

Combining the off-target effects of widely available NRF2-inducing drugs may result in new
therapies for Acute Mountain Sickness

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ABSTRACT

Under normal physiology, the brain and the vasculature form a tight, controlled junction commonly known as the blood-brain barrier; which only allows the flow of nutrients and other essential compounds. However, under stressed conditions, this barrier gets loose, allowing serum and large proteins to flow indiscriminately through it. This can lead to brain edema, which if untreated, can be fatal. This is precisely the primary symptom associated with Acute Mountain Sickness (AMS), a disturbance affecting 60% of individuals ascending to high altitude. Reactive oxygen species (ROS) have a well-defined role in the development of this pathology. They are thought to provide the chemical stress needed to advance the disease. In the past, drugs with antioxidant properties have been used to partially alleviate the symptoms associated with AMS¹. In this study we focus on increasing the body's innate antioxidant status, by up-regulating the genes responsible for this antioxidant response. One of the ways to do this is by targeting the NRF2 pathway, which is responsible for 95% of the body's antioxidant response. Bovine brain microvascular endothelial cells (BBMEC) were treated with Methazolamide, Nifedipine and Protandim, and NRF2 levels were determined. The antioxidant state of BBMECs was determined using an amplex red assay, in order to elucidate a correlation between the drugs and NRF2 induction. In addition, Sprague Dawley rats were treated with the same drugs, and blood-brain barrier permeability and NRF2 induction were determined. Treatment with these drugs increased NRF2 stabilization both *in vivo* and *in vitro*. Additionally, treated rats showed reduced vascular leak compared to a control. An assessment of ROS depletion *in vitro* revealed an increased antioxidant state in treated BBMECs. Our data suggest that

drugs that induce the antioxidant response through the NRF2 pathway may be good candidates to abate the symptoms associated with AMS.

Key words: acute mountain sickness, NRF2, vascular leak, edema, off-target effects

INTRODUCTION

Acute mountain sickness (AMS) is a syndrome that affects 60% of individuals ascending into high altitude. AMS reduces athletic performance by inducing pulmonary and cerebral vascular leak, which in turn cause the headache and nausea associated with this syndrome. If left untreated, this syndrome can prove fatal, by deteriorating into pulmonary and/or cerebral edema. Previous studies suggest that hypoxia triggers the release of reactive oxygen species (ROS), thus providing the chemical stress needed for the development of AMS and vascular leak. The organism's natural response to this "hypoxic insult" is to modulate the Nuclear factor (erythroid-derived 2)-related factor 2 (NRF2) pathway, which controls the transcription of about 90% of the body's antioxidants. NRF2 is constantly degraded by KEAP1 dependent ubiquination. However, in the presence of ROS stress, it is stabilized by the inactivation of KEAP1, thus allowing for cytoplasmic and subsequent nuclear accumulation of NRF2, where it can act on the antioxidant response element (ARE) to transcribe oxidative stress genes. Previous in-vitro experiments in our laboratory have shown that drugs (Methazolamide, Nifedipine and Protandim) used (individually or in combination) to abate AMS symptoms have the off-target effect of inducing NRF2 activation. Using an Evan's blue (EB) assay, these results were associated to an in-vivo model by determining the contribution these drugs have in reducing brain vascular leak.

Our studies have shown the contribution Methazolamide, Nifedipine and Protandim have in reducing pulmonary and cerebral vascular leak and inducing the stabilization of NRF2. In-vitro, we performed an Amplex red assay with bovine brain microvascular endothelial cells; by analyzing H₂O₂ production between treatments and untreated control, we determined the effect these drugs have on ROS depletion. We evaluated the drugs' ability to stabilize NRF2, using immunohistochemistry (IHC) in BBMVECs.

In-vivo, we performed IHC to study the rodent's response to NRF2 activating drugs; brain/vascular permeability of treated animals was compared to control using fluorescein sodium and factor 8 counterstaining. We will used western blot technique to quantify these results not only for NRF2 stabilization, but also for the expression of "downstream" (HO-1) proteins in order to elucidate-the molecular pathways drugs use to stabilize NRF2.

We hypothesized that through the stabilization of NRF2, Methazolamide, Nifedipine and Protandim (individually and in combination) will reduce vascular endothelial cell permeability by increasing the levels of genes responsible for an oxidative stress response both in cells and in tissues.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats ($n = 22$) weighting 380–450g and 8 –10 weeks of age were obtained from a commercial vendor (Charles River, Wilmington, MA) and housed in the University of Colorado Health Sciences Center's Center for Laboratory Animal Care (elevation: 5,280 ft). Animals were allowed access to food and water ad libitum, and kept on a 16:8-h day-

night cycle. The animals were allowed to adjust to their environment for at least 7 days before starting any experiment. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver School of Medicine.

Cell cultures

Bovine brain micro endothelial cells (BBMEC) were obtained from Cell Applications, INC. (No. B840K-05) and cultured in BBMEC growth medium (No. B819-500; Cell Applications, INC.). For all experiments BBMEC were used between passages 4-8, grown to confluence, and cultured under standard conditions. For each experiment, BBMEC were randomly divided into three groups: normoxia control, hypoxia control, and hypoxia treated. Hypoxia cell treatments consisted of three subgroups: Methazolamide (100ug/mL), Nifedipine (7ug/mL), and combo (100ug/mL-Meth, 2.5ug/mL-NFP). Data were collected from two separate cell culture preparations on the same day (n=2).

Hypoxic Incubation

Cell cultures were placed in an anaerobic cell culture chamber (3% O₂) equipped with an antechamber, which ensured that cells remain in a constant hypoxic environment. All necessary hydrogen peroxide assays were conducted inside the hypoxic chamber to avoid fluctuations in oxygen concentration.

Hydrogen peroxide measurement assay

An indirect measure of O₂⁻ (ROS) production was obtained by assessing hydrogen peroxide production using an Amplex red kit (10-acetyl-3,7-dihydrooxphenoxazine, A-22188; Molecular Probes, Eugene, OR, USA). The reaction of Amplex red with horseradish peroxidase results in resorufin, a compound with an absorption and fluorescence emission maxima of 571 and 585 nm. BBMEC were seeded into 96-well plates, and exposed to either control or treated conditions. Cell media was replaced after 24 hrs, H₂O₂ production was measured and normalized to a standard curve.

Immunohistochemical staining for NRF2

BBMEC were seeded into an eight well microscope slide. After treatment, cells were immuno-stained for 24 hrs. at 4°C with a polyclonal rabbit anti-rat NRF2 antibody (1:100, Abcam PLC, Cambridge, MA), and with a polyclonal rabbit anti-rat Von Willebrand factor antibody (1:100, Abcam PLC, Cambridge, MA). Slides were mounted Vectashield with DAPI (Vector Labs) and visualized with a Nikon Eclipse Ti fluorescence microscope.

In Vivo Methods

Experimental groups

The vascular leak study consisted of five animal groups ($n = 20$) in hypoxic (alt 18,000 ft.) conditions: control (5:1 PEG400 in 70% EtOH); methazolamide treated (7mg/kg); nifedipine treated (10mg/kg); combined treated (2mg/kg methazolamide, 10 mg/kg nifedipine) treated; and

Protandim treated (20 mg/kg). The drugs were administered by a daily intraperitoneal injection for 4 days.

Sodium Fluorescein Permeability Study

On day 5 of the vascular leak study, 20 rats were injected intravenously with 1 mL of 2% sodium fluorescein (NaFl) tracer (Sigma, St. Louis, MO) dissolved in 0.9% saline. The tracer was allowed to circulate for 10 min to obtain peak fluorescence in animal brains². At 10 min, all intravascular marker was washed out by cardiac perfusion with PBS. The brains were removed, frozen in OCT then sliced into 4-μm serial cryostat sections. The slides were preserved at -4°C until used for immunohistochemistry.

Tissue harvesting

Animals were anesthetized with intraperitoneal sodium pentobarbital followed by cardiac perfusion with 10cc PBS. Brain was either snap frozen or fixed in formalin for 24 hours, then transferred to EtOH before being sent to histology for tissue embedding.

Western blot

Nuclear and cytoplasmic brain protein was isolated from homogenized tissue using a NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Isolated nuclear and cytoplasmic proteins were fractioned by electrophoresis using a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), and blocked with

5% dry milk in Tris buffered saline Tween 20 (TBST). They were incubated with monoclonal rabbit anti-mouse NRF2 primary antibody (1:1000, Abcam PLC, Cambridge, MA), monoclonal rabbit anti-mouse HO-1 primary antibody (1:1000, Pierce Biotechnology, Rockford, IL, USA), and β-actin (1:1000, Sigma, St. Louis, MO), diluted in 5% milk protein in TBST overnight at 4°C. The membrane was then probed with goat anti-rabbit horseradish peroxidase-conjugated IgG antibody (1:2000, Chemicon, Temecula, CA, USA). Blots were developed using the Supersignal West Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

Immunohistochemistry

For the NRF2 activation study; paraffin-embedded brain slices were immuno-stained for 24 hours at 4°C with a polyclonal rabbit anti-rat NRF2 antibody (1:100, Abcam PLC, Cambridge, MA), and with a polyclonal rabbit anti-rat Von Willebrand factor antibody (1:100, Abcam PLC, Cambridge, MA). For the NaFl permeability study, OCT-embedded brain slices were incubated overnight at 4°C with a polyclonal rabbit anti-rat Von Willebrand factor antibody (1:100, Abcam PLC, Cambridge, MA). Brain slides were mounted using Vectashield with DAPI (Vector Labs) and visualized with a Nikon Eclipse Ti fluorescence microscope.

RESULTS

Methazolamide, nifedipine and Protandim increased brain NRF2 stabilization in vivo.

Changes in NRF2 protein expression in rodent brain tissue were observed following exposure to the NRF2-inducing drugs methazolamide, nifedipine, and a combination of both. In **Figure 1**, a

significant increase in nuclear NRF2 activation was observed in the western blot when compared to control. A densitometry analysis performed on a representative blot showed significant increases in NRF2 stabilization in treated animals compared to control. This is coupled to a nuclear to cytosolic NRF2 ratio analysis, which shows an increased ratio in treated rats compared to control. In addition **Figure 2**, depicts in vivo NRF2 stabilization in representative slides of control and treated groups. A significant qualitative increase of NRF2 presence between treated and control was observed.

Methazolamide, nifedipine and Protandim increased hypobaric hypoxia-induced brain HO-1 activation via the NRF2 pathway in vivo. Changes in HO-1 protein expression in rodent brain tissue were observed following exposure to the NRF2-inducing drugs methazolamide, nifedipine, and a combination of both. In **Figure 3**, a significant increase in nuclear HO-1 activation can be observed in treated groups compared to control. A densitometry analysis performed on a representative blot showed significant increases in HO-1 stabilization in treated animals compared to control.

Methazolamide, nifedipine and Protandim decreased hypobaric hypoxia-induced brain vascular leak in vivo. When examining immuno-stained rodent brain samples under fluorescence microscopy, a large “leak episode” is observed around the control vessel. When compared to the treated samples, a significant decrease in blood-brain permeability can be inferred. **Figure 4** shows representative photographs of each group.

Methazolamide and nifedipine increased hypoxia-induced BBMEC NRF2 activation in vitro. **Figure 5**, shows the changes in NRF2 stabilization in BBMEC following exposure to the NRF2-inducing drugs methazolamide, and a combination of methazolamide and nifedipine. A significant increase in nuclear NFR2 stabilization is observed in treated samples when compared to the control.

Methazolamide, nifedipine and Protandim decreased hypoxia-induced BBMEC H₂O₂ production in vitro. **Figure 6** shows the changes in H₂O₂ production in BBMEC following exposure to the NRF2-inducing drugs methazolamide, nifedipine, a combination of both and Protandim was observed. A significant decrease in H₂O₂ production is observed in treated samples when compared to the control.

DISCUSSION

The results presented above suggest that by targeting NRF2 for up-regulation, the organism's innate antioxidant status is increased. Our NRF2 western blot paired with the HO-1 western and the in-vivo NaFl studies suggest a strong correlation between NRF2 up-regulation, decreased oxidative stress and decreased hypobaric hypoxia-induced vascular leak. Our findings of increased HO-1 transcription further support the notion that these drugs are increasing the organism's innate antioxidants in an NRF2-dependent manner. In our findings we saw increased HO-1 nuclear expression. This is likely due to an incomplete separation of the nuclear and cytosolic components during the extraction process. We propose that these drugs decrease vascular leak through a non-injurious pathway. Our findings with the in vivo microscopy

techniques suggest that the treated animals experienced less vascular leak, which translates into a reduction of the symptoms associated with AMS. Furthermore, our in vitro data strongly supports the idea of having the decrease in vascular permeability as a direct cause of increased NRF2 activation. The fact that we saw a decreases in H₂O₂ production in vitro strongly suggests that NRF2 up-regulation is occurring by a pathway other than the one induced by ROS, since this injurious pathway is the natural route for inducing nuclear NRF2 in the organism. (**Supp. Figure 1**).

In the future, it is essential to delve into the mechanisms behind the abatement of vascular leak by these drugs as the end result. In addition, our team is working on obtaining in vitro data for vascular permeability using BBMEC cell inserts. As mentioned earlier, we believe NRF-2 inducing drugs act through a non-injurious pathway that needs to be elucidated.

REFERENCES

1. Bailey, D. M., Bärtsch, P., Knauth, M., Baumgartner, R. W., 2009, Emerging concepts in acute mountain sickness and high---altitude cerebral edema: from the molecular to the morphological, *Cellular and Molecular Life Sciences*, v. 66, p.3583-3594
2. J Appl Physiol. 2009 Oct;107(4):1348-56.

AKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Fig. 1 A) Changes in NRF2 protein expression in rodent brain tissue following exposure to the NRF2-inducing drugs Methazolamide, Nifedipine, and a combination of both. A significant increase in nuclear NRF2 activation can be observed when comparing treated groups to control. B) Densitometry analysis performed on NRF2 protein expression, comparing treated groups to control. A significant increase in nuclear NRF2 activation in the treated groups is observed. C) Nuclear to cytosolic NRF2 ratio. When dividing nuclear concentration NRF2 percent over that of cytosol, an increase in the ratio between both compartments of the cell is observed. This embodies the translocation of NRF2 from cytosol to nucleus.

Fig. 2 A) Changes in HO-1 protein expression in rodent brain tissue following exposure to the NRF2-inducing drugs Methazolamide, Nifedipine, and a combination of both. A significant increase in nuclear HO-1 activation can be observed when comparing treated groups to control. B) Densitometry analysis performed on HO-1 protein expression, comparing treated groups to control. A significant increase in nuclear HO-1 activation in the treated groups is observed.

Fig. 3 In vivo NRF2 activation. Changes in NRF2 activation in rodent brain tissue following exposure to the NRF2-inducing drugs Methazolamide, Nifedipine, a combination of both and Protandim. A significant increase in nuclear NRF2 stabilization is observed in treated samples when compared to the control.

