ABSTRACT

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Myocardial infarction and stroke are two major causes of death and disability. As such, novel interventions are needed to reduce the incidence and severity of these events, and improve post-event rehabilitation. I have developed a potentially novel therapy by combining two interventions that independently exert neuro- and cardio-protective effects - exercise and remote ischemic preconditioning (RIPC) - in the form of a unique exercise paradigm, cyclical blood flow restriction exercise (cyclical BFRE). I hypothesized that an acute bout of cyclical BFRE would augment the release of factors that mediate the protection associated with exercise and RIPC when performed independently (nitric oxide and cytokines). A concern about clinical application of BFRE is the potential amplification of the exercise pressor reflex, causing an unsafe rise in arterial pressure. To address this, I further hypothesized that exercise-induced elevations in plasma norepinephrine, arterial pressure, and cerebral blood flow would be 1) attenuated with cyclical blood flow restriction resistance exercise, due to the use of lower workloads, but 2) augmented with cyclical blood flow restriction aerobic exercise, due to increased exercise pressor reflex activation. Fifteen healthy human subjects completed 5 experiments: RIPC, aerobic exercise with and without cyclical blood flow restriction, and resistance exercise with and without cyclical blood flow restriction. A standard thigh cuff pressure of 220 mmHg was used for all restriction protocols.

The major findings from these studies are: 1) an acute bout of cyclical BFRE does not increase release of nitric oxide and key anti- and pro-inflammatory cytokines when blood sampling is performed immediately post-exercise, 2) there is high inter-subject variability in the degree of blood flow restriction achieved with a standardized cuff pressure, 3) cyclical blood flow restriction resistance exercise elicits an attenuated increase in sympathetic activity compared to conventional resistance exercise, 4) cyclical blood flow restriction aerobic exercise elicits an exaggerated increase in sympathetic activity compared to conventional aerobic exercise, but the cyclical reperfusions resulted in lower arterial pressures. This work has laid the groundwork for future studies utilizing individualized cuff pressures in both healthy and clinical populations, as well as training studies to assess the long term adaptations that may result from cyclical BFRE.

POTENTIAL THERAPEUTIC BENEFITS OF CYCLICAL BLOOD FLOW RESTRICTION EXERCISE: A NOVEL ADAPTATION OF REMOTE ISCHEMIC PRECONDITIONING

Justin Sprick, M.S.

APPROVED:

Caroline A. Rickards, Ph.D., Major Professor

J. Thomas Cunningham, Ph.D., Committee Member

Styliani Goulopoulou, Ph.D., Committee Member

Robert T. Mallet, Ph.D., Committee Member

Tara Tovar-Vidales, Ph.D., University Member

Steve W. Mifflin, Ph.D., Chair, Department of Physiology and Anatomy

---

Johnny J. He, Ph.D., Dean, Graduate School of Biomedical Sciences

POTENTIAL THERAPEUTIC BENEFITS OF CYCLICAL BLOOD FLOW RESTRICTION EXERCISE: A NOVEL ADAPTATION OF REMOTE ISCHEMIC PRECONDITIONING

DISSERTATION

Presented to the Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

By

Justin Daniel Sprick

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LIST OF ABBREVIATIONS

ACSM	American College of Sports Medicine
ANOVA	Analysis of Variance
BDNF	Brain derived neurotrophic factor
BFRE	Blood flow restriction exercise
BMI	Body mass index
C-$BFRE_{AERO}$	Cyclical blood flow restriction aerobic exercise
C-$BFRE_{RES}$	Cyclical blood flow restriction resistance exercise
CE	Conventional exercise
CE_{AERO}	Conventional aerobic exercise
CE_{RES}	Conventional resistance exercise
CHO	Chinese hamster ovary
CRP	C-reactive protein
CSA	Cross sectional area
DAG	Diacylglycerol
dHb	Deoxygenated hemaglobin
ECG	Electrocardiogram
eNOS	Endothelial nitric oxide synthase
$etCO_2$	End-tidal carbon dioxide
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilation

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HbO_2	Oxygenated hemaglobin
HR	Heart rate
HR_{max}	Maximal heart rate
IL-6	Interleukin-6
IL-10	Interleukin-10
IP3	Inositol triphosphate
IR	Ischemia-reperfusion
IRB	Institutional Review Board
MAP	Mean arterial pressure
MCAv	Middle cerebral artery velocity
MPTP	Mitochondrial permeability transition pore
mRS	Modified Rankin Scale
MSNA	Muscle sympathetic nerve activity
NE	Norepinephrine
NIRS	Near infrared spectroscopy
NO	Nitric Oxide
OPRD	Delta opioid receptor
PCAv	Posterior cerebral artery velocity
PE	Phycoerythrin
PI	Principal investigator
PI3	Phosphatidylinositol-3
PLC	Phospholipase C

REC	Recovery
RER	Respiratory exchange ratio
1RM	1-repetition maximum
RIPC	Remote ischemic preconditioning
SAA	Serum amyloid-A
ScO_2	Cerebral oxygen saturation of the frontal cortex
sICAM-1	Soluble intercellular adhesion molecule-1
SFA	Superficial femoral artery
SPECT	Single-photon emission computed tomography
sVCAM-1	Soluble vascular adhesion molecule-1
TACSM	Texas chapter of the American College of Sports Medicine
THC	Total hemoglobin concentration
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen activator
TPR	Total peripheral resistance
VCO_2	Carbon dioxide production
VO_2	Oxygen consumption
VO_{2max}	Maximal oxygen consumption
VO_{2peak}	Peak oxygen uptake

CHAPTER I

LITERATURE REVIEW

Myocardial infarction (i.e., heart attack) and stroke are two of the leading causes of potentially preventable death and disability nationwide, with approximately ~795,000 people experiencing either their first, or a recurrent heart attack or stroke each year (6). Additionally, the risk of stroke more than doubles for each successive decade after the age of 55, a projection that is associated with a dramatic increase in the costs of caring for these patients (85). Extensive resources have been invested into exploring therapies to reduce the severity (e.g., recombinant tissue plasminogen activator, tPA; cardiac catheterization) (84), and incidence (e.g., diet and exercise) (38) of stroke and heart attacks, and also in rehabilitating patients who have survived these events (8, 60). Despite these advancements, however, someone in the US will suffer from a heart attack or stroke every 40 seconds, and stroke still remains a leading cause of serious long-term disability (6).

While much of the injury to the heart and brain occurs as a direct result of tissue ischemia, paradoxically, damage can also result from the restoration of flow during the reperfusion period; a phenomenon known as “ischemia-reperfusion injury” (53). The mechanisms underlying this phenomenon are complex, and involve the engagement of plasma membrane bound ion transporters (49, 80), dysregulation of intracellular calcium signaling (7, 80), reactive oxygen species formation (4, 56, 130), and the activation of pro-inflammatory cytokines (18, 31, 122, 130). Collectively, all of these events culminate in the opening of the mitochondrial permeability transition pore (MPTP), a hallmark of ischemia-reperfusion injury that ultimately leads to tissue necrosis and cell death (36, 53). Based on its critical role in

mediating cell death resulting from ischemia-reperfusion, the MPTP has been an important target for cardio- and neuro-protective therapies (36, 37, 50, 94). While the cellular mechanisms underlying ischemia-reperfusion injury are beginning to be illuminated, the consequences of heart attack and stroke still place a serious health and economic strain on society. As such, more directed therapies are needed that can both reduce the incidence of these events, and also be used to rehabilitate patients who have suffered from them. The purpose of the studies described herein is to explore a potential therapy, cyclical blood flow restriction exercise (BFRE), which could potentially be implemented to meet this need. This therapy combines two interventions that are both known to independently reduce the incidence and severity of ischemia-reperfusion injury: remote ischemic preconditioning (RIPC), and exercise.

In this chapter, I will first review the origins and applications of RIPC, and the mechanisms underlying its signal transduction. Next, BFRE will be described, and connections to RIPC will be made. This background material is important as it directly relates to *Specific Aim 1* of my dissertation project. In the second part of this chapter, key concerns regarding the safety of BFRE will be highlighted as it specifically relates to *Specific Aim 2* of my dissertation project.

History of Ischemic Preconditioning:

Ischemic preconditioning describes the phenomenon in which brief cycles of sub-lethal ischemia provide protection to a vital organ in the face of a subsequent ischemia-reperfusion injury. Murry et al. first described this phenomenon in 1986, with a canine model that was "preconditioned" with 4 x 5-min cycles of coronary circumflex artery occlusion separated by 5-min of reperfusion (40-min total), prior to a sustained 40-min occlusion-reperfusion of this same artery. When assessing infarct size as a function of area at risk, the preconditioned group

exhibited an infarction only ~25% of that observed in the control group who were subjected to the same 40-min ischemic insult, but in the absence of preconditioning (81). Importantly, there were no differences in the area at risk or collateral blood flow between groups, indicating equivalent regional perfusion. This initial observation gave rise to several other investigations utilizing this same ischemic preconditioning principle (82, 83, 112). It wasn't until 1993, however, that the first description of "remote ischemic preconditioning" (RIPC) was published by Pryzklenk et al. (91). Also in a canine model, the circumflex artery was once more preconditioned using the same paradigm described by Murry et al. (4 cycles of 5-min of ischemia and 5-min of reperfusion). In contrast to the Murry et al. study, however, these investigators then induced a sustained occlusion (1 hour) and reperfusion (4.5 hours) to the left anterior descending coronary artery, rather than the circumflex, i.e., the preconditioning stimulus was "remote" from the ischemia-reperfusion injury. When examining the infarct size as a function of the area at risk, the animals treated with RIPC exhibited approximately one third of the infarct size observed in the control group (6 ± 2% in RIPC versus 16 ± 5% in control). Again, there were no differences in the area at risk between groups (91). For the first time, the results of this work demonstrated that the preconditioning stimulus need not be applied directly to the same vascular bed subjected to prolonged ischemia-reperfusion, but could also confer this protection when administered to a nearby or *remote* location.

In 2002, Kharbanda and colleagues expanded on the cardiac RIPC paradigm by applying the preconditioning stimulus to a limb, rather than an adjacent coronary vessel (54). In this seminal study, pigs were subjected to four cycles of 5-min hind limb occlusion-reperfusion prior to 40-min of sustained occlusion and reperfusion of the left anterior descending coronary artery. The animals subjected to this novel RIPC paradigm exhibited a substantially smaller infarct size

compared to the control group (~26% in RIPC versus ~53% in controls). Again, there were no differences in left ventricular mass or area at risk between groups. These early studies in animal models served as the proof of concept that gave rise to the multitude of clinical trials that would follow.

The first clinical trial utilizing RIPC in humans was performed by Cheung et al. in 2006 (19). These investigators assessed the effects of pre-operative limb RIPC in children undergoing repair of congenital heart defects. A blood pressure cuff around the upper arm was inflated to a supra-systolic pressure in accordance with the same cyclical paradigm previously used in animal models (4 x 5-min of ischemia-reperfusion). The control subjects underwent a sham procedure in which the blood pressure cuff was placed on the arm but remained deflated. Cardiac troponin was assessed from an arterial blood sample post-operatively as a biomarker of cardiac damage. While both groups exhibited an increase in cardiac troponin, this increase was attenuated in the RIPC group compared to the control group, indicating a cardio-protective effect. The following year, the first clinical trial using RIPC in adults was performed. Hausenloy et al. subjected patients to pre-operative RIPC of 3 x 5-min cycles of arm cuff inflation-deflation (to 200 mmHg) prior to undergoing coronary artery bypass graft surgery (40). Much like the Cheung et al. study (19), plasma troponin concentration was the primary end-point of interest, and was interpreted as an indicator of cardiac necrosis. While cardiac troponin increased in both groups, the total troponin increase over the 72 hours following surgery was attenuated in the RIPC group by approximately 43% when compared with the control group (40).

Since these early clinical trials, RIPC has been applied to numerous other pre-operative interventions including heart valve replacement (16), laparoscopic partial nephrectomy (46), infrarenal abdominal aortic aneurysm repair (120), percutaneous coronary intervention (101),

and carotid endarterectomy (120). Although the results of RIPC trials have not been uniformly favorable (39, 47, 89, 92, 113), divergent findings between studies have raised additional experimental questions, and have highlighted potential pitfalls associated with RIPC prescription. Importantly, comorbidities such as hypercholesterolemia (55, 118) and diabetes (59, 114, 116, 125), sleep disturbances (11), medication use (107, 132), and pre-operative anesthesia (57, 58, 132), have all been identified as potential factors that can diminish the preconditioning signal, resulting in a reduced or abolished protective effect. Additionally, advanced age has also been associated with an attenuated response to RIPC (5, 125); this is an especially concerning observation given that aging is independently associated with cardiovascular and cerebrovascular disease risk (6, 85). It is imperative that these limitations be considered as the use of RIPC in acute settings continues to be optimized. While the study of these potential confounds is still in its infancy, elucidating the mechanisms underlying the impaired signal transduction of RIPC will facilitate a more personalized and optimized approach to RIPC prescription. Ultimately, it is envisioned that patients who are more or less likely to benefit from this therapy can be identified and treatments can be adjusted accordingly.

Remote Ischemic Preconditioning "Training" as a Therapy

In addition to the acute scenarios previously described, RIPC has also been applied to more prolonged settings. In this context, RIPC has been used prophylactically to enhance peripheral vascular function (51, 52), and reduce the incidence of stroke in high risk patients (73, 74). These novel applications highlight a relatively recent paradigm shift for RIPC that circumvents some of the potential limitations previously highlighted. That is, if RIPC can be adopted into a daily regimen to promote healthy aging, it is possible that this therapy could be

implemented before the onset of diseases that may interfere with its signal transduction. For example, Jones et al. demonstrated that 7-d of daily arm RIPC (4 cycles of 5-min ischemia and 5 min reperfusion) induced a ~1.8% improvement in brachial artery flow mediated dilation (FMD; an index of conduit artery endothelial function) in young healthy males (51). Remarkably, these vascular improvements also extended to the contralateral arm which did not receive RIPC treatment (and of the same magnitude as the arm treated with RIPC). Furthermore, the improvements in FMD were still present even 8 days post-intervention. While seemingly small, this 1.8% enhancement in FMD is clinically relevant in relation to cardiovascular disease risk reduction. As highlighted in a meta-analysis consisting of 14 studies, a 1% increase in FMD is associated with a 13% reduction in the risk of future adverse cardiovascular events (48). Importantly, the observation that the vascular improvements also translated to the contralateral arm suggests a humoral component underlying the protective effects of RIPC signaling. A follow up study by the same group of investigators also explored the effects of 8 weeks of upper arm RIPC applied three times weekly. Similarly, enhancements in brachial artery FMD were apparent beginning at week 2 (5.6% for RIPC vs. 4.6% for control), and these improvements persisted throughout the 8 week intervention (5.2% with RIPC vs 4.1% for control); baseline FMD was 4.3% for both conditions. This follow up study provides further support for the use of RIPC as a long-term therapy, and also demonstrates that a lower frequency of application (three times weekly versus daily) is still a sufficient dose to promote positive vascular adaptations.

This novel application of RIPC has also been tested in clinical populations. Meng et al. treated 38 older stroke survivors (~60 years of age) with symptomatic atherosclerotic intracranial stenosis to twice daily bilateral arm RIPC, performed as 5 cycles of 5-min occlusion and 5-min reperfusion with a cuff pressure of 200 mmHg (73). All subjects had experienced a stroke or

transient ischemic attack within 30 days prior to being enrolled in the study, and the RIPC treatment was carried out over a period of 300 days. Intriguingly, the incidence of recurrent stroke at the end of the intervention was only 7.9% in the treatment group compared to 26.7% in age-matched controls. This 70% reduction in stroke incidence was also accompanied by enhanced cerebral blood flow, and a shortened recovery time in those subjects who did suffer a stroke when compared to controls. Specifically, ~76% of patients in the treatment group exhibited improved cerebral perfusion as measured with single-photon emission computed tomography (SPECT) imaging at day 300 compared to only ~53% in the control group at the same time point. The proportion of patients achieving a 0-1 on the modified Rankin Scale (mRS; indicating their ability to carry out all usual activities independently) was ~66% in the treatment group compared to only ~13% in the control group at day 90 (73); mRS results at day 300 were the same between groups. These promising results provided the impetus for a subsequent study by the same investigators in octo- and nonagenarians, also with symptomatic intracranial arterial stenosis (74). The same RIPC paradigm (twice daily bilateral upper limb RIPC) was performed for the duration of 180 days. Again, this treatment reduced the incidence of recurrent stroke and transient ischemic attack to ~13% at the end of the 180 days in the RIPC group compared to ~36% in the age-matched controls who underwent sham RIPC. Inflammatory biomarkers including C-reactive protein and interleukin (IL)-6 were also lower in the treatment group compared to controls at day 30 (the final time point for blood sample analysis) (74).

Combined, these promising results in cerebrovascular health outcomes with RIPC training raise the question of whether similar improvements could be exhibited in cardiac health outcomes. Although data from cardiac rehabilitation in humans is lacking, a recent animal study explored the effects of applying hind-limb RIPC every other day beginning two weeks after

myocardial infarction in Sprague-Dawley rats (3). These investigators demonstrated a reduction in myocyte apoptosis in the RIPC group that was accompanied by improvements in left ventricular developed pressure, and an upregulation of mitochondrial respiratory chain complexes I-IV (3). Collectively, these studies provide support for the notion that RIPC could be used as a preventative therapy, and a rehabilitative tool for at-risk and aged populations.

Signal Transduction of Remote Ischemic Preconditioning

The mechanism underlying transmission of the preconditioning signal from the remote location (i.e., the limb) to the target tissue (i.e., the heart or brain) has been the topic of much investigation. In general, two separate ideas have emerged to explain this process: neurogenic transmission, and humoral transmission. **Neurogenic:** In this model, it is postulated that afferent nerves from the site of the RIPC stimulus relay a signal to the central nervous system which then confers protection to the target organ via efferent nerve activation (27, 67, 71, 72). This efferent nerve stimulation is thought to activate protein kinase C-epsilon in the target tissue which can be protective through two distinct mechanisms: 1) activation of mitochondrial ATP-sensitive potassium channels (123), and, 2) induction of cardio-protective genes (128). **Humoral:** In the humoral model, circulating blood-borne factors are released systemically in response to the RIPC stimulus, and these factors then travel to the target organs where they then exert their protective effects (34, 75, 93, 124). Some work also suggests that both mechanisms (neurogenic and humoral) are required for the protective phenotype to be present (62, 88). While all aforementioned viewpoints are grounded in experimental evidence, the precise mediators underlying the protection associated with RIPC, and the potential cross-talk between these

mediators is still a matter of intense investigation and debate. In this review, however, I will focus primarily on the humoral mechanism as this directly relates to my dissertation project.

Numerous circulating factors have been implicated in mediating the cardio- and neuro-protective effects of RIPC. These mediators include adenosine (45, 109), bradykinin (29, 98), prostaglandins (2, 97), various micro-RNAs (12, 61, 100), heat shock proteins (30, 133), endocannabinoids (106), nitric oxide (NO) metabolites (25, 93), cytokines (1, 14, 15), and endogenous opioids (75, 99, 109, 131). This list is continuously expanding as more factors are implicated with ongoing research activities. For the purpose of this project, however, this discussion will be focused on the anti-inflammatory cytokine IL-10, the NO metabolites nitrate and nitrite, and endogenous opioids (figure 1).

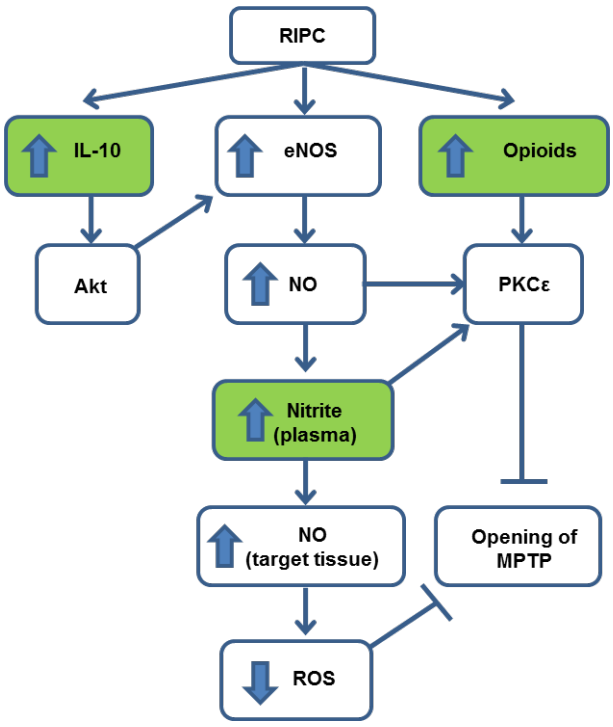

Figure 1: Proposed mechanisms of protection conferred through humoral mediators of RIPC. Mediators directly assessed in this dissertation project are highlighted in green. All mediators ultimately converge on the inhibition of the opening of the mitochondrial permeability transition pore (MPTP). IL-10: Interleukin-10; NO: Nitric oxide; PKCε: Protein kinase C-epsilon; ROS: Reactive oxygen species. eNOS: Endothelial nitric oxide synthase

IL-10

Support for the role of IL-10 in the protective effect conferred by RIPC stems from animal models in which IL-10 activity was either pharmacologically inhibited through the use of an antibody directed towards the IL-10 receptor, or genetically ablated through the use of IL-10 knock out mice (15). In both sets of experiments, mice underwent hind limb RIPC, performed as three cycles of 5-min femoral artery ligation and reperfusion via a micro-vessel clip. Twenty-four hours later, the animals were subjected to sustained left coronary artery ligation and reperfusion. The mice treated with RIPC exhibited infarcts approximating one-third the size of those observed in the control group. Treatment with the IL-10 receptor antibody, however, completely abolished this effect. When the same RIPC protocol was performed in IL-10 knock out mice, there was no reduction in infarct size following the ischemia-reperfusion injury. Treatment with exogenous IL-10, however, restored the protection that was previously demonstrated in wild type animals.

