DISSERTATION

COMPENSATORY RESPONSES TO OXIDANT STRESSES IN VITRO AND IN VIVO

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Shadi Khademi

Department of Health and Exercise Science

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Doctoral Committee:

Advisor: Karyn L. Hamilton

Benjamin F. Miller
Matthew S. Hickey
Ronald B Tjalkens

ABSTRACT

COMPENSATORY RESPONSES TO OXIDANT STRESSES IN VITRO AND IN VIVO

Emerging evidence shows that reactive oxygen species (ROS) are not merely damaging agents causing random destruction to cell structure and function, but that they act as modulators of physiological processes (such as cell adaptation to physical exercise) by regulating gene transcription and protein synthesis. The exact redox signaling pathways involved in cell adaptations to oxidative stress are unknown. Since various stimuli can induce oxidative stress under different conditions in vivo and in vitro, different models are warranted to study the cell signaling pathways involved in compensatory responses to oxidative stress. The following investigation comprises a series of experiments with the overall aim of elucidating the role of redox sensitive pathways in inducing cellular responses to oxidative stress in vitro and in vivo.

The experiments tested the general hypothesis that changes in the redox state of the cell, through hypoxia, contractile activity or direct application of hydrogen peroxide (H_2O_2), would cause antioxidant compensatory responses and cell adaptations. The specific aims of the experimental series were: 1) to determine whether pulmonary edema, evoked by cerebral hypoxia in the presence of systemic normoxia, will be accompanied by sympathetic activation, increased oxidative stress, and upregulation of endogenous antioxidant pathways, 2) to determine whether electrical stimulation (Es) induced contractile activity of cultured murine myotubes would induce energetic stress, redox sensitive signaling, and mitochondrial biogenesis, and 3) to determine whether treatment with H_2O_2 would result in a greater rate of mitochondrial biogenesis compared to control, and whether the increase would be maintained during co-

treatment with either an exogenous antioxidant (vitamin C) or a nuclear erythroid 2 -related factor 2 (Nrf2) activator that increases transcription of endogenous antioxidants.

Studies in aim 1 demonstrated that under pathologic conditions such as isolated cerebral hypoxia with systemic normoxia, tissue specific patterns of compensatory responses to the hypoxic stressor exist. Further, this study showed that the differences in lung and brain redox signaling pathways during hypoxia can have different systemic outcomes through modulation of the sympathetic nervous system (SNS). Studies in aim 2 demonstrated a unique model of contractile activity in vitro, which was successful in simulating the cellular adaptations to a single bout of endurance exercise such as greater rate of cytosolic protein synthesis, upregulation of antioxidants and mitochondrial protein markers as well as AMP activated protein kinase (AMPK). Studies in aim 3 demonstrated that H_2O_2 did not increase mitochondrial biogenesis. Further, increasing Nrf2 activation maintained the rate of mitochondrial protein synthesis during H_2O_2 treatment, while treatment with the exogenous antioxidant failed to restore the H2O2 induced decreases in mitochondrial biogenesis during H_2O_2 treatment.

Collectively, we have used different models of oxidative stress in vitro and in vivo to evaluate some of the mechanisms involved in cell adaptations responses. Findings from these experiments provide insight into understanding the role of redox signaling in pathologic and non-pathologic circumstances and can help future therapeutic recommendations for battling the consequences of oxidative stress on health.

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CHAPTER I - INTRODUCTION AND EXPERIMENTAL AIMS

Oxidative stress is a term often used to indicate a condition in which a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses has damaging effects in many tissues and organs [1]. In fact, there is an established link between increased oxidative stress and the pathogenesis of an array of human diseases as well as biological aging [2, 3]. In addition, it is important to understand the role of ROS in regulation of cellular responses to oxidative stress since ROS, as byproducts of normal cellular metabolism, act as modulators of physiological processes in healthy organisms as well as mediating pathophysiological consequences of a variety of diseases.

Neurogenic pulmonary edema (NPE) is an acute pulmonary edema occurring after a central neurologic insult, and is believed to occur independent of a cardiogenic contribution. NPE can occur following stroke, traumatic brain injury and seizure, and represents a major public health concern due to high morbidity (40-50%) and mortality (7%) [4]. Cerebral hypoxia is a common feature of many neuropathologies such as stroke, brain injury and seizure, suggesting that hypoxia may be an important factor in the pathogenesis of NPE [5]. Currently, the underlying mechanism(s) of NPE are poorly understood.

Nuclear erythroid 2 -related factor 2 (Nrf2), the master regulator of endogenous antioxidant genes, is a redox sensitive transcription factor that becomes activated upon exposure to oxidants [6]. Nrf2 induces transcription of antioxidant response element (ARE)-bearing genes including phase II antioxidants such as hemeoxygenase-1 (HO-1) and superoxide dismutase 1 (SOD1) [7, 8]. Through transcriptional activation of these genes, Nrf2 activates cellular pathways that protect against oxidative injury. It has been shown that Nrf2 can be activated by oxidants produced during hypoxia [9]. In addition, HO-1 is induced in the rostral ventrolateral

medulla during chronic hypoxia and it is associated with cardiorespiratory adaptations during hypoxia [10, 11]. Given the evidence of ROS generation during hypoxia, and based on studies that have suggested a role for oxidative stress in induction of pulmonary edema, we were particularly interested in exploring the role of oxidative stress and compensatory antioxidants in both cerebral and global hypoxia-induced pulmonary edema.

A great deal of epidemiological data has shown that exercise decreases the incidence of oxidative stress- associated diseases [12]. Several pieces of evidence have shown that ROS are crucial for the activation of the key transcription factors and co-activators to induce adaptive responses to higher metabolic demand as happens during exercise, including enhanced antioxidant defense and oxidative damage repairing systems [13, 14]. Recent studies show that the ROS produced during a bout of exercise can contribute to training adaptations such as mitochondria biogenesis [15, 16], although this concept lacks consensus [17, 18]. ROS-mediated mitochondrial biogenesis likely occurs via the upregulation and transcriptional regulatory activity of redox sensitive transcriptional factors and co-activators such as nuclear respiratory factor (NRF-1) [19] as well as peroxisomal proliferator-activated receptor [gamma] coactivator-1 alpha (PGC1-α) [20, 21]. While PGC1-α has been proposed as one of the main transcriptional regulators of mitochondrial biogenesis [22, 23], some studies suggest that PGC1-α is not mandatory for exercise adaptation responses [24-28], implying that other pathways may be sufficient to increase mitochondrial biogenesis independent of PGC1-α. Nrf2 is a potential candidate for an activator of mitochondrial biogenesis because the NRF-1 gene, which encodes a protein responsible for transcriptional control of a range of mitochondrial proteins, contains an ARE [29]. It has also been shown that acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium [30]. Whether Nrf2 contributes to

exercise-induced skeletal muscle mitochondrial biogenesis has not been thoroughly investigated.

Understanding the specific molecular mechanisms that result in exercise-induced cellular responses such as mitochondrial biogenesis has been complicated by the fact that during contraction the muscle fibers are exposed to numerous metabolic and mechanical stimuli [31]. Therefore, a model of muscle contraction in vitro could enable researchers to analyze the effect of excitation/contraction on gene expression and/or proteomic specific to mitochondrial biogenesis under conditions where nutrient/oxygen supply (akin to blood flow) can be better controlled. Electrical stimulation (E-stim), which has been applied successfully to stimulate skeletal muscle cells in vitro, has been shown to effectively elicit both resistance exercise and endurance exercise-like contractions in cultured skeletal muscle cells [32, 33]. Although the intracellular signaling associated with mitochondrial biogenesis has been assessed in electrically stimulated cultured muscle cells [32, 33], the synthesis of new mitochondrial proteins (the definition of "mitochondrial biogenesis") was not assessed.

As stated above, it has been shown that ROS produced during exercise can stimulate mitochondrial biogenesis, while simultaneous oral administration of antioxidants such as vitamin C (Vit C) blunts the exercise-induced adaptation of muscle mitochondria [12, 17, 34]. We and others have shown that treatment with a variety of phytochemicals results in Nrf2 activation and upregulation of endogenous antioxidants [35-38]. However, the role of endogenous antioxidants in mitochondrial biogenesis is not known.

Mitochondrial biogenesis has been assessed by a variety of methods such as mitochondria-specific mRNA, mitochondrial protein expression, enzyme activity, and mitochondrial density [39]. Although mitochondrial biogenesis has been assessed by these methods, strictly speaking biogenesis refers to the making of new mitochondria [40]. Therefore,

it is debatable whether some commonly used methods measure mitochondrial biogenesis. Recently, Miller and Hamilton have developed methods to measure mitochondrial protein synthesis and have applied these techniques to a variety of tissues in vivo [41-44]. To date, these methods have not been applied in vitro.

This body of work collectively tests three specific hypotheses aimed at elucidating the role of oxidative stress on cellular responses associated with hypoxia, contractile activity, and H_2O_2.

Overall Hypothesis: We tested the overall hypothesis that changes in the redox state of the cell, through hypoxia, contractile activity or direct application of H_2O_2, would cause antioxidant compensatory response and cell adaptations.

Specific Aims

Experiment 1: To determine whether pulmonary edema, evoked by cerebral hypoxia in the presence of systemic normoxia, will be accompanied by systemic sympathetic activation, increased oxidative stress, and up regulation of endogenous antioxidant enzymes in brain and lung (compensatory antioxidants).

Experiment 2: To determine if E-stim-induced contractile activity of cultured skeletal muscle cells will induce energetic stress, redox sensitive signaling, and mitochondrial biogenesis.

Experiment 3: To determine if 1) treatment with H_2O_2 will result in a greater rate of mitochondrial biogenesis compared to control, 2) compared to control, the greater rate of mitochondrial biogenesis after H_2O_2 treatment will be blunted by Vit C treatment, and 3) compared to control, the greater rate of mitochondrial biogenesis after H_2O_2 treatment will not be blunted by treatment with a Nrf2 activator.

CHAPTER II – MANUSCRIPT I

Hypoxia mediated pulmonary edema: Potential influence of oxidative stress and sympathetic activation [1]

Summary

Neurogenic pulmonary edema (NPE) is a non-cardiogenic form of pulmonary edema consequent to stroke, traumatic brain injury and seizure. NPE is a public health concern due to high morbidity and mortality, yet the mechanism(s) are unknown. Hypothesis: Pulmonary edema, evoked by cerebral hypoxia in the presence of systemic normoxia, will be accompanied by sympathetic activation, increased oxidative stress, and compensatory antioxidant mechanisms. Methods: Using an updated Moss's model, thirteen Walker hounds were randomly assigned to receive 2 hours of cerebral hypoxia (SaO_2 ~55%) with systemic normoxia (SaO_2 ~90%) (CH; n=6), cerebral and systemic (global) hypoxia (SaO_2 ~60%) (GH; n=4), or cerebral and systemic normoxia (SaO_2 ~90%) (CON; n=3). Femoral venous (CH and CON) perfusate was delivered from a cardiopulmonary bypass circuit to the brain and GH was induced by reducing the inhaled oxygen concentration to 10% to maintain the SaO_2 at ~60%. Lung wet weight to lung dry weight ratios (LWW/LDW) were assessed as an index of pulmonary edema in addition to hemodynamic measurements. Plasma catecholamines were measured as markers of sympathetic nervous system (SNS) activity. Plasma lipid peroxidation and total glutathione, along with carbonyl modification of brain and lung proteins, were quantitated as indicators of oxidative

[1] Shadi Khademi*, Melinda Frye†, Kimberly Jeckel†, Thies Schroeder‡, Eric Monnet[+], Dave Irwin◊, Patricia Ann Cole[+], Christopher Bell*, Benjamin Miller*, Karyn Hamilton*†. *Translational Research on Aging and Chronic Disease Laboratory, Department of Health and Exercise Science., [+]Department of Clinical Sciences., †Department of Biomedical Sciences., Colorado State University, Fort Collins, CO 80523 USA. ‡Department of Radiation Oncology, Duke University Medical Center, Durham, NC 27710 USA. ◊ Cardiovascular Pulmonary Research, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045 USA.

stress, and brain and lung compensatory antioxidants were measured with immunoblotting. Results: LWW/LDW was greater in CH and GH compared to CON. Pulmonary artery pressure (PAP) was significantly higher in CH and GH compared to CON. Expression of hemeoxygenase-1 (HO-1) in brain was higher in CH compared to GH and CON, despite no significant difference in lipid peroxidation in the brain and lung in CH and GH compared to CON. Up regulation of endogenous antioxidant enzymes (compensatory antioxidants) in the lungs of CH and GH were similar to CON. Increases in catecholamine levels showed a trend to be higher in CH and GH compared to CON. Conclusion: Cerebral hypoxia, in the face of systemic normoxia, is not associated with an increase in oxidative stress and compensatory antioxidant enzymes in lung, suggesting oxidative stress did not contribute to an induction of NPE. However, SNS activity may have a role in induction of pulmonary edema.

Introduction

Neurogenic pulmonary edema (NPE) is an acute pulmonary edema occurring after a central neurologic insult, and is believed to occur independent of a cardiogenic contribution. NPE can occur following stroke, traumatic brain injury and seizure, and represents a major public health concern due to high morbidity (40-50%) and mortality (7%) [4]. Cerebral hypoxia is a common feature of many neuropathologies such as stroke, brain injury and seizure [5], suggesting that hypoxia may be an important factor in the pathogenesis of NPE. Currently, the underlying mechanism(s) of NPE are poorly understood.

The etiology of NPE is difficult to explore in human subjects. In animal models, NPE has been studied by applying increased intracranial pressure (ICP) [45, 46], inducing whole body hypoxia [47], or triggering the area of the A1 catecholamine neurons in the ventrolateral medulla by applying bilateral electrolytic lesions [48]. Though all of these models result in pulmonary

edema, the contribution of a cardiogenic source of the edema is difficult to confirm/deny, either because cardiac indices were not measured or because they exhibited significant changes during experimentation. Therefore, based on currently published findings, it is not possible to draw conclusions about the etiology of NPE in these models without consideration of a cardiogenic source.

To date, only a few studies have sought to investigate the mechanistic link between cerebral hypoxia and the induction of pulmonary edema. In 1976, Moss and Stein pioneered a canine model in which cerebral hypoxia was induced by directing venous perfusate to the brain in the presence of pulmonary and systemic normoxia, resulting in pulmonary edema [49]. More recently, our group modified the model by directing both arterial and venous perfusate to the brain to confirm that a hypoxic brain perfusate could lead to formation of pulmonary edema while avoiding the complications associated with venous CO_2 and pH [50]. However, in this experiment, alterations in ICP and cerebral blood flow (CBF) during hypoxia were not assessed. An important protective feature of the cerebral circulation is the ability to maintain CBF over a wide range of cerebral perfusion pressure (CPP), a physiological phenomenon known as cerebral autoregulation [51, 52]. On initial exposure to PaO_2 levels below a certain threshold (~40-45 mmHg), resultant tissue hypoxia produces cerebral vasodilation [52] leading to an increase in CBF. Hypoxia-induced increases in mean arterial pressure (MAP), stimulation of neuronal pathways, angiogenesis, release of adenosine, endothelium-derived nitrogen oxide (NO) and a variety of cytokines are additional factors acting to increase CBF and, overall, may cause loss of cerebral autoregulation [51, 52]. Loss of cerebral autoregulation may lead to overperfusion and increased ICP. Elevated ICP increases pulmonary vascular permeability to protein in dogs, contributing to NPE [46]. Because our previous study did not examine potential effects of CBF

alterations on induction of pulmonary edema, in the current study we modified our technique and controlled CBF. Further, since CPP is directly influenced by ICP [53], we monitored ICP to allow for better interpretation of CPP alterations.