Fig. 4 In vivo vascular leak assessment. Changes in blood-brain barrier permeability can be assessed using the ability of the fluorescent tracer NaFl to cross the barrier as reference

between control and treated groups. A large “leak episode” is observed at the bottom left area outside the vessel of the control animal. Comparing this to the treated groups, a significant decrease in leak is observed.

Fig. 5 In vitro NRF2 activation. Changes in NRF2 stabilization in BBMEC following exposure to the NRF2-inducing drugs methazolamide, and a combination of methazolamide and Nifedipine. A significant increase in nuclear NRF2 stabilization is observed in treated samples when compared to the control.

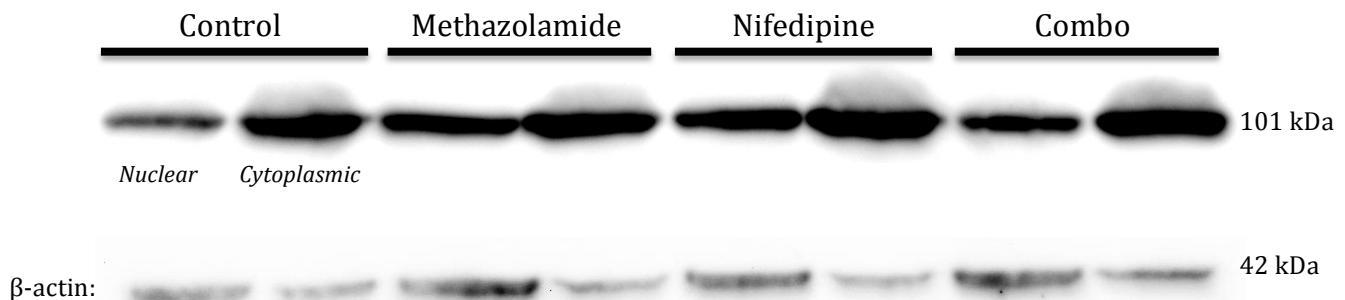
Fig. 6 A) In vitro ROS quantification. Untreated brain micro endothelial cells showed increased H₂O₂ production in hypoxic conditions compared to a normoxic control. **B)** Changes in ROS production in BBMEC following exposure to the NRF2-inducing drugs Methazolamide, Nifedipine, a combination of both and Protandim are observed. A significant decrease in H₂O₂ production is observed in treated samples when compared to the control.

Supplementary Fig. 1 Adapted from Taguchi et al. Gene Cell **16**:123 (2011). Proposed pathway for NRF2 activation. The injurious (ROS-mediated) pathway is shown, as well as a possible mechanism of action though which NRF2-inducing drugs act.

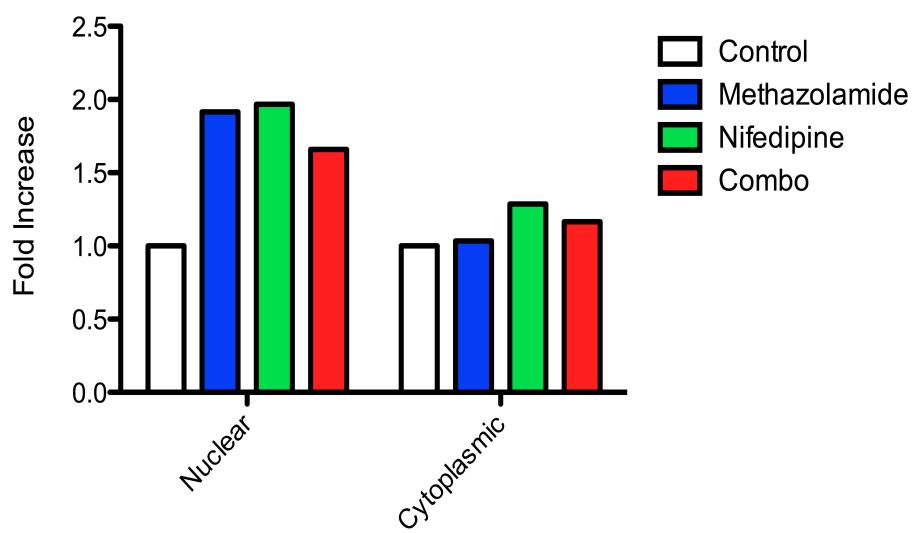
Supplementary Fig. 2 A) BMEC were treated with Methazolamide, Nifedipine, and Protandim in order to determine NRF2 induction at different concentrations. B) Evans blue assay performed on rats treated with Methazolamide, Nifedipine and Protandim in order to determine the drugs’ effect on vascular permeability.

Rat Brain Tissue

A. S



B.



C.

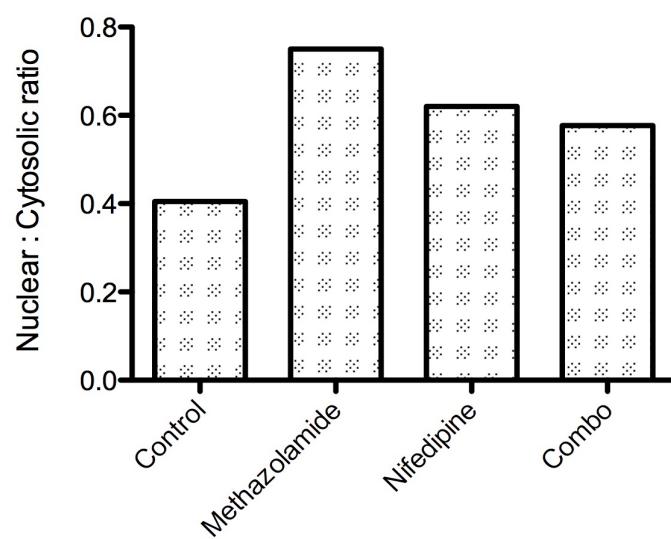
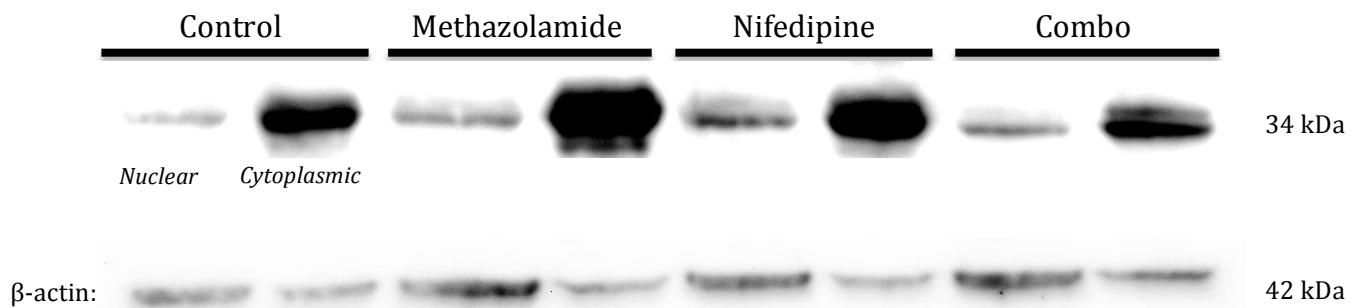


Figure 1

A.



B.