Additional experiments in this study using isolated perfused heart preparations (i.e., the Langendorff preparation) facilitated further insight into the intracellular events underlying this protection (15). Hearts were perfused with Krebs-Henseleit buffer with and without addition of exogenous IL-10, prior to induction of global ischemia and reperfusion. In a subset of heart preparations, the IL-10 receptor antibody or a phosphatidylinositol-3 (PI3) kinase (Akt) inhibitor were also added to the preparation. As IL-10 utilizes PI3 kinase signaling, the authors hypothesized that addition of this inhibitor would also abolish the cardio-protective effects of IL-10. In line with the previous observations *in vivo,* exogenous IL-10 induced a protective effect following ischemia and reperfusion, manifest by a decreased infarct size that was accompanied

by an attenuated reduction in left ventricular developed pressure. These effects were reduced by the addition of the IL-10 receptor antibody and by the P13 kinase inhibitor.

Next, these investigators sought to determine the source of the IL-10 release in response to RIPC and to elucidate the molecular mechanism responsible for its production. Mice were once again exposed to femoral artery RIPC as previously described, and IL-10 concentrations were quantified in both plasma and myocardial tissue (15). RIPC resulted in an increase in both plasma concentrations and myocardial protein concentrations of IL-10, with the latter being blocked by addition of the IL-10 receptor antibody. In a final set of experiments in the same study, the gastrocnemius muscle (downstream of the site of the RIPC stimulus) was excised and subjected to western blot analysis 24 hours after RIPC. This analysis revealed that IL-10 expression was upregulated in the gastrocnemius, and this effect was abolished if the animals were co-treated with a STAT3 inhibitor. As STAT3 is a transcription factor that has previously been implicated in IL-10 production (104), the authors aimed to link this molecular mechanism to the IL-10 produced in response to RIPC (15). Collectively, this elegant series of experiments highlights the essential role of IL-10 in mediating the cardio-protective effects of RIPC, and also points toward PI3k/Akt in the downstream signaling cascade (see figure 1). The source of IL-10 appears to be the skeletal muscle tissue subjected to repeated bouts of ischemia and reperfusion, and STAT3 activation is required for its production (15).

In summary, these experiments strongly support the notion that IL-10 is associated with the cardio-protective effects of RIPC. Plasma concentrations and myocardial protein concentrations of IL-10 increased in response to RIPC, and these responses were blocked by pharmacological inhibition of the IL-10 receptor (15). RIPC failed to reduce infarct size in IL-10 knock out mice, but this effect was restored by exogenous IL-10 treatment (15), and muscle IL-

10 expression was upregulated in response to upstream application of RIPC (15). *While IL-10 is essential in mediating the cardio-protection afforded by RIPC, it is unexplored in relation to cyclical BFRE. This knowledge gap will be addressed in Specific Aim 1 by quantifying plasma IL-10 concentrations in response to cyclical BFRE to determine the potential role of IL-10 in vital organ protection with this therapeutic approach*.

Nitric Oxide (NO)

NO has been implicated as a key mediator underlying the cardio-protection observed following RIPC (23, 42, 93, 115). The rationale for release of NO with RIPC is based on the shear stress-mediated activation of endothelial nitric oxide synthase (eNOS) that results from rapid cuff deflation and reperfusion (93). As NO has a relatively short half-life (several seconds), it is thought that circulating nitrite, a metabolite of NO, serves as the NO reservoir within the circulation (23, 115). The half-life of nitrite is considerably longer than NO (~40 min) (24), thus allowing it to circulate in the blood for more extended periods of time. After nitrite is formed, it circulates in the blood until it is taken up into cardiac myocytes, where it is reduced back to NO via myoglobin (23). NO is then used to facilitate S-nitrosation, a reversible post-translational modification which occurs on complex I of the mitochondrial electron transport chain (20). There are multiple cysteine residues on complex I that are subject to S-nitrosation. Although some degree of S-nitrosation occurs during normal physiological conditions, it occurs at a much greater rate during periods of low mitochondrial respiration, such as during ischemia (20). This is thought to be due to the location of a specific cysteine residue on one subunit of complex I. Specifically, Cys39, located on the ND3 subunit, undergoes a conformational change during periods of hypoxia which increases its susceptibility to S-nitrosation (32, 35). Once formed, this

modification locks the conformation of complex I into a low activity state, and slows the flux of electrons through the chain during the transition from ischemia to reperfusion (20). This decreased flux of electrons through complex I attenuates the production of reactive oxygen species that typically characterizes ischemia-reperfusion (20). As aberrant formation of reactive oxygen species is a well-established mediator of MPTP opening (78, 86, 110), increases in circulating nitrite inhibits this deleterious cascade via S-nitrosation of complex I (see figure 1).

In support of NO signaling in the protection induced by RIPC, Rassaf et al. subjected mice to 4 cycles of 5-min hind-limb ischemia and reperfusion prior to sustained myocardial ischemia reperfusion injury (93). The RIPC protocol resulted in a ~50% reduction in infarct size compared to the control group who underwent no intervention. This reduction in infarct size was completely abolished, however, when the mice were treated with a nitrite scavenger (carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide) (93). The same experiment was repeated in eNOS knock-out mice, and similarly, there was no protection observed following the RIPC intervention. To explore the role of cardiac myoglobin in reducing the circulating nitrite back to NO within the ischemic tissue, this experiment was repeated once more in myoglobin knock-out mice. Plasma and cardiac nitrite concentrations increased in response to the preconditioning stimulus, but this was not accompanied by a reduction in infarct size in the myoglobin knock-out mice; in fact, the infarct size was increased in response to RIPC in these mice. Of note, RIPC also resulted in an increase in S-nitrosation of complex I in the wild type mice. This was accompanied by a reduction in complex I activity and hydrogen peroxide formation, findings that were not observed in the knock-out mice.

Finally, in this same study, Rassaf et al. subjected human volunteers to four cycles of brachial artery RIPC via cuff inflation. One group underwent RIPC as it is conventionally

prescribed, with rapid arm cuff deflation and reactive hyperemia during the reperfusion periods. In the experimental group, however, rather than just rapidly deflate the cuff, an ultrasound probe was placed just distal to the cuff and pressure was gradually released during the reperfusion periods. The objective of this procedure was to reduce the shear stress mediated increase in NO resulting from the reactive hyperemia. Lastly, a control group was included which was not subjected to any form of RIPC (with or without reactive hyperemia). As expected, conventional RIPC elicited a ~61% increase in plasma nitrite concentrations, while the group without reactive hyperemia exhibited no change in plasma nitrite. Plasma from all three groups was then transferred to isolated mouse heart preparations (Langendorff preparation) that were subjected to ischemia-reperfusion. Treatment with the plasma from the RIPC group (with reactive hyperemia) reduced infarct size from ~47% (control group that was not subjected to any form of RIPC) to ~31%. This reduction in infarct size was completely abolished, however, if the preparation was also co-treated with sulfanilamide, a nitrite scavenger. Additionally, there was no reduction in infarct size in the hearts treated with plasma from individuals who underwent RIPC without reactive hyperemia, but when exogenous nitrite was added into the preparation, cardio-protection was restored, reducing infarct size from ~50% to ~25% (93).

This series of experiments highlights the role of the NO-dependent signaling component of RIPC. NO is released in response to shear stress, and is transported throughout the systemic circulation in the form of nitrite. Nitrite is then reduced back to NO by myoglobin in the heart, allowing it to subsequently modify complex I of the electron transport chain via S-nitrosation. This post translational modification results in an attenuated formation of hydrogen peroxide (a reactive oxygen species) during the reperfusion period, due to a decreased activity of complex I. The observation that this protection is transferable between species (human to mouse) strongly

supports the humoral model of RIPC protection. Furthermore, the fact that the signaling was completely absent in eNOS and myoglobin knock-out mice confirms the critical role of these oxygen binding proteins in transducing NO across tissues. Lastly, the ability of nitrite scavengers to abolish the RIPC-induced protection, whereas exogenous nitrite can restore it, provides further support for this hypothesis. Collectively, these observations strongly support the role of NO in underlying the cardio-protective effects of RIPC. *As such, in Specific Aim 1 plasma concentrations of nitrite, an NO metabolite, will be quantified in response to cyclical BFRE to determine the potential role of NO in vital organ protection with this therapeutic approach.*

Endogenous Opioids

A further category of humoral mediators implicated in RIPC signaling are the endogenous opioids. Importantly, within the context of the investigations described for this dissertation project, these mediators are common to the protective effects elicited by both exercise (10, 26, 75, 76) and RIPC (68, 75, 109). Endogenous opioids trigger the commonly reported sensation of "runners high" that is often described with exercise (9, 126). However, they may also underlie some of the negative findings associated with translation of RIPC into clinical practice, as anesthetics commonly used during surgery also exert their effects through opioidergic signaling (43, 58). All of the endogenous opioids are formed from larger parent compounds, with site-specific cleavage resulting in the formation of distinct subtypes. These subtypes are the endorphins, dynorphins, and enkephalins, with each exhibiting different affinities for the distinct receptor subtypes at which they bind. Endorphins exhibit high affinity for mu (μ) receptors, dynorphins for kappa (κ) receptors, and enkephalins for delta (δ) receptors (119). As the δ-opioid receptor is the specific subtype that has primarily been implicated in

cardio-protection from RIPC (10, 76), this receptor and its ligands (i.e., the enkephalins) will be the focus of this discussion.

In general, there are two distinct agonists of the δ-opioid receptor, both of which belong to the enkephalin family; met-enkephalin and leu-enkephalin. While enkephalins are conventionally understood to be co-stored with catecholamines in the adrenal gland and post-ganglionic neurons (44, 127), more recent work has demonstrated that these molecules can be produced directly by myocytes, where they exert their effects in an autocrine and paracrine fashion (26, 76). Once a ligand is bound, the δ-opioid receptors utilize G-protein mediated signaling to ultimately activate protein kinase C-epsilon, which subsequently inhibits MPTP formation (see figure 1), likely through the activation of mitochondrial ATP-sensitive potassium channels (41, 119).

The role of the δ-opioid receptor in mediating the cardio-protection induced by RIPC has been demonstrated in a number of studies. These investigations have most commonly used cross species transfer of plasma from animals or humans treated with RIPC to demonstrate *in vitro* cardio-protection in either cell culture or isolated heart models (75, 99, 109). This protection can be attenuated or abolished if co-treated with opioid receptor antagonists (75, 99). Interestingly, several studies have even demonstrated that combined morphine and RIPC work synergistically to decrease infarct size in animal models of myocardial ischemia reperfusion injury (68, 121). Surprisingly, very few studies have directly quantified the increase in plasma or myocardial concentrations of endogenous opioids as a result of RIPC. Younes et al. did demonstrate that myocardial ischemic preconditioning applied directly to a coronary vessel (rather than to a remote limb) increased the cardiac availability of met-enkephalin precursors (129). A separate group of investigators also reported a direct cardio-protective effect of met-enkephalin and leu-

enkephalin application in isolated cardiac myocytes (111). This protection, however, resulted from treatment with synthetic versions of these compounds, rather than those derived *in vivo* (111), and was explored outside the context of RIPC. While studies directly quantifying plasma concentrations of these peptides in response to RIPC are lacking, this is potentially due to the technical limitations associated with these measurements. Resting concentrations of enkephalins in the blood are typically very low (21, 96) as these compounds are subject to rapid degradation by circulating endogenous peptidases (79).

Importantly in the context of this dissertation project, δ-opioid receptor agonists have also been associated with the cardio-protective effects of exercise. Exercise has long been known to confer cardio-protection, and recent work has highlighted the role of δ-opioid receptor agonists in mediating this effect. Borges et al. demonstrated that 60-min of treadmill exercise at 70% maximal oxygen consumption (VO_{2max}) for 4 days, reduced infarct size by approximately 34% in a rat model of myocardial ischemia-reperfusion (10). This protective effect was still present when the animals were treated with μ or κ receptor antagonists, but was abolished when treated with the δ-opioid receptor antagonist naltrindole (10). Similarly, Miller et al. (76) reported a reduction in infarct size in response to coronary artery ligation after three consecutive days of treadmill running in Sprague-Dawley rats. These effects were also attenuated when animals were co-treated with naltrindole. Pro-enkephalin mRNA expression was quantified in cardiac tissue as well as the soleus muscle, and revealed that while cardiac pro-enkephalin mRNA expression doubled in response to exercise, soleus pro-enkephalin mRNA expression remained unchanged. These findings suggest a cardiac origin of enkephalins in response to exercise (76).

Both exercise and RIPC elicit cardio-protection via an opioid-dependent mechanism. A recent study highlighted this potential overlap by exposing human subjects to either brachial

artery RIPC or an acute bout of maximal aerobic exercise, separated by 1 week (75). Following each experiment, plasma was collected and perfused through a 12-14 kDa dialysate filter to isolate the small compounds (i.e., endogenous opioids). The filtrate was then perfused through an isolated rabbit heart preparation (Langendorff preparation) prior to sustained 40-min regional ischemia, followed by 2 hours of reperfusion. Plasma from both treatments conferred a reduction in infarct size, which was accompanied by an improvement in post-ischemic left ventricular developed pressure. These effects were abolished when the hearts were co-treated with the non-specific opioid receptor antagonist naloxone (75). Collectively, these findings strongly support the notion that both exercise and RIPC can independently confer cardio-protective effects in the face of ischemia-reperfusion injury, and that these effects are likely mediated through an opioid-dependent mechanism. *As such, in Specific Aim 1 will seek to develop a novel technique for the measurement of delta opioid receptor agonist activity. Establishing this technique will eventually allow for delta opioid receptor agonist activity to be quantified in response to cyclical BFRE, to determine the potential role of delta opioid receptor agonists (i.e. the enkephalins) in vital organ protection with this therapeutic approach.*

Combined, these observations raise an important question that provides the impetus for this dissertation project. As exercise and RIPC can both independently facilitate cardio-protection via endogenous opioid release (75), and as many of the other factors associated with RIPC protection are also released in response to exercise [i.e., IL-10 (13, 22, 77), NO (17, 28, 117)], it is possible that these protective effects could be additive when combining these two interventions via cyclical BFRE.

Blood Flow Restriction Exercise (BFRE)

The novel exercise modality that uses limb blood flow restriction, similar to RIPC, is known as BFRE. As BFRE is inherently characterized by both blood flow restriction and exercise, it likely shares many similarities in the signal transduction and systemic effects of both conventional exercise (CE) and RIPC that may ultimately lead to vital organ protection. BFRE was originally developed as a method to augment muscle growth, and a hallmark of its application is the use of lower training loads compared to CE (63, 66). The mechanisms underlying the muscle hypertrophic effects of BFRE are still a matter of ongoing investigation, but are likely related to heightened growth hormone signaling, cell swelling, and altered patterns of neuromuscular recruitment (64-66). While virtually all of the BFRE literature has been focused on exercise performance and musculoskeletal rehabilitation, the potential parallel to RIPC remains relatively unexplored.

The Exercise Pressor Reflex

One concern that has been raised about the use of BFRE in the rehabilitative setting is the potential for an augmentation of the exercise pressor reflex, which could cause an unsafe rise in sympathetic outflow and arterial pressure (103). The exercise pressor reflex originates within the muscle, and is associated with increased activity of type III and type IV afferent nerves which are responsive to mechano- and metabo-stimulation (102). Upon stimulation, afferent neural signals are relayed to the central nervous system, resulting in an increase in efferent sympathetic nerve activity. This increase in sympathetic nerve activity results in increased cardiac output, contractility, and total peripheral resistance, all of which facilitate an increase in arterial pressure (103). This feedback loop is normally beneficial as it facilitates an increase in the supply of

nutrient-rich blood to meet the demands of the exercising skeletal muscle, while also facilitating the removal of metabolic by-products (102). A concern with BFRE, however, is that application of an external pressure stimulus (i.e. the occlusive cuffs), combined with the trapping of metabolic byproducts within the muscle with cuff inflation, may increase activation of this reflex (103). The combined increase in activation of type III and IV afferent nerves could cause an even greater increase in sympathetic nerve activity and arterial pressure, which would be undesirable in at-risk patients (e.g., patients with hypertension). As such, much of the previous literature focusing on the hemodynamic responses to BFRE has sought to address this concern.

Madarame et al. (69) subjected patients with ischemic heart disease to 4 sets of bilateral knee extensions with and without blood flow restriction (200 mmHg) applied to the upper thighs. As expected, the BFRE condition did elicit a greater increase in plasma norepinephrine, supporting the notion of a greater sympathetic activation with blood flow restriction. Importantly, however, the relative exercise intensity was the same in both conditions (20% of 1 repetition maximum; 1RM), whereas BFRE is typically performed at a lower relative workload than what would be conventionally prescribed (63). Using a more conventional approach, Poton and Polito (90) compared the hemodynamic responses to knee extension performed at low intensity (20% 1RM) with blood flow restriction and at high intensity (80% 1RM) performed without blood flow restriction. As expected, the high intensity condition induced greater increases in arterial pressure (90), suggesting that the augmented exercise pressor reflex with BFRE can be offset through a reduction in the relative workload.

While studies assessing the responses to aerobic BFRE are less common than those exploring resistance BFRE, several investigations have compared the hemodynamic responses to an acute bout of aerobic BFRE (95, 105, 108). Renzi et al. (95) observed greater elevations in

arterial pressures in response to 5 x 2-min bouts of treadmill walking at 3.2 km/h with blood flow restriction (160 mmHg) applied to the upper thighs compared to a control condition at the same exercise intensity without blood flow restriction. Similarly, Staunton et al. (105) compared treadmill walking (4 km/h) with and without blood flow restriction in both young and older adults. The BFRE condition elicited a greater increase in mean arterial pressure (MAP) compared to the control condition (~7 mmHg difference) with no differences observed between the two age groups. Lastly, Sugawara et al. (108) compared 5 bouts of 2-min constant treadmill walking at 3.2 km/h with and without blood flow restriction applied to the upper thighs (160 mmHg). Similarly, arterial pressures of both central and peripheral arteries were greater with BFRE compared to the control condition in which subjects exercised without blood flow restriction. While findings from all of these investigations support the notion of an augmented exercise pressor reflex with BFRE, it is important to point out that the relative intensities between BFRE and control groups were not matched based on heart rate or VO_{2max} between conditions in any of these studies. It is common practice, and recommended by the American College of Sports Medicine to prescribe exercise at intensities based on a percentage of maximal heart rate (HR_{max}) or VO_{2max} for both healthy and clinical populations (87). *As such, in the studies designed for this dissertation project, I sought to compare the sympathetic and hemodynamic responses to an acute bout of aerobic cyclical BFRE when exercise intensities are matched for relative heart rate responses between conditions (Specific Aim 2).*

Cerebrovascular Responses to Blood Flow Restriction Exercise

There have been very few studies assessing the cerebrovascular responses to BFRE. This is surprising, given that BFRE is often utilized by elderly populations who may be at a higher

risk for cerebrovascular disease. Ganesan et al. (33) did assess frontal lobe cerebral oxygen saturation (ScO_2) responses to bilateral knee extensions with and without BFRE; cerebral blood flow was not measured. Both groups performed three sets at 50% 1RM to volitional fatigue, with and without bilateral upper thigh cuff inflation (100 mmHg). ScO_2 was measured via cerebral near infrared spectroscopy (NIRS), which primarily detects oxy- and deoxy-hemoglobin concentrations in venous (75%) rather than arterial (20%) and capillary (5%) blood (70). As such, an increase in ScO_2 would be indicative of a decreased oxygen extraction, since less oxygen would be taken up by the tissue (i.e. the brain), and thus more left over on the venous side of the circulation. While the magnitude of the increase in ScO_2 reported by Ganeson et al. was greater during the BFRE condition, interpretation of this finding is limited without concomitant measures of cerebral blood flow. That is, an increase in ScO_2 could be the result of a greater increase in supply (i.e. cerebral blood flow), which was not measured, or a decrease in metabolic demand in relation to the control condition. Unfortunately, without knowledge of cerebral blood flow responses together with ScO_2, this study is inconclusive. *In this dissertation project I will seek to fill this knowledge gap by assessing both the cerebral blood flow and oxygenation responses to an acute bout of cyclical BFRE.*

Modalities of Training

My overall goal for this dissertation project is to develop a therapy, cyclical BFRE, that can be ultimately be implemented in a cardiac- or stroke-rehabilitation setting. As such, the guidelines for rehabilitation in these settings incorporates both aerobic and resistance training modalities, with each modality conferring its own distinct benefits (8, 87). Aerobic exercise promotes an increase in maximal aerobic capacity, improves vascular function, and improves

functional outcomes such as walking speed and efficiency (8). In comparison, resistance exercise increases muscle strength and endurance, and reduces cardiac demands during simple activities such as lifting objects (8). Collectively, regular participation in both exercise modalities facilitates independence and improves the ability to perform activities of daily living. *As such, in this dissertation project I compared CE with cyclical BFRE for both aerobic and resistance training. Such an approach was adopted to facilitate translation into a rehabilitative setting and to understand how this novel form of exercise could be best integrated into the current rehabilitation guidelines.*

Summary

Based on the commonalities between exercise and RIPC in regards to the cardio- and cerebro-protection they afford, I sought to explore the potential additive benefits of both therapies through development of the cyclical BFRE paradigm. Specifically, I compared the release of protective blood-borne factors that have been associated with both exercise and RIPC when combining both interventions into cyclical BFRE. To address the potential concerns regarding an augmented exercise pressor reflex with BFRE, I also comprehensively assessed the sympathetic, hemodynamic, and cerebrovascular responses to an acute bout of RIPC, CE, and cyclical BFRE. If cyclical BFRE elicits an augmented release of the blood-borne factors that are thought to mediate the enhanced cardio- and neuro-protection demonstrated by exercise and RIPC alone, then this type of therapy could potentially be applied to clinical settings such as cardiac- and stroke rehabilitation.