Whole body hypoxia acts directly on systemic vascular smooth muscle, causing vasorelaxation and resultant hypotension [54]. Systemic hypotension, in turn, is known to result in baroreceptor-mediated sympathetic nervous system (SNS) activation and release of the catecholamines norepinephrine and epinephrine [54]. SNS activation in response to global hypoxia causes a systemic compensatory response including increases in heart rate (HR) and myocardial contractility leading to an increase in cardiac output (CO) [51, 54]. Use of animal models of NPE reveal that pronounced activation of the SNS is a necessary prerequisite for the development of NPE [55]; thus, it is possible that in certain instances hypoxia induces a hyperstimulated SNS which contributes to NPE. The role of SNS activation in induction of pulmonary edema following isolated cerebral hypoxia, with controlled CBF, is not known. In the current study, we measured plasma catecholamines to further understand the impact of the SNS, and associated hemodynamic alterations, on induction of pulmonary edema in response to isolated cerebral hypoxia compared to global hypoxia.

In addition to stimulating the SNS, hypoxia promotes generation of reactive oxygen species (ROS), leading to increased oxidative damage to lipids, proteins, and DNA [2]. ROS production that exceeds cellular and extracellular enzymatic and nonenzymatic antioxidants can disturb reduction-oxidation (redox) equilibrium [3]. There is an established link between increased oxidative stress and the pathogenesis of a number of human diseases [2, 3]. Several studies have described the potential role of oxidants in the pathogenesis of acute lung injury [56-58]. It is therefore possible that cerebral hypoxia induces excessive circulating brain derived

ROS that impair pulmonary endothelial barrier function and contribute to the development of NPE.

The endogenous cellular antioxidant defense system consists of approximately 400 proteins that maintain the reducing environment [6]. The nuclear factor erythroid 2 related factor 2 (Nrf2) belongs to the Cap'n'collar/basic region leucine zipper (CNC-bZIP) transcription factor family and is the master regulator of antioxidant defenses [6]. After oxidization of cysteine residues on the cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap1), Nrf2 dissociates from Keap1, translocates to the nucleus and binds to the antioxidant response element (ARE) in target genes. Through transcriptional activation of ARE-bearing genes involved in antioxidant defense, such as the phase II antioxidants superoxide dismutase (SOD), NAD (P) H: quinone oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1), Nrf2 activates cellular pathways that protect against oxidative injury [7, 35, 37].

Nrf2 can be activated by oxidants produced during hypoxia [9]. It has been shown that HO-1 is induced in the rostral ventrolateral medulla during chronic hypoxia and it is associated with cardiorespiratory adaptations during hypoxia [10, 11]. Further, Sharma et al, have shown that during hypoxia, SOD1 expression was increased in the cortex to neutralize the hypoxia-induced ROS generation [59]. Given the evidence of ROS generation during hypoxia, and based on studies that have suggested a role for oxidative stress in induction of pulmonary edema, we were particularly interested in exploring the role of oxidative stress and compensatory antioxidants (HO-1, NQO1 and SOD1) in both cerebral and global hypoxia-induced pulmonary edema.

In the present study we refined our existing cerebral hypoxia-induced NPE model by controlling CBF and monitoring ICP to further delineate the role of a cardiogenic source in the

induction of pulmonary edema, and we investigated the role of oxidative stress in this form of pulmonary edema. We hypothesized that pulmonary edema, evoked by cerebral hypoxia in the presence of systemic normoxia, will be accompanied by sympathetic activation, increased oxidative stress, and compensatory antioxidant responses. Finally, we compared and contrasted the responses to cerebral and global hypoxia-induced pulmonary edema.

Methods

Experimental design

All surgical procedures and methods were approved by Colorado State University Animal Care and Use Committee (protocol numbers: 09-1351A and 11-2663A) and adhered to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Thirteen male adult Walker hounds (25 – 30 kg), 4 years of age, were housed in individual stalls, provided water and food ad libitum, and randomly assigned to one of three groups: control (CON, n=3), cerebral normoxia with systemic normoxia (SaO_2 ~90%); cerebral hypoxia (CH, n=6), cerebral hypoxemia (SaO_2 ~55%) with systemic normoxia (SaO_2 ~90%); global hypoxemia (GH, n=4), systemic hypoxemia (SaO_2 ~60%).

Surgical procedure

Anesthetic and surgical procedures were adapted from those previously described [50] reflecting a modification to the original model developed by Moss and Stein [49]. Following an overnight fast, the dogs were sedated with fentanyl (10 μg/kg SQ), atropine (0.04 mg/kg SQ) and midazolam (0.2 mg/kg SQ) 30 minutes prior to anesthetic induction. Anesthesia was induced by propofol (2.5 mg/kg IV) and maintained by fentanyl (0.01 mg/kg IV). Once a surgical plane of anesthesia was confirmed, animals in the GH group were intubated and placed on a volume ventilator (10 breaths/minute) with 100% oxygen and isoflurane (1.0-2.0%) during surgical

isolation of the jugular veins, carotid, and femoral arteries. A catheter was placed underneath the meninges to estimate ICP. ICP was recorded every 15 minutes on a pressure monitor (Marquet 7000, Fridley, MN). CPP (mmHg) is calculated by subtracting the ICP (mmHg) from the MAP (mmHg). Dogs were instrumented with a 7.5 Fr Swan Ganz catheter floated in the pulmonary artery from a jugular vein. CO was measured by thermodilution [60] with 10 ml of iced saline. Pulmonary arterial pressure (PAP) and capillary wedge pressure (Pwedge) were assessed using a Swan-Ganz catheter inserted into the pulmonary artery. A catheter was placed in a dorsal pedal artery to measure MAP. Stroke volume (SV) was the quotient of CO (L/min) divided by HR (bpm). Systemic vascular resistance (SVR) was the quotient of MAP divided by CO and presented as the product of dyne and time relative to $cm^{\wedge 5}$ ($dyn*s/cm^5$). Pulmonary vascular resistance (PVR) was calculated by the subtraction of PAP from Pwedge divided by CO and presented as the product of dyne and time relative to $cm^{\wedge 5}$ ($dyn*s/cm^5$). A 2 mm flow probe (Transonic, Transonic Systems, Inc., Ithaca, NY) was placed around each carotid artery to measure blood flow. Data were collected at baseline and then every 30 minutes during the experiment.

In the GH group SaO_2 was clamped at 60% by having dogs breath a 10% oxygen, nitrogen balance mixture. Then we would bleed in room air at 21%, as needed, until SaO_2 was at a steady state of 60%. In CH and CON groups, a femoral vein was cannulated with a 14 Fr cannula and connected to the inlet port of a cardiopulmonary bypass pump (Roller pump, Cobe, Lakewood, CO, USA). Both carotid arteries were cannulated with an 8 Fr cannula and connected to the pump outlet with a "Y" connector. When venous and arterial isolations were achieved, the animals were weaned to 21% O_2 and ventilation rate adjusted to 8 - 10 breaths/minute to maintain normal systemic partial pressure of CO_2 and oxygen saturation (SaO_2:

92-95%). Both vertebral arteries were dissected at the level of 7th cervical vertebrae. Tourniquets were preplaced around each vertebral artery.

After normal carotid blood flow was achieved from the cardiopulmonary bypass pump, carotid arteries were perfused with femoral venous blood desaturated to 60% for the CH group or saturated to 92-95% for the CON group. The tourniquets around the vertebral arteries were pulled to prevent entry of the normoxic blood to the brain. Blood flow of the CH and CON groups was matched to the increase of blood flow registered in the dogs in the GH group. All animals underwent 2 hours of hypoxia (or normoxia in the CON group).

Blood and tissue acquisition

Blood (6 ml) was drawn from the dorso-pedal arterial and jugular venous and internal carotid catheters at baseline, 30, 60, 90 and 120 minutes of hypoxia or normoxia. Samples were transferred into chilled vials, one set containing Ethylenediaminetetraacetic acid (EDTA) (1.8 mg K3 EDTA per 1 ml of blood) and the other set containing 0.3 M ethylene glycol tetraacetic acid (EGTA) and 0.3 M glutathione plus EDTA (1.8 mg K3 EDTA per 1 ml of blood). Plasma was separated by centrifugation (4°C, 14,000 x g; 10 minutes) immediately, and stored at -80°C until assayed. At the end of 2 hours of hypoxia/normoxia, the animals were euthanized with sodium pentobarbital (10 ml IV) and the heart and lungs were removed by median sternotomy. The left caudal lung lobe was removed, weighed, and oven dried (65°C until stable weight was achieved) for lung wet weight to lung dry weight (LWW/LDW) ratios as indices of pulmonary edema. The right caudal lobe was frozen in liquid nitrogen and stored at -80°C for further analysis. Remaining lung lobes were fixed in 10% formalin for 24 hours, placed into 70% ethanol, paraffin embedded and cut into 4μm sections for histological analyses. Brainstem, cerebellum, left half of the cerebral cortex and myocardium, removed from the middle of the left ventricular free wall, were removed, snap frozen in liquid nitrogen, and stored at -80°C for later

analysis. A coronal section through the cortex around the prefrontal lobe and a sample of left ventricular free wall was prepared for histological analyses in a manner identical to that used for lung tissue.

Western blot analyses

Approximately 50 mg of frozen brain cortex and lung were homogenized (Next Advance Inc, Averill Park, NY, USA) in 1 ml of ice-cold buffer (40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 100 mM NaCl, 1% TritonX-100, 1 mM EDTA, pH 7.4) with protease and phosphatase inhibitors (Halt, Thermo Fisher, Rockford, IL, USA). Samples were centrifuged (4°C, 10,000 x g, 10 minutes) and protein concentration of the supernatant was determined using a bicinchoninic acid assay (Thermo Fisher, Rockford, IL, USA). Samples were heat denatured in Laemmli buffer, separated using 10% SDS-PAGE, transferred to nitrocellulose paper, and incubated in 5% milk in TBST (Tris-buffered saline with tween) for 1 hour prior to immunoblotting. To prepare the nuclear extracts, 40 mg of cerebral cortex and lung tissue were homogenized in 200 volumes of Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit buffer (Thermo Fisher, Rockford, IL, USA). 20 µg of the nuclear protein (lung and brain) was then prepared for immunoblotting as described above. Antibodies were purchased from Abcam (Cambridge, MA, USA, HO-1 # Ab13248, NQO-1 # Ab2346), Santa Cruz Biotechnology (Santa Cruz, CA, USA, Nrf2 #SC-1302, SOD1# SC-8637) and Millipore (EMD Millipore Corporation, Billerica, MA, USA; amyloid precursor protein A4 (APPA4) # MAB348SP). Blots were incubated overnight at 4°C with primary antibodies diluted 1:200 in TBST, washed in TBST, and incubated with HRP-conjugated secondary antibody diluted 1:1000 in 5% milk in TBST for 1 hour at room temperature (anti-rabbit for Nrf2, anti-goat for SOD-1 and NQO-1 and anti-mouse for HO-1) followed by chemiluminescence detection (West Dura; Pierce, Rockford, IL, USA). Images were captured and densitometry conducted using a UVP

Bioimaging system (Upland, CA, USA). Equal loading was verified using Ponceau Staining as well as actin antibodies (SC-8432; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry (IHC) and myocardial histology

Aforementioned 4 μm thick paraffin sections from brain cortex were mounted on poly-L-lysine slides. Slides were dewaxed and sections rehydrated by immersion in ethanol (100%, 95%, and 70%) and then distilled water. After washing, sections were preincubated in PBS supplemented with 0.5% BSA and 10% normal horse serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour, then incubated overnight with mouse monoclonal anti-APPA4 antibody diluted 1:100 in PBS containing 0.5% BSA and 15% normal horse serum. The sections were then incubated for 1 hour with 1:1000 biotin-labeled anti-mouse secondary antibodies (Santa Cruz, CA, USA), followed by streptavidin-biotin-horseradish peroxidase solution containing 3′, 3′-diaminobenzidine (DAB) tetrahydrochloride dihydrate (Dakocytomation, CA, USA) and hydrogen peroxide. Finally, the sections were counterstained with hematoxylin. Signal density was quantified using Image J software (NIH, USA).

Aforementioned sections of left ventricle were stained with hematoxylin and eosin at the Colorado State University Diagnostic Laboratory. A section from each dog was examined using light microscopy by a boarded veterinary pathologist (PC) who was naïve as to the experimental condition, and observed for normal structures as well as the presence of pathologic lesions.

Cardiac troponin-I (cTnI)

The Ultra-Sensitive Dog cTnI ELISA Kit (Life Diagnostics Inc., West Chester, PA) was used to measure cTnI, a circulating marker of myocardial injury and/or ischemia, in EDTA systemic arterial samples at time 0 and 2 hours of study.

Assessment of oxidative stress: Carbonylated proteins of lung and brain tissues were detected and analyzed following derivatization of protein carbonyl groups with 2, 4-

dinitrophenylhydrazine, using the OxyBlot kit (Millipore, Billerica, MA, USA). Immunodetection was performed using 15 μg of protein per lane (3μg/μl) and primary antibody directed against dinitrophenylhydrazone (Millipore, Billerica, MA, USA). To measure malondialdehyde (MDA) concentrations, 25 mg of lung and brain tissue was quantitated using a TBAR assay kit (Cayman Chemical, Ann Arbor, MI, USA) per manufacturer recommendations. The total glutathione (GSH) content of the brain and lung tissue was determined by means of a GSH assay kit (Cayman Chemical, Ann Arbor, MI, USA). Using this system, oxidized GSH is converted to GSH by GSH reductase, and is included in the total GSH measured.

Plasma catecholamines

Plasma obtained from the blood that was collected into tubes with EGTA/glutathione was used to quantitate circulating norepinephrine and epinephrine with enzyme-linked immunosorbent assay (ELISA) (Rocky Mountain Diagnostics Inc., CO, USA).

Statistical analyses

Hemodynamic data from all 13 dogs were included in analyses (CH=6, GH=4, CON=3). Unless otherwise noted, the remaining analyses were conducted using data from 12 dogs (tissues were not collected from the first CH procedure). Statistical analyses were performed with the SAS (version 9) statistical package (SAS, Cary, NC) and SPSS (IBM, version 19). Inter-subject variability in baseline hemodynamic measurements was controlled for by considering baseline measurements as covariates. Data were analyzed by one-way repeated measures of analysis of variance (ANOVA). Western blot, IHC and catecholamine data were analyzed by one-way ANOVA. Statistical significance was established a priori at $p < 0.05$.

Results

In dogs assigned to the CH group, femoral and carotid SaO_2 were measured to verify that

cerebral hypoxia was established in the presence of systemic normoxia. As expected, SaO_2 was lower in carotid versus femoral arterial blood in CH. Global hypoxia resulted in a significantly lower femoral SaO_2 compared to CH and CON. Carotid SaO_2 was significantly lower in both CH and GH compared to CON (Figure 2.1, $p < 0.05$). Therefore, the model was successful in induction of isolated cerebral hypoxia.

Gross lung assessment at necropsy and LWW/LDW ratios were used to evaluate the presence of pulmonary edema. Compared to CON, the LWW/LDW ratio was greater in CH and GH animals (Figure 2.2, $p < 0.05$). In addition, gross specimens of lung showed areas of petechiae that were more visible in CH and GH compared to CON (Figure 2.3). These findings suggest that pulmonary edema was established after 2 hours of either global hypoxia or isolated cerebral hypoxia.

Measured and calculated hemodynamic measurements are presented in Table 2.1. Mean CO across all time-points was greater in GH compared to CH; the greater CO values for GH compared with CON did not attain statistical significance ($p = 0.07$). These differences were attributed to an increase in the SV of GH animals. Our data showed no difference in MAP between GH and CON animals, but MAP was decreased in CH compared to CON animals. SVR was lower in GH compared to CON. Pulmonary arterial pressure was greater in both hypoxic groups compared to CON, but Pwedge was similar across groups. Mean ICP was not different among groups; however, CPP was lower in CH compared to CON and GH.