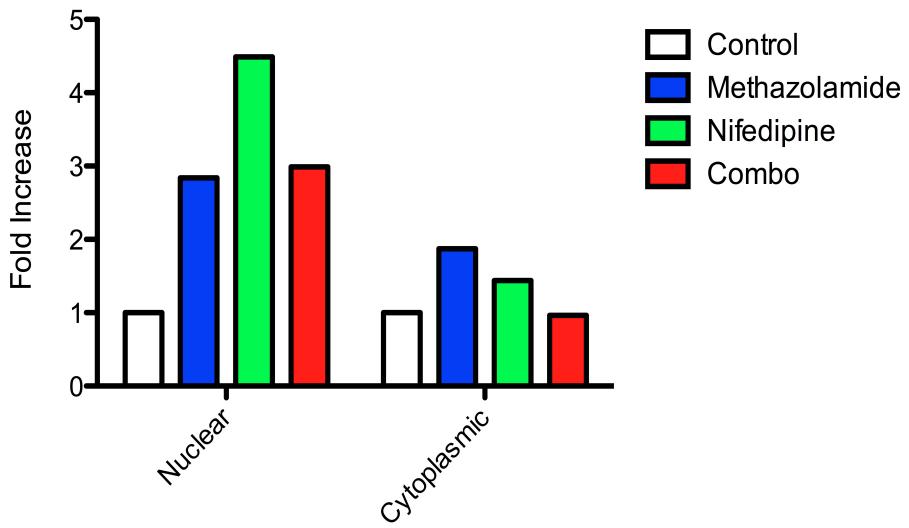


Figure 2

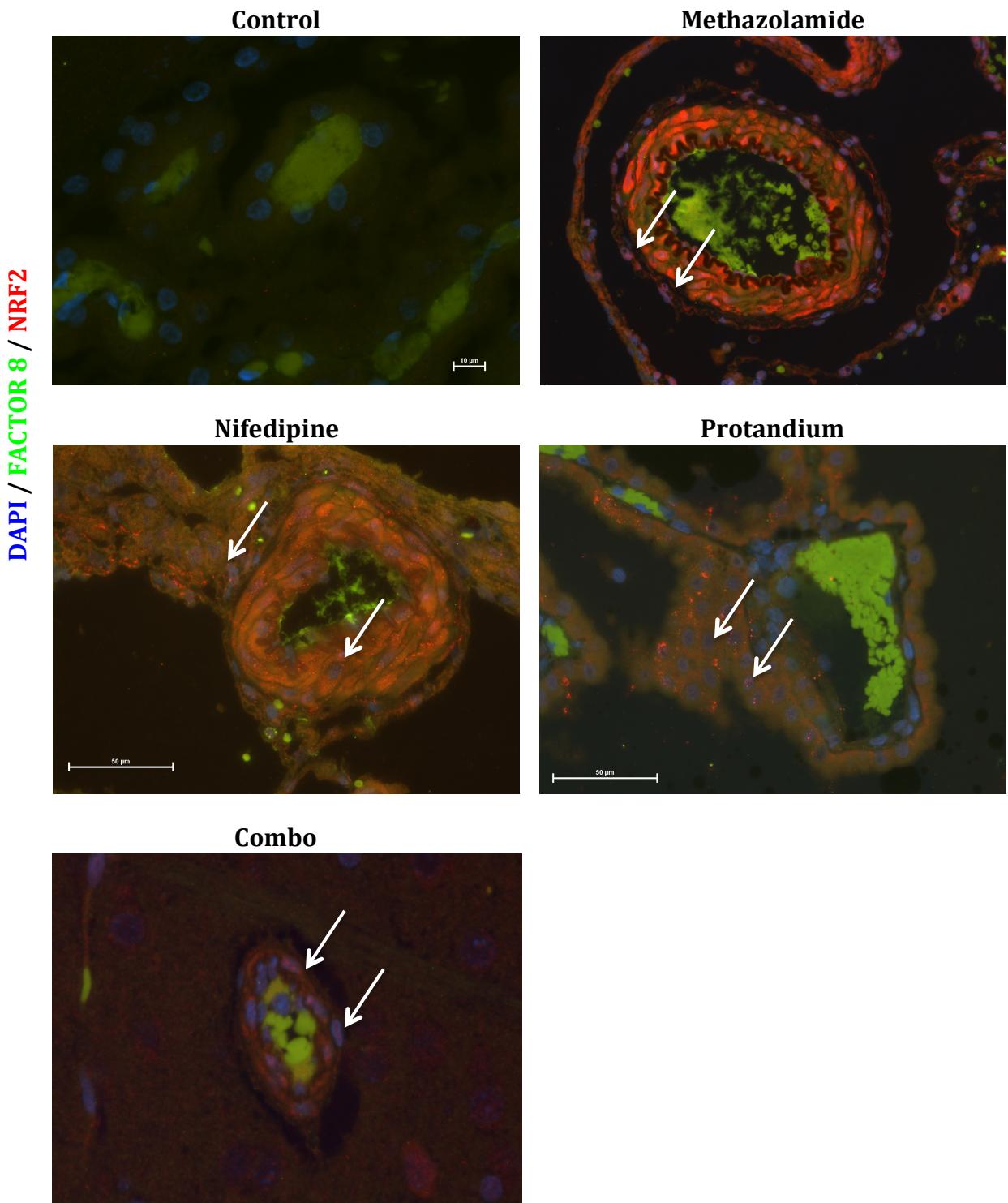


Figure 3

DAPI / NaFl / FACTOR 8

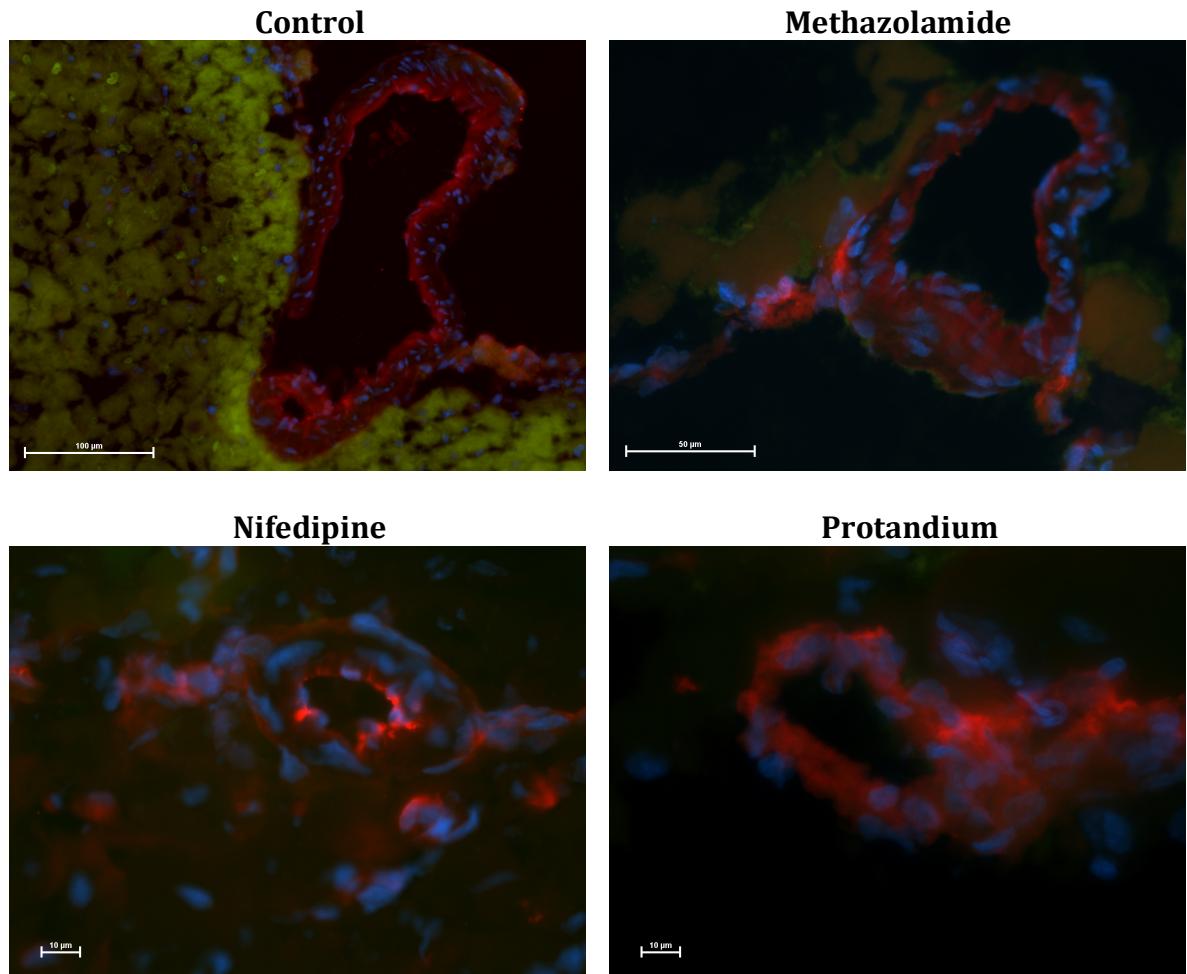


Figure 4

A. BMEC

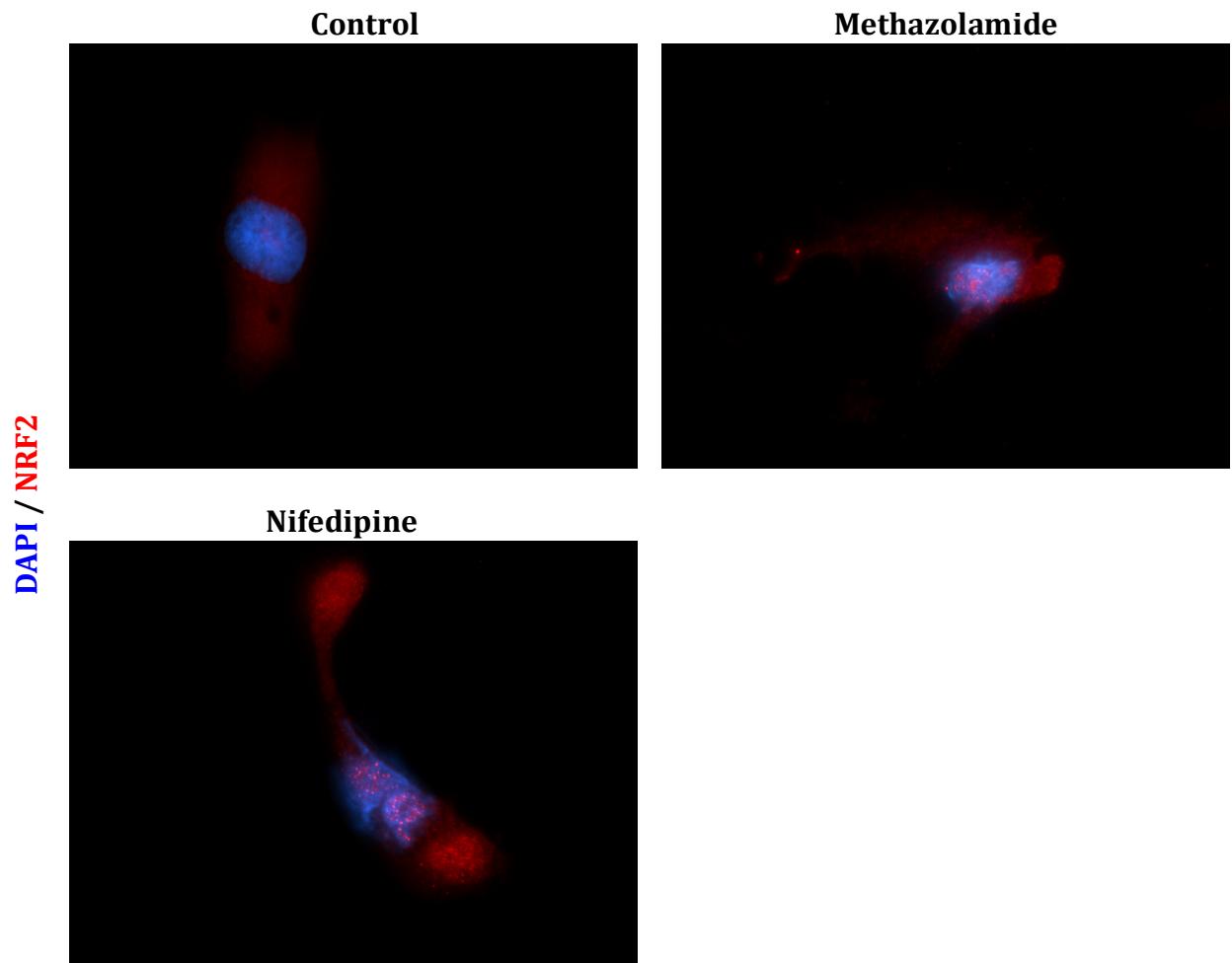
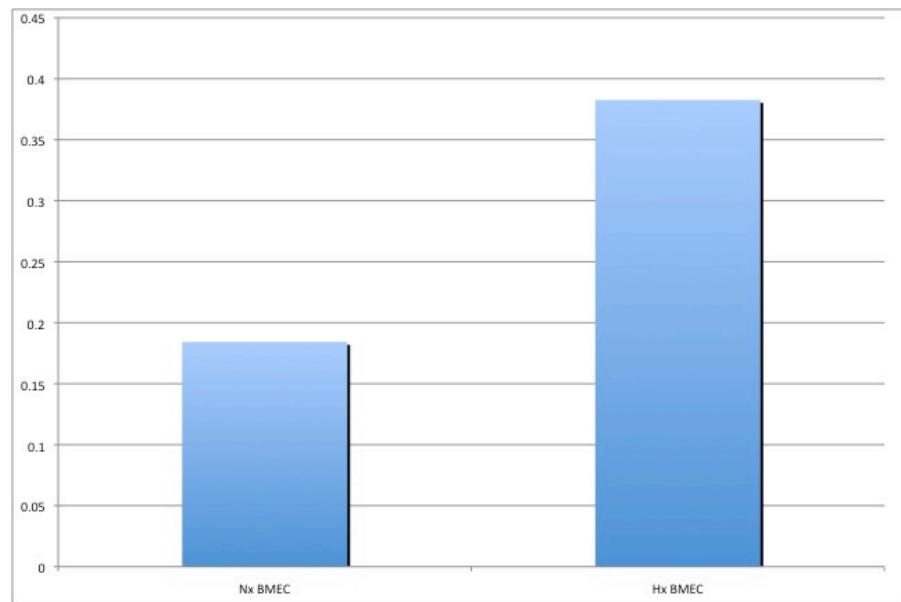


Figure 5

A. BMEC



B.

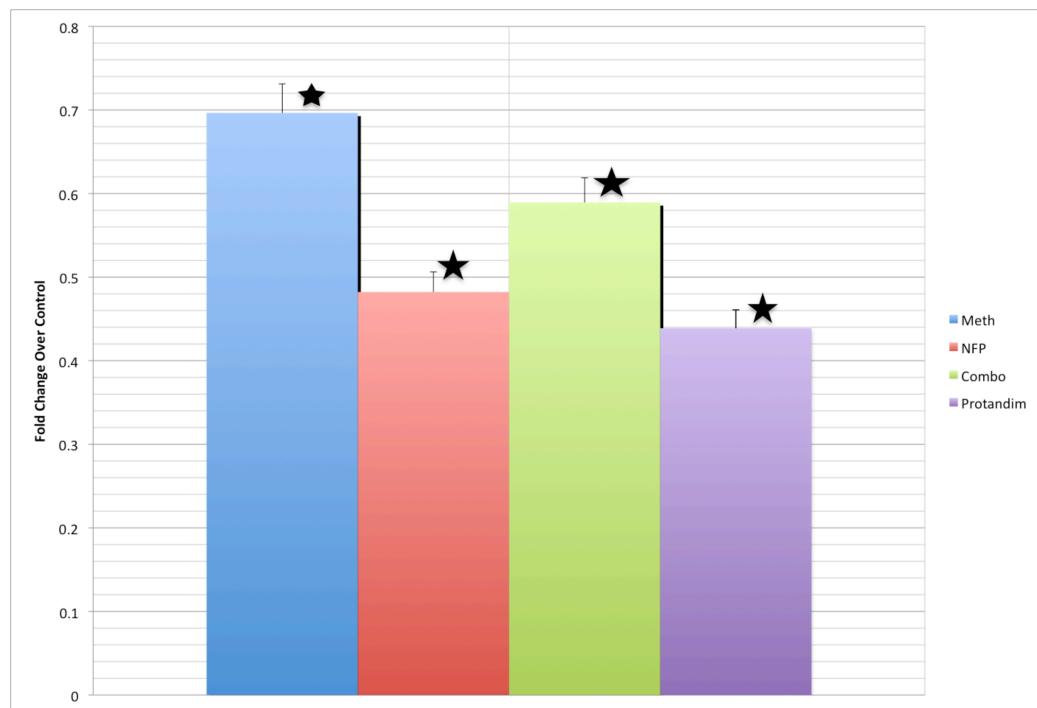
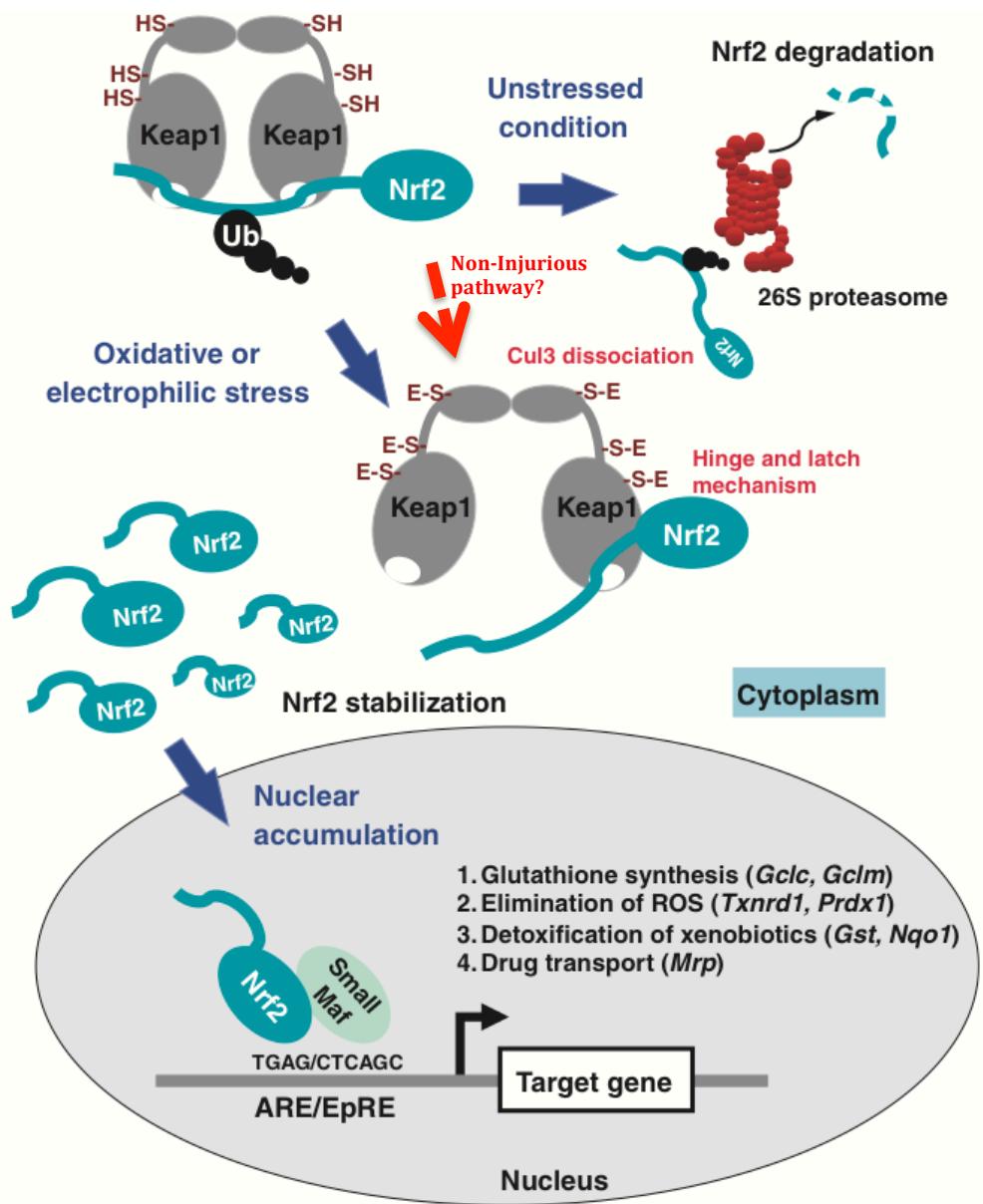
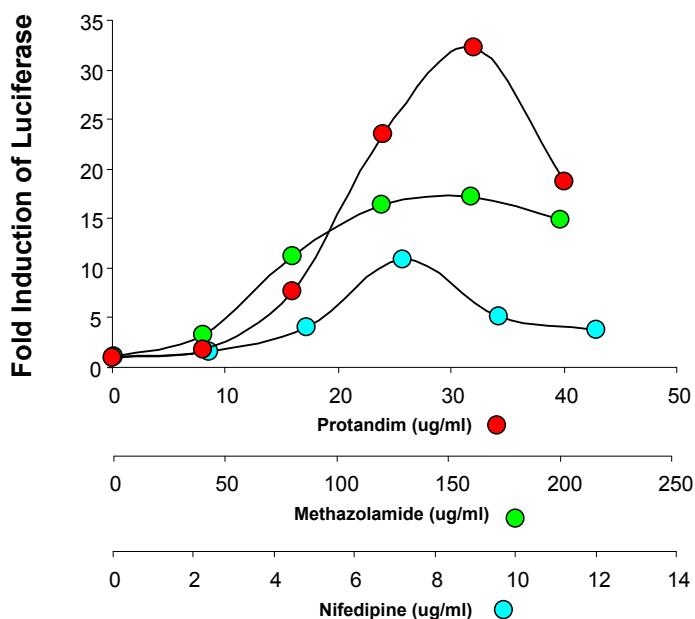


Figure 6

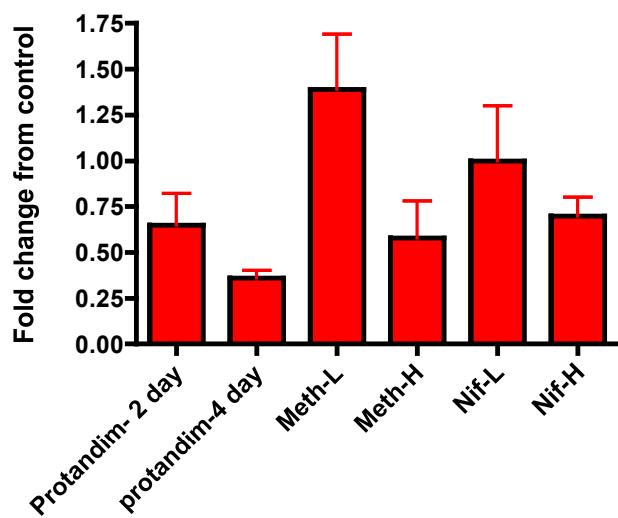


Supp. Fig. 1

A.