SPECIFIC AIMS

Specific Aim 1: To compare the effects of cyclical BFRE, CE, and RIPC alone, on the release of circulating factors previously demonstrated to be protective in the face of ischemia-reperfusion injury.

Hypothesis 1: I hypothesized that an acute bout of cyclical BFRE (resistance or aerobic) will augment the release of circulating neuro- and cardio-protective factors compared with CE or RIPC alone.

Approach 1: To address this hypothesis, I analyzed blood samples for NO metabolites, IL-10, and delta-opioid receptor agonist activity after an acute bout of CE (aerobic and resistance), cyclical BFRE (aerobic and resistance), and RIPC alone in healthy human subjects.

Specific Aim 2: To address the concern about an amplification of the exercise pressor reflex with BFRE, the hemodynamic and cerebrovascular responses were compared between CE (aerobic and resistance), cyclical BFRE (aerobic and resistance), and RIPC.

Hypothesis 2: I hypothesized that the exercise-induced elevation in plasma norepinephrine, arterial blood pressure, and cerebral blood flow would be 1) attenuated with cyclical blood flow restriction resistance exercise, due to the use of lower workloads, and 2) augmented with blood flow restriction aerobic exercise, due to increased engagement of the exercise pressor reflex.

Approach 2: To address this hypothesis, I comprehensively assessed and compared the hemodynamic and cerebrovascular responses to an acute bout of cyclical BFRE (aerobic and resistance), CE (aerobic and resistance), and RIPC in healthy human subjects.

OVERALL EXPERIMENTAL DESIGN

A series of common experiments were conducted in healthy human subjects to address these two specific aims. All subjects were required to visit the laboratory for one familiarization session, one maximal exercise testing session, and 5 experimental sessions (7 visits in total), with at least one month between each experimental session (repeated measures, randomized block design). All experimental methods within each visit are described in detail in the subsequent chapters. Based on the data presented by Rassaf et al. (93), in which RIPC in humans induced an increase in plasma nitrite concentrations of 10 ± 4 nM (mean ± SD), a sample size of 10 healthy human subjects was required to achieve statistical power >0.8 with an α of 0.05.

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CHAPTER II

Combining Remote Ischemic Preconditioning and Aerobic Exercise: A Novel Adaptation of Blood Flow Restriction Exercise

Justin D. Sprick & Caroline A. Rickards

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ABSTRACT

Remote ischemic preconditioning (RIPC) can attenuate tissue damage sustained by ischemia-reperfusion injury. Blood flow restriction exercise (BFRE) restricts blood flow to exercising muscles. We implemented a novel approach to BFRE with cyclical bouts of blood flow restriction-reperfusion, reflecting the RIPC model. A concern about BFRE, however, is potential amplification of the exercise pressor reflex, which could be unsafe in at-risk populations. We hypothesized that cyclical BFRE would elicit greater increases in sympathetic outflow and arterial pressure than conventional exercise (CE), performed at the same relative intensity. We also assessed the cerebrovascular responses, due to potential implementation of BFRE in stroke rehabilitation. Fourteen subjects performed treadmill exercise at 65-70% HR_{max} with and without intermittent BFR (4x5-min intervals of bilateral thigh-cuff pressure followed by 5-min reperfusion periods). Mean arterial pressure (MAP), plasma norepinephrine (NE), and middle and posterior cerebral artery velocities (MCAv and PCAv) were compared between trials. As expected, BFRE elicited higher [NE] compared to CE (1249±170 vs 962±114 pg/ml; P=0.06). Unexpectedly, however, there were no differences in MAP between conditions (overall P=0.33), and MAP was 4-5 mmHg lower with BFRE vs. CE during the reperfusion periods ($P \leq 0.05$ for reperfusion periods 3 and 4). There were no differences in MCAv or PCAv between trials

($P \geq 0.22$), suggesting equivalent cerebro-metabolic demand. The exaggerated sympatho-excitatory response with BFRE was not accompanied by higher MAP, likely due to the cyclical reperfusions. This cyclical BFRE paradigm could be adapted to cardiac- or stroke-rehabilitation, where exercising patients could benefit from the cardio- and cerebro-protection associated with RIPC.

INTRODUCTION

Remote ischemic preconditioning (RIPC) is a therapeutic approach that has been developed to attenuate the damage incurred by ischemia-reperfusion injury (23, 39). Characterized by cyclical occlusions and reperfusions of a remote limb, this therapy has been used in numerous clinical trials in diverse patient populations, including patients undergoing repair of congenital heart defects, coronary artery bypass grafting, and primary percutaneous coronary intervention (6, 16, 44). As all of these clinical scenarios are associated with an elevated risk of ischemia-reperfusion injury, RIPC is applied pre-operatively to reduce the severity of this injury if and when it occurs.

Recently, a number of studies have demonstrated the efficacy of RIPC when used prophylactically in patients with significant carotid artery stenosis (30, 31). Five cycles of 5-min bilateral upper arm ischemia and reperfusion, applied twice daily over the course of 180-300 days increased cerebral blood flow and decreased the incidence of stroke in these high-risk patients (30, 31). These improvements in cerebrovascular function are consistent with other recent work demonstrating that daily RIPC therapy can also promote improvements in peripheral vascular function (i.e., flow mediated dilation and cutaneous vascular conduction) (21, 22). These documented benefits in vascular health raise the question of whether RIPC therapy could

be used as an adjunct to aerobic exercise, which also facilitates improvements in vascular function (10) and can reduce the severity of ischemic injury (9, 14).

A novel form of exercise which shares some similarities with RIPC, is known as blood flow restriction exercise (BFRE) (24, 26, 36, 42). Originally designed to promote muscular hypertrophy, this exercise modality uses restrictive bands or cuffs to reduce blood flow to the active muscles (24). Although most investigations using BFRE have focused on resistance training (26, 27), studies using aerobic exercise have also reported muscular hypertrophy (3, 42) and improved aerobic capacity (2, 36). A key concern that has recently been raised with use of BFRE, however, is the potential for an amplification of the exercise pressor reflex, due to greater stimulation of the type III (mechano-sensitive; via cuff compression) and type IV (metabo-sensitive; via cuff restriction) afferent nerves (45). While several studies have reported elevated heart rate (HR) and arterial pressure responses with aerobic BFRE compared to a control condition performed at the same absolute workload (41, 46, 47) (i.e., matching physical work), no studies to date have assessed the sympathetic responses to BFRE when HR is matched between conditions (i.e., matching physiological work). Given that exercise prescriptions for both healthy and clinical populations are typically based on a relative intensity which represents a percentage of maximal HR (HR_{max}) or HR reserve (1), this is the approach utilized in the present investigation.

The vast majority of studies of aerobic BFRE have utilized short exercise bouts (~2 min) with *continuous* limb blood flow restriction (41, 42, 46, 47), which is the traditional methodological approach for BFRE. One recent study did explore the HR, muscle deoxygenation and pulmonary responses to *intermitten*t BFRE (upright cycling), but arterial pressures and/or other measures of the exercise pressor reflex were not reported (7). Furthermore, to our

knowledge, no studies have assessed the cerebral blood flow and cerebral oxygenation responses to aerobic BFRE; assessment of these responses is necessary for potential implementation within a stroke-rehabilitation setting. Due to our interest in combining RIPC with exercise, the aim of the present investigation was to assess the sympathetic, hemodynamic, and cerebrovascular responses to a novel form of BFRE, which superimposes the RIPC-like paradigm of cyclical blood flow restriction and reperfusions with steady state aerobic exercise via treadmill walking. We hypothesized that there would be an augmentation of the exercise pressor reflex with BFRE compared to conventional exercise (CE) when performed at the same relative HR intensities.

METHODS

Subjects

Young, healthy volunteers participated in this study conducted at the University of North Texas Health Science Center (UNTHSC), in Fort Worth, TX. All experimental procedures were conducted in accordance with a protocol approved by the UNTHSC Institutional Review Board (IRB #2014-149). Prior to participation, all subjects underwent a medical history evaluation, including seated and standing 12-lead ECG and blood pressure measurements, and were cleared to participate by a physician. Subjects did not routinely use any nicotine products (including tobacco cigarettes, electronic cigarettes, chewing tobacco). Prior to each experiment, subjects abstained from caffeine, alcohol, dietary supplements, medications, and exercise for 24 hours, and fasted for at least 8 hours (overnight). Female subjects completed a urine pregnancy test to ensure they were not pregnant. All subjects underwent a familiarization session in which they were shown all equipment and experimental procedures that would be performed in the subsequent experimental sessions. Each subject gave written informed consent to participate in

this study. All subjects also participated in a resistance BFRE study reported in a companion manuscript. The RIPC protocol that was performed in both studies was identical.

Maximal Exercise Testing

All subjects underwent a 1-repetition maximum (1RM) test on a leg press machine prior to a maximal aerobic exercise test (data presented in a related manuscript). As the maximum load of the leg press machine is 184 kg, individuals who had a 1RM greater than 184 kg were excluded from participation in this study. Following a 1 hour rest period (15), peak oxygen uptake (VO_{2peak}) was assessed via a treadmill test to volitional fatigue (TMX428CP, TrackMaster, Newton, KS) in accordance with the Bruce Protocol (1). HR (wireless strap; Polar, H1 Series, Polar Electro Oy, Kempele, Finland) and expired gases were collected and analyzed via a metabolic cart (TrueOne, ParvoMedics, Sandy, UT). HR_{max} and VO_{2peak} were determined from this testing. HR_{max} was used to calculate the relative exercise intensities for the subsequent experimental conditions (65-70% of HR_{max}). In order to recruit a relatively homogeneous subject population in regards to cardiovascular fitness, only subjects with a VO_{2peak} between 30-50 ml/kg/min were included for participation in this study.

Experimental Protocols

At least 2 weeks after completion of the maximal exercise testing session, subjects reported back to the laboratory for 3 experimental sessions (randomized) separated by at least 1 month each. The three sessions were: 1) Blood Flow Restriction Aerobic Exercise (BFRE), 2) Conventional Aerobic Exercise (CE), and 3) Remote Ischemic Preconditioning (RIPC). Female subjects were tested in the early follicular phase of their menstrual cycle (first 4 days measured

by self-report) and completed a urine pregnancy test at the start of each visit to the laboratory to ensure they were not pregnant. All session were performed in the morning in a thermo-neutral laboratory (temperature = 23.2 ± 0.1^0C, humidity = 53.2 ± 2.2%, barometric pressure = 744.2 ± 0.6 mmHg).

Instrumentation

Upon arrival to the laboratory, subjects were encouraged to empty their bladder to ensure optimal comfort and to limit the potential confounding effects of increased sympathetic nervous system activation with bladder distension (11). Subjects were instrumented with a standard II lead ECG (shielded leads, cable, and amplifier, AD Instruments, Bella Vista, NSW, Australia) for measurement of R-R intervals, and calculation of HR. A separate wireless HR monitor (wireless strap; Polar, H1 Series, Polar Electro Oy, Kempele, Finland) that was interfaced with the metabolic cart was also used to verify that subjects were within their target HR range (65-70% HR_{max}) for the duration of the experiment; ECG data was used for HR analysis. Non-invasive arterial pressure and stroke volume (via the pulse contour method (19)) were measured via finger photoplethysmography (Finometer^{TM}, Finapres Medical Systems, Amsterdam, The Netherlands). This arm was placed in a sling for stability, to ensure accurate detection of the blood pressure waveform throughout the exercise bout. The Finometer was placed on the left arm for most subjects, and was on the same arm within each subject for both treadmill experiments. For some subjects, the Finometer was placed on the opposite arm for the RIPC experiment as this condition was included in the current study, and in the study reported in a companion paper. Bilateral transcranial Doppler ultrasound (ST3, Spencer Technologies, Seattle, WA) was used to measure middle cerebral artery velocity (MCAv) and posterior cerebral artery

velocity (PCAv). The MCAv and PCAv signals were always obtained on opposite sides of the head, which was variable between subjects, but the same within each subject across all experiments. Cerebral oxygen saturation of the frontal cortex (ScO_2) was measured via near-infrared spectroscopy (NIRS; OxiplexTS, ISS Inc., Chaimpaign-Urbana, IL) and was calculated as the quotient of oxygenated hemoglobin to total hemoglobin concentration (THC) multiplied by 100; THC was calculated as the sum of oxygenated hemoglobin and deoxygenated hemoglobin. Cerebral oxygenation measurements were only measured on one side of the forehead, and were always selected to be the same side as the TCD-derived MCAv measurement to ensure that regional oxygenation and perfusion were measured on the same side. A venous catheter was inserted into an antecubital vein of the arm contralateral to the blood pressure measurements for collection of venous blood samples. As previously indicated, this arm was the same side for both treadmill experiments, but sometimes the opposite arm for the RIPC experiment. During the blood flow restriction protocols (BFRE and RIPC sessions), 5 cm wide inflatable pressure cuffs (SC5^{TM}, D.E. Hokanson, Bellevue, WA) were placed around both upper thighs, secured in place with tape, and connected to an inflation system (E20 Rapid Cuff Inflation System, D.E. Hokanson, Bellevue, WA).

Blood Flow Restriction Aerobic Exercise Session (BFRE)

This session was used to determine the effects of BFRE on sympathetic, hemodynamic, and cerebrovascular responses. Following instrumentation, subjects underwent a 15-min seated baseline, after which they were moved onto the treadmill for the following 40-min exercise bout. At the start of exercise, the thigh cuffs were rapidly inflated to 220 mmHg and the treadmill speed was adjusted to a target of 4 km/h (2.5 mph). While the treadmill speed remained constant

(by design), the incline (% grade) was adjusted to achieve the target HR intensity corresponding to 65-70% of HR_{max}. Thus, the target HR intensity was maintained by adjusting the incline setting only. The cuffs remained inflated to 220 mmHg for 5-min followed by a 5-min deflation and reperfusion period. This cyclical occlusion and reperfusion protocol was repeated for 4 cycles throughout the 40-min of exercise. After the 40-min of exercise was completed, the subjects immediately commenced a 5-min active recovery period during which the treadmill speed was reduced to 2.4 km/h (1.5 mph) and the incline was reduced to 0^0. Three blood samples (10 ml) were collected throughout the protocol; 1) 5-min into baseline ("pre"); 2) at the end of the second occlusion period ("mid"), and; 3) at the end of the final reperfusion period ("post").

Conventional Aerobic Exercise Session (CE)

This session was used to compare the sympathetic, hemodynamic, and cerebrovascular responses of BFRE to CE. This protocol was performed in exactly the same manner as the BFRE session with the exception that there were no thigh cuffs applied and hence no occlusive stimulus. Exercise intensity was set at 65-70% of HR_{max}, achieved with a treadmill speed of 4 km/h (2.5 mph) and variable incline, as previously described. Blood samples were collected at the same time points as the BFRE condition (pre, mid, and post).

Remote Ischemic Preconditioning Session (RIPC)

This session served as a control condition to isolate the effects of the repeated cuff occlusions and reperfusions, independent of exercise. Following instrumentation, subjects completed a 15-min seated baseline. The thigh cuffs were then rapidly inflated to 220 mmHg for 5-min, followed by rapid deflation and reperfusion for 5-min. This occlusion/reperfusion

protocol was repeated 4 times over 40-min, followed by a 5-min recovery period. Blood samples were collected at the same time points as the exercise conditions (pre, mid, and post).

Data Analysis

All continuous waveform data (ECG, arterial pressure, stroke volume, MCAv, PCAv, ScO_2, THC, $etCO_2$) were recorded at 1000 Hz (PowerLab/Labchart, AD Instruments, Bella Vista, NSW, Australia) and analyzed offline via specialized software (WinCPRS, Absolute Aliens, Turku, Finland). R-wave detection was performed on the ECG signal, and used to determine the timing of each cardiac cycle. MAP, mean MCAv, and mean PCAv were calculated as the area under the curve from the arterial pressure and cerebral blood velocity waveforms. Cardiac output was calculated as HR multiplied by stroke volume, and total peripheral resistance (TPR) was subsequently calculated as MAP divided by cardiac output. For the baseline period, minutes 5-10 were averaged. For the 40-min of exercise, all hemodynamic variables were averaged for each 5-min period; these time intervals corresponded to each occlusion and reperfusion period, and recovery. The data were evaluated in this way so that each occlusion and reperfusion period could be analyzed independently, and compared with the matching time point during the CE condition with no occlusive stimulus, and with the RIPC condition without the exercise stimulus.

Whole blood was collected in EDTA tubes treated with glutathione as a preservative (1.23 mg glutathione/1 ml whole blood) and centrifuged at 1500 RPM for 15-min at 4^0C. Plasma was separated and snap-frozen in liquid nitrogen, then stored at -80^0C until analyzed. Norepinephrine (NE) was measured in duplicate via enzyme linked immunosorbent assay (BA E-6200, Rocky Mountain Diagnostics, Colorado Springs, CO). Only duplicate samples with a

coefficient of variation less than 15% were included in the final analysis. As a result, N=9 for NE data. Hematocrit was also assessed from the "pre" blood sample to ensure equivalent hydration status between conditions.

Statistics

Two-way repeated measures ANOVAs (factor 1: time, factor 2: condition - BFRE, CE, RIPC) were used to compare the effects within and between each condition over time. A one-way (condition only) repeated measures ANOVA was used to compare baseline hematocrit between conditions. Tukey post-hoc tests were performed when a significant interaction was indicated by the ANOVAs. For comparison of treadmill incline, a paired t-test was performed between the BFRE and CE conditions. Exact P-values are reported for all comparisons. Unless otherwise stated, all data are presented as mean ± standard error (SE).

RESULTS

Twenty-three subjects were recruited to participate in this study. Of these 23 subjects, 2 were excluded due to VO_{2peak} <30 ml/kg/min, 3 were excluded due to a 1RM >184 kg, 2 were excluded due to medication use, and 2 withdrew due to scheduling or personal reasons. As a result, 14 subjects completed all three experimental conditions (8M/6F, age 28 ± 2 years, height 170 ± 3 cm, weight 71 ± 3 kg, BMI 24.5 ± 0.7 kg/m^2). The average HR_{max} was 189 ± 2 bpm, and the average VO_{2peak} was 35.8 ± 1.8 ml/kg/min. There were no differences in baseline hematocrit between the three conditions (P=0.68). During the two exercise trials (CE and BFRE), all subjects achieved the target HR intensity of 65-70% HR_{max}, and were able to maintain this intensity throughout the entire 40-min of exercise (figure 1A). In one case, the treadmill speed was adjusted below 4 km/h during the occlusion periods to maintain the subject's HR within the

target range. For one other subject, the treadmill incline was not recorded due to a technical failure. As a result, N=13 for treadmill incline. While the treadmill speed remained constant at 4 km/h (by design), subjects were able to reach this target HR intensity at a lower treadmill incline during BFRE compared to CE (7.3 ± 0.3^0 for CE vs. 6.0 ± 0.3^0 for BFRE, $P<0.001$).

Stroke volume and cardiac output increased with exercise for both CE and BFRE ($P<0.001$), and remained elevated above baseline throughout the exercise periods (figure 1B&C). While cardiac output remained elevated above baseline throughout the exercise bouts ($P<0.001$), stroke volume progressively fell throughout the exercise bout for both CE and BFRE ($P\leq0.03$ from 25-min into exercise vs. onset of exercise). While there was no change in cardiac output throughout the RIPC session ($P\geq0.99$), stroke volume fell below baseline values during the final 3 occlusion periods ($P\leq0.07$), suggesting a decreased venous return with limb occlusion. TPR decreased with exercise for both CE and BFRE ($P<0.001$), and while there were no differences between CE and BFRE ($P=0.73$), TPR was lower for both exercise conditions compared to RIPC throughout the intervention ($P\leq0.001$) (figure 1D).

In support of our hypothesis, plasma [NE] increased with exercise ($P<0.001$ for both BFRE and CE) and was higher with BFRE compared to CE at the "post" time point ($P=0.06$; figure 2A). [NE] did not increase from baseline with RIPC ($P\geq0.91$). MAP increased with exercise for both CE and BFRE ($P<0.001$), and was higher with BFRE during the first occlusion period compared to CE ($P=0.08$) (figure 2B). MAP remained elevated with CE, with no differences between minute 5 and 40 of exercise ($P\leq0.18$ vs. 5-min time point). In contrast, MAP progressively fell with BFRE throughout the exercise period ($P\leq0.02$ vs. 5-min time point), and was lower than CE during the 3rd and 4th reperfusion periods ($P\leq0.05$). Interestingly, MAP also increased progressively with RIPC ($P=0.04$ vs. baseline by the final reperfusion period), reaching

values equivalent to the BFRE condition by the 3rd reperfusion period (P≥0.46). Based on the observation that BFRE elicited a lower MAP response than CE during a number of the reperfusion periods when all 3 conditions were included in the ANOVA (BFRE, CE, RIPC), a separate 2-way ANOVA was conducted between CE and BFRE only to isolate the MAP responses to the two exercise conditions independent of RIPC. This analysis revealed that during BFRE, MAP was still initially higher during the first occlusion period compared with CE (P=0.03), but then was lower during all 4 reperfusion periods (P≤0.05) and during recovery (P=0.07).