Presence of cerebral hypoxia was evaluated by quantifying the expression of hypoxia-sensitive protein APPA4 in the brain by immunoblotting and immunohistochemistry (Figures 2.4A and 2.4B, respectively). There was almost a 3-fold increase in APPA4 DAB staining in CH compared to CON, although data did not reach statistical significance.

Light microscopic examination of cardiac left ventricular tissue sections revealed no observable pathologic lesions in CON, CH and GH after 2 hours of treatment conditions. Cell and matrix morphology was normal in all sections in all animal groups. Further, there was no group difference in cTnI, a marker of myocardial injury and/or ischemia (data not shown).

Presence of oxidative stress in lung and brain tissues was evaluated by assessing lipid peroxidation and protein carbonylation. No group differences in these markers of oxidative stress were detectable after 2 hours of treatment conditions (data not shown).

To assess Nrf2 activation, the nuclear to cytoplasmic ratio of this protein in lung and brain was measured in all three groups. No group differences in the nuclear to cytoplasmic ratio of Nrf2 were detectable after 2 hours of treatment conditions (data not shown).

To detect whether there was a hypoxia-induced upregulation of ARE-regulated phase II antioxidants, expression of Nrf2, HO-1, NQO1, and SOD1 were measured in lung (Figure 2.5) and brain (Figure 2.6) of all three animal groups. There was no difference in Nrf2 expression in lung or brain, nor in lung HO-1 or brain SOD1, across treatment groups. Expression of NQO1 in the lungs of CH and GH animals was significantly less than CON (Figure 2.5C). Brain HO-1 expression was greater in CH compared to CON and in CH compared to GH (Figure 2.6C).

To assess SNS activity, catecholamine concentrations in blood collected from the jugular vein were measured at baseline and after 60 minutes of hypoxia/normoxia. Compared with CON, the magnitude of increase in norepinephrine was greater in CH ($p = 0.07$) and GH ($p = 0.10$) although this increase did not attain statistical significance (Figure 2.7A). Similarly, compared with CON, the increase in epinephrine at 60 minutes in CH, although physiologically relevant, was not statistically significant ($p = 0.10$) (Figure 2.7B). Norepinephrine and epinephrine concentrations increased between baseline (time 0) to 60 minutes in nearly all CH

and GH individual canines, while both catecholamines remained unchanged in the CON dogs (data not shown).

Discussion

Cerebral hypoxia is a common feature of many neuropathologies [9], yet limited data are available on the mechanisms that induce pulmonary edema in the presence of hypoxic brain insults. The current study represents an appreciable advancement of previous methods [49, 50] due to consideration of ICP and controlling for CBF. In the presence of similar CBF, both cerebral and global hypoxia resulted in pulmonary edema. We also investigated markers of systemic SNS activation and oxidative stress in lung and brain tissues in this model. Findings of the present study suggest that 2 hours of cerebral or global hypoxia in the presence of pulmonary hypoxia was not sufficient to induce oxidative stress, however, our data suggest involvement of sympathetic activity in induction of pulmonary edema.

Novelty of the model

Several important and novel aspects of the model are noteworthy. This experiment was the first to monitor ICP in the canine model during cerebral hypoxia, allowing for calculation of CPP. Even though ICP was measured indirectly by probe placement in the subdural space rather than the ventricles, it allowed us to assess the magnitude of change in ICP. Our data suggest that CPP values were in the physiologic range (50-160 mmHg) [52], therefore reflective of a functional autoregulation system. This study was also the first to control CBF during an isolated cerebral hypoxia model by matching CBF during both cerebral and global hypoxia. We found that CBF does not appear to be a major determinant of development of pulmonary edema, that is, pulmonary edema may occur in the absence of changes in CBF.

Pulmonary hemodynamics

It has been suggested that an increase in PAP contributes to the pathogenesis of NPE [61]. We found PAP was significantly greater in CH and GH compared to CON group. Our data did not support previous findings of Irwin et al. [50] in which they showed no significant difference between PAP in cerebral hypoxia compared to the normoxic animals. The absence of greater afterload in pulmonary vasculature in our study, indicated by a lack of differences in Pwedge between the CH and GH compared to CON, along with the absence of significant myocardium pathology and circular marker of myocardium injury cTNI in all groups, supports previous findings that cardiogenic component is absent in an induction of NPE [50]. Our data suggest that increased pressure within the pulmonary circuit likely contributes to pulmonary edema in our model thus, vascular permeability alone in the absence of significant rise in PAP as observed by Irwin et al. [50] does not explain the development of edema in this model.

Systemic hemodynamics

Sensing of hypoxia occurs through specialized chemoreceptor cells located in bifurcation of carotid artery that regulate cardiovascular and ventilatory rates [62]. Hypoxia in general decreases the vascular resistance, and CO is subsequently altered so that physiologic MAP is maintained [54]. As shown in the current study, GH led to greater CO compared to CH. Augmented CO in GH group was attributed to an increase in SV since the HR in the GH animals was not different compared to CON and CH. MAP in GH and CON was not significantly different, which implies a CO compensation for hypoxic vasodilation. In our study, the hypoxic perfusate to the brain bypassed the chemoreceptors such that hypoxia was not detected by the chemoreceptors and MAP was significantly attenuated in CH compared to CON. Yet, oxygen saturation in carotid artery was lower in CH compared to CON, which was an indication of hypoxia in the brain. Together we have found that systemic compensatory responses to hypoxia

are absent in our isolated cerebral hypoxia model.

SNS activity

Acute hypoxia has been well-established as a sympathetic activator [54]. However, the role of SNS activity in development of NPE in isolated cerebral hypoxia compared to global hypoxia is not known. To evaluate the systemic SNS activity, plasma catecholamine concentrations were quantified. Since catecholamine concentrations are the net result of secretion, spill-over, reuptake and excretion, comparison of concentrations at baseline and 60 minutes was performed to better characterize the net change in each canine. Increases in catecholamines, CO, and SV in GH suggest that the physiological chemoreceptor response may be activated through SNS in this group.

A 6-fold increase in norepinephrine in CH compared to CON suggests that SNS activity may be higher with cerebral hypoxia compared to normoxia. An increase in SNS activity in CH occurred in the presence of increased PAP. The fact that sensing hypoxia in CH models did not occur via the activation of carotid chemoreceptors indicates that SNS activation in the CH group did not occur through this pathway. Neurons that normally contribute to oxygen sensing are located in the rostral ventrolateral medulla, the caudal hypothalamus, the pre-Bötzinger complex and the nucleus tractus solitarius [10]. In the absence of a carotid chemoreceptor response, our findings suggest oxygen sensing in these higher centers are involved in SNS activation and subsequent increase in PAP that contribute to the formation of pulmonary edema. Whether other factors are involved in SNS activation will require further investigation.

Role of oxidative stress in NPE

Increases in SNS activity due to oxidative stress have been suggested [63-65], and it has been shown that sympathetic activity has a role in generation of NPE in several animal models [66-68]; but whether oxidative stress accompanied by sympathetic activation are involved in

generating increased PAP or pulmonary edema is unclear. In our study, markers of oxidative stress and antioxidant response were not higher in lung tissue after 2 hours of hypoxia. Other studies do not support this finding [69]. However, brain HO-1, a phase II antioxidant downstream of Nrf2, was significantly higher in CH compared to CON. In vitro data from our lab have shown that Nrf2 translocation to the nucleus, an indicator of Nrf2 activation, occurs within 15 minutes of an oxidative challenge [37]. Nevertheless, Nrf2 nuclear localization is not sustained and there is a controlled mechanism for nuclear export of Nrf2 [70]. We were therefore not surprised by the absence of Nrf2 activation after 2 hours of hypoxia, yet higher levels of brain HO-1 in CH compared to CON suggest Nrf2 was activated earlier in the experiment. Nrf2 activation in the absence of oxidative stress markers suggests a protective role of Nrf2 in induction of oxidant induced pulmonary edema. The suggestion of a Nrf2 protective role is supported by the finding that Nrf2 (-/-) mice have a greater increase in the lung wet/dry weight ratio following head injury compared to wild-type Nrf2 (+/+) [71].

Higher levels of brain HO-1 in isolated cerebral hypoxia in the current study suggest that redox sensitive signaling in the brain could contribute to activation of the SNS and induction of pulmonary edema. This finding is supported by previous reports. For example, hypoxia acts directly on brainstem neurons, as opposed to only carotid body inputs which are carried within the carotid sinus nerve to the nucleus tractus solitarius and the brainstem respiratory centers [72]. Knowing that hypoxia can induce ROS production [73], it is possible that brain hypoxia activates higher centers of SNS through redox signaling pathways, which leads to increased PAP and induction of pulmonary edema. Also, in the presence of elevated hypothalamic NADPH oxidase activity (a marker of oxidative stress), the expression level of tyrosine hydroxylase, an enzyme responsible for the synthesis of cathecholamines, was higher in the nucleus tractus solitarius of

the brain stem [74]. These data, along with previous findings that HO-1 is induced in hypoxia-sensitive brain regions [11, 75] are consistent with our data suggesting that HO-1, a compensatory response to oxidative stress, could act as a sensor in brain hypoxia and may have a role in induction of SNS activity.

Limitations of the study

There are additional issues pertaining to the present study that warrant further discussion. First, we recognize the fact that all of our hemodynamic measurements were performed in anesthetized animals. However, as none of the procedures described could have been performed in the absence of anesthesia and thus, we feel this is an acceptable limitation. Second, on a technical note, ICP was not determined directly, rather it was estimated from subdural probe placement. This approach is not without precedent, indeed, it has been validated against gold-standard techniques [76]. Third, several of the differences we report did not attain statistical significance; this may be due in part to the relatively small sample size and hence insufficient statistical power. However these were expensive and technically challenging experiments and previous studies also were limited in the sample size [49, 50]. Lastly, while all of our data may not be statistically significant, we do believe them to be physiologically relevant.

Conclusions

In conclusion, the present study suggests that increased SNS activity and PAP, in the absence of CBF alterations, contributes to pulmonary edema in isolated cerebral hypoxia. Furthermore, isolated cerebral hypoxia for 2 hours induces HO-1 in the brain, suggesting redox sensitive signaling and a compensatory antioxidant response to hypoxia. In this way, it seems that HO-1 may be an oxygen sensor in the brain, leading to compensatory activation of the SNS, and induction of pulmonary edema through a rise in PAP.

Table 2.1: Hemodynamic measurements during experimentation. Data represent mean ± SEM of measures taken every 30 min under sham operated animals (CON), cerebral hypoxia (CH), and global hypoxia (GH). *p < 0.05 compared to CON, #p < 0.05 CH compared to GH. Baseline values were measured prior to induction of experimental conditions. The last column in the table represents the mean ± SEM of all time- points.

		Baseline	30 min	60 min	90 min	120 min	Mean ± SEM at all timepoints
HR (bpm)	CON	116±5	118±14	134±19	122±22	124±9	119±15
	CH	111±4	116±12	107±5	117±10	124±9	114±10
	GH	90±6	111±12	98±11	101±13	103±9	111±15
MAP (mmHg)	CON	65±6	78±12	85±1	82±2	83±8	86±4
	CH	84±11	82±6	67±6	74±4	73±6	73±2*
	GH	81±3	80±2	77±3	79±2	77±2	78±3
PAP (mmHg)	CON	15±1	14±3	15±1	14±2	15±1	15±2
	CH	22±4	22±2	20±2	23±1	25±1	23±1*
	GH	22±2	28±2	24±2	29±3	29±3	27±2*
Pwedge (mmHg)	CON	11.7±0.6	12.0±0.6	11.7±0.7	12.3±1.2	12.3±0.9	12.5±0.9
	CH	14.2±0.9	13.2±0.8	13.0±0.5	12.3±0.9	12.5±0.9	12.7±0.5
	GH	15.8±1.3	15.0±1.2	13.8±1.3	14.8±0.3	15.0±0.4	14.4±0.7
CO (L/min)	CON	2.6±0.3	3.1±0.8	3.3±0.7	3.2±0.9	3.7±0.6	3.8±0.5
	CH	4.3±1.2	4.3±1.0	4.1±0.7	4.1±0.4	3.8±0.3	3.9±0.3
	GH	3.9±0.4	5.4±1.0	5.1±0.4	5.2±0.7	5.1±0.7	5.2±0.4#
ICP (mmHg)	CON	12±3	10±2	11±2	11±3	11±3	14±2
	CH	21±3	22±3	21±2	21±3	22±3	17±1
	GH	11±2	7±2	10±4	9±3	11±5	13±2
SV (ml)	CON	23.3±3.4	29.3±11.5	26.8±8.3	28.6±9.5	34.1±9.9	35.4±4.3
	CH	40.8±12.4	39.9±8.7	39.7±7.3	35.9±3.5	31.7±3.1	35.7±2.9
	GH	44.8±6.1	48.2±3.9	53.2±4.3	52.7±7.0	50.2±5.7	48.4±3.6*#
PVR (dyn*s/ cm^5)	CON	1.7±0.9	3.1±0.8	1.5±0.4	2.6±0.7	2.5±0.5	2.4±0.7
	CH	2.1±0.5	3.6±1.9	2.1±0.6	2.6±0.4	3.2±0.6	2.8±0.5
	GH	1.3±0.2	1.1±0.6	1.8±0.5	1.3±0.9	1.5±0.7	1.6±0.6
SVR (dyn*s/ cm^5)	CON	24.8±0.9	26.9±4.1	29.6±8.6	29.3±6.5	24.3±7.1	27.6±3.2
	CH	27.2±5.2	26.0±7.3	18.3±3.5	19.2±2.7	19.5±2.0	19.8±2.3
	GH	21.9±3.7	16.0±2.6	15.5±1.7	16.4±2.9	15.9±2.8	17.3±2.8*
CPP (mmHg)	CON	53±10	68±14	74±3	71±4	72±6	74±3
	CH	63±12	60±5	45±6	53±4	51±7	52±2*#
	GH	70±5	74±4	68±6	70±3	66±5	67±3

Figures

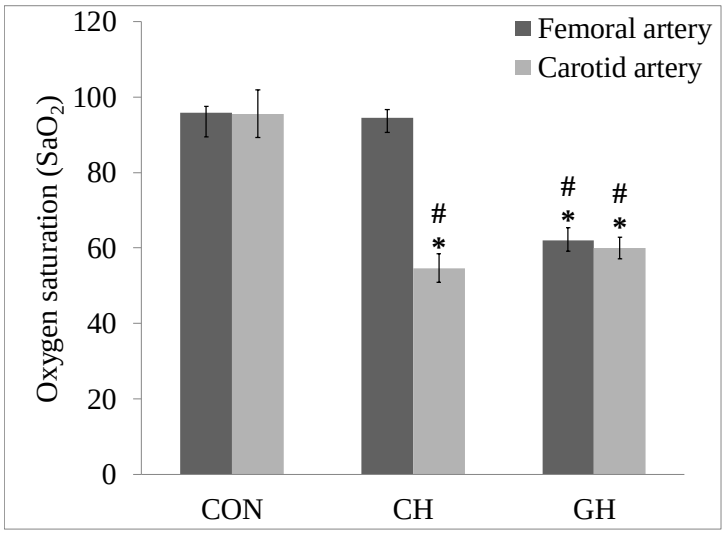

Figure 2.1. Arterial oxygen saturation (SaO_2) of blood from femoral and carotid arteries in CON, CH and GH animals after 2 hours of hypoxia. (*$p < 0.05$ compared to CON femoral artery and CON carotid artery, #$p < 0.05$ compared to CH femoral artery). Data presented as mean ± SEM.