B.



Supp. Fig. 2

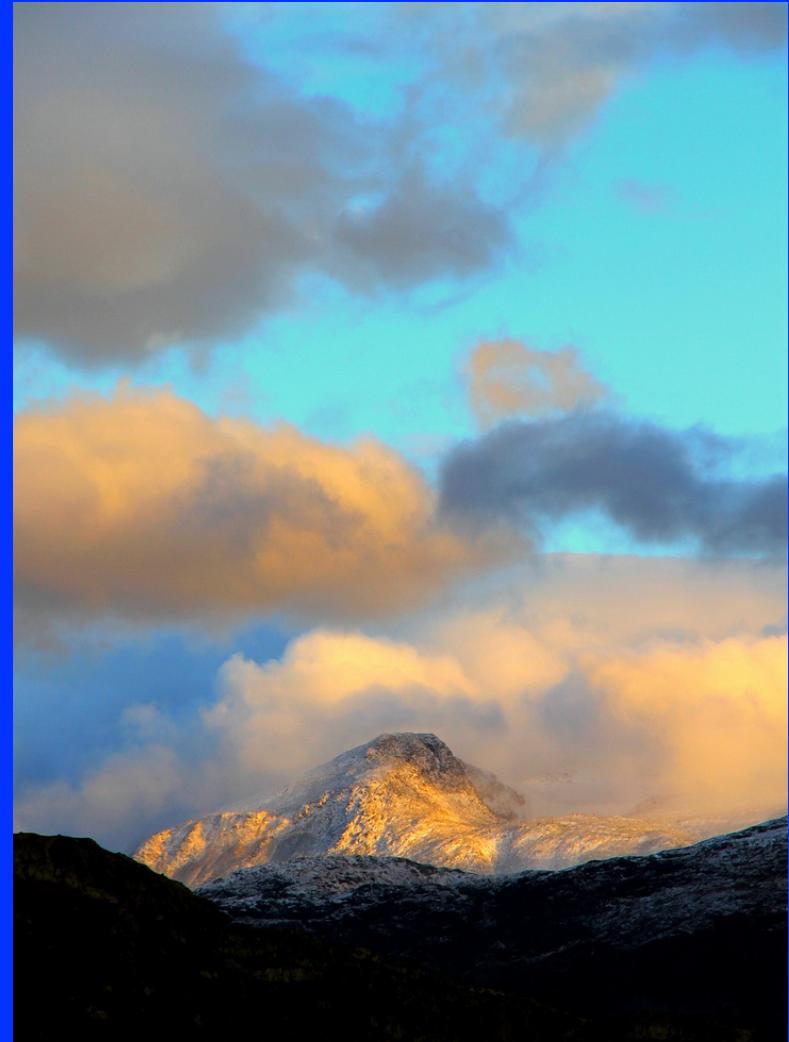
Combining the off-target effects of widely available NRF2-inducing drugs may result in new therapies for Acute Mountain Sickness

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Acute Mountain Sickness

- Affects 60% of individuals ascending above 8000 ft.
- Syndrome characterized by:
 - Pulmonary vascular leak
 - Leads to HAPE
 - Brain vascular leak
 - Leads to HACE
- Severe symptoms: shortness of breath, and headaches that do not respond to analgesics.



What causes AMS?



- Hypobaric hypoxia
- Physical stress.
- Chemical stress.
- Reactive oxygen species are thought contribute towards the development of brain vascular leak.

Evidence suggests

- Reactive oxygen species
 - Promote edema
 - Act as pro-inflammatory agent
 - Scavenge NO
- ROS as possible target for:
 - Treating the symptoms associated with AMS.
- Antioxidants might abate AMS
 - Up-regulation of antioxidant genes as therapy.

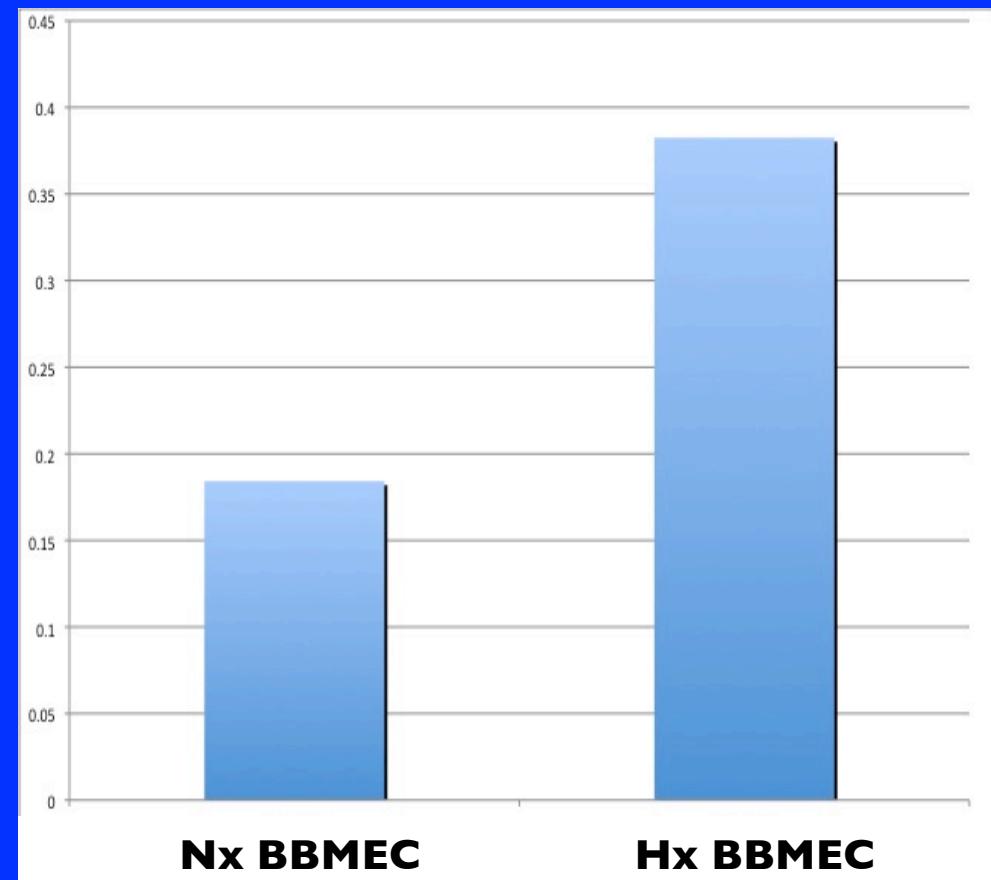
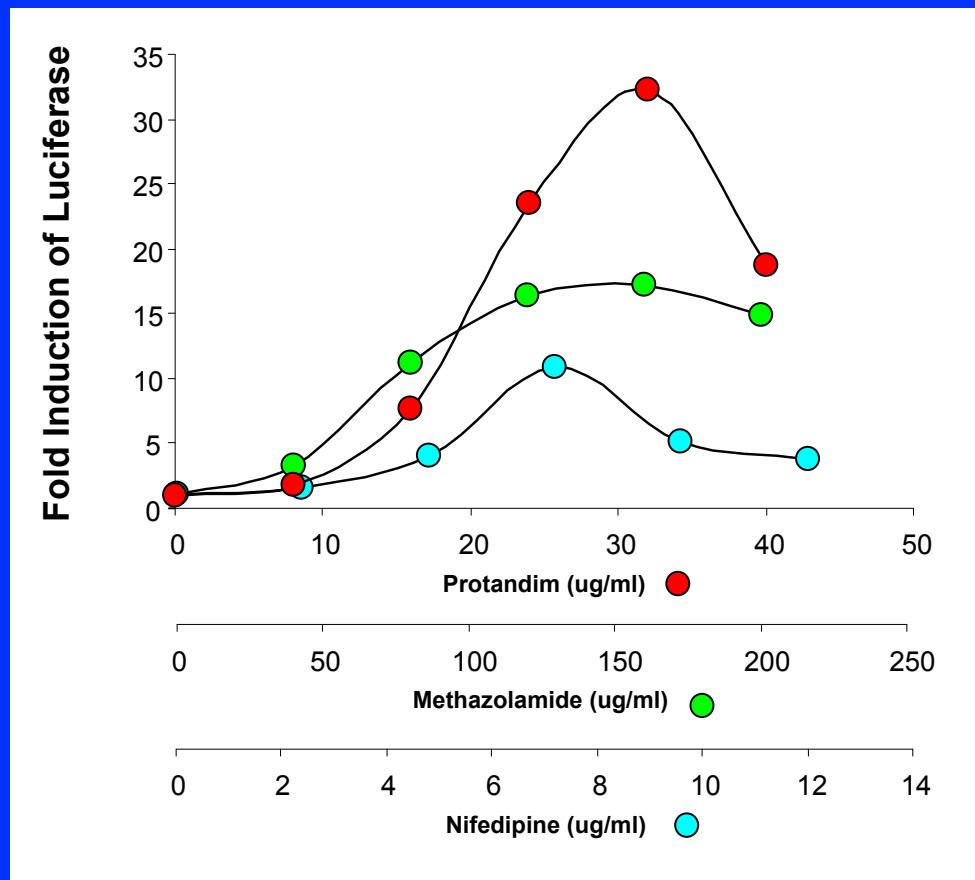