Mean MCAv increased from baseline with the onset exercise (P<0.001), then progressively fell throughout exercise for both CE and BFRE (P<0.001 from 15-min into exercise vs. onset of exercise), with no differences between these two conditions (P=0.99) (figure 3A). For mean PCAv, N=7 due to the inherent difficulties of acquiring and maintaining the PCA signal throughout each of the three experimental sessions. Mean PCAv also increased from baseline with the onset of exercise (P<0.001) and remained elevated throughout the exercise bout for CE (P≥0.42 vs. onset of exercise), but decreased progressively towards baseline with BFRE (P≥0.09 vs. baseline by occlusion 3). Overall, there were no differences in mean PCAv between the two exercise conditions (P=0.22). Unexpectedly, mean PCAv was slightly higher at baseline with BFRE compared to RIPC (P=0.02), but not between CE and RIPC, or between BFRE and CE (both P≥0.25). End tidal CO_2 increased with the onset exercise for both CE and BFRE (P≤0.001) and remained higher during both exercise conditions compared to RIPC throughout the intervention (P≤0.03) (figure 3C). There was an overall time by condition effect (P=0.05) for ScO_2, and this appears to be primarily driven by the exercise conditions (P=0.07 for BFRE vs. RIPC; P=0.10 for CE vs. RIPC), with no difference between BFRE and CE (P=0.98) (figure 3D).

DISCUSSION

In the current investigation we assessed the sympathetic, hemodynamic, and cerebrovascular responses to an acute bout of steady state aerobic BFRE with cyclical occlusions and reperfusions, reflecting the RIPC paradigm. The major findings were: 1) in support of our hypothesis, we observed an increase in sympathetic activity with BFRE, evidenced by greater plasma [NE]; 2) despite this increased sympathetic drive, arterial pressures were lower with the BFRE condition compared to CE; 3) although HR was matched between conditions, the target HR intensity was achieved with a lower treadmill incline under the BFRE condition; and, 4) there were no differences in cerebral blood flow or cerebral tissue oxygenation responses between the CE and BFRE conditions.

The greater sympathetic drive exhibited with BFRE supports the notion that there is indeed augmented activation of the exercise pressor reflex with BFRE. Despite this, however, MAP was actually lower with BFRE compared to CE during all 4 reperfusion periods and during recovery. The reasons for this are likely related to 1) the cyclical nature of the occlusions and reperfusions that are unique to this model, combined with 2) a reduction in venous return resulting from the occlusions. With each reperfusion, the reactive hyperemia that results from rapid release of thigh cuff pressure would potentiate the exercise-induced functional sympatholysis at the level of the active muscles. As both exercise and reactive hyperemia independently cause the release of vasoactive mediators, the combination of these two stimuli could potentially result in an augmented vasodilation. In support of this viewpoint, Horiuchi et al. demonstrated that sympathetically mediated vasoconstriction elicited via a cold pressor test, was further attenuated during moderate-intensity handgrip when this exercise bout was preceded by ischemic preconditioning of the muscle bed (17). In a related study, Mulliri et al. also found

that ischemic preconditioning induced via arm cuff inflation can reduce the magnitude of the metaboreflex-mediated increase in arterial pressure during post-exercise muscle ischemia which was applied after 3-min of rhythmic handgrip (34). While the occlusive stimulus was applied before, rather than during the exercise bouts in these studies, it is certainly plausible that the attenuated arterial pressure response observed with BFRE in the present investigation was due to the superimposition of the RIPC model with exercise, resulting in an enhanced local vasodilation with each reperfusion period. Unfortunately, our only measure of vascular resistance in the current investigation is TPR, so we are unable to determine if local vasodilation of the active muscle beds was enhanced during each reperfusion period; this phenomenon certainly requires further investigation.

While stroke volume was not different between BFRE and CE, the decreased stroke volume observed during occlusion only with the RIPC condition suggests that the restrictive stimulus of the cuffs was sufficient to decrease venous return. This decrease in stroke volume with cuff inflation was offset by the addition of exercise during the BFRE condition, however, most likely due to engagement of the skeletal muscle pump. Although partial restriction of venous return with cuff inflation might still be occurring with BFRE, it is possible that the increased sympathetic activity we observed with BFRE (reflected in the NE response) could increase cardiac contractility (12), subsequently increasing stroke volume and compensating for any decreases in venous return induced by cuff inflation. Several investigations have reported lower stroke volume with BFRE compared to a control condition where subjects exercised at the same absolute workload (41,46,47). In these studies, however, continuous vascular occlusion was used which may elicit a greater restriction of venous return, compared with the cyclical occlusion-reperfusion paradigm used in our investigation.

To our knowledge, this is the first investigation to superimpose an RIPC-like paradigm of 4 x 5-min cycles of limb blood flow restriction/reperfusion with steady state aerobic exercise. This is in contrast to most BFRE protocols which use a continuous occlusive stimulus throughout the exercise bout (41,42,46,47). One recent study did utilize an *intermittent* BFRE protocol (7), but the duration and frequency of the occlusive stimulus was quite different to traditional RIPC protocols (10 x 2-min bouts of exercise with occlusion, each separated by 1-min of active recovery without occlusion). Furthermore, the increased muscle deoxygenation and metabolic strain reported with intermittent BFRE in that study are not directly related to the sympatho-excitatory responses explored in the current investigation. In regards to aerobic BFRE with *continuous* occlusion, several studies have demonstrated that treadmill walking with blood flow restriction resulted in a greater increase in MAP compared to a non-occlusive control condition performed at the same treadmill speed (3.2-4 km/h) – i.e., the same absolute workload (41, 46, 47). Not surprisingly, HR was consistently higher with BFRE compared to the control condition, i.e., subjects were exercising at a higher *relative* intensity with BFRE, due to constant stimulation of the exercise pressor reflex with a continuous occlusive stimulus. In the present investigation, HR was matched between trials to ensure that the same relative intensity was achieved in both conditions. As exercise prescriptions are typically based on a percentage of HR_{max} or HR reserve, we opted to match HR between conditions to be consistent with current recommendations (1).

It is noteworthy that although HR was matched between conditions, the treadmill incline required to elicit the target HR response was lower in the BFRE condition compared to CE (lower absolute workload). We speculate that this response stems from augmented activation of the exercise pressor reflex with the occlusive stimulus, eliciting an elevation in HR.

Consequently, in order to maintain HR within the predetermined target range (i.e. 65-70% HR_{max}), the treadmill incline had to be reduced in the BFRE condition. The lower treadmill incline we observed with aerobic BFRE is analogous to the lower training intensities traditionally utilized in blood flow restriction resistance exercise (20% of 1RM rather than 65% of 1RM) (24). This finding could have implications for application of BFRE to the rehabilitation setting, where individuals may benefit from the lower mechanical stress associated with a decreased treadmill incline, particularly elderly individuals and/or patients with musculo-skeletal injuries. If the same benefits known to be associated with conventional aerobic exercise are also apparent with cyclical BFRE performed at a lower absolute intensity, this would make this novel adaptation of BFRE especially appealing.

To our knowledge, this is also the first investigation to assess cerebral blood flow and cerebral oxygenation responses to aerobic BFRE. These assessments are important given that this exercise modality could potentially be applied to cerebrovascular (stroke) rehabilitation, where an augmented activation of the exercise pressor reflex could cause an undesirable increase in cerebral perfusion pressure. Cerebral blood flow responses to conventional aerobic exercise are well characterized (35). Generally, cerebral blood flow increases with increasing exercise intensity up to ~60% of VO_{2peak}, in parallel with increases in metabolically-derived arterial CO_2 (33, 35). As exercise intensity increases above 60% of VO_{2peak}, hyperventilation-induced reductions in arterial CO_2 cause cerebral vasoconstriction and a subsequent reduction in cerebral blood flow (35). In the present investigation, we observed cerebral blood flow responses in the anterior (indexed by mean MCAv) and posterior (indexed by mean PCAv) circulations that are consistent with this model, as these responses also tracked changes in $etCO_2$ (used as a surrogate for arterial CO_2). Metabolic production of CO_2 combined with increases in MAP resulted in an

initial increase in cerebral blood flow with exercise which was then followed by a progressive decrease due to hyperventilation induced hypocapnia. This response was expected as the exercise intensities utilized in this study are above 60% of VO_{2peak}, the level of exercise previously reported to cause a hypocapnia-induced cerebral vasoconstriction (35). While MAP was lower at multiple time points during the BFRE condition compared with CE, this pattern of response was not reflected in cerebral blood flow. Since we recruited only young, healthy subjects in the present study, the relative stability of cerebral blood flow despite variations in arterial pressure is likely due to intact cerebral autoregulation (49). Future studies are required, however, to determine if this stability of cerebral blood flow is still maintained in clinical populations such as stroke patients, who often exhibit impairments in cerebral autoregulation (5).

We also observed similar frontal lobe oxygen saturation (ScO_2) responses between the two exercise conditions. As the cerebral NIRS signal is understood to primarily be indicative of oxygen saturation of the venous blood rather than tissue (i.e. prefrontal cortex) oxygen saturation (29), alterations in ScO_2 can be suggestive of changes in cerebral oxygen extraction. That is, a decrease in ScO_2 would indicate either a reduction in O_2 supply (i.e., cerebral blood flow), and/or an increase in O_2 demand (i.e. cerebral oxygen consumption). By coupling this metric with simultaneous measurements of cerebral blood flow (via velocity), the balance of cerebral oxygen supply and demand can be assessed. As there were no differences in cerebral blood flow and ScO_2 responses between exercise conditions, we interpret these outcomes to suggest an equivalent cerebro-metabolic demand. As it has been suggested that the exercise-induced increases in cerebral blood flow are a key feature underlying the enhancements in cerebrovascular function associated with exercise (28), these findings have promising implications for application of BFRE to stroke rehabilitation, where patients may benefit from

the lower arterial pressures and equivalent cerebrovascular responses compared to conventional exercise paradigms.

There are several methodological considerations that need to be mentioned as they relate to interpretation of these findings. First, assessment of cerebral blood flow via transcranial Doppler ultrasound relies on the assumption that the diameter of the insonated artery remains constant. Recent studies using high resolution magnetic resonance imaging, however, have demonstrated that periods of pronounced hyper- (+9 mmHg) or hypo-capnia (-13 mmHg) can induce diameter changes in the MCA (8, 48). Since the end-tidal CO_2 responses observed in the present study are much lower in magnitude (approx. +5 mmHg) than those reported in these aforementioned studies, changes in diameter are not likely. Furthermore, even if vessel diameters were increasing in response to elevations in arterial CO_2, the reported MCAv measurements would actually be underestimating rather than overestimating changes in cerebral blood flow. While the effects of changes in arterial CO_2 on PCA diameter are still unknown, the observation that there were no differences in cerebral blood velocity between the two exercise conditions, diminishes the potential confounds of this limitation.

Second, our NIRS-derived cerebral oxygenation data is limited as we are only able to assess oxygen saturation of the frontal lobe. While more invasive techniques could provide an evaluation of overall cerebral oxygen consumption (i.e., performing arterial-venous blood sampling across the brain), we have minimized the potential confounds of this limitation by simultaneously measuring cerebral blood velocity within the same brain region being assessed by NIRS (via MCAv). Another possible confounding factor related to the cerebral NIRS measurement is the potential for contamination from increases in skin blood flow, which would be expected during exercise (32). However, by using a spatially resolved NIRS sensor consisting

of four emitters placed at varying distances from the detector, extraneous measurements from extracranial sample volume (i.e., skin, muscle, fat) can be removed from the NIRS signal.

Third, the measurement of plasma NE as an index of overall sympathetic drive does not represent the total NE that is released from post-synaptic neurons. While performing direct measurements of muscle sympathetic nerve activity (MSNA) would be ideal, the repeated measures design of this study combined with the inherent difficulty of maintaining MSNA signals throughout 45-min of upright treadmill exercise necessitated assessment of sympathetic activity via plasma NE. Additionally, it is generally accepted that venous NE is an appropriate marker of overall sympathetic activity (13), particularly during the steady state exercise protocol employed in this investigation. Furthermore, we cannot rule out the potential role of pain and/or discomfort from the occlusive cuffs on sympathetic outflow, independent of the exercise protocols. Previous work has demonstrated that RIPC can induce pain with different cuff pressures (43), which could be manifest in an increase in circulating catecholamines. While we did not directly quantify pain in our investigation, subjects did not indicate that inflation of the cuffs was painful per se (although some indicated tolerable discomfort), and no experiments were terminated due to pain.

Fourth, while the one-month intervening period between experiments was necessary to control for menstrual cycle phase in our female subjects, we acknowledge that this prolonged length of time could have increased the variability of responses. However, as the order of experiments was randomized, and the key findings were statistically robust, this extended duration of participation did not appear to influence the outcomes of this study.

Fifth, the RIPC session was conducted with the subjects in the upright, seated position, while the two exercise conditions were performed with the subjects in the upright standing

position. These postural differences could have influenced hemodynamic and sympathetic responses, independent of the interventions. As the RIPC session was 1 hour in duration, however, we opted to keep the subjects in the seated position to ensure that they were as relaxed and comfortable as possible. This posture was a compromise between completely supine and upright. Having subjects in the upright posture for this length of time would very likely have increased sympathetic nervous system activity, and engagement of the muscle pump, both potential confounding factors that we were aiming to avoid.

Finally, the variability in cuff sizes and occlusive pressures used between BFRE investigations makes it difficult to compare outcomes across studies. Other investigations have used cuffs ranging from 3 cm to 18 cm in width (7, 37, 46, 50), while others do not report the width of the cuff at all (41, 47). Several studies, including the present investigation use a standardized pre-determined occlusive pressure across all participants (4, 47), while others use very individualized approaches to determine the target pressures for each subject, including consideration of limb circumference, limb adiposity, and arterial pressure (7, 46). As differences in cuff width (20), cuff pressure (25), as well as the individual physical and physiological characteristics of each subject (18) can all have a profound influence on the degree of arterial occlusion achieved, these variables should be considered when comparing findings across studies. As this was the first investigation to superimpose the cyclical RIPC-like paradigm with steady state aerobic exercise, we opted to use the same absolute cuff pressure for each subject, which was selected based on pressures commonly used in RIPC studies (16, 21, 22) and those reported within the BFRE literature (2-4). It is important to note, however, that most RIPC studies use a cuff pressure and limb (usually the upper arm) that would result in complete arterial occlusion, which is in contrast to the partial blood flow restriction used in our study (with

cuffs around the upper thighs). As highlighted by Spranger et al. (45), differences in cuff width would likely have a dramatic effect on the activation of the exercise pressor reflex, with wider cuffs resulting in a greater activation. These considerations highlight the need for future studies to identify the optimal cuff widths and pressures and limb/s that should be utilized in this RIPC-like cyclical BFRE paradigm.

PERSPECTIVES AND SIGNIFICANCE

In this investigation we utilized a novel BFRE paradigm which superimposes the cyclical RIPC model of 4 cycles of 5-min occlusion and 5-min reperfusion with steady state aerobic exercise at a relative intensity of 65-70% of HR_{max}. While this model resulted in increased sympathetic activity compared to conventional exercise, this response was not accompanied by higher arterial pressure, potentially due to the cyclical nature of the occlusions and reperfusions that is unique to this model. Furthermore, although HR was matched between conditions, this target was reached with a lower treadmill incline during the BFRE condition, and with an equivalent cerebro-metabolic demand. A key question that remains to be answered is whether the benefits associated with RIPC can also be achieved through the use of this novel exercise paradigm. A recent review by Quindry is promising in this regard, highlighting numerous similarities in the signal transduction pathways underlying cardio-protection facilitated by both exercise and RIPC, including K_{ATP} channels, endogenous opioids, and circulating cytokines (40). Furthermore, as repeated RIPC and exercise training can both independently promote improvements in vascular health (21, 22) and reduce the damage incurred by ischemia-reperfusion injury (38), future studies are necessary to explore the possibility that these benefits could be additive within the context of this novel BFRE paradigm. Moreover, as the majority of

RIPC studies utilize a restrictive stimulus that most likely results in complete restriction of blood flow, future work should also explore whether complete cessation of blood flow is required to elicit the protective effect, or if only partial restriction (as was used in this investigation), can still result in cardio- and cerebro-protective effects, in combination with, or independently of exercise.

This model of exercise could potentially be explored in clinical settings such as cardiac- and stroke rehabilitation, where patients are already exercising, and could also benefit from the positive responses associated with RIPC, including reduced tissue damage from ischemia-reperfusion injury; future longitudinal studies are required, however, before this application could be made.

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FIGURES

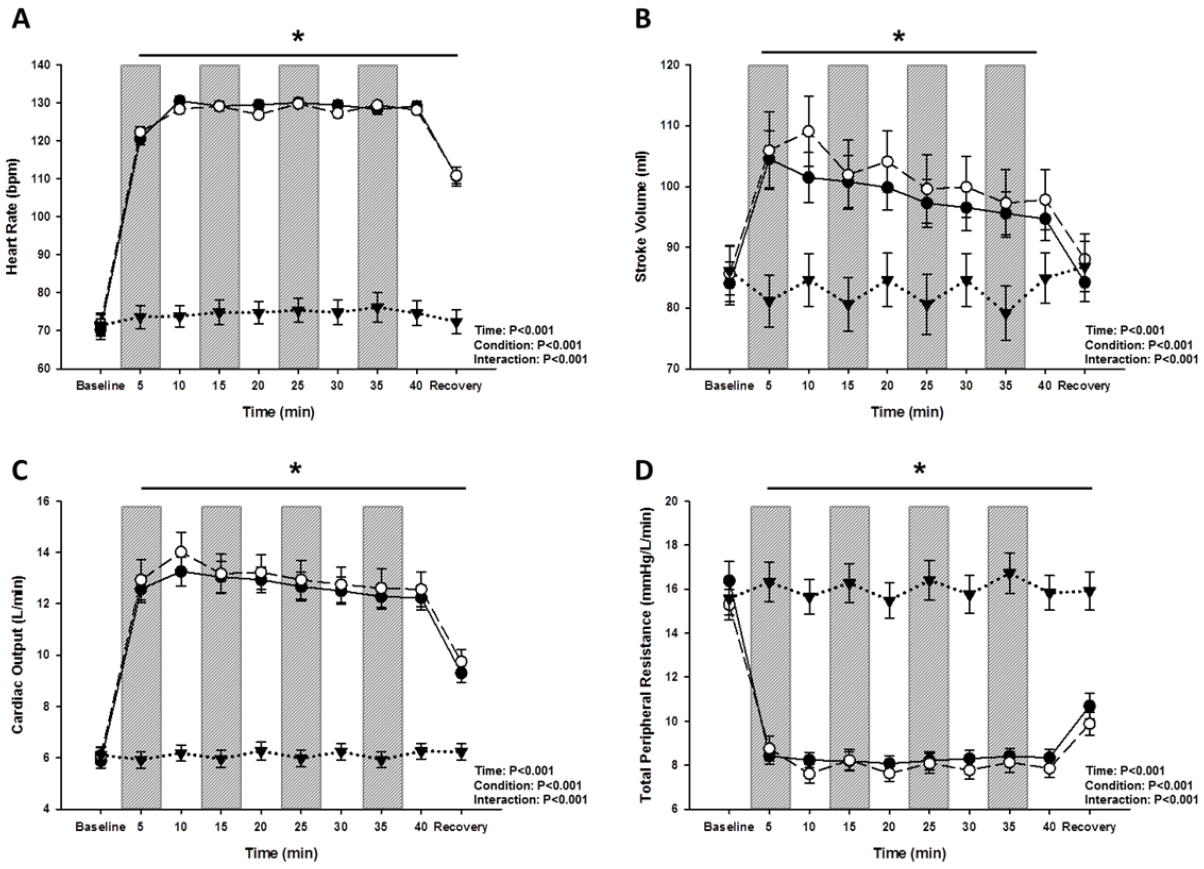

Figure 1: Hemodynamic responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Occlusion periods denoted by vertical grey bars. Heart rate (Panel A) increased with exercise and was higher with CE and BFRE compared to RIPC throughout the intervention ($P<0.001$) with no differences between CE and BFRE ($P\geq0.55$). Stroke volume (Panel B) increased with exercise for both CE and BFRE ($P<0.001$) and was higher than RIPC throughout the intervention ($P<0.001$). Cardiac output (Panel C) increased with exercise ($P<0.001$) and was higher than RIPC throughout the intervention ($P<0.001$). Total peripheral resistance (Panel D) decreased with exercise and was lower compared to RIPC throughout the intervention ($P<0.001$). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *, $P\leq0.03$ for CE and BFRE vs RIPC.

