**	**36 ± 13**[h]	**13 ± 9**	**ETRA**
Dexamethasone	0–10	<2	0.1	1	70 ± 19[i]	75 ± 27[i]	Cort
Theophylline	0–10	<2	30	60	+18 ± 6	(2) ± 15	Methxyn

Boldface signifies drugs that showed ability to induce Nrf2 activation. CAI, carbonic anhydrase inhibitor; CCB, calcium channel blocker; PDE, phosphodiesterase inhibitor; ETRA, endothelin receptor A antagonist; Cort, corticosteroid; Methxyn, methylxanthine; act., activator; in., inhibitor.

[a] Numbers in parentheses signify increased leak.

[b] Protandim—rats were all treated with 10 mg/kg. Low dose is considered to be rats treated on the day of altitude exposure and at 24 h (two doses) and high dose is considered to be rats treated 2 days before altitude exposure, the day of, and at 24 h after (four doses).

[c] $p = 0.001$ vs vehicle control.

[d] $p = 0.051$ vs vehicle control.

[e] $p = 0.019$ vs vehicle control.

[f] $p = 0.04$ vs vehicle control.

[g] $p = 0.044$ vs vehicle control.

[h] $p = 0.045$ vs vehicle control.

[i] $p = 0.03$ vs vehicle control.