Previous Data

In vitro

Nifedipine and
Methazolamide induce
NRF2

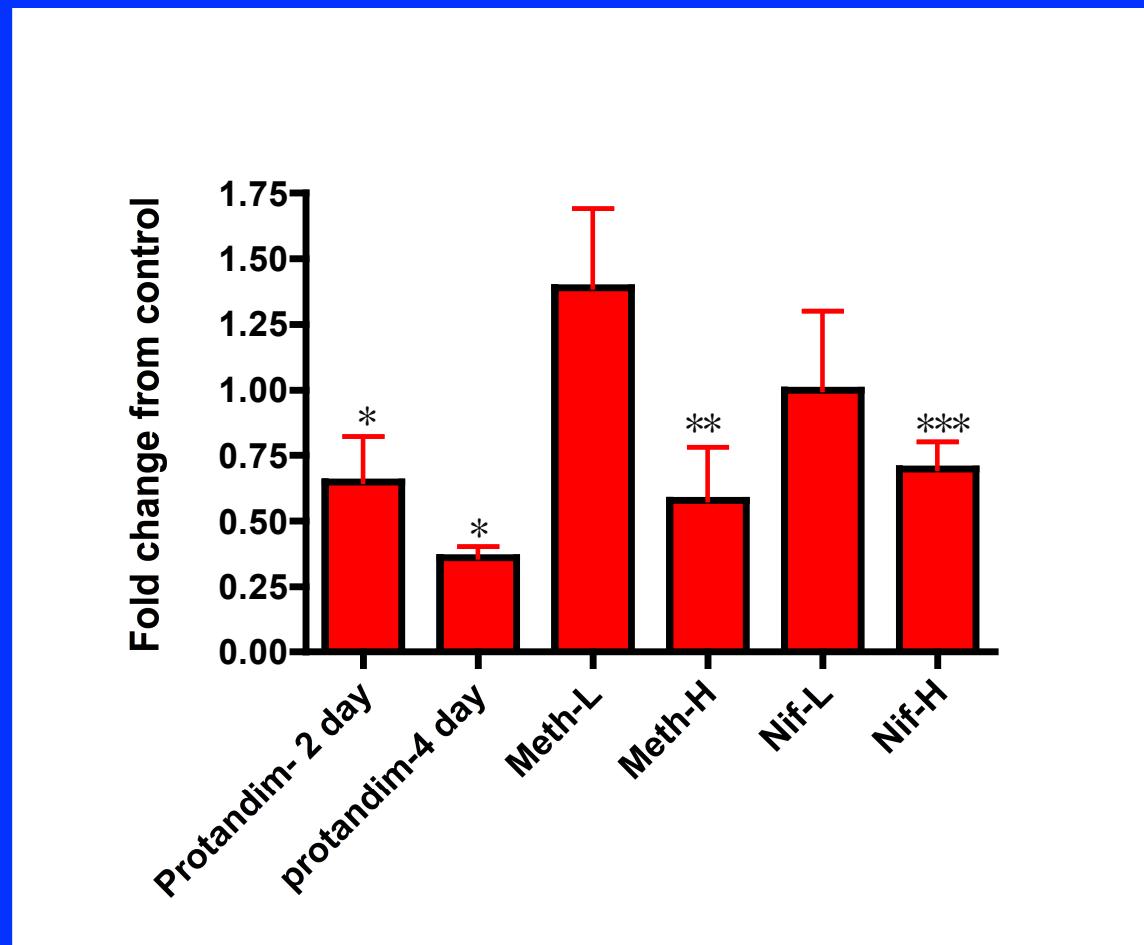
Hypoxia induces H_2O_2
production



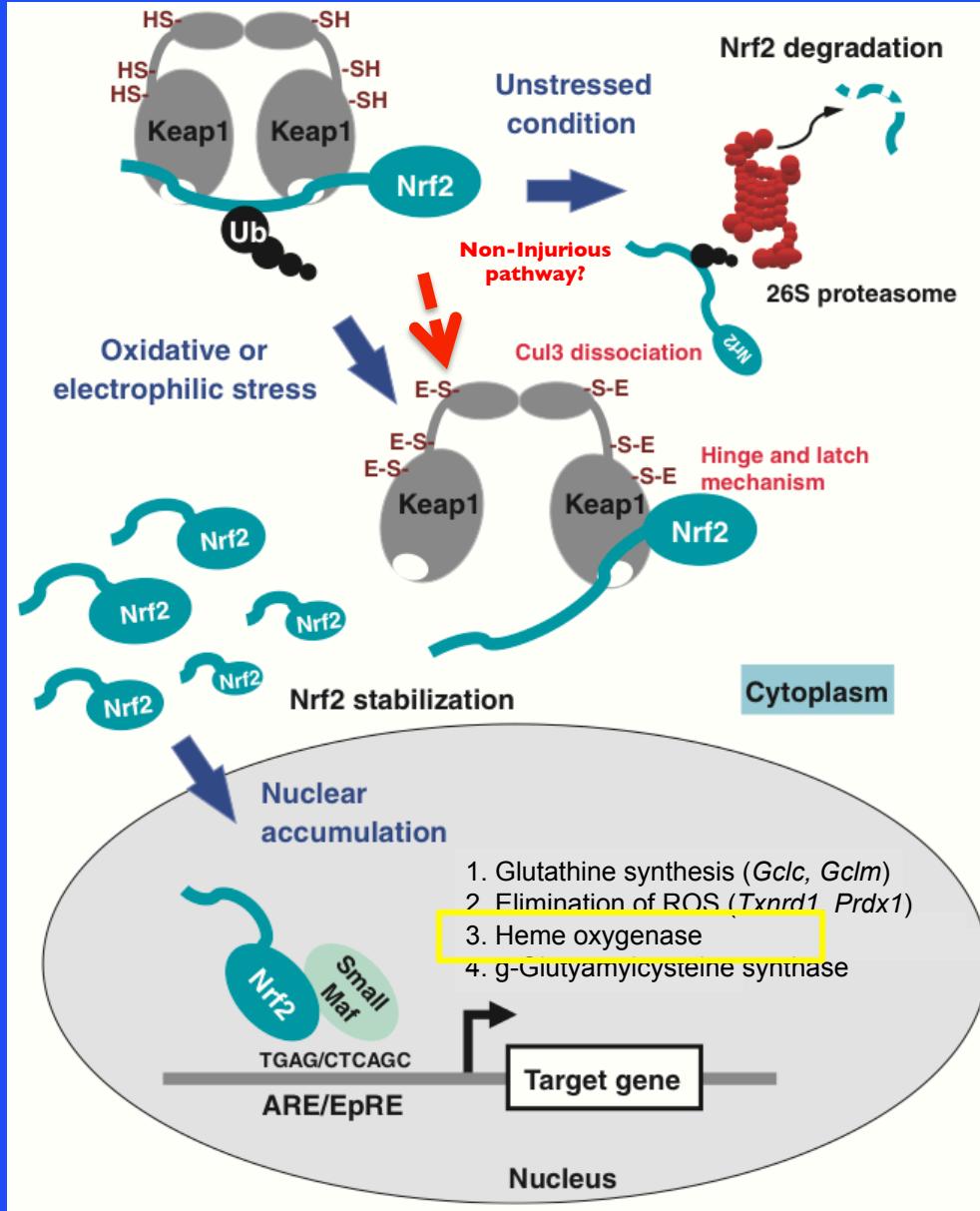
Previous Data

In vivo

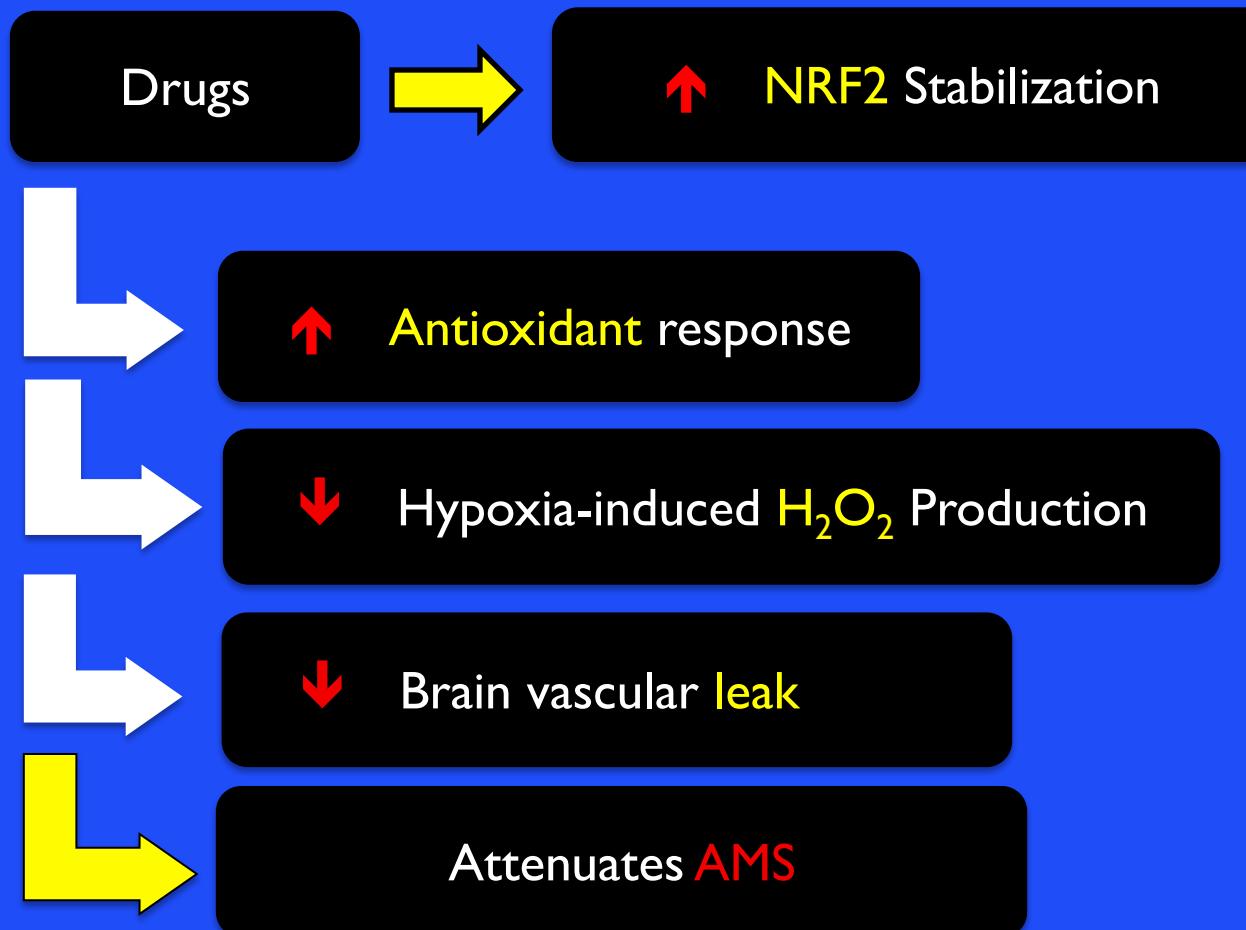
Drugs that activate NRF2 decrease high altitude-induced brain vascular leak



NRF2 Stabilization



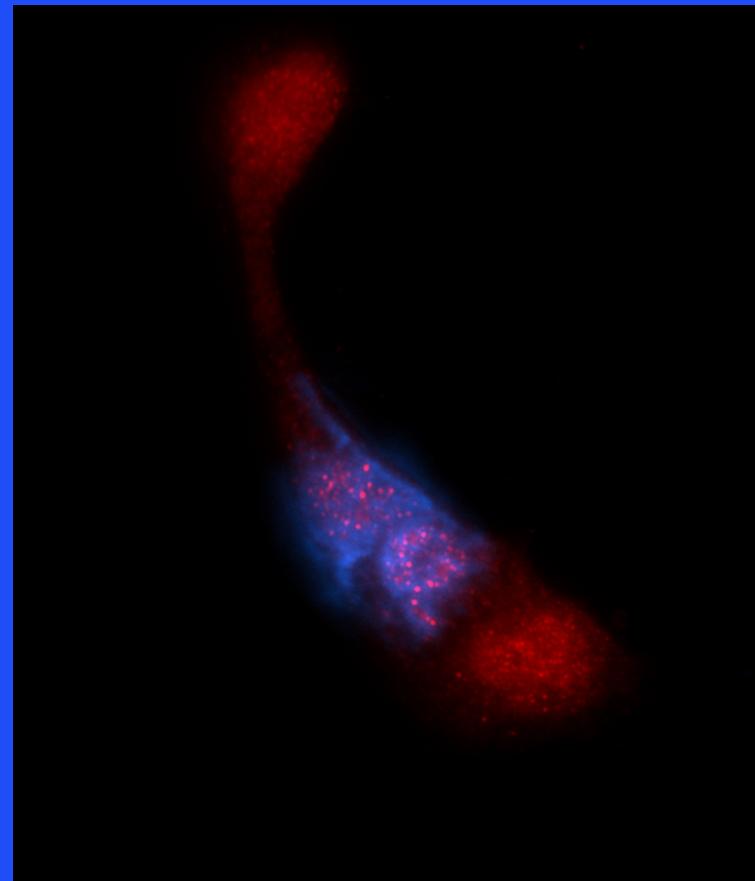
Hypothesis



Methods

In vitro approach

- Used BBMECs to study the contribution Meth, Nif and Prot, have in:
- NRF2 stabilization
Immunohistochemistry
- ROS depletion
Amplex red



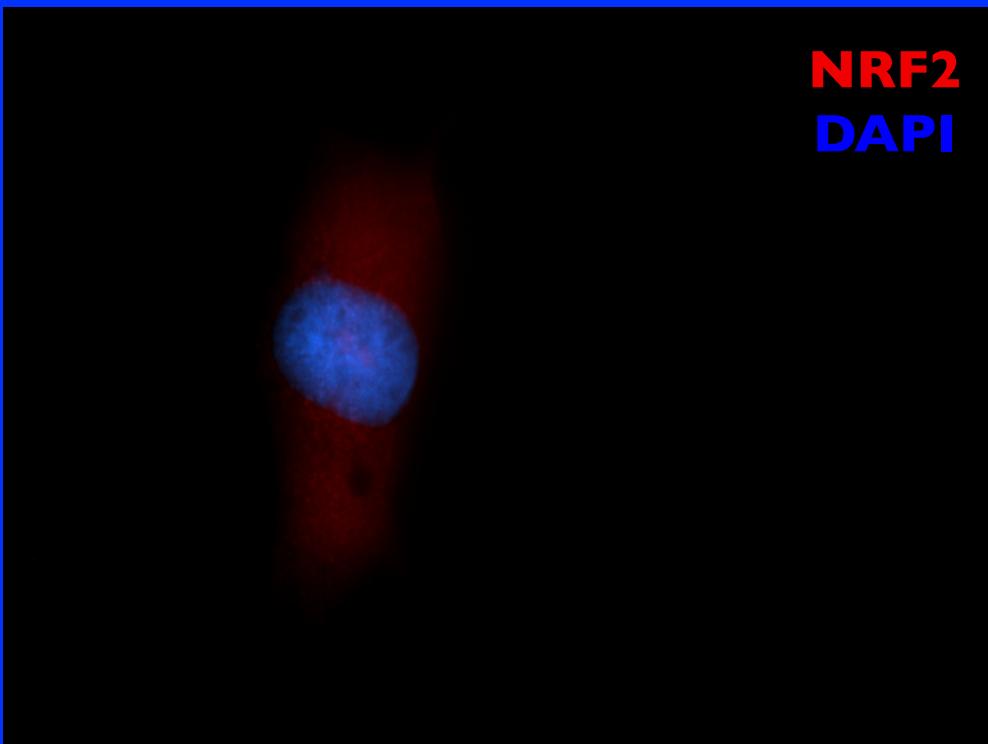
Methods



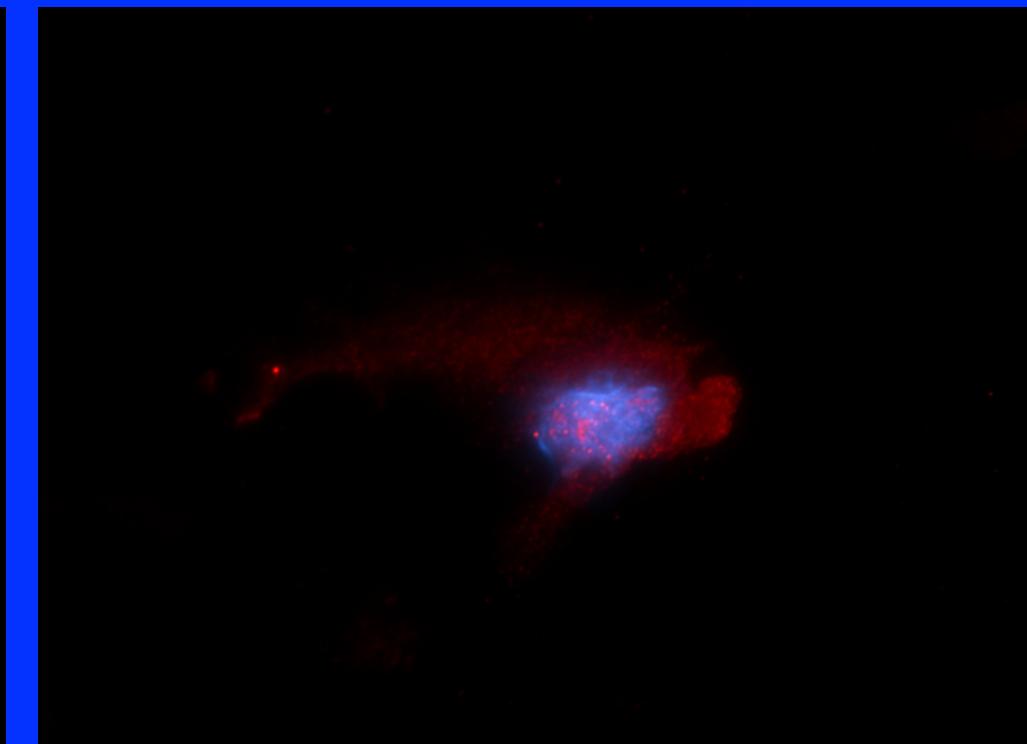
In vivo rat model

- Use **IHC** to determine NRF2 induction by drugs.
- Quantify NRF2 and downstream induction by **western blotting** technique.
- Brain vascular permeability will be assessed using **fluorescein sodium** and **immunofluorescence microscopy**.