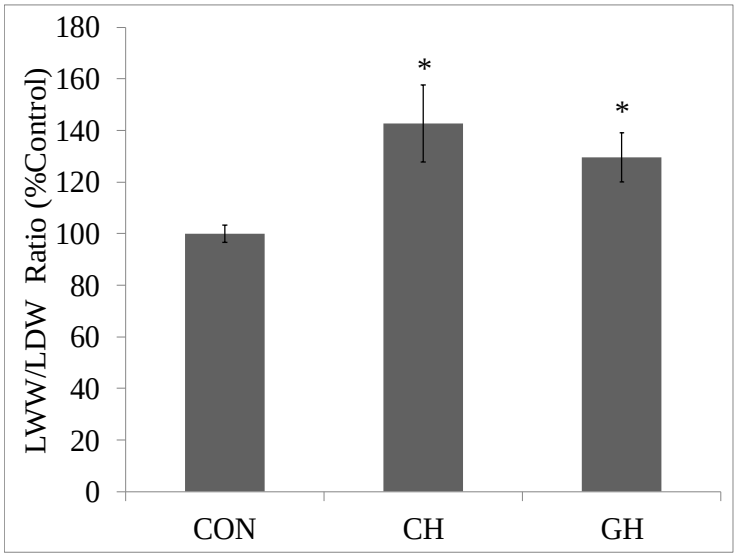

Figure 2.2. Lung wet weight to dry weight (LWW/LDW) ratio. Two hours of CH and GH increased the LWW/LDW ratio, indicating increased pulmonary vascular leak (*$p < 0.05$

compared to CON). Data presented as mean ± SEM.

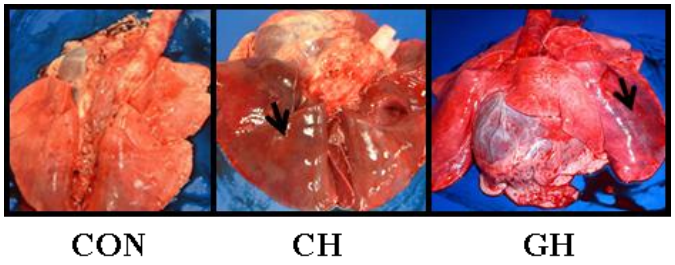

Figure 2.3. Areas of petechiae visible on gross examination of lung tissue. Petechiae, indicated by arrows, are more visible in CH and GH compared to CON.

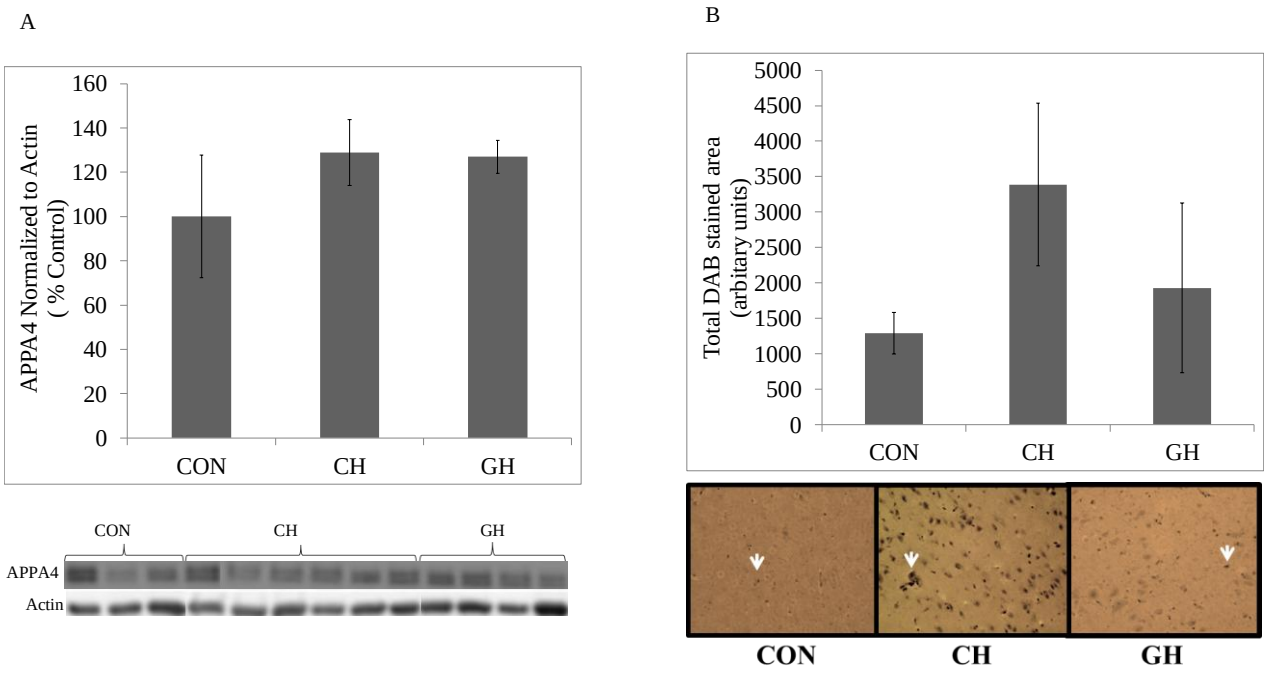

Figure 2.4. Expression of amyloid precursor protein A4 (APPA4) in the brain cortex after 2 hours of normoxia (CON), CH and GH. A. APPA4 expression detected by Western blotting. B. APPA4 expression detected by immunohistochemistry. Arrows indicate positive DAB staining. Data presented as mean ± SEM.

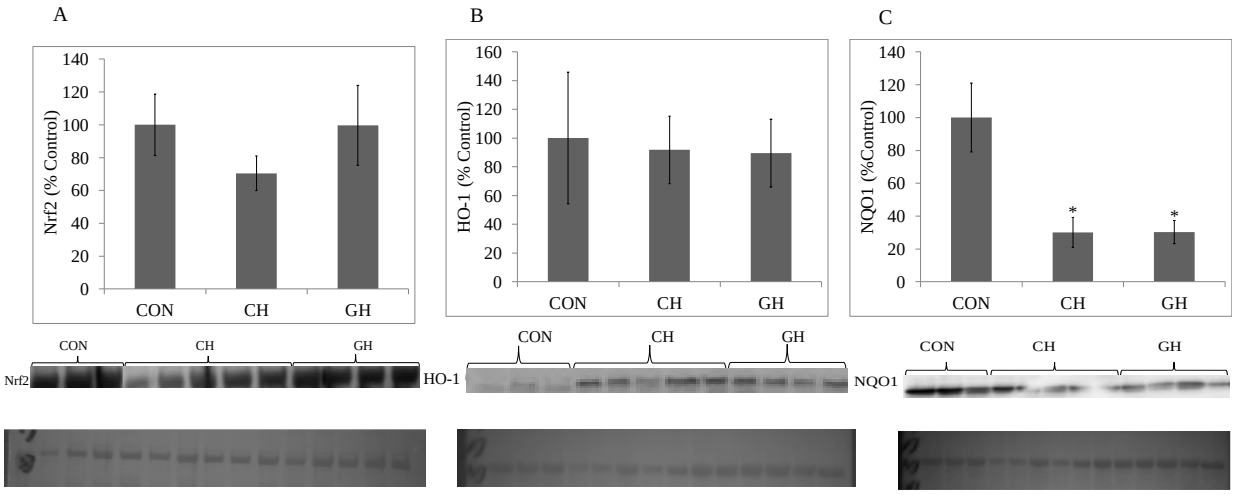

Figure 2.5. Nrf2 and phase II antioxidant protein expression in lung tissue, measured by Western blotting. Bar graphs (top) reflect the ratio as % CON while bottom images are representative immunoblots and ponceau staining, since actin was not detectable as a loading control. Nrf2 (A) and HO-1 (B) expression was similar across treatment groups. C. NQO1 expression was decreased in CH and GH compared to CON (*$p < 0.05$). Data presented as mean ± SEM.

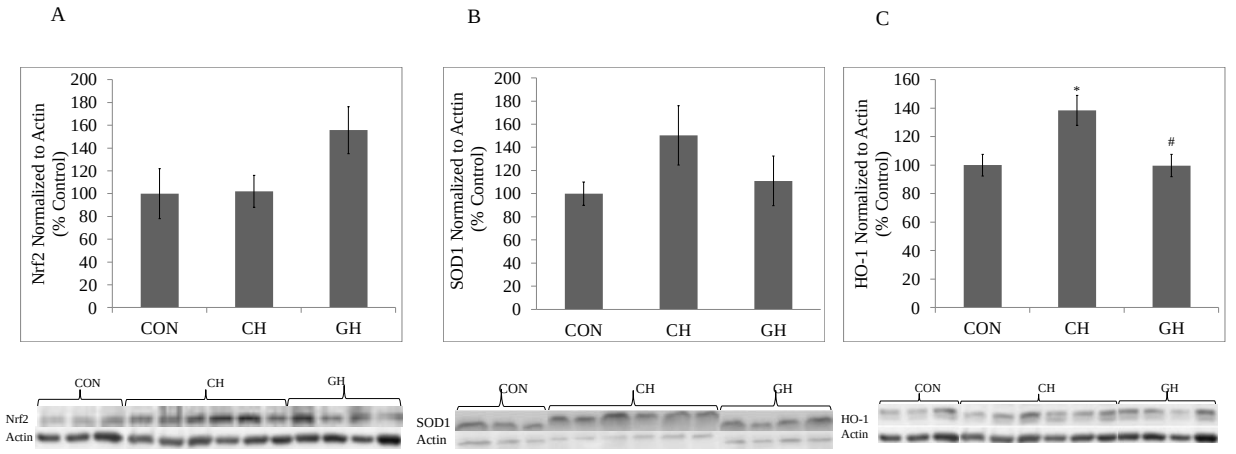

Figure 2.6. Nrf2 and phase II antioxidant protein expression in brain tissue, measured by Western blotting. Bar graphs (top) reflect the protein content normalized to actin and expressed as % CON while bottom images are representative immunoblots and actin as a loading control protein. A. Nrf2 expression in CON, CH and GH. B. SOD1 expression in CON, CH and GH

animals. C. HO-1 expression in CON, CH and GH. *p < 0.05 compared to CON. #p < 0.05 compared to CH. Data presented as mean ± SEM.

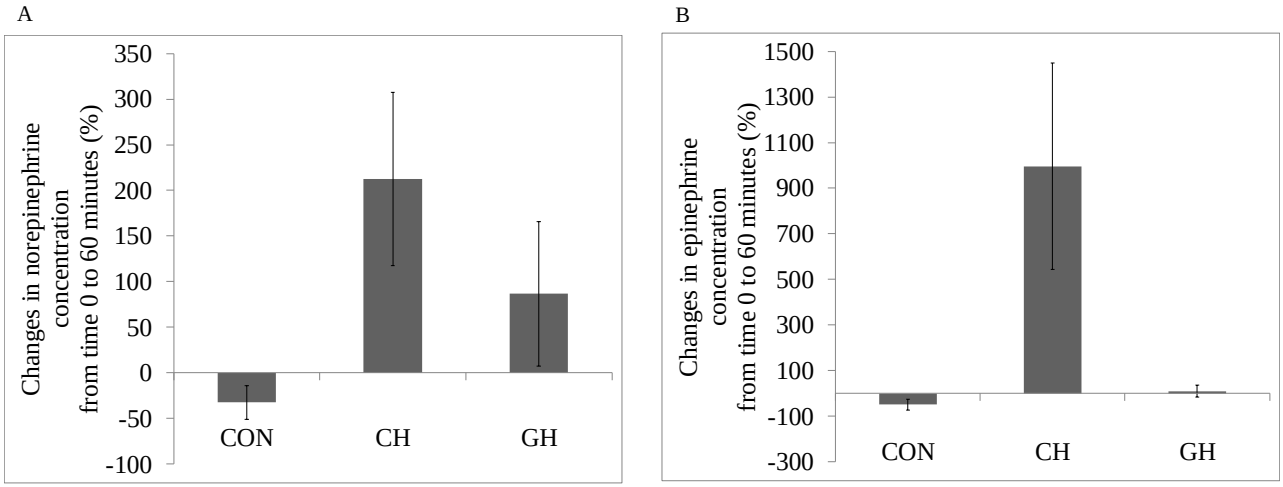

Figure 2.7. Percent changes in catecholamine concentrations in venous blood extracted from the jugular vein after 60 minutes of treatment. A. Percent change of norepinephrine concentrations from baseline to time 60 in CON, CH and GH groups. B. Percent change of epinephrine concentrations from baseline to time 60 in CON, CH and GH groups. Data presented as mean ± SEM.

CHAPTER III – MANUSCRIPT II

A development of an in vitro model for studying endurance-like exercise in C2C12 myotubes[2]

Summary

Despite progress in the redox biology of exercise, the role of redox sensitive pathways in cellular adaptation such as mitochondrial biogenesis is poorly understood. Understanding the specific molecular mechanisms that result in exercise-induced cellular responses such as mitochondrial biogenesis has been complicated by the fact that during contraction the muscle fibers are exposed to numerous metabolic and mechanical stimuli. Therefore, there is a need for a model of muscle contraction in vitro. To provide evidence that an in vitro model of electrically stimulated myotubes is capable of simulating the adaptive response to a single bout of endurance exercise, C2C12 myotube contraction was induced by electrical stimulation (E-stim). Hypothesis: E-stim induced contractile activity of cultured murine myotubes would induce energetic stress, redox sensitive signaling, and mitochondrial biogenesis in a manner qualitatively comparable to endurance exercise conducted in an intact organism. Methods: C2C12 myotubes cultured onto 6-well plates received E-stim with an electric pulse of 50 V at 10 Hz for 4 s at 3.6 s intervals and were contracted for 3 hrs. To determine if the E-stim had aerobic exercise-like responses, we assessed protein synthesis rates with deuterium oxide (D_2O) in addition to assessing activation of pathways indicative of energetic stress or oxidative stress at 0 hr, 3hr and 6 hrs post E-stim. Results: The sarcoplasmic protein synthesis rate was higher in E-stim compared to CON. There was a 20% increase in AMP activated protein kinase (AMPK)

[2] Shadi Khademi*, Benjamin Miller*, Karyn Hamilton*. *Translational Research on Aging and Chronic Disease Laboratory, Department of Health and Exercise Science, Colorado State University, Fort Collins, CO 80523 USA

mRNA levels in E-stim compared to CON. HO-1 protein level was significantly higher at all timepoints of E-stim compared to CON. In addition, protein expression of peroxisomal proliferator-activated receptor [gamma] coactivator-1 (PGC-1) alpha (PGC1-α) and cytochrome c oxidase subunit IV (COXIV) was significantly increased 6 hrs post E-stim compared to CON. Conclusion: redox sensitive signaling and new protein synthesis are two major outcomes of induced contractile activity in electrically stimulated C2C12 myotubes. These findings are in accordance with cellular adaptations that occur after a single bout of endurance exercise in vivo. Thus, this model is an applicable in vitro system for the analysis of intracellular events evoked by muscle contraction. Using this model will allow for investigating the role of redox signaling pathways in cellular response to exercise such as mitochondrial biogenesis.

Introduction

Skeletal muscle exhibits remarkable adaptive capabilities in response to exercise or chronic contractile activity [77, 78]. Central to the plasticity and metabolic flexibility of skeletal muscle are the mitochondria [79]. It is well known that physical activity, particularly endurance type exercise, induces mitochondrial biogenesis [77]. Mitochondrial biogenesis induced by physical activity has a physiologically relevant health benefit, since reduction in mitochondrial content and function are observed in a variety of metabolic diseases such as type 2 diabetes, sarcopenia and aging [80-82].

Mitochondrial biogenesis has been assessed by a variety of methods such as mitochondrial protein mRNA, mitochondrial protein expression, enzyme activity, and mitochondrial density [39]. Although mitochondrial biogenesis has been assessed by these methods, strictly speaking biogenesis refers to the making of new mitochondria. Therefore, it is debatable whether many of the commonly used methods measure mitochondrial biogenesis.

Recently, Miller and Hamilton proposed that the best determination of mitochondrial biogenesis is measuring the synthesis of new mitochondrial proteins [40], since the making of new mitochondrial protein is associated with expansion of the mitochondrial reticulum. This group has developed methods to measure mitochondrial protein synthesis and have applied these techniques to a variety of tissues in vivo [41, 43, 44]. To date, these methods have not been applied in vitro.