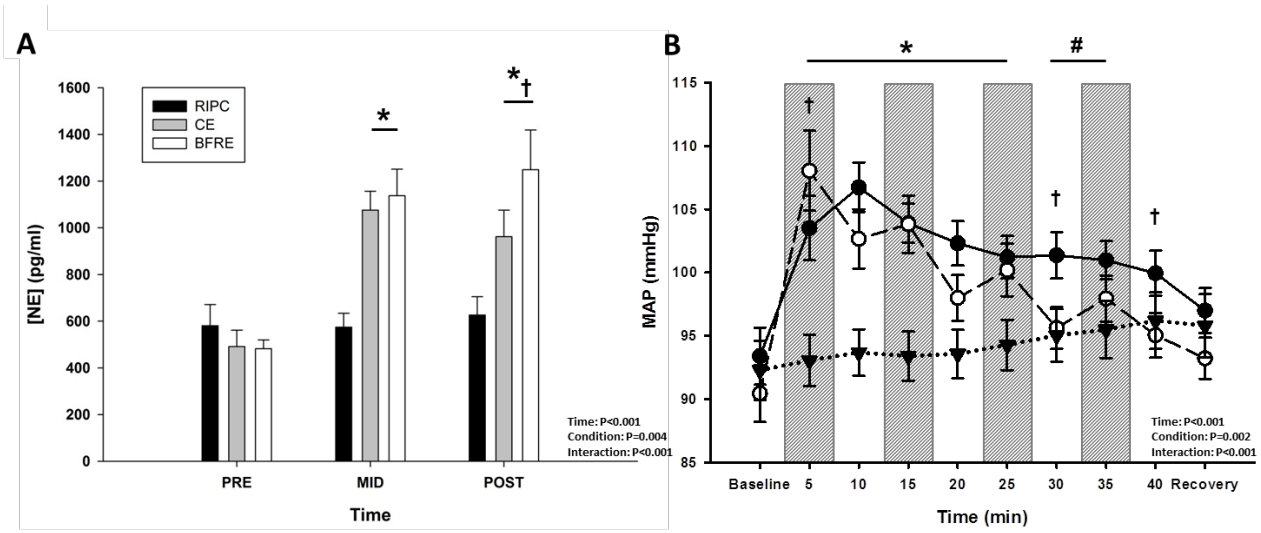

Figure 2: Plasma Norepinephrine ([NE]; Panel A, N=9) responses to remote ischemic preconditioning (RIPC, black bar), conventional exercise (CE, grey bar), and blood flow restriction exercise (BFRE, white bar). [NE] increased with exercise (P≤0.001 for both BFRE and CE) and was higher with BFRE compared to CE and RIPC at the "post" time point (P≤0.06).*, P≤0.03 for CE and BFRE vs RIPC within time point; †, P=0.06 for CE vs BFRE within time point. Mean arterial pressure (MAP; Panel B) responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Occlusion periods denoted by vertical grey bars. MAP increased with exercise for both CE and BFRE (P≤0.005). MAP remained elevated for CE (P≥0.18 vs. exercise onset) but fell progressively with BFRE throughout the intervention (P≤0.02 vs. exercise onset). MAP increased with RIPC, with no difference between BFRE and RIPC by the 3rd reperfusion period (P≥0.46). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *, P≤0.09 for CE and BFRE vs RIPC; #, P≤0.04 for CE vs RIPC; †, P≤0.08 for CE vs BFRE.

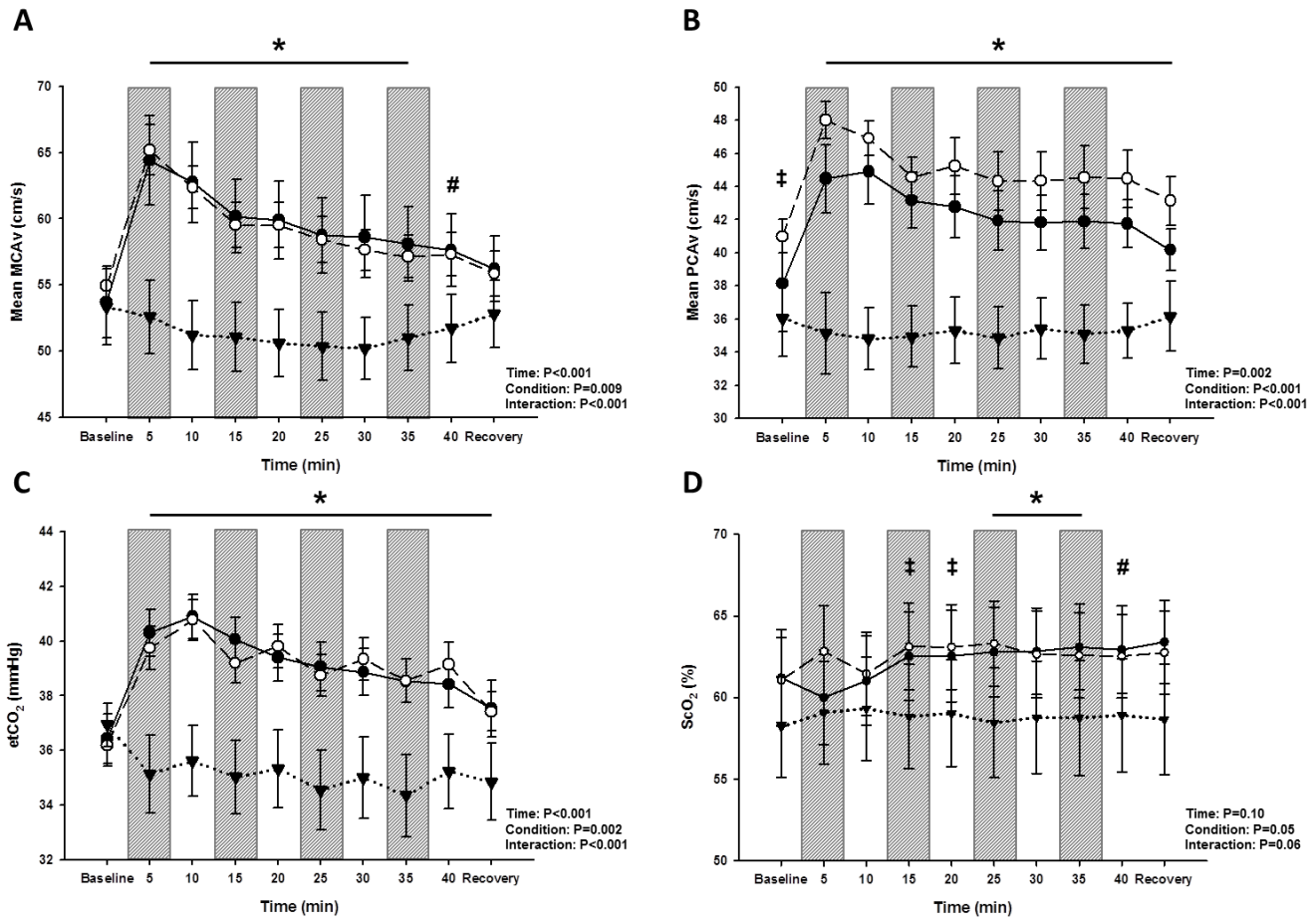

Figure 3: Regional cerebral blood velocity, cerebral oxygenation, and end-tidal CO_2 responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Occlusion periods denoted by vertical grey bars. Mean middle cerebral artery velocity (MCAv; Panel A), mean posterior cerebral artery velocity (PCAv; Panel B, N=7), and end tidal CO_2 ($etCO_2$; Panel C) increased over time with exercise ($P<0.001$) and were higher with CE and BFRE compared to RIPC ($P<0.02$). There was an overall condition effect ($P=0.05$) for frontal lobe cerebral oxygen saturation (ScO_2; Panel D, N=12); ScO_2 was higher for CE and BFRE than RIPC at several time points ($P\leq0.09$). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *, $P\leq0.09$ for CE and BFRE vs RIPC; #, $P\leq0.08$ for CE vs RIPC; ‡, $P\leq 0.07$ for BFRE vs RIPC.

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CHAPTER III

Cyclical Blood Flow Restriction Resistance Exercise: A Potential Parallel to Remote Ischemic Preconditioning?

Justin D. Sprick & Caroline A. Rickards

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ABSTRACT

Remote ischemic preconditioning (RIPC) is characterized by the cyclical application of limb blood flow restriction and reperfusion, and has been shown to protect vital organs during a subsequent ischemic insult. Blood flow restriction exercise (BFRE) similarly combines bouts of blood flow restriction with low-intensity exercise and thus could potentially emulate the protection demonstrated by RIPC. One concern with BFRE, however, is the potential for an augmented rise in sympathetic outflow, due to greater activation of the exercise pressor reflex. Due to the use of lower workloads, however, we hypothesized that BFRE would elicit an attenuated increase in sympathetic outflow (assessed via plasma norepinephrine (NE) and mean arterial pressure (MAP)), and middle cerebral artery velocity (MCAv) when compared with conventional exercise (CE). Fifteen subjects underwent two leg-press exercise interventions: 1.BFRE-220 mmHg bilateral thigh occlusion at 20% 1 rep-max (1RM), and; 2.CE-65% 1RM without occlusion. Each condition consisted of 4 x 5-min cycles of exercise, with 3 x 10-reps in each cycle. 5-min of rest and reperfusion (for BFRE) followed each cycle. MAP increased with exercise ($P<0.001$), and was 4-5 mmHg higher with CE vs. BFRE ($P\leq0.09$). Mean MCAv also increased with exercise ($P<0.001$) and was higher with CE compared to BFRE during the first bout of exercise only ($P=0.07$). Plasma [NE] increased with CE only ($P<0.001$), and was higher than BFRE throughout exercise ($P\leq0.02$). The attenuated sympathetic response combined with

similar cerebrovascular responses suggest that cyclical BFRE could be explored as an alternative to CE in the clinical setting.

INTRODUCTION

Ischemic preconditioning is a therapy that has demonstrated utility in protecting vital organs in the face of ischemia-reperfusion (IR) injury. First described by Murray et al. in 1986 (43), this technique was characterized by brief cycles of sub-lethal ischemia applied to a coronary artery, which then protected the heart from subsequent IR injury. Consequently, a number of investigators reported that this cyclical application of sub-lethal ischemia and reperfusion could also be administered to a "remote" limb, rather than a coronary vessel, and still provide cardio-protection within this IR injury setting (23, 49). This phenomenon of preconditioning from a distance became known as "remote ischemic preconditioning" (RIPC) and served as the foundation for the clinical trials that followed (5, 16).

Since this initial characterization, use of RIPC has expanded into multiple avenues of clinical investigation in patient populations. These applications include coronary artery bypass graft surgery (13, 16), aortic valve surgery (45, 57), and percutaneous coronary intervention (2, 9, 53). All of these applications have a common limitation, however - the IR injury is known or planned prior to initiation of the preconditioning stimulus, thus potentially limiting the use of RIPC in clinical practice. One potential solution to this constraint is to incorporate RIPC into current therapies utilized by patients at high risk of ischemic injury. Recently, two clinical trials have demonstrated that daily RIPC can be used prophylactically to decrease the incidence of stroke and increase cerebral blood flow in patients with significant carotid artery stenosis (37, 38); this is a novel implementation of RIPC that warrants further exploration. Another important target population for such an intervention would be individuals undergoing cardiac or stroke

rehabilitation. Exercise, including resistance exercise, is a cornerstone of rehabilitation for cardiac (6, 22, 35) and stroke patients (3, 17, 56), and thus would be an ideal target for implementation of an RIPC-like stimulus.

Blood flow restriction exercise (BFRE) is a unique exercise paradigm that is characterized by limiting blood flow to the working muscles by the use of a restrictive cuff (30). This technique was originally developed to augment the muscle hypertrophic response to resistance exercise (30). A hallmark of resistance BFRE is the use of much lighter workloads than typically prescribed - intensities of 20-30% of 1 repetition maximum (1RM) (30), compared with 65-70% 1RM for conventional resistance exercise (1). Accordingly, BFRE has been adapted to numerous special populations who can benefit from the use of these lighter workloads, including the elderly (21, 25, 52, 59), patients at risk for osteoarthritis (51), and individuals with ischemic heart disease (31). Another potential application of BFRE that has yet to be explored is stroke rehabilitation; to our knowledge, the cerebrovascular responses to this novel mode of exercise training have not been comprehensively assessed. Traditionally, the occlusive stimulus used during BFRE is applied continuously, during both the exercise and rest phases. Due to the parallels between BFRE and RIPC, this form of exercise training could potentially emulate the protection facilitated by RIPC, if performed in the same manner, with cyclical blood flow restriction and reperfusions; this is the approach utilized in the present investigation. Due to stimulation of type III (mechano-sensitive; via cuff compression) and type IV (metabo-sensitive; via cuff restriction) afferent nerves with application of the restrictive stimulus, however, there is some concern that BFRE could result in greater activation of the exercise pressor reflex, leading to an unsafe rise in arterial pressure (54). Conversely, due to the use of lower workloads, and the cyclical nature of the occlusive stimulus used in the present

study, we hypothesized that BFRE would elicit an attenuated increase in sympathetic outflow, manifest in a blunted rise in plasma catecholamines and arterial pressure.

METHODS

Subjects

Young healthy volunteers participated in this study conducted at the University of North Texas Health Science Center (UNTHSC), in Fort Worth, TX. All experimental procedures were conducted in accordance with a protocol approved by the UNTHSC Institutional Review Board (IRB #2014-149). Prior to participation, all subjects underwent a medical history evaluation, including seated and standing 12-lead ECG and blood pressure measurements, and were cleared to participate by a physician. Subjects did not routinely use any nicotine products (including tobacco cigarettes, electronic cigarettes, chewing tobacco). Prior to each experiment, subjects abstained from caffeine, alcohol, dietary supplements, medications, and exercise for 24 hours, and fasted for at least 8 hours (overnight). Female subjects completed a urine pregnancy test to ensure they were not pregnant. All subjects underwent a familiarization session in which they were shown all equipment and experimental procedures that would be performed in the subsequent experimental sessions. Each subject gave written informed consent to participate in this study. Fourteen of these subjects also participated in an aerobic BFRE study reported in a companion manuscript. The RIPC protocol that was performed in both studies was identical.

Maximal Exercise Testing

All subjects underwent a 1RM test on a leg press machine (VR3, Cybex, Medway, MA) in order to determine the load required for the submaximal intensities used in the subsequent

experimental sessions. As the maximum load of this leg press machine is 184 kg, individuals who had a 1RM greater than 184 kg were excluded from participation in this study. Following a 1-hour rest period, peak oxygen uptake (VO_{2peak}) was assessed on a treadmill (TMX428CP, TrackMaster, Newton, KS) in accordance with the Bruce Protocol (1). Testing was terminated when subjects reached volitional fatigue. Expired gases were collected and analyzed via a metabolic cart (TrueOne, ParvoMedics, Sandy, UT). To ensure a relatively homogenous subject pool in regards to cardiorespiratory fitness, only subjects with VO_{2peak} values between 30-50 ml/kg/min were included for participation in this study.

Experimental Protocols

At least 2 weeks after completion of the maximal exercise testing session subjects reported back to the laboratory for 3 experimental sessions (randomized) separated by at least 1 month each. The three sessions were: 1) Blood Flow Restriction Resistance Exercise (BFRE), 2) Conventional Resistance Exercise (CE) and, 3) Remote Ischemic Preconditioning (RIPC). Female subjects were tested in the early follicular phase of their menstrual cycle (first 4 days determined by self-report), and completed a urine pregnancy test at the start of each visit to the laboratory to ensure they were not pregnant. All sessions were performed in the morning in a thermo-neutral laboratory (temperature = 23.1 ± 0.1^{0}C, humidity = 49.6 ± 2.4%, barometric pressure = 744.0 ± 0.9 mmHg).

Instrumentation

Upon arrival to the laboratory, subjects were encouraged to empty their bladder to ensure optimal comfort, and to limit the potential confounding effects of increased sympathetic nervous

system activation with bladder distension (11). Subjects were instrumented with a standard lead II ECG (shielded leads, cable, and amplifier, AD Instruments, Bella Vista, NSW, Australia) for measurement of R-R intervals, and calculation of heart rate (HR). Non-invasive arterial pressure and stroke volume (via the pulse contour method (18)) were measured via finger photoplethysmography (Finometer^{TM}, Finapres Medical Systems, Amsterdam, The Netherlands). This arm was also placed in a sling for stability, to ensure accurate detection of the blood pressure waveform during exercise. The Finometer was placed on the right arm for most subjects, and was on the same arm within each subject for all experiments. Bilateral transcranial Doppler ultrasound (ST3, Spencer Technologies, Seattle, WA) was used to measure middle cerebral artery velocity (MCAv) and posterior cerebral artery velocity (PCAv). The MCAv and PCAv signals were always obtained on opposite sides of the head, which was variable between subjects, but the same within each subject across all experiments. Cerebral oxygen saturation of the frontal cortex (ScO_2) was measured via near-infrared spectroscopy (NIRS; OxiplexTS, ISS Inc., Chaimpaign-Urbana, IL). This technique quantifies oxygenated hemoglobin (HbO_2) and deoxygenated hemoglobin (dHb) (33), allowing for calculation of total hemoglobin (THC; HbO_2 +dHb) and ScO_2 as [(HbO_2/THC) · 100]. Cerebral oxygenation measurements were only measured on one side of the forehead , and was always selected to be the same side as the TCD-derived MCAv measurement to ensure that regional oxygenation and perfusion were measured on the same side. A venous catheter was inserted into an antecubital vein of the arm contralateral to the blood pressure measurements for collection of venous blood samples. For most subjects, this was the same arm for all three experiments. During the blood flow restriction protocols (BFRE and RIPC sessions), 5 cm wide inflatable cuffs (SC5^{TM}, D.E. Hokanson, Bellevue, WA)

were placed around both upper thighs and connected to an inflation system (E20 Rapid Cuff Inflation System, D.E. Hokanson, Bellevue, WA).

Blood Flow Restriction Resistance Exercise Session (BFRE)

This session was used to determine the effects of BFRE on sympathetic, hemodynamic, and cerebrovascular responses. Following instrumentation, a 15-min baseline period commenced during which subjects were seated on the leg press machine with their feet placed flat on the ground. During the final minute of rest, subjects placed their feet onto the leg press platform (knee at a ~90^0 angle), and 10-s prior to the commencement of exercise, the bilateral thigh cuffs were rapidly inflated to a target pressure of 220 mmHg. The exercise session was 40-min in duration, divided into 4 x 10-min blocks (figure 1). Each 10-min block consisted of a 5-min exercise period with cuffs inflated, and a 5-min recovery/reperfusion period with cuffs deflated. The exercise period consisted of 3 sets of 10 repetitions with a load corresponding to 20% of 1RM. Each repetition was performed at a pace of 4-s/repetition (2-s concentric contraction, 2-s eccentric contraction; timed via a metronome), and each set was separated by 1-min rest periods. The thigh cuffs remained inflated throughout the 5-min exercise period, including the 1-min rest periods. At the end of the 5-min exercise period, the thigh cuffs were rapidly deflated, subjects placed their feet back on the floor, and rested quietly for a 5-min reperfusion period. An additional 5-min recovery period followed completion of the 40-min of exercise. Three blood samples (10 ml) were collected throughout the protocol; 1) 5-min into baseline (“pre”); 2) at the end of the second exercise block (“mid”), and; 3) at the end of the final reperfusion period (“post”).

Conventional Resistance Exercise Session (CE)

This session was included to compare sympathetic, hemodynamic, and cerebrovascular responses with BFRE exercise to resistance exercise as conventionally prescribed (65% of 1RM). This session was performed in exactly the same manner as the BFRE session, but without use of the inflatable thigh cuffs and with a higher leg press load of 65% of 1RM, per conventional guidelines (1). The timing of the exercise periods and rest periods were identical to the BFRE condition (see figure 1), and blood samples were collected at the same time points (pre, mid, and post).

Remote Ischemic Preconditioning Session (RIPC)

This session served as a control condition to isolate the effects of repeated occlusions and reperfusions independent of exercise. Following instrumentation, subjects completed a 15-min seated baseline. The thigh cuffs were then rapidly inflated to 220 mmHg for 5-min, followed by rapid deflation and reperfusion for 5-min. This inflation/deflation protocol was repeated 4 times over 40-min, followed by a 5-min recovery period. Blood samples were collected at the same time points as the BFRE session (pre, mid, and post).

Data Analysis

All continuous waveform data (ECG, arterial pressure, SV, MCAv, PCAv, ScO_2, THC, $etCO_2$) were recorded at 1000 Hz (PowerLab/Labchart, AD Instruments, Bella Vista, NSW, Australia) and analyzed offline via specialized software (WinCPRS, Absolute Aliens, Turku, Finland). R-wave detection was performed on the ECG signal, and used to determine the timing of each cardiac cycle. MAP, mean MCAv, and mean PCAv were calculated as the area under the

curve for the arterial pressure and cerebral blood velocity wave forms. Cardiac output was calculated as HR multiplied by stroke volume, and total peripheral resistance (TPR) was subsequently calculated as MAP divided by cardiac output. For the baseline period, minutes 5-10 were averaged. All variables were calculated for each 5-min block of exercise, and the first 4-min of each intervening reperfusion period; subjects were moving their feet into position for the subsequent exercise block during the final 1-min of each reperfusion period, so this was not included in the analysis. The data were evaluated in this way so that each occlusion and reperfusion period could be analyzed independently, and compared with the matching time point during the CE condition with no occlusive stimulus, and with the RIPC condition without the exercise stimulus.

Whole blood was collected in EDTA tubes treated with glutathione (1.23 mg glutathione/1 ml whole blood) as a preservative and centrifuged at 1500 RPM for 15-min at 4^0C. Plasma was separated and snap-frozen in liquid nitrogen, then stored at -80^0C until analyzed. Norepinephrine (NE) was measured in duplicate via enzyme linked immunosorbent assay (BA E-6200, Rocky Mountain Diagnostics, Colorado Springs, CO). Only duplicate samples with a coefficient of variation less than 15% were included in the results. As a result, N=9 for NE data. Hematocrit was also assessed with from the "pre" blood sample to ensure equivalent hydration status between each trial.

Statistics

Two-way repeated measures ANOVAs (factor 1: time, factor 2: condition - BFRE, CE, RIPC) were used to compare the effects of each condition over time. A one-way (condition only) repeated measures ANOVA was used to compare baseline hematocrit between conditions. Tukey

post-hoc tests were performed when a significant interaction was indicated by the ANOVAs. Exact P values are reported for all comparisons. Unless otherwise stated, all data are presented as mean ± standard error (SE).