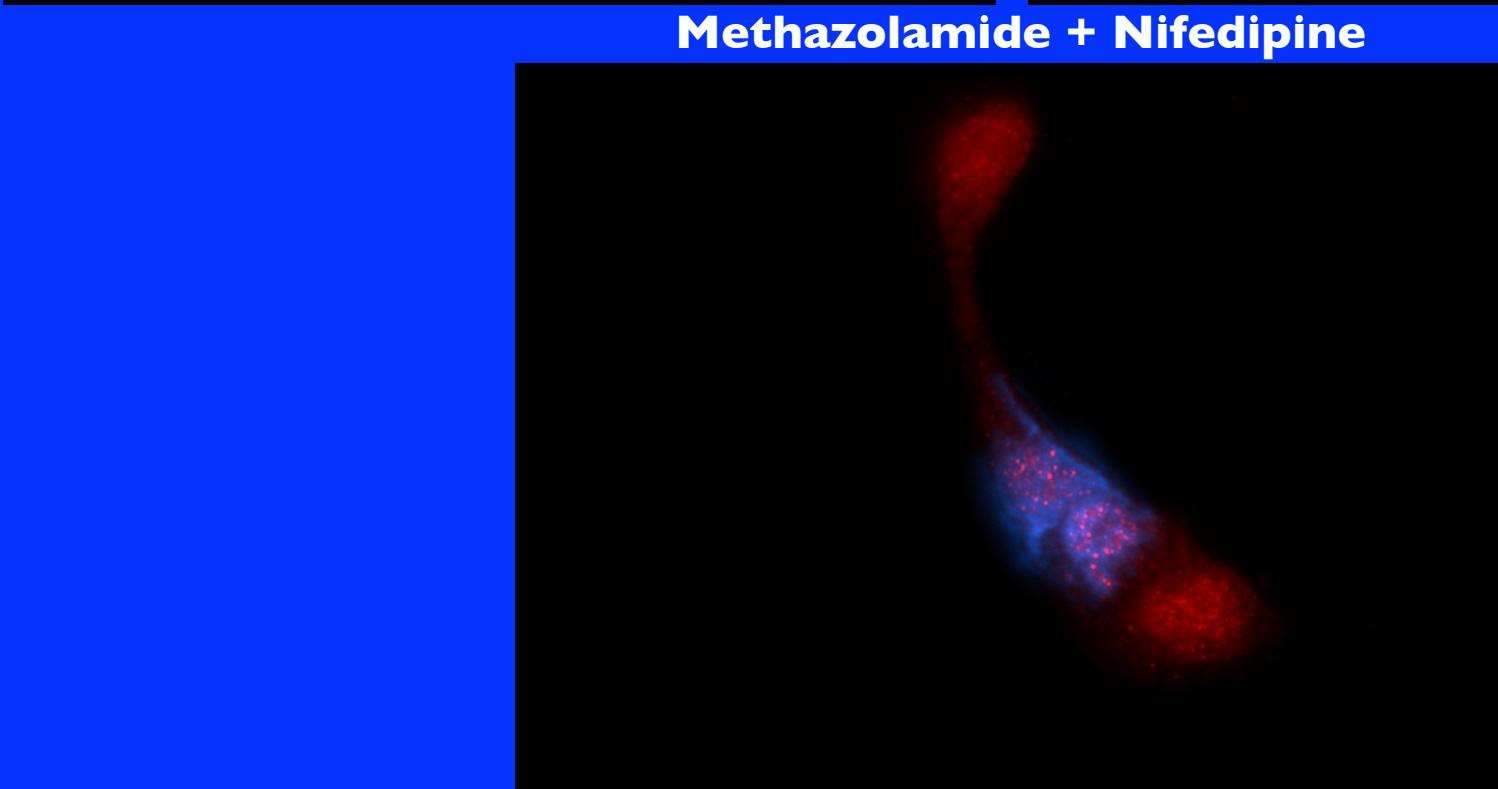
Control



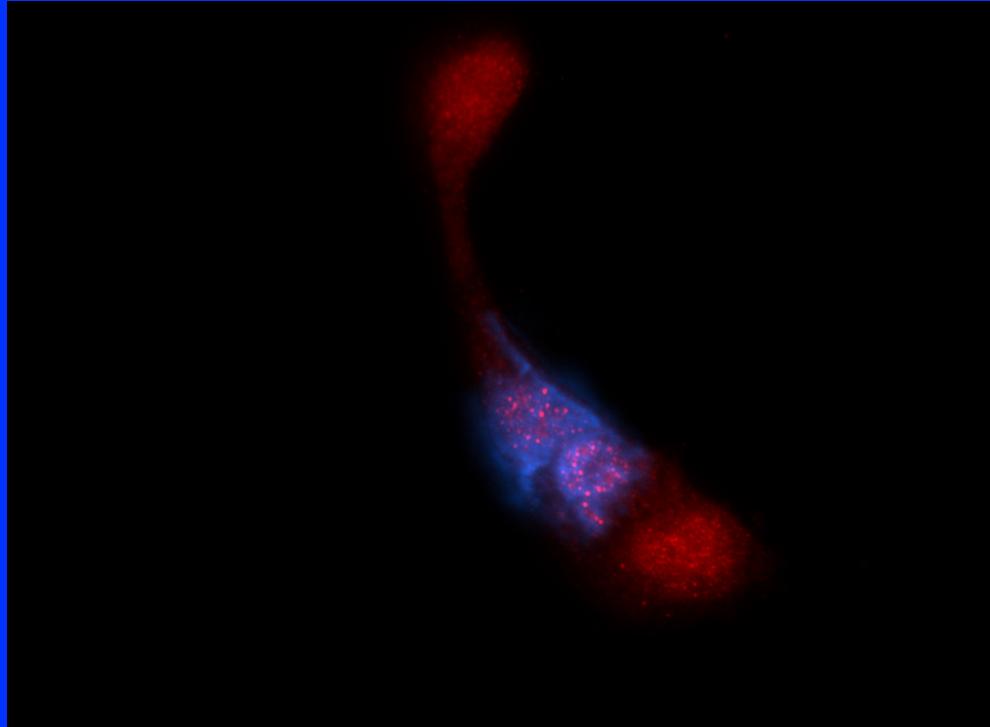
In Vitro NRF2



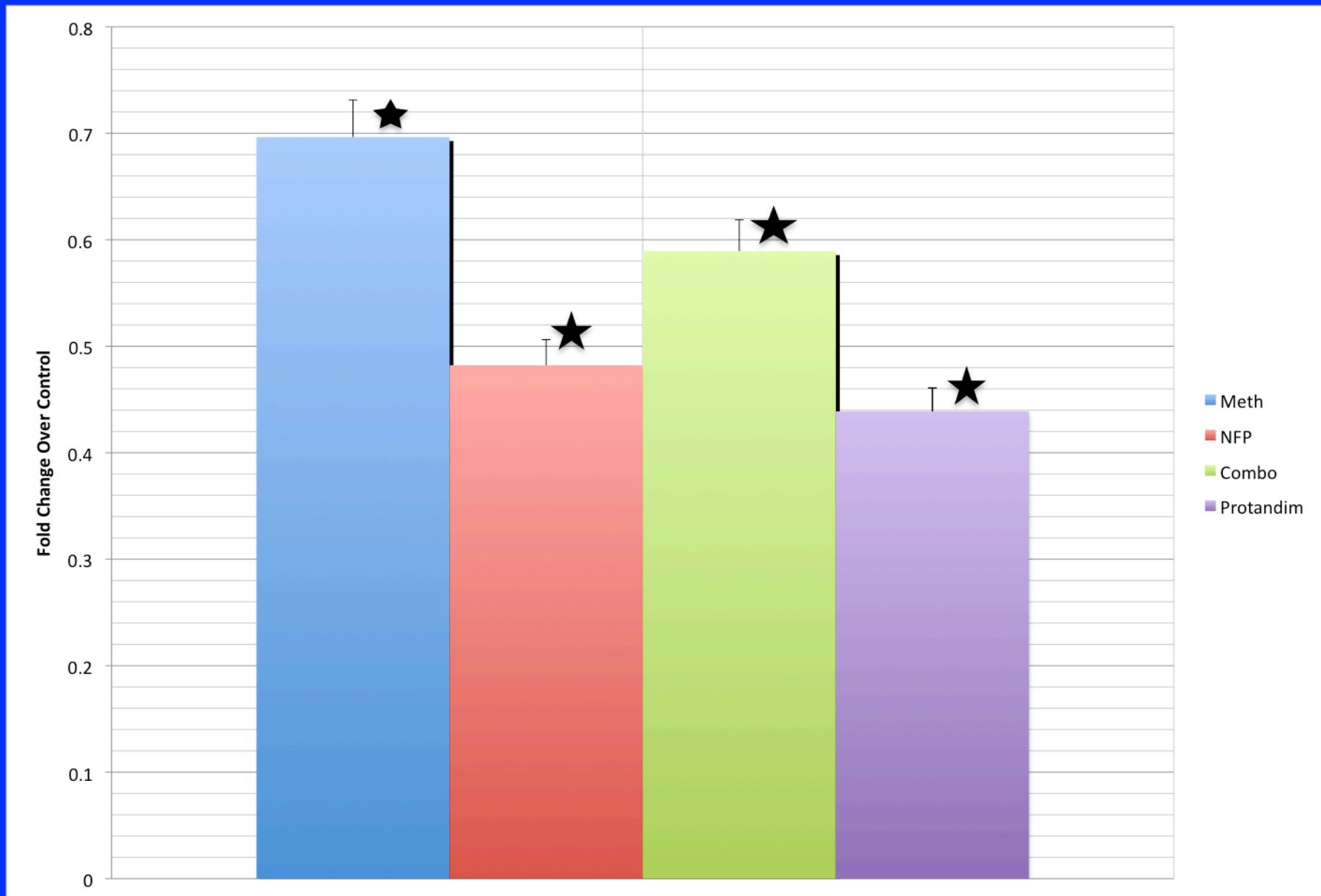
Methazolamide



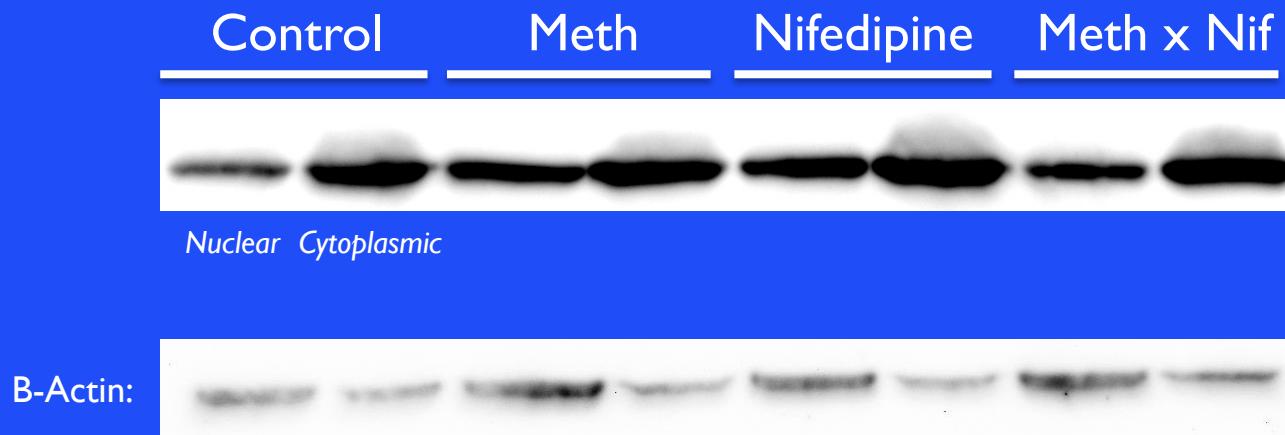
Methazolamide + Nifedipine



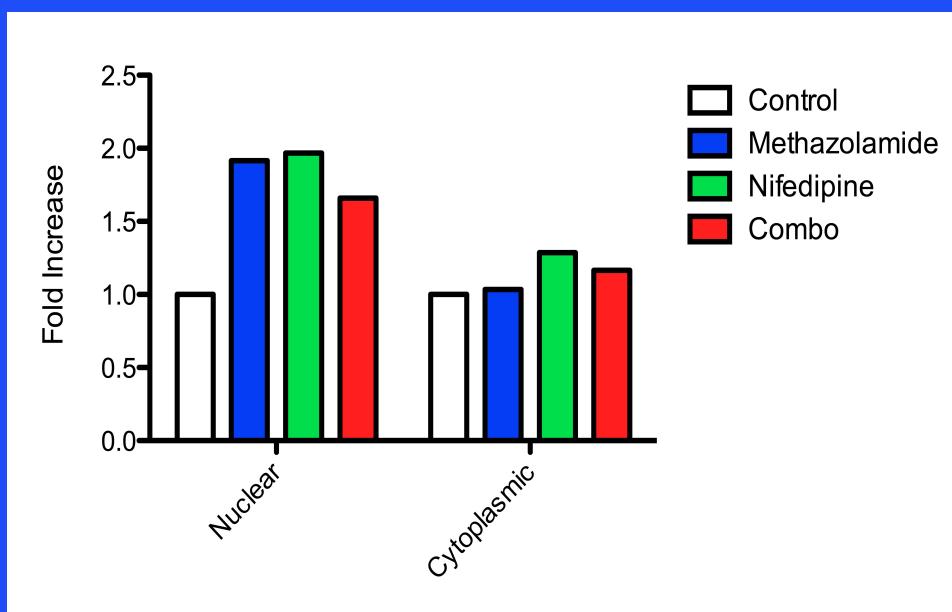
ROS Production



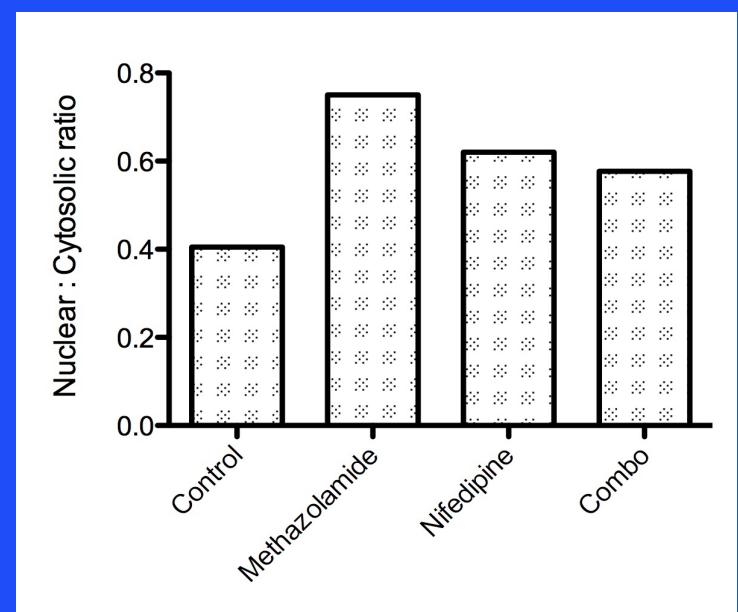