Multiple molecular events play a role in exercise-induced mitochondrial biogenesis [22]. One of the anticipated effects of exercise is the activation of AMP activated protein kinase (AMPK), which is known as the energy sensor of the cell [83]. It has been shown that acute contractile activity activates AMPK [84], which further promotes the phosphorylation and activation of one of the major players of mitochondrial biogenesis, transcriptional coactivator peroxisomal proliferator-activated receptor [gamma] coactivator-1 (PGC-1) alpha (PGC1-α) [85]. Through interaction of PGC1-α with nuclear respiratory factor-1 (NRF-1), which has binding sites in the promoter of nuclear encoded mitochondrial proteins, as well as mitochondrial transcription factor A (Tfam), the dual genomic regulation of mitochondrial biogenesis can be coordinated [86]. While PGC1-α has been proposed as one of the main transcriptional regulators of mitochondrial biogenesis [22, 23, 87], some studies suggest that PGC1-α is not mandatory for exercise adaptation responses [24-27] implying that other pathways may be sufficient to increase mitochondrial biogenesis independent of PGC1-α.

Physical exercise leads to enhanced formation of reactive oxygen species (ROS) [13, 14]. Recent studies show that the ROS produced during a bout of exercise can contribute to training adaptations such as mitochondrial biogenesis [88, 89], although this concept lacks consensus [90]. ROS-mediated mitochondrial biogenesis likely occurs via the upregulation and

transcriptional regulatory activity of redox sensitive transcriptional factors and co activators such as NRF-1 [19] and Tfam [15, 16] as well as PGC1-α [20, 21].

Nuclear erythroid 2 -related factor 2 (Nrf2) is a redox sensitive transcription factor that becomes activated upon exposure to oxidants [91]. Nrf2 induces transcription of antioxidant response element (ARE)-bearing genes including phase II antioxidants such as hemeoxygenase-1 (HO-1) and superoxide dismutase 1 (SOD1) [6, 8]. Besides its role as a master regulator of the antioxidant response, Nrf2 is a potential candidate for an activator of mitochondrial biogenesis because ARE sequences in the promoter region of the NRF-1 gene have been reported in murine cardiac cells [29]. It has also been shown that acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium [30]. Whether Nrf2 contributes to exercise-induced skeletal muscle mitochondrial biogenesis has not been thoroughly investigated.

Electrical stimulation (E-stim) has been applied successfully to stimulate skeletal muscle cells in vitro [92, 93]. Recently, a new bioreactor, which is portable while still maintaining the precision needed for physiological stimulation, was described [32, 33]. The inventors of this device have proposed that it is able to elicit both resistance exercise and endurance exercise contraction protocols in skeletal muscle cells. Although the authors assessed intracellular signaling associated with mitochondrial biogenesis, the making of new mitochondrial proteins was not assessed.

Understanding the specific molecular mechanisms that result in exercise-induced cellular responses such as mitochondrial biogenesis has been complicated by the fact that during contraction the muscle fibers are exposed to numerous metabolic and mechanical stimuli [31]. Therefore, a model of muscle contraction in vitro could enable researchers to discriminate intracellular signals of mitochondrial biogenesis from those that originate in the extracellular

environment. Additionally, since the mitochondria are not the only source of ROS produced during contractile activity [94, 95], an in vitro model of muscle contraction could be valuable for identifying the specific signaling and adaptive roles of ROS formed in different cellular compartments [95, 96]. Thus, using the novel bioreactor to model exercise in vitro could help us to elucidate the intracellular signaling mechanisms involved in myocyte adaptation to contractile stimuli with a level of experimental control not possible in vivo.

The purpose of our study was to simulate skeletal muscle contraction similar to endurance exercise in vitro by E-stim and to determine whether the induced contractile activity elicits outcomes consistent with those induced by endurance exercise. We used deuterium oxide (D_2O) to measure mitochondrial protein synthesis rate and assessed activation of the energetic sensor AMPK, to explore endurance exercise like effects. Further, to determine whether redox sensitive pathways were an adaptive response to the contraction, expression of Nrf2 regulated proteins was assessed. We hypothesized that E-stim induced contractile activity of cultured murine myotubes would induce energetic stress, redox sensitive signaling, and mitochondrial biogenesis. If successful, the ability to simulate exercise activity in vitro could allow further understanding of the cellular factors that determine mitochondrial biogenesis.

Methods

Overall study design

C2C12 murine skeletal muscle cells (ATCC, Manassas, VA, USA) were seeded on six-well culture dishes (BD Biosciences, San Jose, California ,USA) in Dulbecco's modified Eagle's medium (DMEM) (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (ATCC, Manassas, VA, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37°C. At 90-95% confluency, differentiation

into myotubes was induced by switching the medium to DMEM supplemented with 10% heat-inactivated horse serum (ATCC, Manassas, VA, USA). For assessing protein and gene expression, cells were harvested at time 0, 3 and 6 hrs post E-stim. To measure protein synthesis rates, cells were given media enriched with 4% deuterium oxide (D_2O) and were harvested at 4, 8 and 12 hrs post stimulation. In all experiments, control (CON) C2C12 myotubes were treated similarly except for not receiving E-stim.

In vitro Es of C2C12 myotubes

The E-stim was carried out using a custom made electrical stimulator previously described in detail [33]. In summary, this bioreactor was designed around a reprogrammable custom E-stim circuit by Donnelly et al., [32] that allows a single circuit to stimulate twelve samples simultaneously. This bioreactor has three constituents, the microcontroller, a DC-DC converter, and a pulse circuit. Prior to the experiments, the chosen E-stim parameters (50 V at 10 Hz for 4 s at 3.6 s intervals) were validated with the use of an oscilloscope and voltage meter. During this validation procedure, the E-stim unit was run three times and checked during 3 hrs of stimulation for deviations from the expected protocol. During the experiments, E-stim was delivered based on the above protocol for 3 hrs.

Assessment of protein expression:

To increase the yield of protein for western blotting, cells harvested from two wells were combined into one tube and total replicates of three were harvested at each time point. Cell extracts harvested at 0, 3 and 6 hrs post E-stim were washed two times with ice-cold PBS and lysed in RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with added protease and phosphates inhibitors (Halt, Thermo Fischer, Rockford, IL, USA). Samples were sonicated and the lysate protein concentration was measured using the bicinchoninic acid assay. Samples containing equal amounts of protein were prepared

in Laemmli sample buffer with 2-mercaptoethanol and heat denatured for 5 min at 98°C. Proteins were resolved on a 10% Tris/glycine SDS–polyacrylamide gel in running buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 and transferred to a nitrocellulose membrane for 75 min at 100 V using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.02% SDS, and 20% methanol, pH 8.3. Nonspecific proteins were blocked by incubating the membrane in 5% nonfat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) at 4°C overnight. Membranes were incubated overnight in primary antibodies from Abcam (Cambridge, MA, USA, HO-1 (1:200) # Ab13248), Cell Signaling (Danvers, MA, USA, AMPK (1:200) # 2531, pAMPK (1:200) # 2532) and Santa Cruz Biotechnology (Santa Cruz, CA, USA; Nrf2 (1:200) # SC722, PGC1-α (1:200) # SC13067, COXIV (1:200) # SC58348) at 4°C, followed by anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000) for 1 hr at room temperature with 30-min washes of TBST between primary and secondary incubations. Proteins were detected with chemiluminescence (West Dura; Pierce, Rockford, IL, USA) followed by densitometric analysis using Vision Works software (Upland, CA, USA). Blots were incubated with primary antibodies against actin (SC8432; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary donkey anti-goat antibody (SC2033, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr at room temperature to verify equal loading. The protein content normalized to actin and expressed as % control.

Assessment of gene expression using Rt-PCR:

For assessment of gene expression, each well represented a replicate and while there were three total replicates, only one sample (n = 1) was used for the PCR array analysis given the restrictive layout of the custom PCR plate. Total RNA was isolated from stimulated and CON C2C12 myotubes using standard Trizol extraction (Invitrogen, Carlsbad, CA, USA) and treated with a column based DNAse kit (RNeasy, Qiagen, Valencia, CA, USA). RNA purity (260/280

and 260/230 > 1.9) and final concentration (80 ng/µl) were verified with a spectrophotometer (Nanodrop, Wilmington, DE, USA). RNA was considered intact by visualizing two distinct bands on a native 1% agarose gel after ethidium bromide staining. RNA was reverse transcribed to cDNA using an Rt2 First Strand Kit (SABiosciences, Frederick, MD, USA). Approximately 2 ng of cDNA and master mix were loaded into each well that contained the appropriate primers. Real-time PCR of nuclear gene transcripts related to mitochondrial biogenesis was performed using a custom PCR array (SABiosciences, Frederick, MD, USA). Each 384 well array included eight gene targets, two reference genes for normalization (β2 macroglobulin (B2M) and TATA box binding protein (Tbp)), a mouse genomic DNA control (to check for for potential contamination) and a reverse transcription control (to verify reverse transcription efficiency). Real-time PCR was performed using a hot-start (10 min at 95°C) followed by 45 cycles of denaturing (15 sec 95°C) and elongation (1 min 60°C, ramp 1°/sec) on a Lightcycler 480 (Roche Diagnostic Corporation, Indianapolis, IN, USA). A melting curve was performed for each plate (60°C for 15 sec followed by 95°C with ramp at 4.8°/sec). The threshold cycle (Ct) for each sample was determined using a second-order derivative maximum and relative quantification calculated using the $2^{-\Delta\Delta}$Ct method [97] using the two separate reference genes B2M and Tbp.

Assessment of mitochondrial protein synthesis

The determination of protein synthesis was carried out in triplicate. The use of D_2O allows for the measurement of protein synthesis in different protein fractions as previously shown by our group [41-44]. In this study we assessed the synthesis of protein in mitochondrial (Mito), sarcoplasmic (Sarc) and mixed fraction (Mixed), which is primarily myofibrillar protein. Myotubes were given media enriched 4% with D_2O 1 hr before the beginning of stimulation and they were harvested at 4, 8 and 12 hrs after the stimulation. Media were collected and cells were washed with PBS twice. The cells were scraped in 1 mL isolation buffer (100 mM KCl, 40 mM

Tris HCl. 10 mM Tris Base, 5 mM MgCl2, 1 mM EDTA, 1 mM ATP, pH 7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA). The cell extract was centrifuged at 800 g for 10 min to pellet mixed protein fraction (Mixed). The supernatant from the low-speed spin was carefully removed and centrifuged (9000g) for 30 min to pellet a mitochondrial enriched fraction (Mito). The resulting supernatant (400 µL) was used as sarcoplasmic fraction (Sarc). The crude Mito pellet was washed and suspended in 200 µL of solution 2 (100 mM KCl, 10mM Tris-HCl, 10mM Tris base, 1mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4), and then centrifuged (8000g, 10 min, 4°C). The pellet was washed a second time, suspended in 100 µL of solution 2 and centrifuged (6000g, 10 min, 4°C). The final Mixed and Mito pellets were washed with 500 µL of 100% ethanol, centrifuged (1000g, 30 sec, 4°C) and rinsed with water (repeated twice). Protein pellets were solubilized in 1 N NaOH (50°C, 15 min) and hydrolyzed into free amino acids (6 M HCl, 120°C, 24 hrs). The hydrolystaes were ion-exchanged, dried under vacuum, and then suspended in 1 mL of 50% acetonitrile, 50mM K_2HPO_4, pH 11. 20 µL of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA) was added, and the sealed mixture was incubated at 100°C for 1 hr. Derivatives were extracted into ethyl acetate, and the organic layer was removed and dried by the addition of solid Na_2SO_4 followed by vacuum centrifugation. Derivatized amino acids were separated by a DB225 gas chromatograph column. The starting temperature was 100°C, increasing 10°C per min to 220°C. Samples were introduced to the mass spectrometer using negative chemical ionization (NCI) with helium as the carrier gas and methane as the reagent gas. The mass-to-charge ratios of 448, 449, and 450, which were representative of the M+0, M+1, and M+2 pentafluorobenzyl-N, N-di (pentafluorobenzyl) alaninate derivative, were monitored by selective ion monitoring (SIM). In all cases, these mass-to-charge ratios

represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^{2}H enrichment was calculated as the abundance of M+1 divided by the sum of the abundance of M+0 and M+1 [98]. The newly synthesized fraction of proteins was calculated from the enrichment of alanine in the hydrolyzed protein divided by the precursor enrichment as estimated from media enrichment using mass isotopomer distribution analysis (MIDA) [99, 100]. To measure the media enrichment, 120 µl of media were placed inverted on a heat block overnight at 80 °C to collect water. The water from the media were proton exchanged by adding 2 µl of 10 M NaOH and 20 µl of acetone and sat at room temperature overnight. The proton exchanged samples were extracted by adding 200 µl of hexane and were introduced to the mass spectrometer using (NCI) with helium as the carrier gas and methane as the reagent gas. The m+0 and m+2 peaks were analyzed using SIM. The deuterium enrichment was calculated by matching the m+2/m+0 with a generated enrichment standard curve. Fractional synthesis rates (FSR in %/hr) were calculated by dividing the fraction new by time and multiplying by 100. Final synthesis rates were calculated as the average of the 4, 8 and 12 hr timepoints.

Statistical analyses

The data presented are the means ± SEM. One-way analysis of variance was used to evaluate the effects of E-stim on protein expression and mitochondrial protein synthesis. Where significant differences occurred, specific differences were determined using LSD post-hoc test and differences were considered significant if $p < 0.05$.

Results

Energetic response to stimulation of C2C12 myotubes

Myotubes were observed to contract synchronously at the 10 Hz frequency and maintained viability (by visual inspection) throughout the contraction protocol. To evaluate whether the E-stim had endurance exercise like effects, we evaluated the AMPK mRNA levels as well as AMPK phosphorylation. Despite lack of sample size to reach statistical significance, AMPK mRNA was 20% higher at 0 hr post E-stim compared to CON (Figure 3.1A). At all timepoints, there were no differences between E-stim and control in the ratio of phosphorylated AMPK to total AMPK, an indicator of AMPK activity, although the ratio tended ($p = 0.1$) to be higher in 3 hrs post Es compared to CON (Figure 3.1B).

Effect of E-stim induced contractile activity on proteins involved in mitochondrial biogenesis and protein synthesis rates

Protein levels of PGC1-α and COXIV were significantly higher at 6 hrs post E-stim compared to CON (Figure 3.2A and 2B). PGC1-α level was significantly higher at 6 hrs post E-stim compared to 3 hrs post E-stim and tended to be higher at 6 hrs post E-stim compared to 0 hr post E-stim ($p = 0.07$) (Figure 3.2A). COXIV level was significantly higher at 6 hrs post E-stim compared to 0 hr post E-stim (Figure 3.2B). The average synthesis rates of all time points tended ($p = 0.08$) to be greater in Sarc compared to CON at 0 hr post E-stim. Also the rate of protein synthesis was not different in Mito and Mixed fractions after E-stim compared to CON (Figure 3.2C).

ROS activated signaling after E-stim induced contractile activity

Nrf2 protein was not different at 0 hr post E-stim compared to CON but it was significantly decreased at 3 hrs and 6 hrs post E-stim compared to CON and 0 hr post E-stim (Figure 3.3A). However, the Nrf2 regulated protein HO-1 was significantly greater at all time

points after E-stim compared to CON (Figure 3.3B) but there was no significant difference in SOD1 expression levels at any time points compared to CON (Figure 3.3C).

Discussion

We hypothesized that E-stim of C2C12 myotubes would increase energetic stress, redox sensitive signaling and mitochondrial biogenesis. Our main findings were that E-stim-induced contraction tended to increase markers of energetic stress, as well as expression of a Nrf2 regulated protein. Further, there was an increase in the synthesis rate of sarcoplasmic proteins, and an increase in PGC1-α expression during recovery from E-stim. Collectively, we present evidence that this in vitro model could be used to contract skeletal muscle cells and reproduce the muscle phenotype that arises after aerobic exercise.