RESULTS

Twenty-four subjects were recruited to participate in this study. Of these 24 subjects, 2 were excluded due to VO_{2peak} <30 ml/kg/min, 3 were excluded due to a 1RM >184 kg, 2 were excluded due to medication use, and 2 withdrew due to scheduling or personal reasons. As a result, 15 subjects completed all three experimental conditions (8M/7F, age 28 ± 1 years, height 170 ± 3 cm, weight 71 ± 3 kg, BMI 24.6 ± 0.7 kg/m^2). The average VO_{2peak} was 35.4 ± 1.7 ml/kg/min, and the average 1RM on the leg press machine was 120 ± 8 kg (264 ± 18 lbs). During the two exercise trials (CE and BFRE), all subjects completed all repetitions at the prescribed workloads (20% 1RM for BFRE and 65% 1RM for CE). The average workloads were 24 ± 2 kg for BFRE and 78 ± 5 kg for CE. There were no differences in baseline hematocrit between the three conditions (P=0.14).

In support of our hypothesis, the increase in plasma [NE] with exercise was higher with CE compared to BFRE and RIPC at both the "mid" (P=0.02) and "post" (P=0.02) time points (figure 2A). Neither BFRE nor RIPC elicited an increase in [NE] (Pre vs. Post: BFRE, P=0.24; RIPC, P=0.31). HR increased with exercise for both CE and BFRE (P<0.001), remaining elevated with BFRE (P≥0.99 vs. start of exercise), and decreasing with CE during each 5-min rest period. (P<0.001 vs. preceding exercise period). Stroke volume, cardiac output, and MAP also increased from baseline with each 5-min exercise bout (P≤0.03; figures 2B and 3A-C) and decreased during the subsequent 5-min rest periods for both CE and BFRE. As expected, these

responses were augmented with CE compared to BFRE during each 5-min exercise period. We observed a higher stroke volume at baseline with RIPC compared to the two exercise conditions; this was unexpected as subjects were in the upright seated posture for all three experimental conditions, but may be due to a slightly lower resting HR (73 ± 3 beats/min) compared with the two exercise conditions (BFRE: 80 ± 3 beats/min; CE: 81 ± 4 beats/min), although this was not statistically distinguishable ($P \geq 0.11$). There were, however, no differences in stroke volume between CE and BFRE at baseline ($P=0.94$). With RIPC, stroke volume decreased from baseline during each occlusion period ($P=0.07$ for occlusion period 1; $P \leq 0.02$ for remaining 3 occlusion periods) and returned to baseline values during the subsequent reperfusion periods ($P \geq 0.99$ vs. baseline; figure 3B). This pattern of response suggests a restriction of venous return with each occlusion. Neither cardiac output nor MAP changed over time with RIPC ($P \geq 0.19$), but HR increased slightly above baseline during the final 2 occlusion periods ($P=0.07$ for occlusion 3 and $P=0.03$ for occlusion period 4; figure 3A). TPR decreased from baseline throughout exercise for the CE condition ($P<0.001$; figure 3D), and was lower for each leg-press period compared with the preceding recovery period ($P<0.001$). TPR was higher for both BFRE and RIPC vs. CE throughout exercise ($P \leq 0.04$), and there were no differences between BFRE and RIPC at any time points ($P \geq 0.14$). Based on the observation that CE appeared to induce greater increases in MAP than BFRE during the first 3 exercise periods, a separate 2-way repeated measures ANOVA was performed to compare CE versus BFRE without the RIPC condition. This analysis also revealed that CE elicited a higher MAP response during the first 3 exercise periods compared to BFRE ($P \leq 0.03$).

Mean MCAv and mean PCAv (figure 4A&B) increased from baseline with each exercise bout for CE ($P<0.001$) and returned back to baseline values during the subsequent rest periods.

For BFRE mean MCAv also increased from baseline with exercise ($P \leq 0.002$) and returned back to baseline values during the subsequent rest periods. For mean PCAv, N=8 due to the difficulties associated with acquiring and maintaining the PCA signal throughout each of the three experiments performed in this study. Mean PCAv increased with exercise with BFRE and remained elevated from baseline during all but the third exercise period ($P \leq 0.002$ for exercise periods 1 and 2; $P=0.08$ for exercise period 4; $P=0.15$ for exercise period 3). While mean MCAv was initially slightly higher with CE compared to BFRE during the first exercise period ($P=0.07$), there were no differences between exercise conditions for the remainder of the intervention ($P \geq 0.27$). This difference at the start of exercise was likely driven by $etCO_2$ which was also higher with CE compared to BFRE at this same time point ($P=0.006$; figure 4C). There were no differences in mean PCAv responses between CE and BFRE at any time points ($P \geq 0.13$), although both were higher than RIPC during the first two exercise periods ($P \leq 0.03$). Interestingly, mean MCAv progressively decreased over time with RIPC ($P \leq 0.05$ vs. baseline by the 2nd reperfusion period), likely driven by $etCO_2$ ($P \leq 0.07$ by the 2nd occlusion period). Although there was a statistically distinguishable difference in ScO_2 during baseline between CE and RIPC ($P=0.07$), the absolute differences were small, 63.8 ± 2.1% vs. 61.2 ± 2.7%. The differences in ScO_2 responses between trials (condition effect, $P<0.001$) is likely driven by the exercise conditions (BFRE vs. RIPC, $P=0.007$; CE vs. RIPC, $P<0.001$), and there was no difference in the overall ScO_2 response between CE and BFRE ($P=0.55$; figure 4D).

DISCUSSION

In this investigation we explored the sympathetic, hemodynamic, and cerebrovascular responses to an acute bout of cyclical blood flow restriction resistance exercise (BFRE), and

compared these responses to conventional resistance exercise (CE), and cyclical blood flow restriction and reperfusion without exercise (i.e., RIPC). The major findings were: 1) in support of our hypothesis, we observed an attenuated increase in sympathetic activity with BFRE compared to CE, as indicated by lower plasma [NE]; 2) also in support of our hypothesis, HR, MAP, stroke volume, cardiac output, and total peripheral resistance responses were attenuated with BFRE compared to CE; and 3) the similar cerebrovascular responses between CE and BFRE suggest equivalent cerebro-metabolic demand between conditions.

The lower plasma NE with BFRE supports our hypothesis that there is an attenuated increase in sympathetic activity with BFRE compared to CE. This is likely due to the lower workloads used in the BFRE condition (20% 1RM versus 65% 1RM). This finding is in contrast to previous work by Madarame et al. who reported higher NE responses to BFRE compared with CE in both healthy (32) and clinical (31) populations. However, both exercise conditions in these studies were performed at the same relative workload (20-30% 1RM) which is not consistent with the comparisons made in our study, and thus could explain these divergent findings. More commonly, BFRE is prescribed at a lower relative workload than CE (28), which is why it is often adapted to elderly populations (4, 46, 59) or other individuals with musculo-skeletal limitations (4, 36, 51). As such, we opted to compare CE to BFRE at the most commonly performed relative workloads (65% 1RM for CE vs 20% 1RM for BFRE). While it is somewhat surprising that the combination of resistance exercise with thigh occlusion (the BFRE condition) did not elicit a greater increase in NE than thigh occlusion alone (the RIPC condition), we speculate that this is due to both the low workloads (20% 1RM) and cyclical nature of the stimulus used in the BFRE trial. Exercise performed at this workload is likely of insufficient intensity to exacerbate the exercise pressor reflex response resulting from the occlusive stimulus

alone. Additionally, this attenuated sympathetic response may be related to the width of the occlusive cuffs (5 cm) and the occlusive pressure (220 mmHg). We speculate that wider cuffs and/or higher occlusive pressures would confer a greater restriction to arterial inflow (29), and a greater stimulation of type III and IV afferents, subsequently eliciting a magnified sympathetic response (reflected by elevated NE release) (54). It should be noted, however, that although higher cuff pressures are required for complete arterial restriction with a narrower cuff, when restrictive pressures are set relative to cuff width and individual subject responses, cuff widths of different sizes can still induce a similar degree of blood flow restriction (42). From the perspective of clinical application, however, this attenuated sympatho-excitatory response with BFRE is desirable in patient populations who are at an increased risk for adverse cardiovascular events.

We also observed an attenuated increase in HR, stroke volume, and MAP with BFRE compared to CE, which was accompanied by large reductions in TPR with CE that were not present with either BFRE or RIPC. Similarly, Poton & Polito (48) reported an attenuated increase in HR and arterial pressures with low intensity resistance BFRE (20% 1RM; unilateral knee extension) in comparison to a high intensity condition performed without blood flow restriction (80% 1RM). The investigators also included a condition where subjects performed low intensity exercise at the same workload as the BFRE condition (20% 1RM), but without blood flow restriction. While the high intensity condition elicited the greatest increase in arterial pressures, the low intensity BFRE condition also induced greater increases in HR and systolic arterial pressure during the final set of exercise compared to the low intensity condition that was performed without blood flow restriction (48). In contrast to our findings, however, there were no differences in stroke volume between conditions, whereas we observed an attenuated increase

in stroke volume with BFRE compared to CE. This discrepancy may be due to differences in the exercise modality - Poton & Polito used unilateral knee extension compared with the bilateral leg press utilized in our investigation. While not assessed in the present investigation, we speculate that application of blood flow restriction to both thighs, rather than just one, would result in a greater decrease in venous return, and subsequently stroke volume. A reduction in venous return with our BFRE condition is supported by data in the RIPC (occlusion only) condition which demonstrates a decrease in stroke volume during all of the occlusion periods (figure 3B).

Although there were no differences in the absolute stroke volumes between BFRE and RIPC, the pattern of responses was quite different between conditions, with stroke volume decreasing with each occlusion period with RIPC, while increasing during the same period with BFRE. This pattern of responses suggests that engagement of the skeletal muscle pump, even with this low intensity exercise, was sufficient to overcome the restriction in venous return resulting from the occlusions. We also observed a pronounced decrease in TPR from the start of exercise through the end of recovery with CE that was not present in the other two conditions. This is likely related to the higher workloads used in the CE condition, as greater increases in metabolic demand would enhance the functional sympatholysis within the exercising muscle, causing a reduction in TPR, and subsequently eliciting increases in regional muscle blood flow.

Although we observed an attenuated increase in arterial pressures with BFRE compared to CE in young healthy subjects, we acknowledge that these responses to BFRE may be different in clinical populations. As highlighted by Spranger et al. (54), several clinical populations including patients with hypertension, heart failure, and peripheral artery disease exhibit augmented activation of the exercise pressor reflex which could be further exacerbated by blood flow restriction training. In support of this perspective, one recent study demonstrated that low

intensity (20% 1RM) leg press performed with blood flow restriction elicited a greater increase in arterial pressures compared to high intensity exercise (65% 1RM) in hypertensive women (47). Importantly, however, this study used a restrictive pressure that resulted in complete arterial occlusion (an uncommon practice for BFRE), which may have augmented the arterial pressure response. Studies investigating the acute hemodynamic responses to RIPC in either healthy or clinical populations are surprisingly scarce. One study by Li et al. did assess the effects of 5 cycles of unilateral arm RIPC (5-min occlusion/5-min reperfusion) on HR and arterial pressure in healthy subjects and subjects with unilateral middle cerebral artery stenosis (24). While there were no changes in HR and arterial pressure in either group during the RIPC stimulus, the authors did report a reduction in mean arterial pressure and HR 30-min after the final cuff deflation period in the healthy subjects that was not present in the patient population. As such, caution should be taken when generalizing our findings to clinical populations; additional investigations are necessary to determine if our cyclical BFRE paradigm would be appropriate in patient groups of interest, such as individuals in cardiac and stroke rehabilitation.

The similarity in cerebrovascular responses between the two exercise trials suggests an equivalent cerebro-metabolic demand. Although mean MCAv was initially higher at the start of CE, this is likely due to the greater metabolic production of CO_2 (reflected in the etCO_2 response), which would stimulate an increase in cerebral blood flow (44). While several studies have assessed the cerebral blood flow responses to resistance exercise using small muscle groups such as hand grip (12, 20, 26), few studies have measured the cerebrovascular responses to resistance exercise of large muscle mass, such as the leg-press modality used in the present investigation. Dickerman et al. demonstrated that one repetition of maximal leg press resulted in a decrease in MCAv (10), potentially due to performance of the Valsalva maneuver, and

subsequent increases in intracranial pressure. In contrast, two other investigations reported increases in MCAv during one set of 10 repetitions of leg press performed at a workload corresponding to estimated 10-rep max (40, 50); importantly subjects were instructed to breathe throughout each leg press, hence avoiding the Valsalva maneuver and increases in intracranial pressure, and subsequently facilitating the observed increases in MCAv. Studies assessing the cerebrovascular responses to an acute bout of RIPC or BFRE are very limited. In the aforementioned RIPC study, Li et al. reported that MCAv and ScO_2 did not change in response to 5 cycles of unilateral arm RIPC in either healthy subjects or in patients with cerebral artery stenosis (24). In one of the only studies assessing cerebral responses to BFRE, Ganesan et al. assessed cerebral oxygen saturation (but not cerebral blood flow) in subjects performing three sets of bilateral knee extensions to volitional fatigue at 50% 1RM with and without blood flow restriction (14). Since the cerebral NIRS signal is predominantly obtained from venous blood (75%), we interpret an increase in deoxy-hemoglobin (dHb) and decrease in oxy-hemoglobin (HbO_2) as an increase in tissue oxygen extraction, particularly if there is no difference in oxygen supply (i.e. MCAv). So, while ScO_2 increased with exercise in both trials, the BFRE condition resulted in higher dHb and lower HbO_2 compared to the non-occlusive conditions, suggesting a greater oxygen extraction with the BFRE condition. As ratings of perceived exertion were also higher with the BFRE condition, the higher cerebral oxygen extraction may be related to this response; this interpretation is limited, however, as cerebral blood flow (oxygen supply) was not assessed. In contrast, while we did not measure ratings of perceived exertion in our study, we did not observe any differences in ScO_2 responses between the two exercise conditions. It is possible that the perceived exertion with low intensity BFRE is comparable to high intensity CE, resulting in these similar ScO_2 responses, although this interpretation is speculative.

There are several methodological considerations that must be addressed when interpreting these findings. First, we utilized transcranial Doppler ultrasound for measurements of cerebral blood velocity, rather than performing direct measures of cerebral blood flow. This technique is dependent on the assumption that the diameter of the insonated artery does not change. Two recent studies, however, have demonstrated that periods of hyper- (+9 mmHg) or hypo-capnia (-13 mmHg) can change MCA diameter (assessed via high resolution MRI) (8, 55). The end-tidal CO_2 responses elicited by the interventions used in the present investigation, however, are lower in magnitude (+5 mmHg) than reported in these studies. Furthermore, even if this degree of hypercapnia did elicit an increase in MCA diameter, measurement of velocity in the MCA would actually result in an underestimation of cerebral blood flow responses. While the effects of perturbations in arterial CO_2 on PCA diameter are unknown, this potential limitation is mitigated by the observation that there were no differences in mean PCAv responses between the two exercise conditions.

Second, our ScO_2 measurement is assessed indirectly via cerebral NIRS. This measurement approach is potentially contaminated by skin blood flow, which would be expected to increase during exercise (39). The spatially resolved NIRS sensor used in this investigation, however, minimizes this limitation by using multiple emitters at varying distances from the detector, allowing blood flow from the skin, muscle, and fat layers to be mathematically removed from the final signal. This technique is also limited by our ability to only assess changes in oxygenation of the frontal lobe tissue. While global measurements of cerebral oxygenation may be more informative, these measurements require invasive (such as arterial-venous blood sampling across the brain) and/or impractical techniques (such as functional MRI) under the dynamic conditions of the exercise protocols used in the present investigation.

Furthermore, by combining our NIRS-derived ScO_2 measurements with simultaneous measurements of cerebral blood velocity within the same region (MCA), this allows high temporal resolution for estimation of oxygen extraction, as previously described.

A third methodological consideration is the use of plasma NE as an index of sympathetic activation, rather than direct recordings of muscle sympathetic nerve activity (MSNA) via microneurography. While MSNA would have been a more direct assessment of sympathetic activity, the repeated measures design that was employed in this study, combined with the technical difficulties of maintaining a nerve recording during exercise, required the use of plasma NE as an index of sympathetic drive. Despite this limitation, however, there is a direct relationship between venous NE and sympathetic neural activity (15), so we are confident that plasma NE is representative of general increases in sympathetic outflow within this investigation. Furthermore, we cannot exclude the potential role of pain resulting from the restrictive stimulus of cuff inflation. This discomfort could potentially increase sympathetic outflow independent of the exercise bouts. While the effect of pain was not directly assessed in our investigation, all subjects were able to tolerate this stimulus and no experiments were terminated due to the effects of pain.

An additional consideration is the length of time between each of the three experiments. Although the one-month intervening period between experiments allowed for control of menstrual cycle phase in our female subjects, this length of time could have also increased the variability in the key outcomes. This potential confound was minimized, however, with our randomized, counterbalanced design, and the robust results (reflected in the statistical comparisons) supports this contention.

When relating our findings to other similar investigations, the width of the occlusive cuff that was used (5 cm in the present study) should be considered, as previously discussed. As highlighted by Loenneke et al. (29), differences in cuff width can have profound influences on the degree of arterial occlusion that is achieved. In a recent review, Spranger et al., also indicated that these differences in cuff width can also directly affect the magnitude of exercise pressor reflex, with wider cuffs resulting in greater activation of type III and IV afferent nerves (54), presumably due to greater vascular occlusion and compression of the muscle mass. Based on these considerations, comparison of our results with other investigations should be performed judiciously if different cuff sizes and occlusive pressures were used. More work is clearly required to determine optimal occlusive pressures and cuff widths to elicit the key target outcomes with BFRE; indeed, these outcomes themselves, in addition to the target population of interest, may dictate variations in the degree of occlusion required. For example, while some RIPC literature has used cuff widths or pressures that would likely result in complete arterial occlusion (19, 37, 38), most of the BFRE literature suggests that submaximal occlusions are likely sufficient to induce muscular hypertrophy (7, 27). Based on these differences between BFRE and RIPC protocols, it is possible that the submaximal occlusions that were used in this investigation may not confer the same degree of cardio- and cerebro-protection as the maximal occlusions more commonly applied with RIPC. As this is the first investigation to model BFRE around the RIPC paradigm, future work should seek to determine if an optimal pressure can be identified that elicits the beneficial effects of both RIPC and BFRE.

Additionally, when relating the findings of our study to other BFRE investigations, the total training volume utilized in the BFRE condition (3 sets of 10 repetitions) should be considered. While we selected this model to match the total volume between the CE and BFRE

conditions, it should be noted that many BFRE studies focusing on muscular hypertrophy have either performed BFRE to failure, or have used 4 sets (first set of 30 repetitions, then 3 sets of 15 repetitions) (34, 58, 59). As such, it is possible that the BFRE protocol used in our investigation would elicit an attenuated muscular hypertrophic response compared to these other models, although this is speculative, and remains to be tested experimentally.

One final consideration in our experimental design is that we did not include a condition with a load corresponding to 20% of 1RM performed without blood flow restriction. The reasons for this are twofold. First, a primary comparison of interest was between BFRE and RIPC, to determine the effects of combining low intensity exercise with RIPC. Second, conventional guidelines recommend resistance exercise loads of 65% 1RM or greater to achieve appreciable gains in muscle mass (1); in comparison, 20% 1RM (when not taken to failure) would not generally be utilized in practice as it may not elicit an optimal physiological benefit. When taken to failure, however, lower loads have been shown to induce a comparable degree of muscular hypertrophy as observed with heavier training loads (41). Furthermore, as previously indicated, one study has compared the hemodynamic responses to unilateral knee extensions performed under three different conditions: low intensity (20% 1RM), low intensity with blood flow restriction (20% 1RM), and high intensity (80% 1RM). As expected, the low intensity BFRE condition elicited a greater increase in sympathetic activity (manifest by a higher HR and arterial pressure) in comparison to the low intensity condition without blood flow restriction. Also as expected, and similar to the results observed in our investigation, the BFRE condition elicited a lower arterial pressure response compared to the high intensity condition (48). These observations support those reported in our investigation, in that BFRE elicited an attenuated increase in sympathetic drive compared to CE, likely due to the use of lower workloads.

PERSPECTIVES AND SIGNIFICANCE

In this investigation we demonstrated an attenuated increase in sympathetic activity and hemodynamic responses with a novel BFRE paradigm, modeled after RIPC. The similarity in cerebrovascular responses also suggests equivalent cerebro-metabolic demand between this model and resistance exercise as conventionally prescribed. Based on these observations, this cyclical BFRE model could potentially be adapted to clinical populations including patients participating in cardiac- or stroke-rehabilitation programs, where individuals might benefit from the RIPC stimulus superimposed with resistance exercise. As previously mentioned, recent clinical trials have already demonstrated that daily RIPC can be used as a preventative strategy to decrease the incidence of stroke and increase cerebral blood flow in patients with significant carotid artery stenosis (37, 38). As many patients participating in rehabilitation programs are already engaging in exercise, this setting would be an ideal target for implementation of an RIPC-like stimulus, such as the novel BFRE paradigm explored in this investigation.