NRF2 Activation



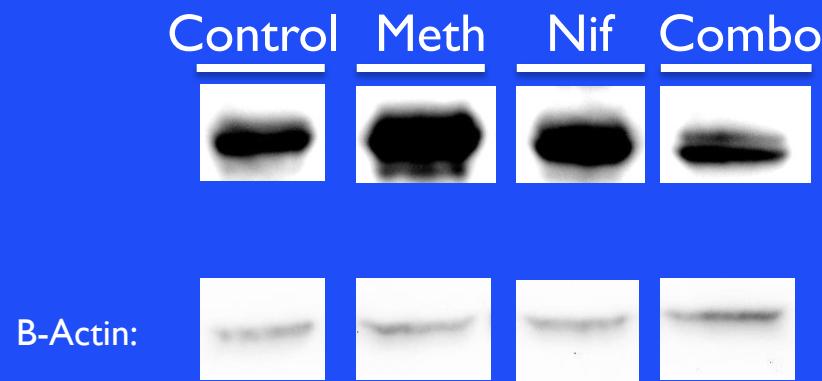
Densitometry



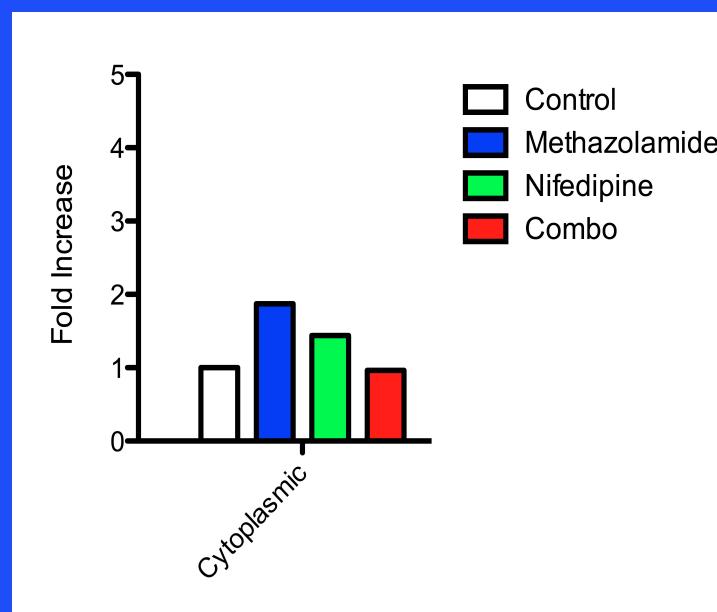
Nuclear to cytosolic ratio

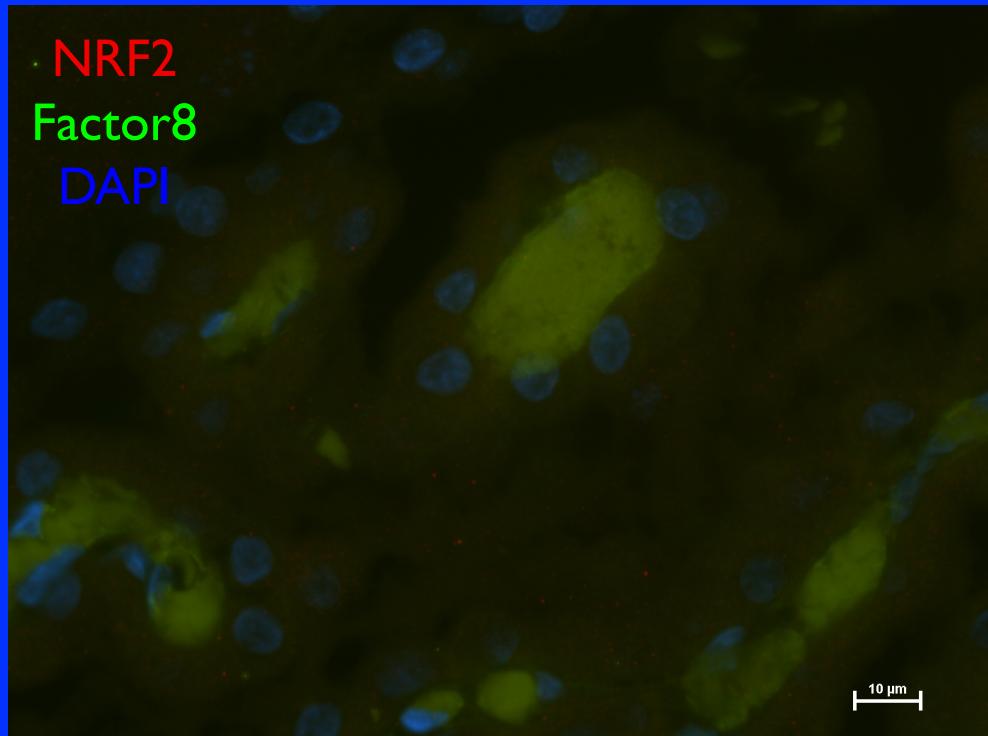
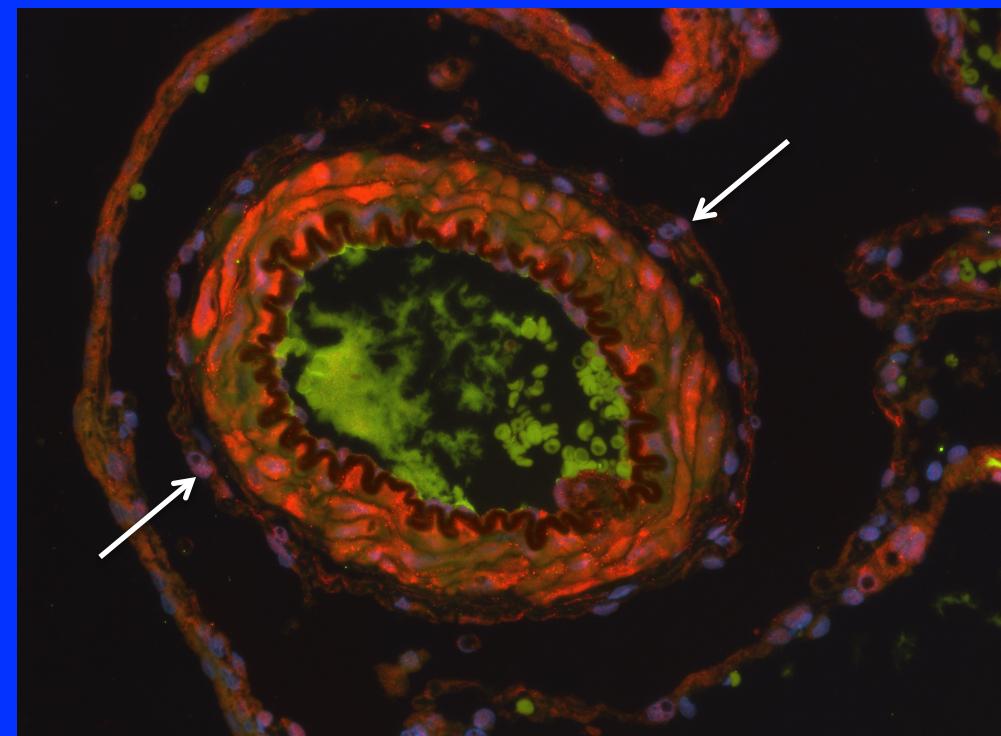
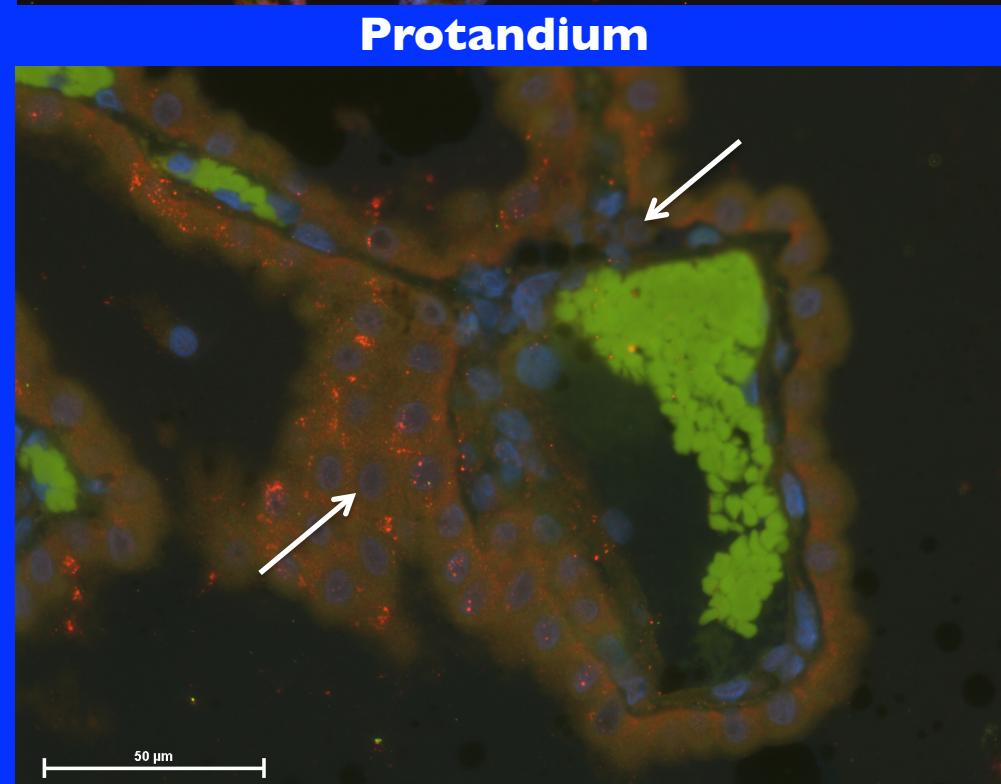
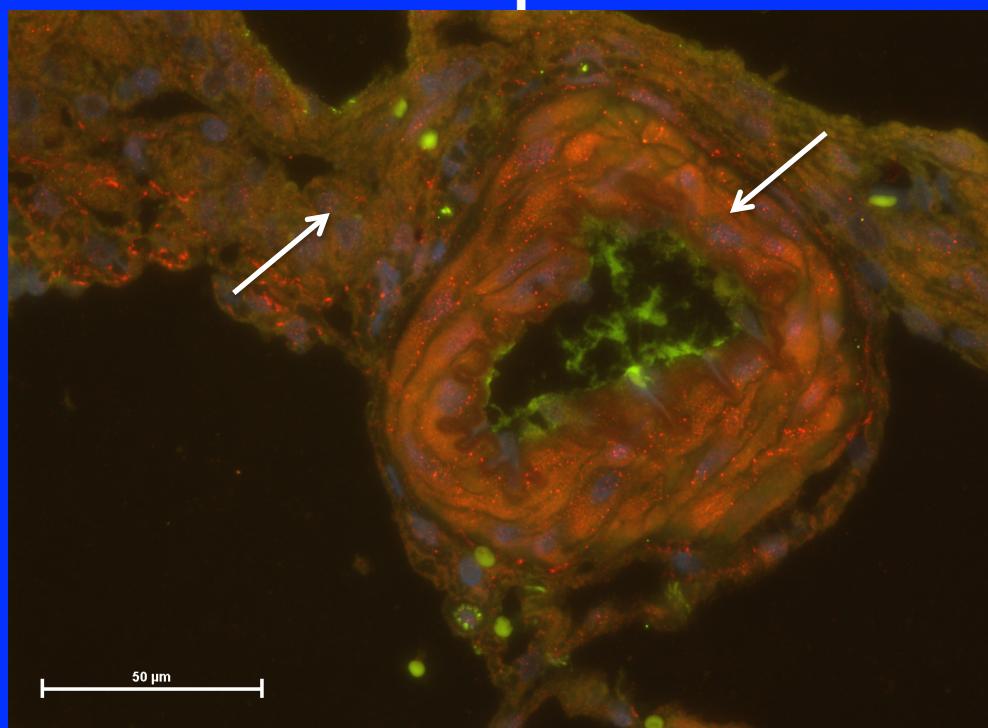
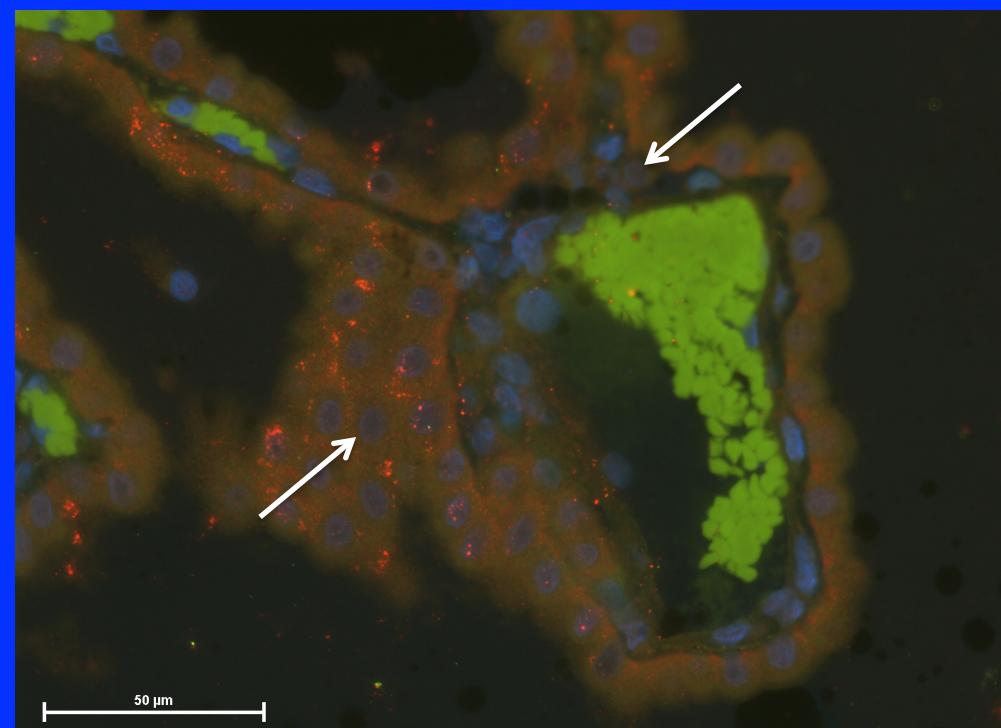