Protein synthesis rates

This study was the first to use D_2O enriched media to assess the rate of protein synthesis in Mixed, Sarc and Mito fractions in vitro. Even though we could not detect a greater rate of protein synthesis in Mito fraction of E-stim compared to CON, rates of protein synthesis were higher in Sarc fractions, which consists of more protein than the other cellular fractions [101], compared to CON. Atherton et al., applied a frequency of 10 Hz for 3 hrs, the same that we have used in our model, on isolated rat muscle, but could not detect an increase rate of protein synthesis [101]. Donnelly et al., also applied the same frequency and duration on C2C12 myotubes but could not detect an increase in rate of protein synthesis either [32]. Atherton et al., used a flooding dose of ^{13}C-labeled proline (20 atoms percent) over 15 min, while Donnelly et al. used (^{35}S) labeled methionine/cysteine for 2 hrs [32, 101]. The disadvantage of short-term labeling, as used in these referenced studies, is that it predominantly only captures rapidly synthesized proteins [100]. By using D_2O, we have measured the rate of new protein synthesis at

the end of 4, 8 and 12 hrs. This extended period, which captures more slowly synthesized proteins, could explain why we detected higher rates of protein synthesis in the sarcoplasmic fraction after contraction while others did not.

Mitochondrial biogenesis

We did not observe an increase in mitochondrial protein synthesis, although this is a primary aerobic exercise response. The absence of an increase in mitochondrial protein synthesis could be explained by differences in the process of making mitochondrial versus sarcoplasmic proteins. Mitochondrial biogenesis requires the cooperation of the nuclear and mitochondrial genomes [102] in addition to the assembly of large hetero-oligomeric protein complexes. A quantitative spatial proteomics analysis of proteome turnover of human cells has shown that majority of mitochondrial proteins have slower turn over than sarcoplasmic proteins [103]. Thus, it could be possible that more time is required for detecting mitochondrial biogenesis after contraction.

Differences in the rate of protein synthesis in mitochondria compared to sarcoplasm in this study is in accordance with previous reports showing that synthesis rates vary among the individual proteins in skeletal muscle and plasma [104, 105]. To our knowledge this is the first study to report the FSR of mitochondrial and sarcoplasmic proteins measured by D_2O under contraction stress in vitro. Yet, understanding the effect of different stressors such as contraction on synthesis rates of individual proteins in vivo followed by exercise requires further study.

Cellular response during recovery

We observed an increase in protein content of PGC1-α and COXIV 6 hrs after E-stim. The PGC-1α protein is recognized for its role as a transcriptional co-activator of downstream targets such as Tfam and NRF1 and therefore has regulatory influences on both nuclear and mitochondrial encoded genes [79]. The possibility that we missed detecting the greater levels of

PGC1-α mRNA after E-stim cannot be excluded. Greater mitochondrial protein content has been observed following a single bout of exercise [106, 107]. There was an increase in nuclear encoded mitochondrial protein, COX IV. Thus we cannot rule out the concept that initial signaling events associated with acute exercise induce transient pulses of increased mRNA abundance that eventually lead to an increase in protein content of some of mitochondrial proteins [22, 108, 109]. Despite not showing a greater rate of mitochondrial protein synthesis till 12 hrs post E-stim, greater rate of sarcoplasmic protein synthesis with greater content of PGC1-α along with an increase in nuclear encoded mitochondrial protein, COXIV, may indicate the beginning of the process of mitochondrial biogenesis upon contraction which requires more time to be developed completely.

Activation of energetic sensor

Contractile activity is a potent energy stressor for cells [84]. A decrease in the ratio of ATP/AMP in muscle cells activates AMPK [110, 111]. We measured that AMPK activation tended to be higher after E-stim. This finding is in accordance with previous studies that show AMPK is activated by exercise in rodents and humans [84, 112, 113]. The activation of AMPK is a well-characterized upstream modulator of PGC-1α gene expression in skeletal muscle [20, 21] that directly phosphorylates PGC-1α, thereby increasing transcriptional activation of the PGC-1α. Thus, AMPK activation upon E-stim suggests that the E-stim model can induce energetic stress like a single bout of endurance exercise. Yet this finding warrants further studies.

Redox signaling

Growing evidence highlights an important role for exercise-induced ROS in regulating cell signaling processes that contribute to adaptations in skeletal muscle after exercise training [12, 13, 88]. For example, Muthusamy et al., have shown that acute exercise stress induces ROS, activates Nrf2/ARE signaling and upregulates antioxidant genes in the myocardium [30].

We found an increase in expression of HO-1, a protein downstream of Nrf2, after E-stim at all time points compared to CON. We use HO-1 as a marker of Nrf2 activation, rather than Nrf2 content, because it provides more insight into transient activation. In vitro data from our lab have shown that Nrf2 translocation to the nucleus, a necessary step of Nrf2 activation, occurs within 15 minutes of oxidative challenge [37] although this nuclear translocation is not sustained [70]. Therefore, it is likely that Nrf2/ARE signaling was induced by ROS during contraction, which led to an increased expression of the antioxidant protein, HO-1. Therefore, we provide evidence that the in vitro model of contraction can induce changes in redox sensitive pathways as has been shown after a single bout of endurance exercise in vivo.

Methodological considerations

Since this was the first use of D_2O enriched media to assess mitochondrial biogenesis in vitro following E-stim, we had to take into account several methodological considerations unique to this model, as well considerations of using D_2O to measure synthesis rates in general. For example, protein content in the cell culture dishes used during E-stim of cells was low. Sizes of cell culture plates could not be changed since they had to fit the bioreactor lead provided by the inventor. However, by pooling two cell culture plates we could successfully increase the protein yield for protein measurements, thus protein content was not a limiting factor.

Conclusions

Induction of contractile activity by E-stim may be a practical model to simulate a single bout of endurance exercise. Here we demonstrate that redox sensitive signaling and new protein synthesis are two major outcomes of induced contractile activity in electrically stimulated C2C12 myotubes. Further development of this valuable in vitro model can help future studies to provide a better understanding of cell adaption mechanisms to contraction.

Figures

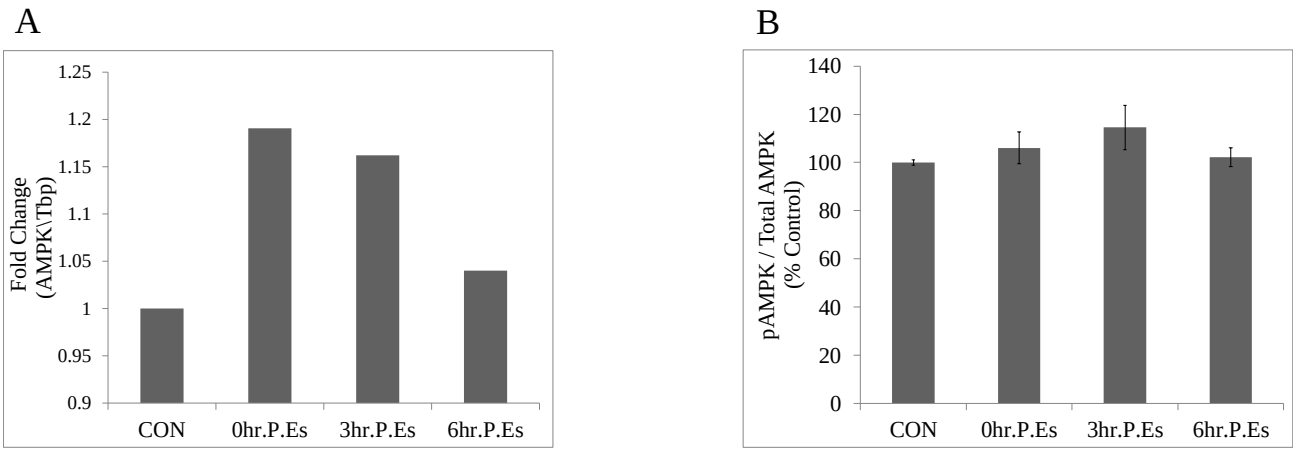

Figure 3.1. Time course of the effect of E-stim induced contractile activity on AMPK mRNA levels and AMPK phosphorylation. A. Changes in AMPK mRNA levels at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. Data are represented as n = 1. B. Expression of pAMPK/Total AMPK by immunoblotting. Data from immunoblotting are mean ± SEM, n = 3.

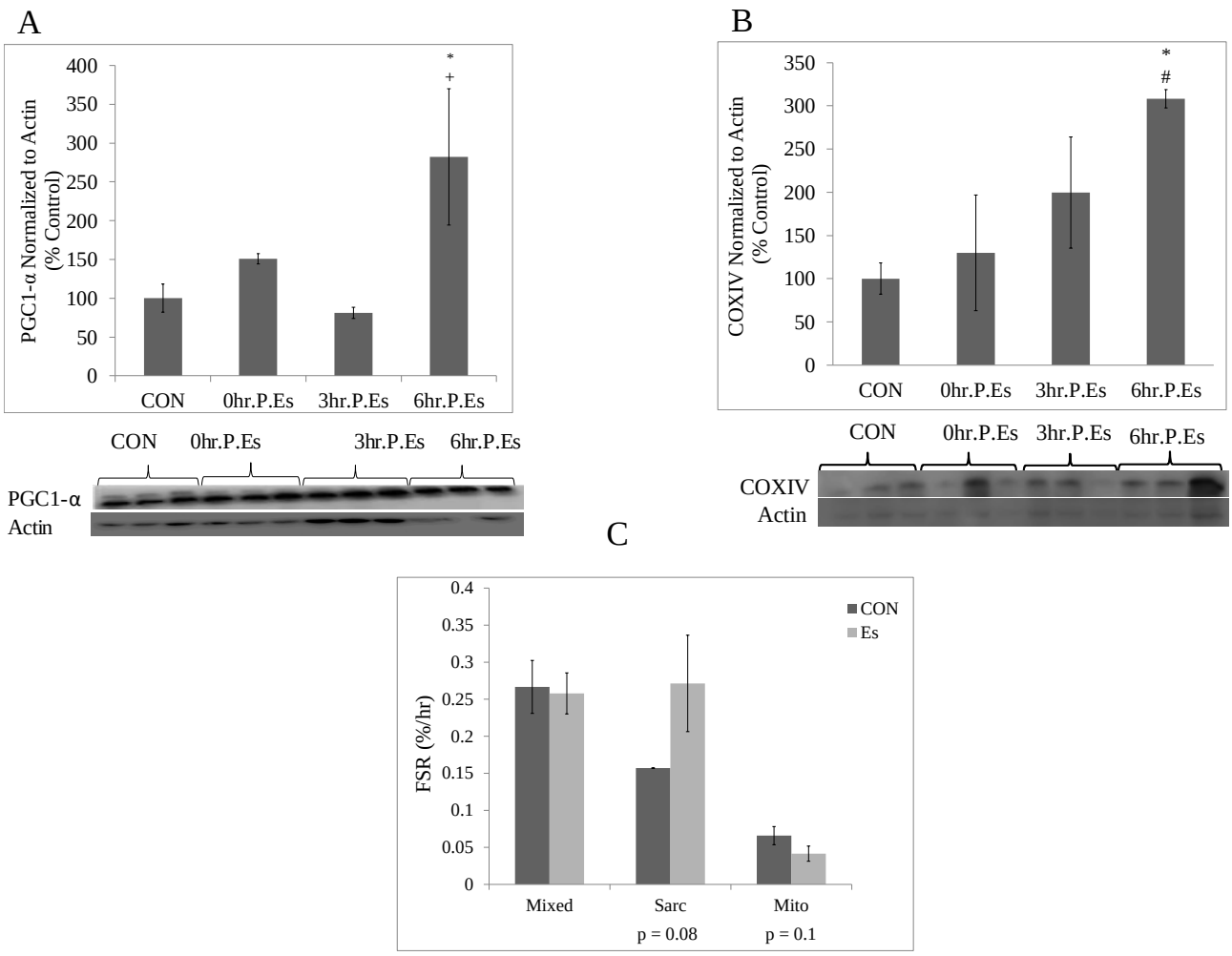

Figure 3.2. Protein levels of transcriptional co-activator and nuclear encoded protein involved in mitochondrial biogenesis, and cellular protein synthesis rates in response to E-stim induced contractile activity. A. PGC1-α protein expression at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. B. COXIV protein expression at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. C. Fraction synthesis rate (FSR) in Mito, Sarc and Mixed compartments of C2C12 myotubes after E-stim induced contractile activity. Data are mean ± SEM, n = 3. *$p < 0.05$ compared to CON. #$p < 0.05$ compared to 0 hr post E-stim. +$p < 0.05$ compared to 3 hrs post E-stim.

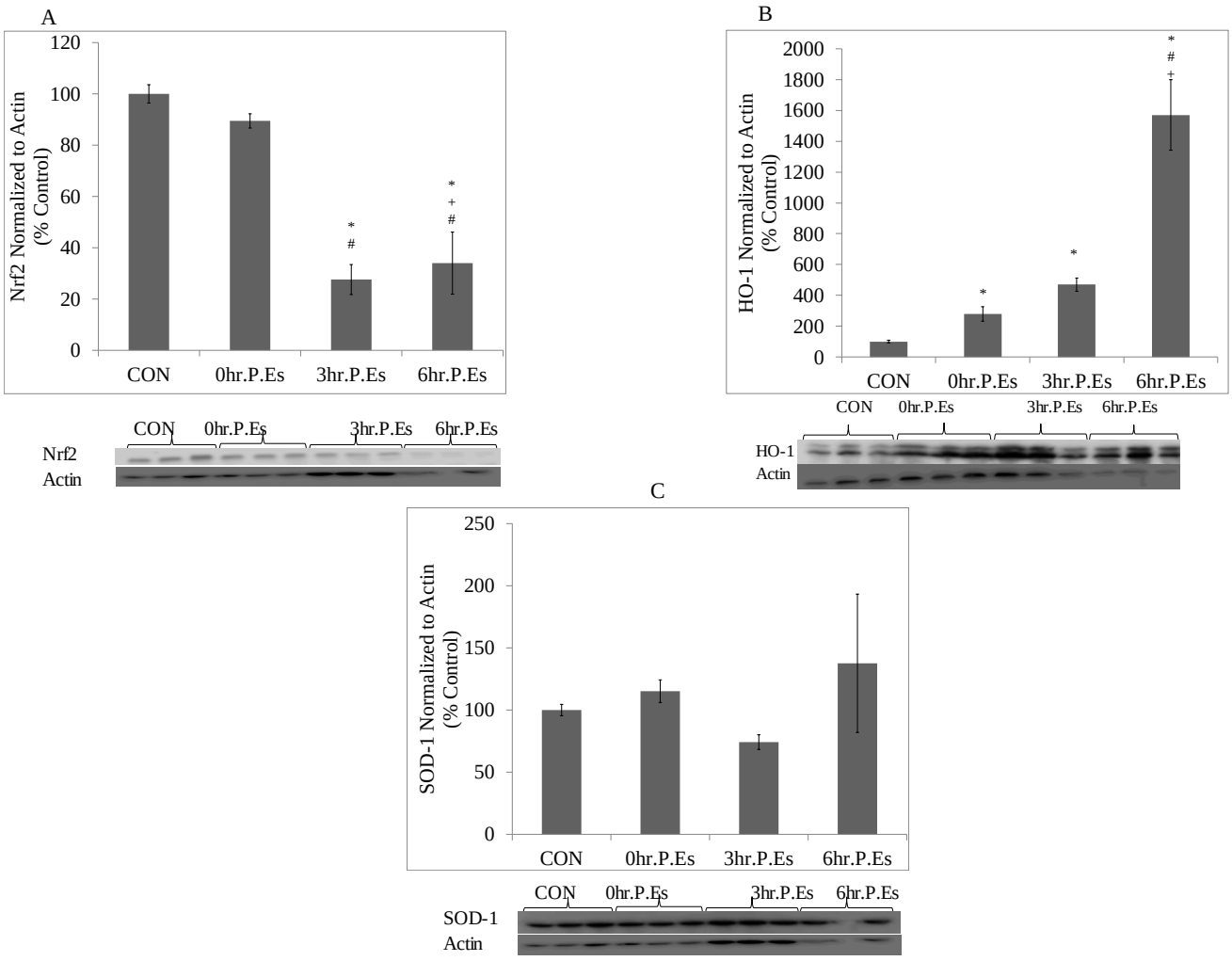

Figure 3.3. Time course of the effect of E-stim induced contractile activity on Nrf2 protein expression and phase II antioxidant compensatory response. A. Nrf2 protein expression at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. B. HO-1 protein expression at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. C. SOD1 protein expression at 0, 3 hrs and 6 hrs post E-stim (P.Es) compared to CON. Data are mean ± SEM, n = 3. *$p < 0.05$ compared to CON. #$p < 0.05$ compared to 0 hr post E-stim. +$p < 0.05$ compared to 3 hrs post E-stim.