A question that remains to be answered, however, is whether the beneficial adaptations to resistance training performed conventionally or with blood flow restriction, are still present when using this novel cyclical BFRE paradigm. For example, muscular hypertrophy and increased strength are well documented with BFRE when performed as traditionally prescribed with continuous vascular occlusion/cuff inflation (21, 27, 58). As we only explored the acute responses to a single cyclical BFRE session, future work is required to determine if exercise training with this novel BFRE paradigm elicits favorable cardiovascular and musculoskeletal adaptations, including muscle hypertrophy, increased bone density, improved vascular function, and reduced resting arterial pressure and heart rate. Similarly, as most RIPC protocols utilize cuff pressures that result in complete cessation of arterial inflow, further work is also needed to

confirm that the cardio- and cerebro-protective effects associated with RIPC can also be extended to the submaximal occlusions that were used in this investigation.

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FIGURES

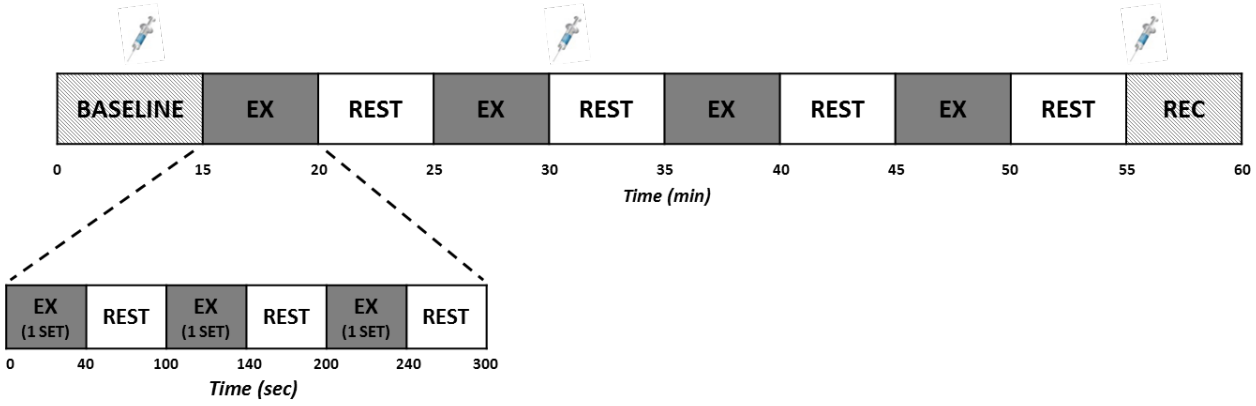

Figure 1: Experimental timeline consisting of a 15-min baseline, 40-min of exercise (4 sets of exercise (EX) and 4 rest/reperfusion periods), and a 5-min recovery period (REC). One 5-min exercise period consisted of 3 sets of exercise with a 1-min rest period between each set; each set consisted of 10 repetitions performed at a load corresponding to 20% (BFRE) or 65% (CE) of 1RM. For the BFRE condition, the occlusive stimulus (cuff inflation) was maintained throughout the entire 5-min of the exercise period. Blood sampling is denoted by syringes.

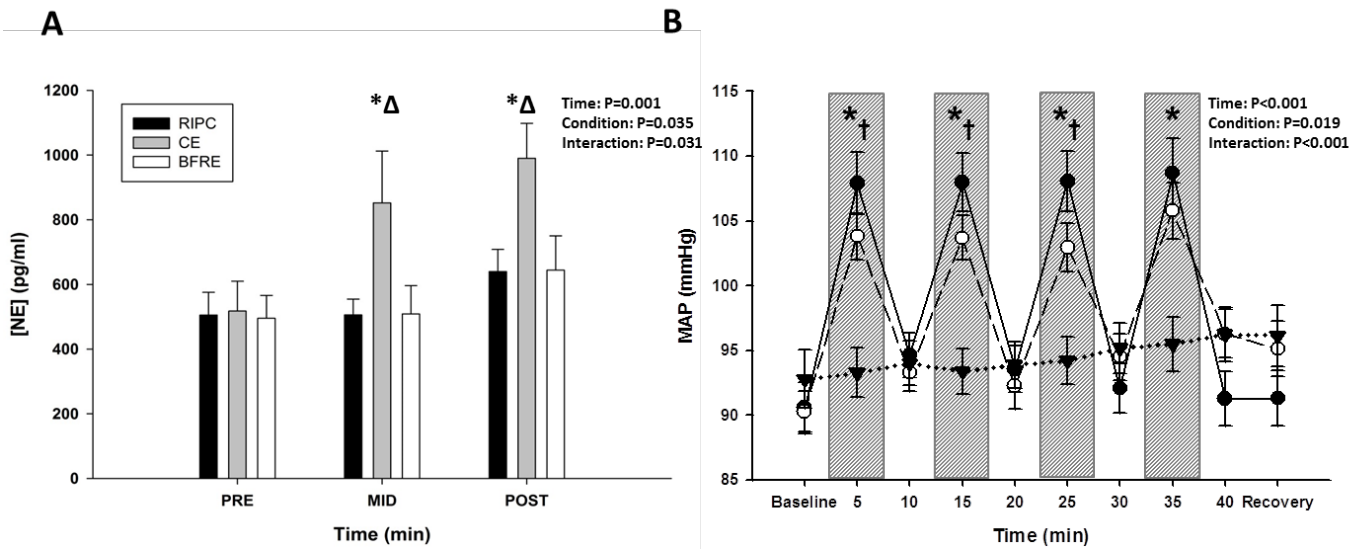

Figure 2: Plasma Norepinephrine ([NE]; Panel A, N=9) responses to conventional exercise (CE, grey bar), blood flow restriction exercise (BFRE, white bar), and remote ischemic preconditioning (RIPC, black bar). NE increased with exercise for CE (*, P≤0.002 vs "pre" time point) and was higher with CE compared to BFRE and RIPC at both the "mid" and "post" time points (Δ, P≤0.02). Mean arterial pressure (MAP; Panel B) responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Exercise bouts (and occlusion periods for BFRE and RIPC) denoted by vertical grey bars. MAP increased with each exercise bout for both CE and BFRE and was higher with CE compared to BFRE during the first 3 exercise blocks (P≤0.09). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *, P<0.001 for CE and BFRE vs RIPC; †, P≤0.09 for CE vs BFRE.

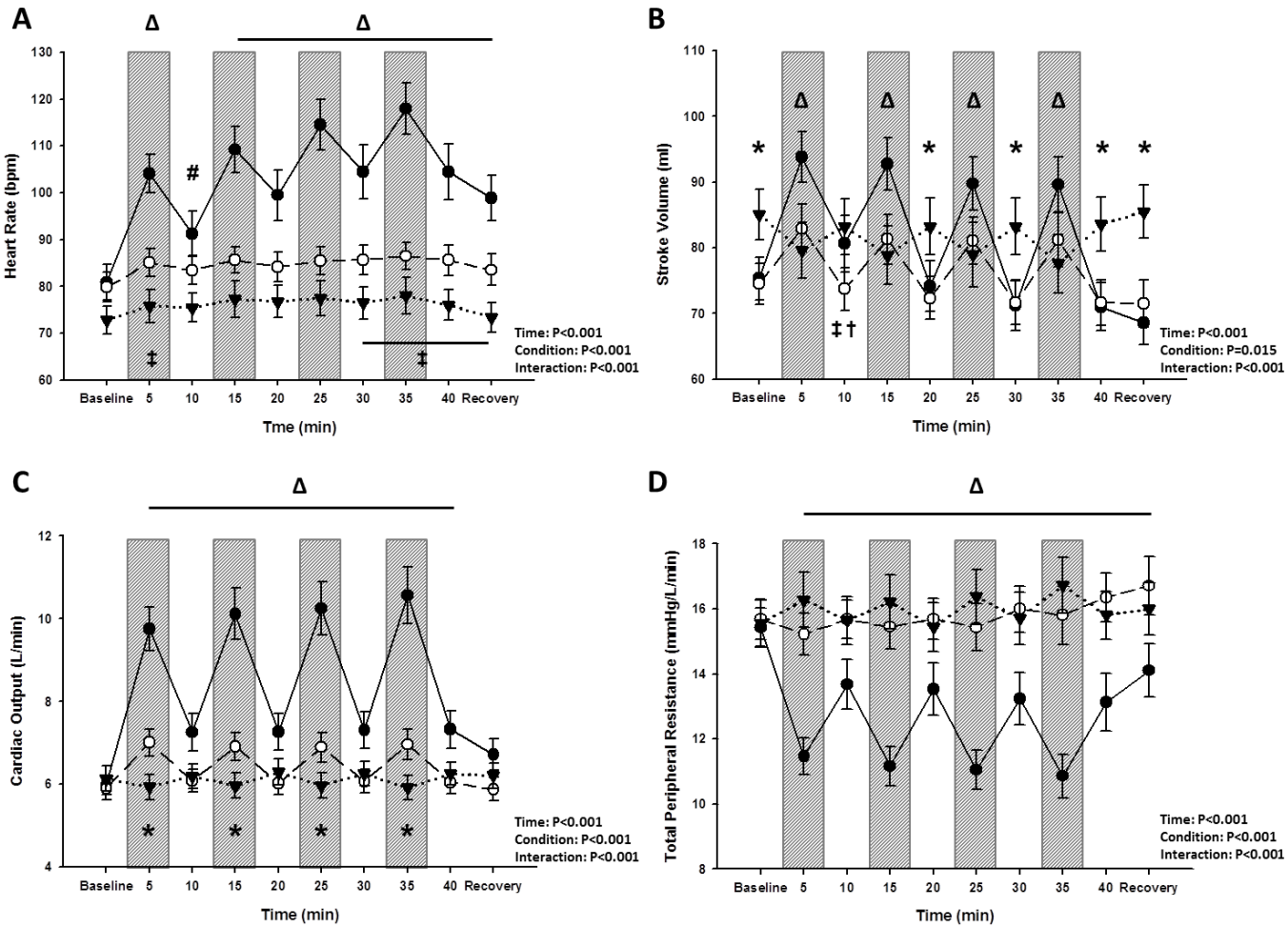
A
Heart Rate (bpm)
Tme (min)
Time: P<0.001
Condition: P<0.001
Interaction: P<0.001
B
Stroke Volume (ml)
Time (min)
Time: P<0.001
Condition: P=0.015
Interaction: P<0.001
C
Cardiac Output (L/min)
Time (min)
Time: P<0.001
Condition: P<0.001
Interaction: P<0.001
D
Total Peripheral Resistance (mmHg/L/min)
Time (min)
Time: P<0.001
Condition: P<0.001
Interaction: P<0.001
Baseline
Recovery

Figure 3: Hemodynamic responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Exercise bouts (and occlusion periods for BFRE and RIPC) denoted by vertical grey bars. Heart rate (Panel A) increased with exercise and was higher with CE compared to BFRE and RIPC during all 4 exercise bouts/occlusion periods (Δ, $P<0.001$). Stroke volume (Panel B) increased with each exercise bout/occlusion period for both CE and BFRE ($P\leq0.007$) and decreased with RIPC during each occlusion period ($P\leq0.07$). Cardiac output (Panel C) increased with each exercise bout/occlusion period for CE and BFRE ($P<0.001$) and was higher with CE compared to BFRE and RIPC from the start of exercise through the last reperfusion period (Δ, $P\leq0.004$). Total peripheral resistance (Panel D) decreased with exercise for CE and was lower with CE compared to BFRE and RIPC throughout the intervention (Δ, $P\leq0.04$). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *, $P\leq0.006$ for CE and BFRE vs RIPC; Δ, $P\leq0.04$ for CE vs RIPC and BFRE; #, $P<0.001$ for CE vs RIPC; †, $P=0.02$ for CE vs BFRE; ‡, $P\leq0.09$ for BFRE vs RIPC.

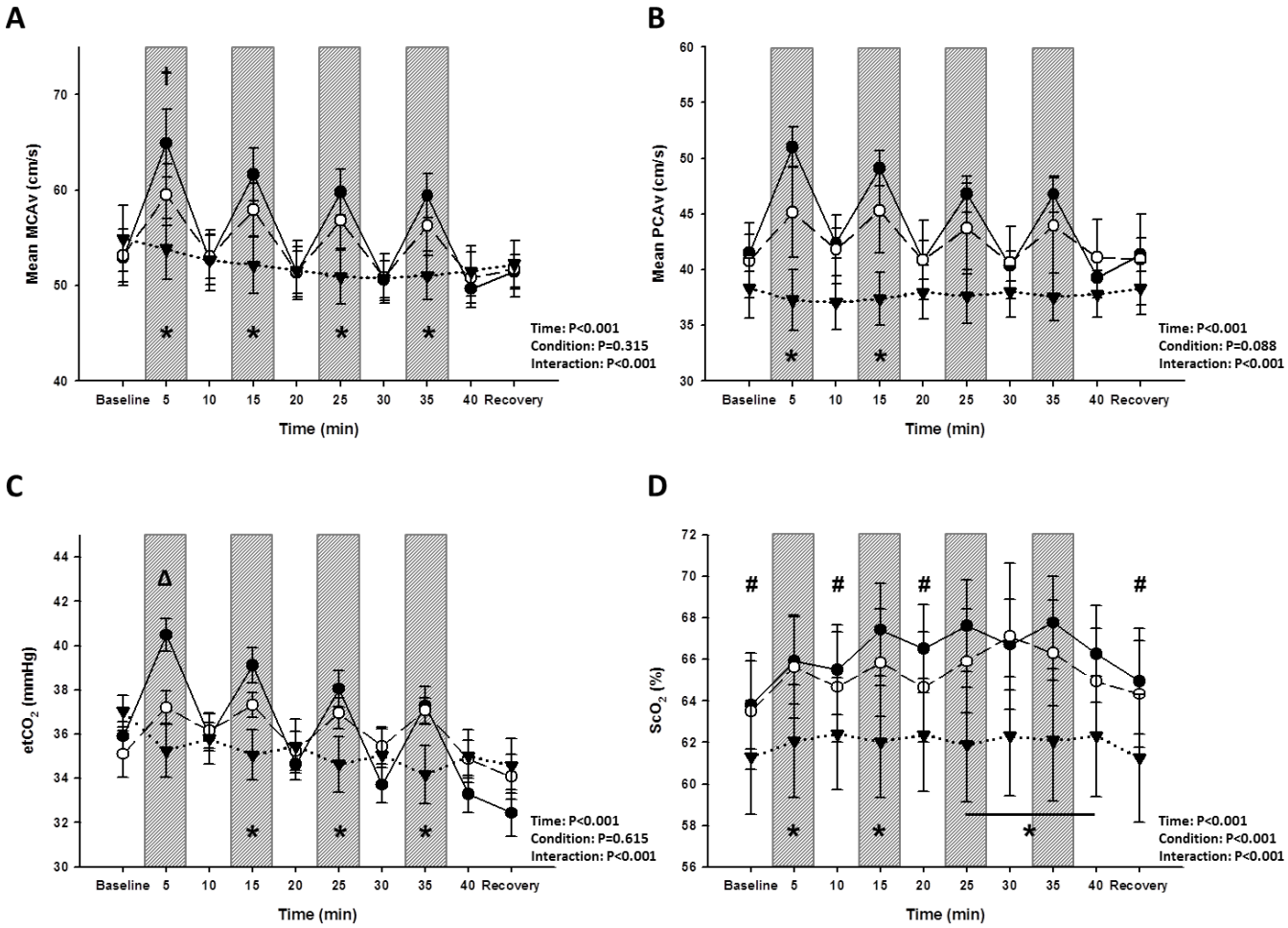
A
Mean MCAv (cm/s)
Time: P<0.001
Condition: P=0.315
Interaction: P<0.001
Baseline 5 10 15 20 25 30 35 40 Recovery
Time (min)
B
Mean PCAv (cm/s)
Time: P<0.001
Condition: P=0.088
Interaction: P<0.001
Baseline 5 10 15 20 25 30 35 40 Recovery
Time (min)
C
etCO2 (mmHg)
Time: P<0.001
Condition: P=0.615
Interaction: P<0.001
Baseline 5 10 15 20 25 30 35 40 Recovery
Time (min)
D
ScO2 (%)
Time: P<0.001
Condition: P<0.001
Interaction: P<0.001
Baseline 5 10 15 20 25 30 35 40 Recovery
Time (min)

Figure 4: Regional cerebral blood velocity, cerebral oxygenation, and end-tidal CO_2 responses to conventional exercise (CE; ●, continuous line), blood flow restriction exercise (BFRE; ○, dashed line), and remote ischemic preconditioning (RIPC; ▼, dotted line). Exercise bouts (and occlusion periods for BFRE and RIPC) denoted by vertical grey bars. Mean middle cerebral artery velocity (MCAv; Panel A, N=13) increased with each exercise period for both CE and BFRE, and was higher with CE and BFRE compared to RIPC (*, $P \leq 0.08$). Mean MCAv was also initially higher with CE compared to BFRE during the first exercise bout/occlusion period (†, $P=0.07$). Mean posterior cerebral artery velocity (PCAv; Panel B, N=8) increased with exercise for CE and BFRE, although there were no differences between exercise conditions ($P \geq 0.13$). Both CE and BFRE were higher than RIPC during the first two exercise periods (*, $P \leq 0.03$). End tidal CO_2 ($etCO_2$; Panel C) increased with exercise for CE and BFRE and was initially higher with CE than BFRE ($P=0.006$). Frontal lobe cerebral oxygen saturation (ScO_2; Panel D, N=14) was different between conditions (overall condition $P<0.001$), with CE and BFRE higher than RIPC at several time points ($P<0.06$). A two-way repeated measures ANOVA was performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post hoc tests were performed when an interaction effect was present. *,$P \leq 0.08$ for CE and BFRE vs RIPC; Δ, $P \leq 0.006$ for CE vs RIPC and BFRE; #, $P \leq 0.07$ for CE vs RIPC; †, $P=0.07$ for CE vs BFRE.

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CHAPTER IV

Substrate Utilization and Energy Expenditure During Aerobic Exercise with Intermittent Blood Flow Restriction

Justin D. Sprick & Caroline A. Rickards

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ABSTRACT

The effects of blood flow restriction exercise (BFRE) on substrate utilization and caloric expenditure have been underexplored. We implemented a novel approach to BFRE, consisting of cyclical bouts of blood flow restriction and reperfusion during steady state treadmill walking; heart rate (HR) intensity was matched with the conventional exercise (CE) control condition. We hypothesized that the hypoxic environment of the muscle, coupled with elevated sympathetic activity with BFRE would result in greater caloric expenditure compared to CE. Thirteen healthy subjects (7M/6F) performed 40-min of treadmill walking at 65-70% of HR_{max} with and without the application of cyclical blood flow restriction (randomized, cross-over design). The BFRE protocol consisted of 4 x 5-min bilateral thigh cuff inflations (220 mmHg cuff pressure), followed by 5-min reperfusion periods. Oxygen consumption (VO_2), carbon dioxide production (VCO_2), caloric expenditure, and respiratory exchange ratio (RER) were compared between trials. The mechanical workload required to elicit the target HR response was lower with BFRE compared to CE, evidenced by a lower treadmill grade (7.3±0.3% vs. 6.0±0.3%, $P<0.001$). Subsequently, CE was associated with a higher VO_2 and VCO_2 compared to BFRE ($P=0.008$ for both), and cumulative caloric expenditure was also slightly higher (CE, 243±17 vs. BFRE, 220±18 kcals, $P=0.01$; average difference of 23.2±7.8 kcals). These responses were not accompanied by a shift in substrate utilization indicated by similar RER between trials ($P=0.84$).

This novel BFRE paradigm could potentially be applied to individuals with musculo-skeletal limitations who could benefit from the lower workloads but similar metabolic responses as CE.

INTRODUCTION

Blood flow restriction exercise (BFRE) is a novel form of training that is characterized by reducing blood flow to the active muscles through the use of a restrictive device (e.g., an inflatable cuff) (18). This exercise modality was developed as a method to augment the muscle hypertrophic response to exercise and, as such, has been applied to diverse populations including athletes (19, 21, 26, 38), the elderly (15, 24, 25), and clinically-relevant groups such as cardiac patients (20). One hallmark of BFRE is the use of low intensity workloads, making it a feasible exercise alternative for individuals suffering from musculoskeletal limitations.

While most investigations on BFRE training have focused on resistance exercise, several studies have also applied BFRE to aerobic training modalities (1, 2, 6, 22, 26). Abe et al. demonstrated that 3 weeks of twice daily treadmill walking (6 days/week) with BFRE resulted in a 4-7% increase in thigh muscle cross sectional area (CSA) (2). This increase in CSA was also accompanied by an 8-10% improvement in isometric strength of the thigh musculature. In a separate study by Abe et al., 8 weeks of low-intensity cycling with BFRE elicited enhancements in both thigh CSA (3.4-5.1%) and VO_{2max} (6.4%) that were not present in the control group who exercised at the same relative intensity (40% VO_{2max}) without blood flow restriction (1). These findings are especially remarkable, since this was the first study to demonstrate concurrent improvements in both aerobic capacity and muscle hypertrophy through a single training modality (1). Park et al. also reported improvements in VO_{2max} (11.6% increase) following just 2 weeks of twice daily BFRE-treadmill walking for 6 days/week (4-6 km/h, 5% grade) (26).

Collectively, these studies support the feasibility and utility of combining blood flow restriction with aerobic exercise to elicit physiological benefits such as muscular hypertrophy, strength, and aerobic capacity.

In addition to these established benefits, aerobic BFRE (compared to exercising without blood flow restriction) could also increase caloric expenditure; very few studies have assessed the effects of BFRE on metabolic parameters. The greater physiological demand imposed by the local hypoxic environment of the muscle, coupled with a greater sympathetic drive resulting from stimulation of the muscle afferent nerves (i.e. the exercise pressor reflex) (33) would potentially stimulate energy mobilization and utilization. Mendonca et al. demonstrated that treadmill walking with blood flow restriction (5 x 3-min bouts at the subject's "optimal walking speed") resulted in a greater increase in VO_2 (10.4%) compared with a control condition (exercising without blood flow restriction)(22). These outcomes could be particularly valuable to populations exercising for weight control.