HO-1 Activation

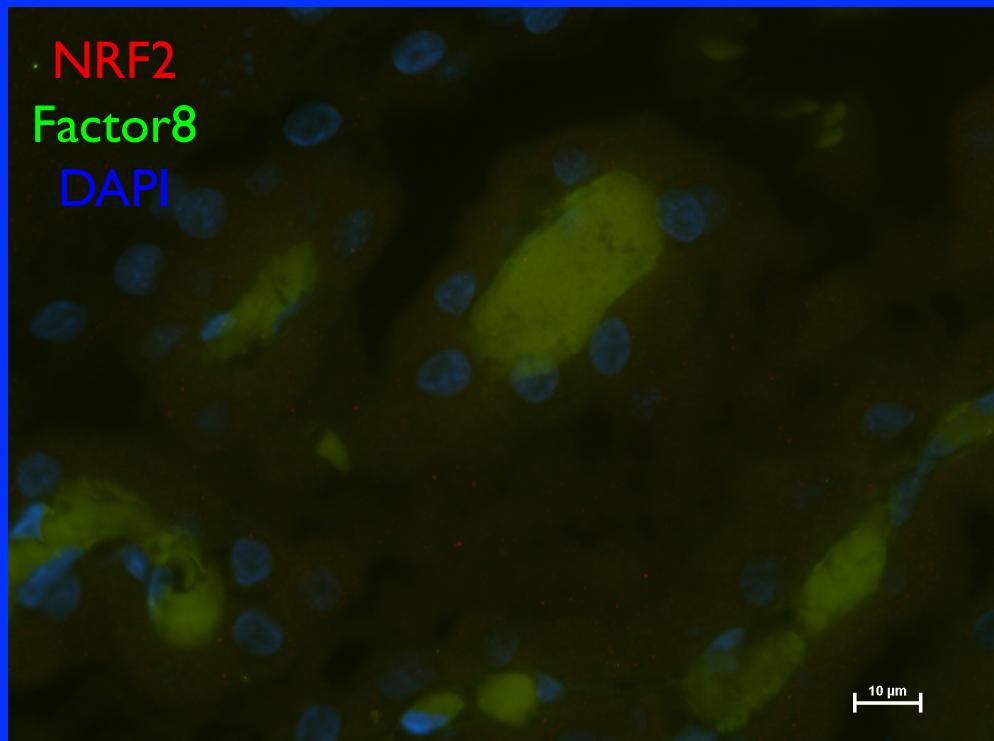


Densitometry

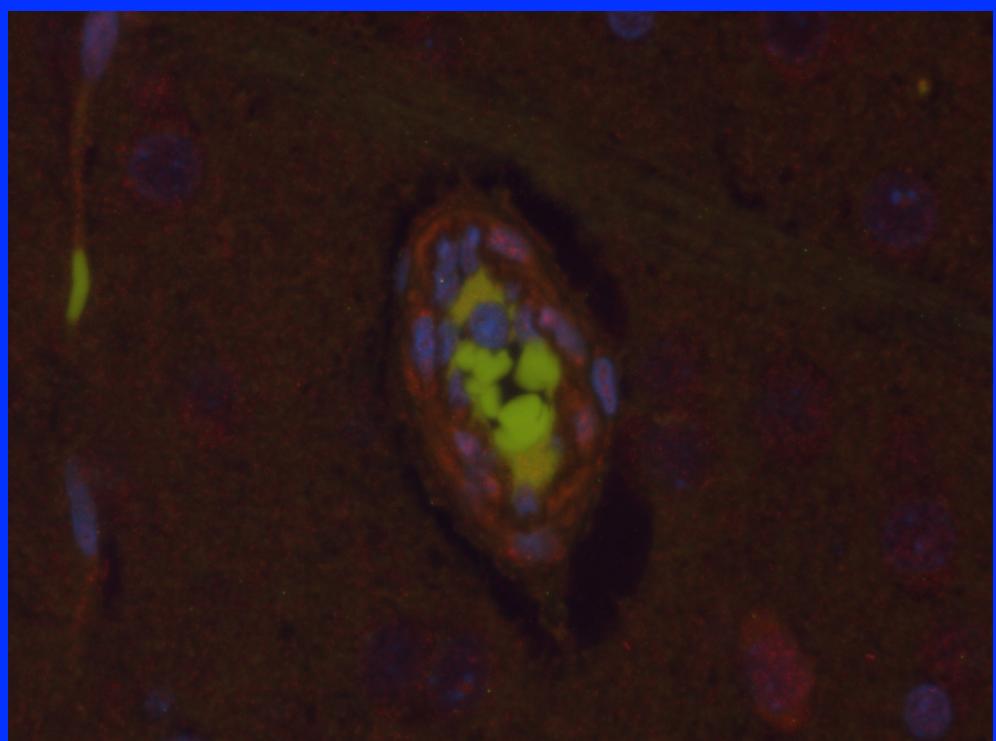


Control**In vivo NRF2****Methazolamide****Nifedipine****Protandium**

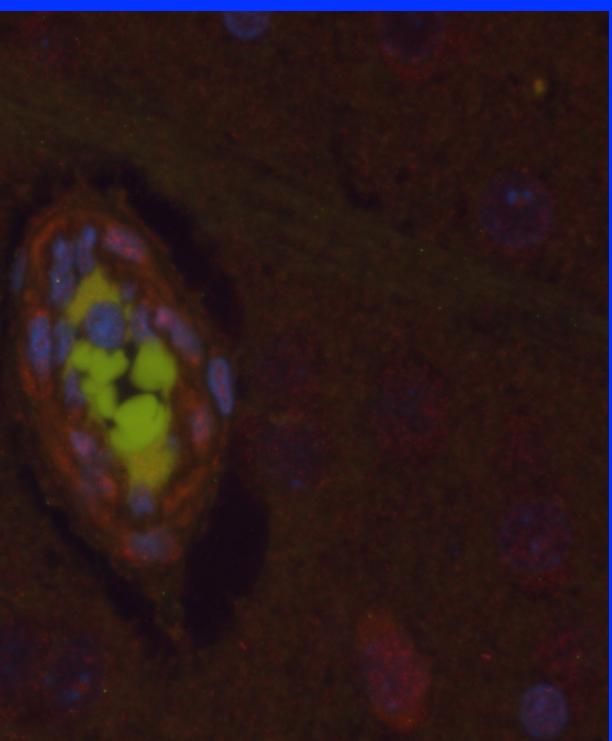
Control



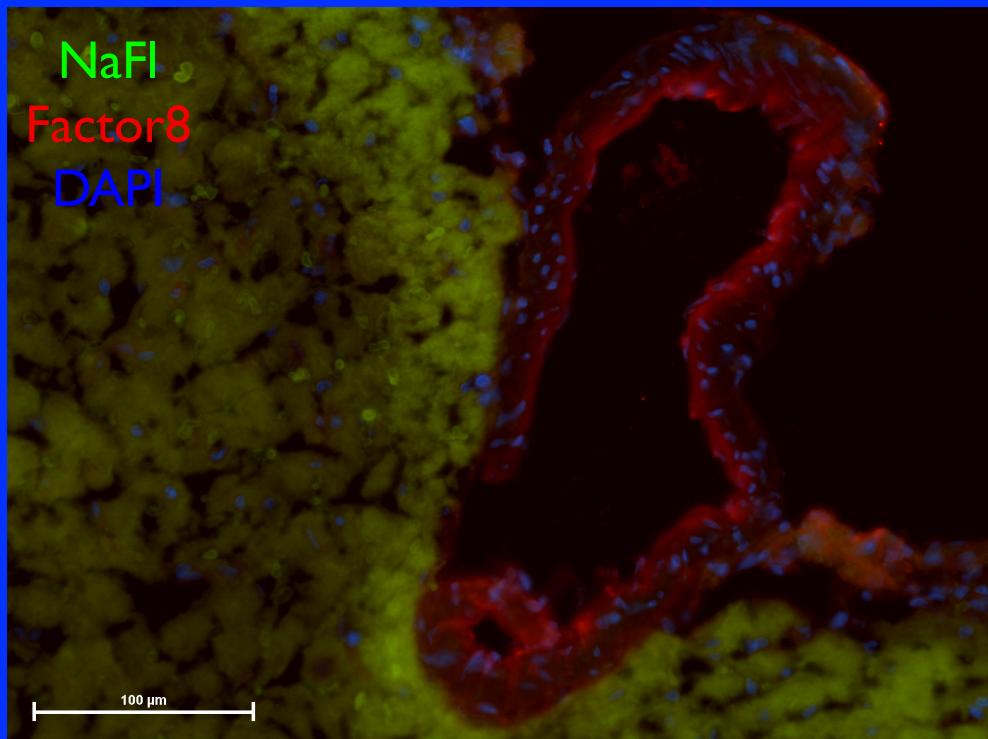
In vivo NRF2



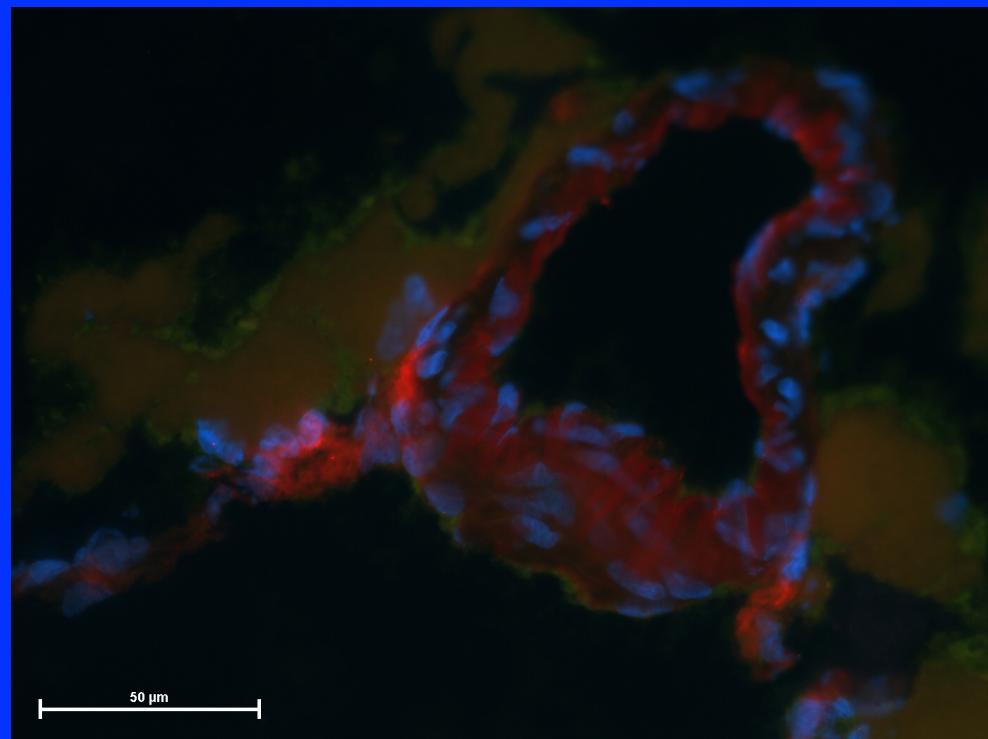
Combo



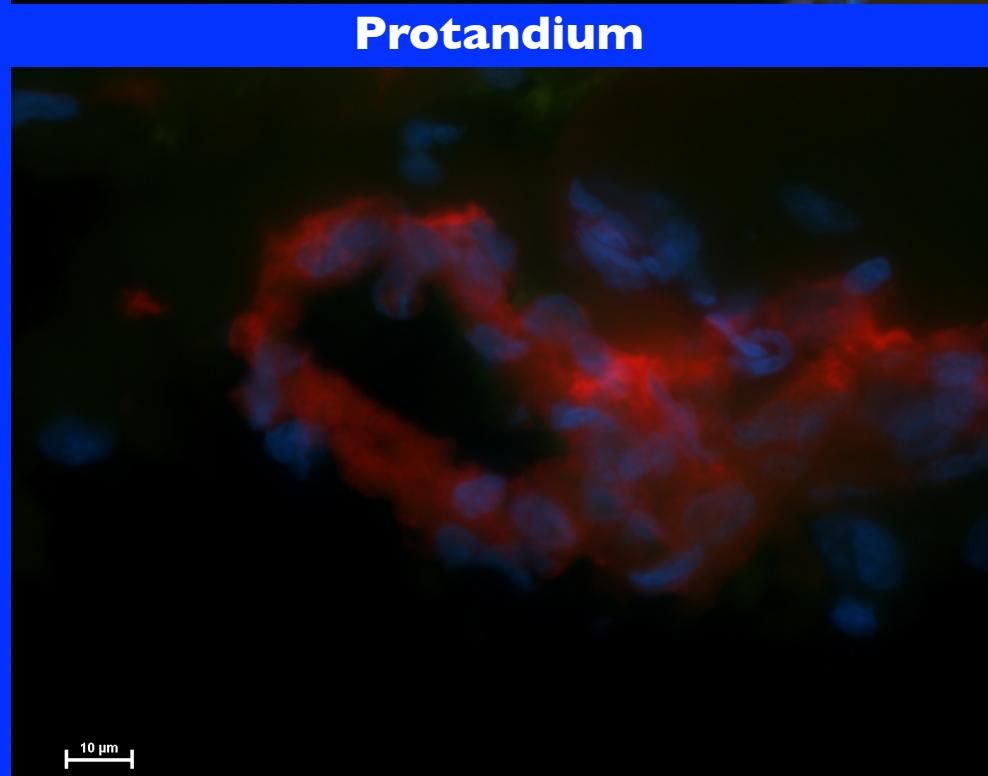
Control



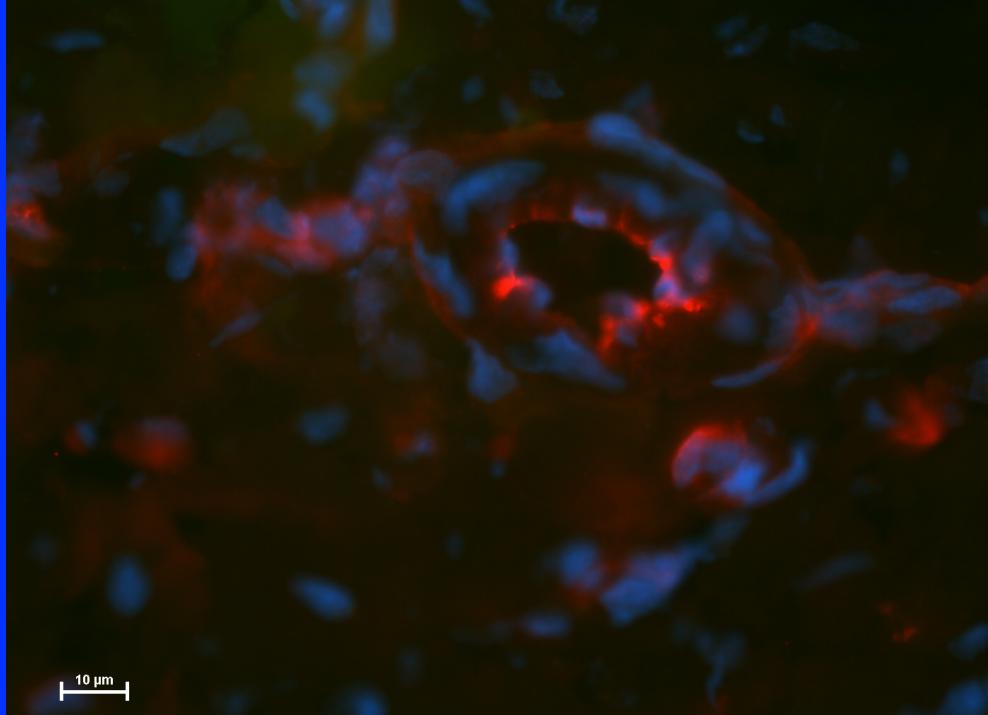
Vascular leak



Methazolamide



Nifedipine



Protandium

Conclusions

- Methazolamide, nifedipine, and Protandim increase the organism's antioxidant status in a **NRF2** dependent fashion.
- NRF2 increase by drugs happens through a non-injurious pathway as evidenced by decreased **H₂O₂** production.
- Drugs had a significant effect in reducing blood-brain permeability *in vivo*, reducing the signs of **AMS**.

Future Studies

- Determine the effects these drugs have on vascular permeability *in vitro*; using cell inserts.
- Look for additional evidence of downstream up-regulation *in vitro*.
- Delve in to the mechanistics behind the non-injurious pathway.
- Screen additional drugs.

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Questions?



“Data don’t make any sense,
we will have to resort to statistics.”