CHAPTER IV – MANUSCRIPT III

Mitochondrial biogenesis in response to endogenous and exogenous antioxidants in myoblasts[3]

Summary

Several studies on rats and humans show blunting of the mitochondrial biogenesis response to exercise with exogenous antioxidant (vitamin C (Vit C)) supplementation. The upregulation of endogenous antioxidant defenses provides the potential for more profound cellular protection than antioxidant vitamin supplementation because of the enhanced ability of enzymatic antioxidants to scavenge ROS compared to traditional antioxidant vitamins. The effect of endogenous antioxidants on mitochondrial biogenic response to exercise has not been studied. Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a transcription factor that activates the endogenous antioxidant system. To understand the effect of endogenous and exogenous antioxidants on mitochondrial biogenic response to hydrogen peroxide (H_2O_2), experiments were performed on myoblasts treated with H_2O_2 combined with Vit C and Nrf2 activator. Hypotheses: treatment with H_2O_2 would result in a greater rate of mitochondrial biogenesis compared to control, and the increase would be maintained during co-treatment with either an exogenous antioxidant (Vit C) or a Nrf2 activator that increases transcription of endogenous antioxidants. Methods: The first experiment was to evaluate the effect of H_2O_2 on protein synthesis rates. Myoblasts were treated with 500 µM H_2O_2 for 1 and 6 hrs and new fractions of mitochondria (Mito), sarcoplasmic (Sarc) and myofibrillar (Mixed) proteins were compared to the media condition. The second experiment was to evaluate the effect of exogenous and

[3] Shadi Khademi*, Benjamin Miller*, Karyn Hamilton*. *Translational Research on Aging and Chronic Disease Laboratory, Department of Health and Exercise Science, Colorado State University, Fort Collins, CO 80523 USA.

endogenous antioxidants on H_2O_2 induced decrements in mitochondrial protein synthesis rates. Myoblasts were treated with 500 µM H_2O_2 alone or co-treated with 50 µM or100 µM Vit C or 100 µg/mL of a Nrf2 activator for 12 hrs to assess the rate of mitochondrial protein synthesis compared to their respective controls. Results: H_2O_2 treatment for 1 hr significantly decreased the synthesis of new proteins in Mito, Sarc and Mixed fractions compared to CON at 12 and 21 hrs. In addition, 12 hrs of treatment with H_2O_2 and Vit C significantly decreased the rate of mitochondrial protein synthesis compared to media and H_2O_2 treated cells. However, H_2O_2 and a Nrf2 activator maintained the rate of mitochondrial protein synthesis compared to the respective controls. Conclusion: treatment with H_2O_2 decreased global protein synthesis, including the mitochondrial fraction (an indicator of mitochondrial biogenesis) in myoblasts. While Vit C treatment did not improve this decrease in mitochondrial biogenesis and in fact worsened this decrease, a Nrf2 activator restored the rate of mitochondrial protein synthesis in myoblasts treated with H_2O_2 to control levels. Findings of this study provide evidence that upregulation of endogenous antioxidants can protect against H_2O_2 induced decrements in mitochondrial biogenesis. Details on Nf2 activation mechanisms involved in selective translation of mitochondrial proteins upon oxidative stress require further investigation.

Introduction

Denham Harman was the first to associate free radicals with aging and established that irradiation of living systems induces mutations, cancer and ageing through production of free radicals [114]. Since this publication, the accumulation of oxidative damage has been one of the most widely accepted causes of aging. However, more recently the free radical theory of aging has been challenged by studies on mice and invertebrates with genetic manipulations in their antioxidant defense system showing that their life span was not different compared to the wild-

type control [115-117]. Moreover, recent studies have shown that increasing generation of reactive oxygen species (ROS), in some cases, increases longevity by acting as pro-survival signals [118-120]. Definitive studies are required to elucidate the role of ROS in the process of aging.

Intracellular ROS are present at low concentration as they are the by-products of normal aerobic metabolism. Under normal conditions, there is a steady-state balance between pro-oxidants and antioxidants [121, 122]. When the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress ensues with consequential damage to DNA, proteins and lipids [1, 123]. ROS can act as a 'double-edged sword' in that ROS overproduction is toxic to cells, but normal ROS production plays an important part in the cell signaling involved in the antioxidant defense network.

There is accumulating evidence that ROS signaling can trigger mitochondrial adaptation. It has been shown that when the complex III inhibitor antimycin A was added to human fibroblasts, thus increasing mitochondrial ROS production, there was an increase in cytochrome c1 and cytochrome b mRNA levels [124]. In agreement with these findings, another study showed that the addition of H_2O_2 to lung fibroblasts led to an increase in the expression of the transcription factor nuclear respiratory factor 1 (NRF-1) [125], which is a critical signal in mitochondrial biogenesis [126]. Moreover, it has been reported that the regulation of the main transcription cofactor of mitochondrial biogenesis, peroxisomal proliferator-activated receptor [gamma] coactivator-1 (PGC-1) alpha (PGC1-α), can be controlled by redox sensitive pathways [20]. Collectively, the adaptive stress-protective mechanism resulting from the induction of ROS-related stress on mitochondria has been defined as 'mitochondrial hormesis' or

'mitohormesis' [127]. Signaling pathways involved in mitohormesis under a variety of cell stressors are not fully understood.

There are several systems including enzymatic, vitamin and thiol-containing molecules to counteract free radical-induced damage [13, 128]. Endogenous antioxidant defense enzymes such as the phase II antioxidants superoxide dismutase (SOD), NAD (P) H: quinone oxidoreductase 1 (NQO1), and heme oxygenase 1 (HO-1) allow the reduction of excess ROS [95]. The master regulator of endogenous antioxidant defenses is nuclear factor erythroid 2 related factor 2 (Nrf2). Nrf2 is a transcription factor which belongs to the Cap'n'collar/basic region leucine zipper (CNC-bZIP) family [6]. After oxidization of cys residues on the cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap1), Nrf2 dissociates from Keap1, translocates to the nucleus and binds to an antioxidant response element (ARE). Through transcriptional activation of ARE-bearing genes involved in antioxidant defense including phase II enzymes, Nrf2 activates cellular pathways that protect against oxidative injury [7, 35].

ROS produced during exercise can stimulate mitochondrial biogenesis [12, 13]. It has been shown that oral administration of exogenous antioxidant Vit C blunts a positive adaptation (mitochondrial biogenesis) during exercise [12, 17, 34]. The unfavorable effects of exogenous antioxidant supplementation have been attributed to the blocking of ROS mediated signaling. Contradictory to these data, other studies on rats and humans have shown that supplementation with Vit C did not prevent the exercise-induced adaptive responses [18, 90]. Discrepancies between these studies that used exogenous antioxidants and aerobic exercise have been attributed to time and quantities of supplementation, time period of measurement, exercise training status, and the choice of outcomes (e.g. mRNA versus phosphorylation or enzyme activity) measured. Many markers of mitochondrial biogenesis have elucidated the transcriptional regulations of this

process. Further studies are required to clarify the translational regulation of mitochondrial biogenesis.

The purpose of our study was to investigate the influence of an upregulation of endogenous antioxidants and treatment with exogenous antioxidants on mitochondrial biogenesis during an oxidant stress. We tested the hypotheses that: 1) treatment with H_2O_2 would result in a greater rate of mitochondrial biogenesis compared to media, 2) as compared to the respective control, the greater rate of mitochondrial biogenesis after H_2O_2 treatment would be blunted by Vit C treatment, and 3) as compared to the respective control, the greater rate of mitochondrial biogenesis after H_2O_2 treatment would not be blunted by treatment with Nrf2 activator.

Methods

Overall study design

C2C12 murine skeletal muscle cells (ATCC, Manassas, VA, USA) were seeded on 60 mm culture dishes (BD Biosciences, San Jose, California ,USA) in Dulbecco's modified Eagle's medium (DMEM) (ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37°C. Experiments were initiated when cells reached 40-50% confluency. Two experiments were performed; H_2O_2 treatment only, and co-treatment of H_2O_2 and Vit C or H_2O_2 and a Nrf2 activator. For protein synthesis measurements (described below), all of the treatments were made in media enriched with 10% deuterium oxide (D_2O). All experiments were carried out in triplicates.

H_2O_2 preparation and H_2O_2 treatment only experiment

To find a right H_2O_2 concentration to treat the myoblasts prior to assessing the mitochondrial protein synthesis, dose response experiment with different H_2O_2 concentrations

were performed. H_2O_2 (30% W/W) was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) and diluted in DMEM at different concentrations (10 μM, 50 μM, 100 μM, 250 μM, 500 μM and 1000 μM). Percent cell death by trypan blue technique was calculated at 4,8,12 and 24 hrs [129]. Since 500 μM H_2O_2 treatment was the highest concentration at which all cells survived for 24 hrs, we used this concentration for the rest of the experiments.

An initial study to assess the effect of H_2O_2 treatment duration on protein synthesis consisted of three experiments. In all experiments cells were washed with sterile PBS at the end of 500 μM H_2O_2 treatment and received fresh 10% D_2O enriched media. Controls (CON) were left untreated for the respective harvest time points. In experiment A, cells were treated with H_2O_2 for one hour and harvested at 1, 3, 6, 12, 21 and 30 hrs. In experiment B, cells treated with H_2O_2 for 6 hrs were harvested at 6, 12, 21 and 30 hrs where in experiment C, cells were first treated with H_2O_2 with no D_2O for 6 hrs and were harvested at 12, 21 and 30 hrs to distinguish the time that the label was applied.

Preparation of Vit C and Nrf2 activator for co-treatment with H_2O_2

To assess the effect of endogenous and exogenous antioxidants on mitochondrial protein synthesis rates, additional experiments were performed in which cells were treated with a combination of H_2O_2 and Vit C or H_2O_2 and Protandim for 12 hrs. Vehicle controls were left untreated for 12 hrs.

A dose-response experiment was conducted to identify the appropriate dose and duration of Vit C treatment. Cells were treated with 10 μM, 50 μM, 100 μM and 200 μM) of Vit C (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM for 2, 6, 8 and 12 hrs followed by microscopic cell morphological assessment. Consistent with previous in vitro studies with Vit C treated C2C12 cells [130, 131], the final concentrations of 50 μM and 100 μM were used for all of the experiments with media as a control.

Protandim is a phytochemical composition containing W. somnifera, B. monniera (45% bacosides), S. marianum, Ca. sinesis (98% polyphenols and 45% (-)-epigallocatechin-3-gallate), and curcumin (95%) from turmeric (Cu. longa) (LifeVantage Corp., Salt Lake City, UT). The five phytochemical components of Protandim have a synergistic effect to induce phase II antioxidant enzymes and protect cells from oxidative stress through activation of the transcription factor Nrf2 [35]. Thus, Protandim is a potent Nrf2 activator that can stimulate the uprgulation of endogenous antioxidants.

Protandim extract was prepared by mixing 500 mg Protandim in 5 mL 95% ethanol. The mixture was rocked overnight at room temperature and then centrifuged (3000g) for 15 min. The resulting supernatant contained ethanol extracted Protandim at a concentration of 100 mg/mL. The Protandim extract was diluted to 100 μg/mL for all of the experiments following initial concentration response experiments that showed 100 μg/mL significantly stimulated phase II antioxidant enzyme (HO-1) protein expression. 95% ethanol used as a vehicle control, at a maximal ethanol concentration in the enriched medium of 0.1% (1 μL in 1 mL).

Assessment of mitochondrial protein synthesis by using of stable isotopes

The use of D_2O allows assessment measurement of protein synthesis in different cell compartments in different tissues as previously showed by our group [41-44]. In this study we assessed the synthesis of protein in mitochondrial (Mito), Sarcoplasmic (Sarc) and mixed fraction (Mixed), which is primarily myofibrillar protein. Cells from aforementioned experiments were scraped in 1 mL isolation buffer (100 mM KCl, 40 mM Tris HCl. 10 mM Tris Base, 5 mM $MgCl_2$, 1mM EDTA, 1mM ATP, pH 7.5) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA). The cell extract was centrifuged at 800 g for 10 min to pellet mixed protein fraction (Mixed). The supernatant from the low-speed spin was carefully removed and centrifuged (9000g) for 30 min to pellet a mitochondrial enriched fraction

(Mito). The resulting supernatant (400 µL) was used as sarcoplasmic fraction (Sarc). The crude Mito pellet was washed and suspend in 200 µL of solution 2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4), and then centrifuged (8000g, 10 min, 4°C). The pellet was washed a second time, suspended in 100 µL of solution 2 and centrifuged (6000g, 10 min, 4°C). The final Mixed and Mito pellets were washed with 500 µL of 100% ethanol, centrifuged (1000g, 30 sec, 4°C) and rinsed with water (repeated twice). Protein pellets were solubilized in 1 N NaOH (50°C, 15 min) and hydrolyzed into free amino acids (6 M HCl, 120°C, 24 hrs). The hydrolystaes were ion-exchanged, dried under vacuum, and then suspended in 1 mL of 50% acetonitrile, 50 mM K_2HPO_4, pH 11. 20 µL of pentafluorobenzyl bromide (Pierce Scientific, Rockford, IL, USA) was added, and the sealed mixture was incubated at 100°C for 1 hr. Derivatives were extracted into ethyl acetate, and the organic layer was removed and dried by the addition of solid Na_2SO_4 followed by vacuum centrifugation. Derivatized amino acids were separated by a DB225 gas chromatograph column. The starting temperature was 100°C, increasing 10°C per min to 220°C. Samples were introduced to the mass spectrometer using (NCI) with helium as the carrier gas and methane as the reagent gas. The mass-to-charge ratios of 448, 449, and 450, which were representative of the M+0, M+1, and M+2 pentafluorobenzyl-N, N-di (pentafluorobenzyl) alaninate derivative, were monitored by selective ion monitoring (SIM). In all cases, these mass-to-charge ratios represented the primary daughter ions that included all of the original hydrocarbon bonds from the given amino acid. ^2H enrichment was calculated the abundance of M+1 divided by the sum of the abundance of M+0 and M+1 [132]. The newly synthesized fraction of cellular proteins (fraction new) was calculated by the enrichment of Alanine in the hydrolyzed protein divided by the precursor enrichment as estimated from media using mass isotopomer distribution analysis

(MIDA) [133]. To measure the media enrichment, 120 µl of media were placed inverted on a heat block overnight at 80 °C to collect water. The water from the media were proton exchanged by adding 2 µl of 10 M NaOH and 20 µl of acetone and sat at room temperature overnight. The proton exchanged samples were extracted by adding 200 µl of hexane and were introduced to the mass spectrometer using (NCI) with helium as the carrier gas and methane as the reagent gas. The m+0 and m+2 peaks were analyzed using SIM. The deuterium enrichment was calculated by matching the m+2/m+0 with a generated enrichment standard curve. Fractional synthesis rates (FSR in %/hr) were calculated by dividing the fraction new by time and multiplying by 100.

Statistical analyses

The data presented are the means ± SEM. One-way analysis of variance was used to evaluate the effects of endogenous and exogenous antioxidants on mitochondrial protein synthesis at 12hrs. When differences existed, LSD used for a post hoc comparison. Two-way analysis of variance with Bonferroni adjustment was used to evaluate the main effect of time and H_2O_2 on fraction of new proteins. In all cases differences were considered significant if $p < 0.05$.

Results

Effect of H_2O_2 treatment on protein synthesis rates

Our first series of experiments determined if there were differences in the effect of 1 hr and 6 hrs treatments with H_2O_2. The fraction of new mitochondrial protein was significantly decreased with 1hr (Figure 4.1A) and 6 hrs treatments with H_2O_2 compared to CON (Figure 4.1D). Since the H_2O_2 treatment response on new fraction of mitochondrial protein was the same at 1 hr and 6 hrs, we further analyzed the one-hour treatment effects on protein synthesis rates in Mixed and Sarc pfractions. One hour H_2O_2 treatment significantly decreased the fraction

of new Sarc and Mixed proteins at 12 and 21 hrs (Figure 4.1B and 1C). Further, there was a significant interaction between time and H_2O_2 treatment (Figure 4.1A-C).

Effect of co-treatment of H_2O_2 and Vit C or H_2O_2 and Nrf2 activator on mitochondrial protein synthesis

12 hrs of H_2O_2 significantly decreased the rate of mitochondrial protein synthesis in myoblasts compared to media only (CON) (Figure 4.2). There was a significant decrease in the rate of mitochondrial protein synthesis in H_2O_2 treated myoblasts with 50 µM Vit C compared to H_2O_2 treated myoblasts alone (Figure 4.2). Conversely, in myoblasts treated with Nrf2 activator (Protandim) and H_2O_2, the H_2O_2-mediated reduction in mitochondrial protein synthesis was completely abolished compared to the respective control (Ethanol and H_2O_2) (Figure 4.2). There was no significant effect of Protandim and H_2O_2 treatment compared to the Ethanol and H_2O_2 treatment (Figure 4.2).

Discussion

We hypothesized that treatment with H_2O_2 would result in a greater rate of mitochondrial biogenesis compared to CON. Further, we hypothesized that as compared to CON, the greater rate of mitochondrial biogenesis after H_2O_2 treatment would be blunted by Vit C treatment but not by treatment with a Nrf2 activator. Our main findings were that H_2O_2 caused a slower rate of mitochondrial biogenesis with short and long term treatments. Moreover, H_2O_2 combined with exogenous antioxidant, Vit C, further blunted mitochondrial biogenesis where H_2O_2 combined with Nrf2 activator maintained the rate of protein synthesis under oxidative stress. This is the first study to use a stable isotope approach to assess the effect of H_2O_2 on rates of protein synthesis, and the impact of exogenous and endogenous antioxidants on the synthetic response to H_2O_2.

Protein synthesis in H_2O_2 induced oxidative stress

Prior to investigation, we hypothesized that a greater rate of mitochondrial protein synthesis with H_2O_2 could have been due to a greater ROS production since H_2O_2 is a known ROS and it has been reported that ROS stimulate mitochondrial biogenesis [20, 125, 134]. However, 500 µM H_2O_2 even in 1 hr significantly decreased the rate of protein synthesis in myoblasts. There are two potential reasons for the lack of H_2O_2 response in mitochondrial biogenesis observed in this study. First, to our knowledge, none of the in vitro studies so far have detected the rate of mitochondrial protein synthesis as an indicator of mitochondrial biogenesis upon H_2O_2 treatment. Irrcher et al., treated the myoblasts for 24 hrs with 300 µM H_2O_2 and reported the greater PGC1-α mRNA levels compared to control as an indicator of mitochondrial biogenesis [20]. Whether an increase in mRNA level of one of the key regulators of mitochondrial biogenesis would necessary translate to a greater rate of mitochondrial protein synthesis is not known.

Further, mitochondria are one major source of ROS production during exercise [135] and mitochondrial originated H_2O_2 can diffuse across intracellular and plasma membranes [135-137]. However, in this study we applied exogenous H_2O_2 as a source of ROS in the cell media. It is not possible to know the dynamics of the H_2O_2 and its propagation in different cellular compartments. Whether exogenous ROS initiates signaling pathways similar to endogenous ROS in the process of cell adaptation is not known. Understanding the effect of different sources of ROS on mitochondrial biogenesis requires further study.

The effect of H_2O_2 treatment on decreasing the rate of protein synthesis in current study supports previous reports from in vivo study by our group that shows under stress condition such as caloric restriction the global rate of protein synthesis is decreased [43]. Further, study on yeast saccharomyces cerevisiae shows that oxidative stress induced by 500 µM H_2O_2 caused

global inhibition of protein synthesis in 15 minutes [138]. Study by Fan et al. [139] shows that while 500 μM H_2O_2 was not lethal for the myoblasts, it did induce slow fragmentation of the mitochondrial reticulum. H_2O_2 induced mitochondrial fragmentation was preceded by decreases in inner mitochondrial membrane potential, suggesting that oxidative stress was induced by H_2O_2. In our current study H_2O_2 treatment was not lethal for the myoblasts. However, H_2O_2 treatment in this study caused lower rates of protein synthesis in myoblasts. Collectively we conclude that H_2O_2 used in our experiment lowered the rate of global protein synthesis by induction of oxidative stress.

Exogenous antioxidant and mitochondrial protein synthesis during oxidative stress

Here we have shown that mitochondrial biogenesis was further blunted by exogenous antioxidant Vit C during H_2O_2 induced oxidative stress compared to the vehicle control. This finding supports previous in vivo studies in humans and rats in that Vit C supplementation blunts exercise-induced mitochondrial biogenesis [17, 34].

Previous in vivo experiments aimed at assess the impact of Vit C treatment on mitochondrial biogenesis measured the protein content of PGC1-α or mitochondrial enzyme (citrate synthase) activity [17, 18, 34, 90]. Although it is important to assess the events that lead to a greater transcription of proteins of interest, as discussed by Hamilton and Miller, biogenesis refers to the making of new mitochondria through translation machinery [140]. Our group has successfully applied D_2O enrichment technique which allows for the assessment of the mitochondrial synthesis rates in vivo [41-44]. In the present studies, we utilize this technique to understand the effect of exogenous antioxidants on mitochondrial biogenesis during oxidative stress in vitro. Applying the D_2O method that allows for assessing the protein synthesis rates help understanding the aspects of protein translation in mitochondrial biogenesis.

Endogenous antioxidant and mitochondrial protein synthesis during oxidative stress

Nrf2 activation, a treatment we have previously reported to increase expression of endogenous antioxidants [35, 37], maintained mitochondrial biogenesis in myoblasts treated with H_2O_2 at levels comparable to the vehicle control. These data are consistent with findings our recent in vivo study showing that upregulation of endogenous antioxidant defenses by Nrf2 activation does not blunt exercise-induced mitochondrial biogenesis, while treatment with Vit C does (unpublished data). These data suggest a role for Nrf2 activation as a means for preventing oxidative stress while permitting redox sensitive signaling resulting in mitochondrial protein synthesis, though the mechanism for such a role remains poorly understood.

Protein synthesis is energetically expensive and under conditions of metabolic stress, increasing protein synthesis does not seem economical [132, 141]. Translational regulation mechanisms exist to selectively promote or inhibit synthesis of specific proteins under stress [42, 43, 133]. For example, it has been shown that mitochondrial biogenesis is maintained during caloric restriction stress despite a decrease in global protein translation in vivo [42, 43]. Data on yeast show that H_2O_2 increases the number of ribosomes associated with a specific subset of mRNAs, which may be protected/targeted for maintained translation [138]. These mRNAs may also represent an mRNA store that could become rapidly activated upon relief of the stress condition. We hypothesize that endogenous antioxidants via Nrf2 activation might have a role in promoting selective translation of some of the mRNAs responsible for mitochondrial biogenesis under stressed conditions. Further investigations are required to test this hypothesis.

Conclusions

Exogenous antioxidant and endogenous antioxidant show different effects on mitochondrial biogenesis in myoblasts under H_2O_2 induced oxidative stress. Here we

demonstrated that H_2O_2 treatment, irrespective of treatment duration, decreased the rate of mitochondrial protein synthesis in vitro. We have also shown that the exogenous antioxidant Vit C further blunted the decrease in mitochondrial biogenesis compared to H_2O_2 alone. Importantly, treatment with a Nrf2 activator abolished the H2O2-induced reduction in mitochondrial biogenesis. These findings highlight the role of Nrf2 in permitting protein translation during stress, and provide a basis for future studies to identify mechanisms involved in mitochondrial biogenesis under stress conditions.

Figures

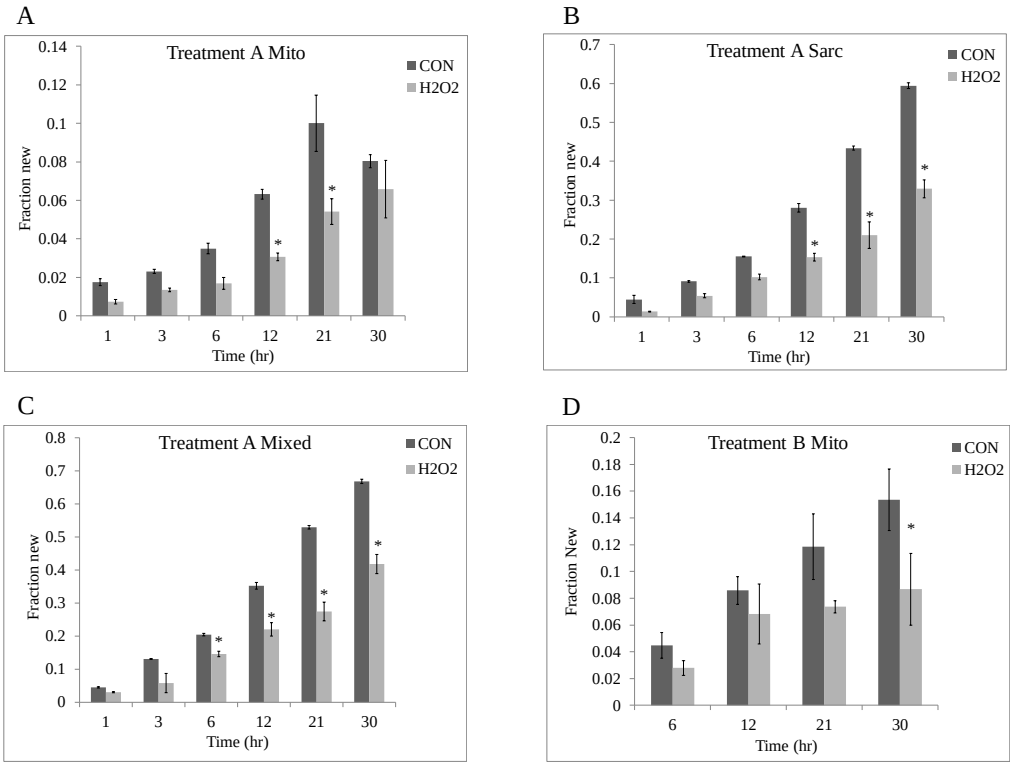

Figure 4.1: Time course of the effect of 1 hr and 6 hrs treatment with H_2O_2 on fraction of new Mito proteins and the effect of 1 hr treatment with H_2O_2 on fraction of new Sarc (B) and Mixed (C) proteins in myoblasts. A. Fraction of new mitochondrial (Mito) protein at 1, 3, 6, 12, 21 and 30 hrs after 1 hr treatment with 500 µM H_2O_2 compared to media (CON). B. Fraction of new sarcoplasmic (Sarc) protein at 1, 3, 6, 12, 21 and 30 hrs after 1 hr treatment with 500 µM H_2O_2 compared to media (CON). C. Fraction of new myofibrillar (Mixed) protein at 1, 3, 6, 12, 21 and 30 hrs after 1 hr treatment with 500 µM H_2O_2 compared to media (CON). D. Fraction of new mitochondrial (Mito) protein at 6, 12, 21 and 30 hrs after 6 hrs treatment with 500 µM H_2O_2 compared to media (CON). Data are mean ± SEM, n = 3. *$p < 0.05$ compared to CON.

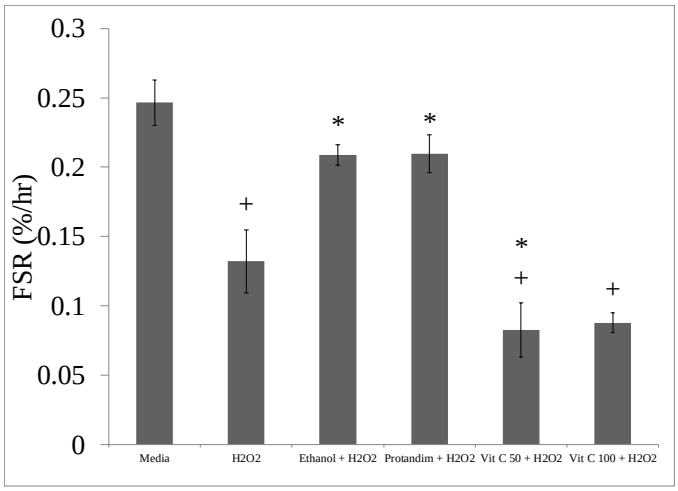

Figure 4.2: Effect of Nrf2 activator (Protandim) and exogenous (Vit C) antioxidants on mitochondrial protein synthesis rate after12 hrs of H_2O_2 treatment in myoblasts. Fraction synthesis rates ((FSR (%/hr)) of mitochondrial proteins in myoblasts treated with 500 µM H_2O_2 only compared to media for 12 hrs as well as FSR (%/hr) of mitochondrial proteins in myoblasts co-treated with 500 µM H_2O_2 and 50 µM or 100 µM Vit C or 500 µM H_2O_2 and 100 µg/mL of a Nrf2 activator (Protandim) for 12 hrs. Media used as a vehicle control for the Vit C and H_2O_2 treatment. Ethanol was used as a vehicle control for the Protandim treatment. Data are mean ± SEM, n = 3. +$p < 0.05$ compared to Media. *$p< 0.05$ compared to H_2O_2.

CHAPTER V – OVERALL CONCLUSIONS

These sets of experiments investigated compensatory responses to oxidative stress in vitro and in vivo. First, we show that increased activation of sympathetic nervous system (SNS) and pulmonary arterial pressure (PAP), in the absence of alterations in cerebral blood flow, contributes to pulmonary edema in isolated cerebral hypoxia. Second, we provide evidence that shows induction of contractile activity by E-stim may be a practical model to simulate a single bout of endurance exercise, even though this finding requires further investigation. In this study, we demonstrate that redox sensitive signaling and new protein synthesis are two major outcomes of induced contractile activity in electrically stimulated C2C12 myotubes. This in vitro model will be valuable in future studies aimed at better understanding mechanisms of cell adaption to contraction. Third, we report different effects of exogenous and endogenous antioxidants on mitochondrial biogenesis in myoblasts treated with H_2O_2. We demonstrated that H_2O_2 treatment irrespective of treatment duration decreased the rate of mitochondrial protein synthesis in vitro. We have also shown that where Vit C blunted mitochondrial biogenesis in response to oxidative stress, Nrf2 activation maintained mitochondrial biogenesis. These findings highlight the role of Nrf2 in protein translation under stress providing the ground work for future studies to understand redox sensitive mechanisms of mitochondrial biogenesis.

Taken together, these projects provide insight into the regulation of cellular response to oxidative stress caused by different stressors. Understanding how such interventions influence adaptive cellular responses such as mitochondrial biogenesis will benefit future recommendations for improving health and longevity.

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