While many investigations comparing BFRE with conventional exercise (CE) have used the same absolute workloads between modalities (often chosen arbitrarily) (2, 16, 32, 36), the American College of Sports Medicine (ACSM) recommends that exercise intensity be prescribed based on a percentage of maximal heart rate (HR_{max}), HR reserve, or VO_{2max}. In the current investigation, we sought to compare the effects of aerobic exercise with and without intermittent blood flow restriction on caloric expenditure and substrate utilization when both exercise bouts are performed at 65-70% of HR_{max}, as per ACSM guidelines. Under these experimental conditions, we hypothesized that BFRE would result in a greater VO_2 and caloric expenditure than CE.

METHODS

Ethical Approval

All experimental procedures were conducted under a protocol approved by the University of North Texas Health Science Center (UNTHSC) Institutional Review Board (IRB #2014-149). Written informed consent was obtained from each subject prior to participation, and all experiments conformed to the standards set by the Declaration of Helsinki.

Subjects

Young, healthy subjects participated in this study conducted at UNTHSC, Fort Worth, TX. Prior to participation in the experimental protocols, all subjects underwent a medical history evaluation, including seated and standing 12-lead ECG and blood pressure recordings, and were cleared to participate by a physician. Subjects did not routinely use any nicotine products (including tobacco cigarettes, electronic cigarettes, chewing tobacco). Before each experiment, subjects abstained from caffeine, alcohol, dietary supplements, medications, and exercise for 24 hours, and were fasted for 8 hours. Female subjects completed a urine pregnancy test to ensure they were not pregnant prior to each experimental session. Subjects underwent a familiarization session in which they were exposed to all of the equipment that would be used in the subsequent sessions. They were also familiarized with the blood flow restriction protocol by having the cuffs inflated to 220 mmHg for 5-min while they walked on the treadmill.

Maximal Exercise Testing

Once cleared to participate, all subjects underwent a maximal aerobic exercise test (VO_{2peak} test) on a treadmill (TMX428CP, TrackMaster, Newton, KS) in accordance with the

Bruce protocol (27). Testing was terminated at volitional fatigue. HR (via wireless strap; Polar, H1 Series, Polar Electro Oy, Kempele, Finland), VO_2 and VCO_2 were measured continuously with a metabolic cart (TrueOne, ParvoMedics, Sandy, UT). Maximum HR (HR_{max}) and VO_{2peak} were determined from this testing session. HR_{max} was used to calculate the relative exercise intensities for the treadmill exercise sessions, set at 65-70% of HR_{max}. In order to recruit a relatively homogeneous subject population in regards to aerobic capacity, subjects were excluded if their VO_{2peak} was <30 or >50 ml/kg/min.

Study Design

At least 2 weeks following completion of the maximal exercise testing, subjects reported back to the laboratory for the first of two experimental sessions (randomized); the two sessions were separated by at least 1 month. Female subjects were tested in the early follicular phase of their menstrual cycle (first 4 days determined by self-report), and completed a urine pregnancy test prior to the commencement of each experiment. All experimental sessions were performed in the morning in a thermo-neutral laboratory (temperature = 23.2 ± 0.1^0C, humidity = 52.6± 3.1 %, barometric pressure = 745.2 ± 0.7 mmHg). Hematocrit was assessed via a baseline blood sample to ensure adequate hydration status for both exercise conditions.

Intermittent Blood Flow Restriction Exercise Session

Upon arriving in the laboratory, subjects were encouraged to empty their bladder to ensure optimal comfort and to limit the potential confounding effects of increased sympathetic nervous system activation (8). Subjects were then instrumented with a wireless HR monitor and fitted with a mask that was attached to the metabolic cart. A 3-lead ECG (shielded leads, cable,

and amplifier, AD Instruments, Bella Vista, VSW, Australia) was also attached for measurement of HR at a sampling rate of 1000 Hz (Powerlab and Labchart, AD Instruments, Bella Vista, NSW, Australia). The wireless HR monitor was used to ensure that subjects stayed within their target HR range (65-70% HR_{max}) during the experiment, while the data collected from the ECG was used for analysis. Noninvasive arterial pressure (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands) was also measured continuously and has been previously published in a related paper (34). In order to apply the blood flow restriction stimulus, inflatable cuffs (5 cm wide; SC5D^{TM}, D.E. Hokanson, Bellevue, WA) were wrapped around the most proximal portion of the subject's thighs, secured with tape, and connected to a rapid inflation system (E20 Rapid Cuff Inflation System, D.E. Hokanson, Bellevue, WA). Subjects underwent a 15-min seated baseline before being moved to the treadmill for the exercise session. The treadmill was connected to the metabolic cart so that the speed and grade could be recorded, and manually adjusted by the operator throughout the exercise session. The treadmill was set at a fixed speed of 4.0 km/h (2.5 mph) while the grade was adjusted so that the physiological workload was within 65-70% of predetermined HR_{max}. The intermittent blood flow restriction stimulus was accomplished by rapidly inflating the cuffs to 220 mmHg for 5-min followed by rapid deflation and 5-min of reperfusion (4 cycles over 40-min of exercise). After completion of the 40-min exercise bout, the treadmill speed was lowered to 2.4 km/h (1.5 mph) and the grade was reduced to 0% for a 5-min active recovery period.

Conventional Exercise Session

Instrumentation was identical to the BFRE session, except that the inflatable cuffs were not applied. Following a 15-min seated baseline, subjects were moved to the treadmill, after

which they began the 40-min of steady-state exercise. Again, the treadmill was set at a fixed speed of 4.0 km/h (2.5 mph) while the grade was adjusted to induce a physiological workload equivalent to 65-70% HR_{max}. Similarly, following completion of the 40-min exercise bout, the treadmill speed was lowered to 2.4 km/h (1.5 mph) while the grade was reduced to 0% for a 5-min active recovery period.

Data Analysis

All data was analyzed offline in 5-min blocks so that each cuff inflation and cuff deflation period could be independently assessed. Breath-by-breath metabolic data analyzed directly from the metabolic cart included VO_2, VCO_2, RER (VCO_2 divided by VO_2) and caloric expenditure. Treadmill speed and incline were also obtained directly from the metabolic cart output. Total caloric expenditure and RER were also calculated for the entire 45-min exercise bout (40-min at 65-70% HR_{max} plus 5-min recovery period) to determine the cumulative effect of each intervention on these two outcome variables. ECG data was analyzed with specialized software (WinCPRS, Absolute Aliens, Turku, Finland). R-waves generated from this signal were detected so that beat to beat HR could be derived from the R-R interval.

Statistics

Two-way repeated measures ANOVAs were used to compare the effects of each exercise condition over time (SigmaPlot 11.0, Systat Software, Inc. San Jose CA, USA).Tukey post-hoc tests were performed to assess differences within and between conditions at specific time points. Paired t-tests were used to compare the treadmill incline, cumulative caloric expenditure, relative VO_2, and RER between conditions over the entire exercise period. Baseline hematocrit between

trials was also assessed via paired t-tests. Unless otherwise stated, all data are presented as mean ± standard error (SE), and exact P values are reported for all comparisons.

RESULTS

Thirteen healthy, non-smoking subjects (7M/6F, age 29 ± 2 years, height 169 ± 2 cm, weight 70 ± 4 kg, body mass index 24.4 ± 0.8 kg/m^2) participated in this study. The average HR_{max} was 189 ± 2 bpm, and the average VO_{2peak} was 36.0 ± 1.9 ml/kg/min. There was no difference in baseline hematocrit between conditions (41 ± 1 % for both conditions, P=0.98). All subjects achieved the target HR intensity of 65-70% HR_{max} in both conditions, and they were able to maintain this intensity throughout the 40-min of exercise (Figure 1A; data previously published (34)). Although the treadmill speed remained constant at 4 km/h in both conditions, subjects achieved this target HR intensity at a lower treadmill incline with BFRE compared to CE (7.3 ± 0.3^0 for CE vs. 6.0 ± 0.3^0 for BFRE, P<0.001; data previously published (34); Figure 1B). For one subject, the treadmill speed was adjusted below 4 km/h (with a grade of 0%) during the occlusion periods of the BFRE trial to prevent their HR from rising above the target range.

Overall, VO_2 and VCO_2 were higher with CE vs. BFRE during the 40-min of exercise (P=0.008, condition effect for VO_2 and VCO_2; Figure 1 C & D). VO_2 and VCO_2 increased at the onset of exercise for both conditions (P<0.001) and remained elevated from baseline throughout the 40-min of exercise (P<0.001). Average relative VO_2 was also higher with CE compared to BFRE throughout exercise (CE, 15.7 ± 0.4 ml/kg/min vs. BFRE, 14.2 ± 0.4 ml/kg/min, P<0.001).

The higher VO_2 and VCO_2 with CE was associated with a higher caloric expenditure at each exercise time point (P=0.007; Figure 2A).Overall, there were no differences in the RER between conditions (P=0.91 for condition; Figure 2B). For both conditions, RER initially decreased at the onset of exercise (i.e., first 5-min, $P \leq 0.03$), then increased above baseline values over the next 5-min of exercise ($P \leq 0.002$ vs. baseline), followed by a progressive decrease, approximating baseline values by the end of exercise bout.

The cumulative caloric expenditure for the entire exercise bout was higher with CE vs. BFRE (243.4 ± 17.4 kcals for CE vs. 220.1 ± 18.4 kcals for BFRE, P=0.01; Figure 2C). Despite this elevated caloric expenditure, however, there was no difference in cumulative RER between conditions (0.89 ± 0.02 for CE vs. 0.88 ± 0.01 for BFRE, P=0.84; Figure 2C), indicating a preferential use of carbohydrates over fat in both trials, with no differences in substrate utilization between conditions.

DISCUSSION

In this investigation we compared the systemic metabolic responses between an acute bout of aerobic cyclical BFRE and CE when both conditions were matched for HR. To our knowledge, this is the first time that the metabolic responses to this novel, cyclical BFRE paradigm have been reported. Contrary to our hypothesis, BFRE elicited an attenuated metabolic response compared to CE, including lower VO_2 and VCO_2 and, subsequently, slightly reduced cumulative caloric expenditure. There were no differences in RER between trials, however, indicating equivalent substrate utilization between conditions.

We originally hypothesized that the hypoxic environment of the muscle with BFRE, coupled with an increased sympathetic drive, would increase caloric expenditure. Cuff inflation

likely decreased the supply of oxygenated blood to the muscle, and also elicited a sympatho-excitatory response due to stimulation of the type III and IV afferent nerve fibers (33); circulating norepinephrine was higher with BFRE (see (34)). This augmented sympatho-excitatory response, however, also provoked an increase in HR which necessitated a decrease in the treadmill grade during the BFRE condition to ensure adequate HR matching between trials. The reduction in treadmill grade reduced the metabolic demand of the active leg muscles, which ultimately caused VO_2 and VCO_2 to decrease with the BFRE condition. The notion that HR would be higher with cuff inflation is supported by previous work by Abe et al. who reported higher HR and VO_2 responses with aerobic BFRE treadmill walking compared to a control walking condition without blood flow restriction at the same absolute intensity (3 km/h) (2). Similarly, Sugawara et al. reported greater HR responses to 2-min bouts of BFRE treadmill walking compared to a control condition performed at the same speed (3.2 km/h) (36). Although we did observe slight differences in HR between conditions at baseline and during the first three cuff deflation periods (≤3 bpm between conditions), it is important to note that HR was maintained within the prescribed intensity of 65-70% HR_{max} in both conditions throughout the entire 40-min of exercise.

The observation that RER remained above 0.85 for the majority of both exercise bouts indicates a preferential usage of carbohydrates over fat (4). As there were no differences in RER between conditions, this suggests equivalent substrate utilization between trials. These results are in contrast to those reported by Karabulut & Garcia who observed a higher RER with BFRE compared to CE during steady state cycling in obese individuals (16). As HR was not matched between conditions, however, the BFRE condition also elicited a greater HR response, and increased caloric expenditure compared with the control condition (16). These differences in

exercise intensity between conditions likely explain the divergent results between the study by Karabulut & Garcia and the present investigation, as higher intensities of exercise are associated with a greater reliance on carbohydrates as fuel rather than fats (7, 14, 31), and hence a higher RER (40). The initial decrease in RER observed at the onset of exercise in both conditions is similar to results reported in other investigations that have assessed acute RER responses at the onset of submaximal exercise (37, 40). Importantly, as a metric of substrate utilization, RER is not valid until a steady-state is reached (11), so this response during the transition phase from rest to exercise may not be reliable. The observation that RER continued to decrease as exercise duration progressed, is consistent with previous reports assessing substrate utilization responses to steady state submaximal exercise (9, 28, 30, 39), and is in line with the general understanding that fatty acid mobilization and utilization increase as a function of exercise duration (7, 31).

The higher caloric expenditure observed with CE compared to BFRE is in contrast to several other investigations that demonstrated greater caloric expenditure with BFRE compared to CE, measured directly (16) or indirectly via reporting of VO_2 (2, 22). While CE did result in greater caloric expenditure than BFRE, the difference between conditions was only ~25 kcals on average (9.9 ± 3.6%) over the 40-min of exercise. When considering the duration of the exercise bout (40-min) and the lower treadmill incline used to elicit this response with blood flow restriction, these findings could still have important implications for individuals who cannot tolerate the higher mechanical workloads (i.e., treadmill incline) associated with conventional exercise modalities. This notion is further reinforced by the equivalent substrate utilization between trials.

There are several methodological considerations that should be addressed. First, although the focus of this investigation was on metabolic responses to BFRE, conditions were matched on

HR, rather than VO_2. As previously described, this HR-matching approach likely explains the reduced metabolic demand observed in the BFRE condition (i.e., lower VO_2 and caloric expenditure). We chose to match intensities based on HR to facilitate translation of cyclical BFRE into an applied setting, where prescribing exercise based on HR responses is common practice (e.g., in the gym or rehabilitation facility). It is important to note, however, that although HR was matched between conditions, the lower oxygen consumption elicited by BFRE may indeed result in an attenuated stimulus for positive physiological adaptations to exercise (e.g. aerobic capacity, mitochondrial density) compared to CE. As this investigation only assessed the acute responses to a single bout of cyclical BFRE, future work should seek to explore the long-term training adaptations induced by regular performance of this novel exercise modality.

Second, it is possible that the use of the same cuff pressures and widths between subjects resulted in inter-individual differences in the degree of blood flow restriction achieved. Other aerobic BFRE studies have used cuffs that range from 8-18 cm (6, 35), and there is no general standard as to the appropriate cuff width or pressure that should be used for BFRE. There is, however, evidence to suggest that varying cuff widths (13) and pressures (17) can influence the degree of blood flow restriction attained. Based on the different approaches used between studies, caution should be taken when comparing our findings to others. In future studies planned in our laboratory, we will be using a cuff inflation system that can be set at a relative pressure based on maximal occlusion pressure for each individual. This approach will ensure that the same relative stimulus is applied to each subject.

Third, while we designed the study with a one-month interval between trials to control for menstrual cycle phase in our female subjects, it is possible that this could have increased the variability in responses between trials. We opted to apply this same one-month break between

experiments for the male subjects to control for between subject variability. As the order of experiments was randomized, however, it is unlikely that this element of our experimental design had an effect on the results.

Finally, as our subject pool consisted of relatively young and healthy individuals, it is possible that these results cannot be extended to more clinically relevant patient populations, who may benefit from the lower workloads used in the BFRE condition. Metabolic responses and substrate utilization patterns have been previously demonstrated to shift with age (23) and vary across disease pathologies (3, 5, 10, 12, 29). As such, future work is required to determine if this novel exercise modality is still relevant for these clinical applications.

Conclusions

In this investigation we utilized a novel aerobic BFRE paradigm consisting of cyclical bouts of blood flow restriction and reperfusion. We demonstrated that this mode of exercise required a lower mechanical workload when both conditions were matched for HR, subsequently eliciting a slightly reduced metabolic demand compared to CE. Despite this reduced demand, however, the differences in caloric expenditure between trials were negligible from a practical standpoint, and there were no differences in substrate utilization between trials. This novel exercise modality could potentially be used by individuals in clinical settings who may find the lower workloads more tolerable than conventional exercise.

ADDITIONAL INFORMATION

Competing Interests: There were no conflicts of interest or competing interests for either author of this manuscript.

AUTHOR CONTRIBUTIONS

This study was performed in the Cerebral and Cardiovascular Physiology Laboratory (PI: Caroline Rickards) at the University of North Texas Health Science Center in Fort Worth, TX. Both JDS and CAR contributed to 1) the conception or design of the work 2) the acquisition, analysis, and interpretation of data, and 3) drafting and revising the manuscript. Both JDS and CAR have approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Lastly, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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FIGURES

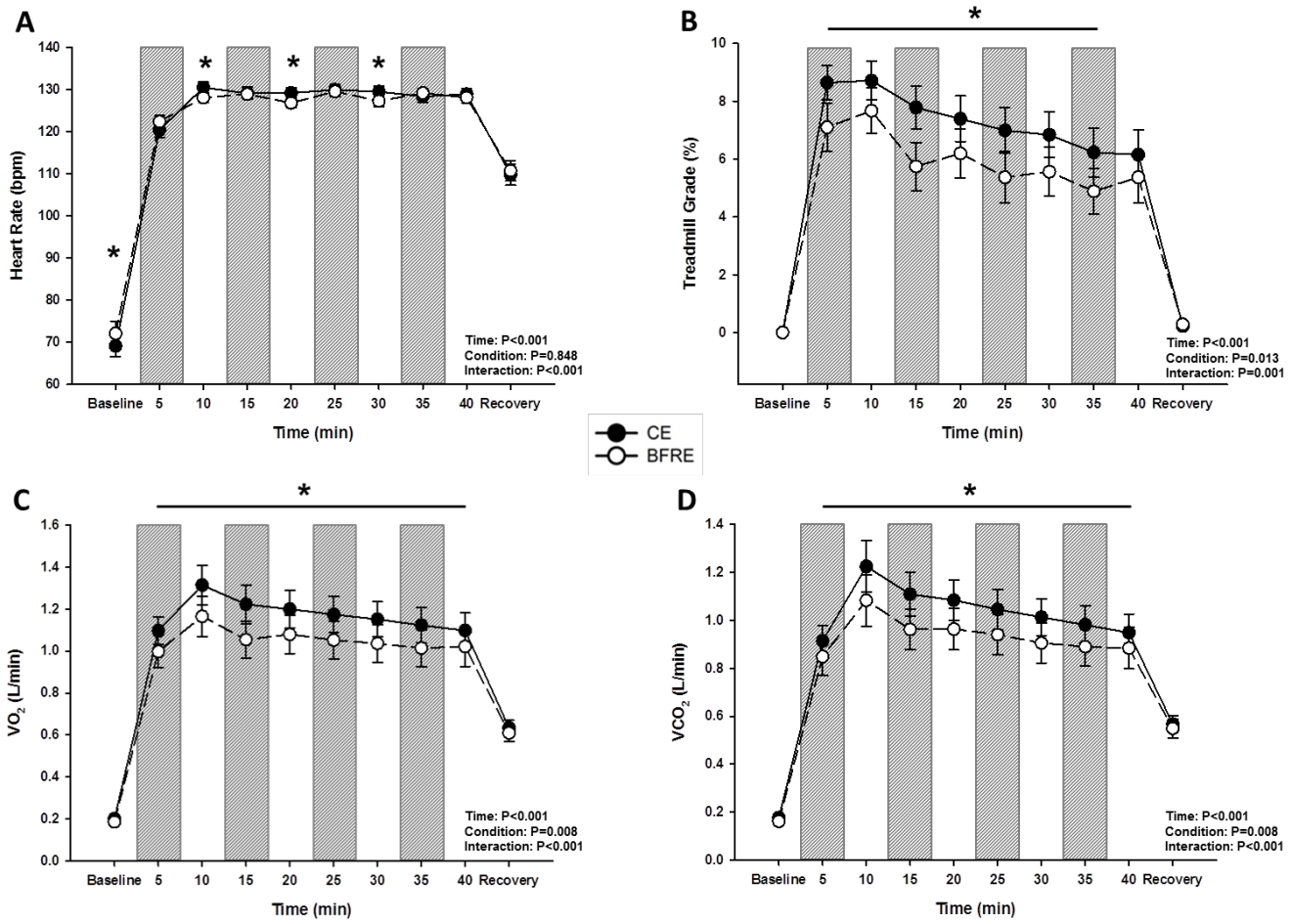

Figure 1: Responses to conventional exercise (CE; ●, continuous line) and blood flow restriction exercise (BFRE; ○, dashed line). Cuff inflation periods denoted by vertical grey bars. Heart rate (Panel A) increased with exercise (P<0.001) and was slightly higher with CE vs. BFRE at baseline and during the first 3 cuff deflation periods (P≤0.08). Treadmill grade (Panel B) was lower with BFRE compared to CE (condition effect, P=0.013). VO_2 (Panel C) and VCO_2 (Panel D) increased with exercise (P<0.001) and were higher with CE compared to BFRE throughout the interventions (condition effect, P=0.008 for both). Two-way repeated measures ANOVAs were performed to compare differences across time (factor 1) and between conditions (factor 2). Tukey post-hoc tests were performed to assess differences between conditions at specific time points. *, P≤0.09 for CE vs. BFRE.

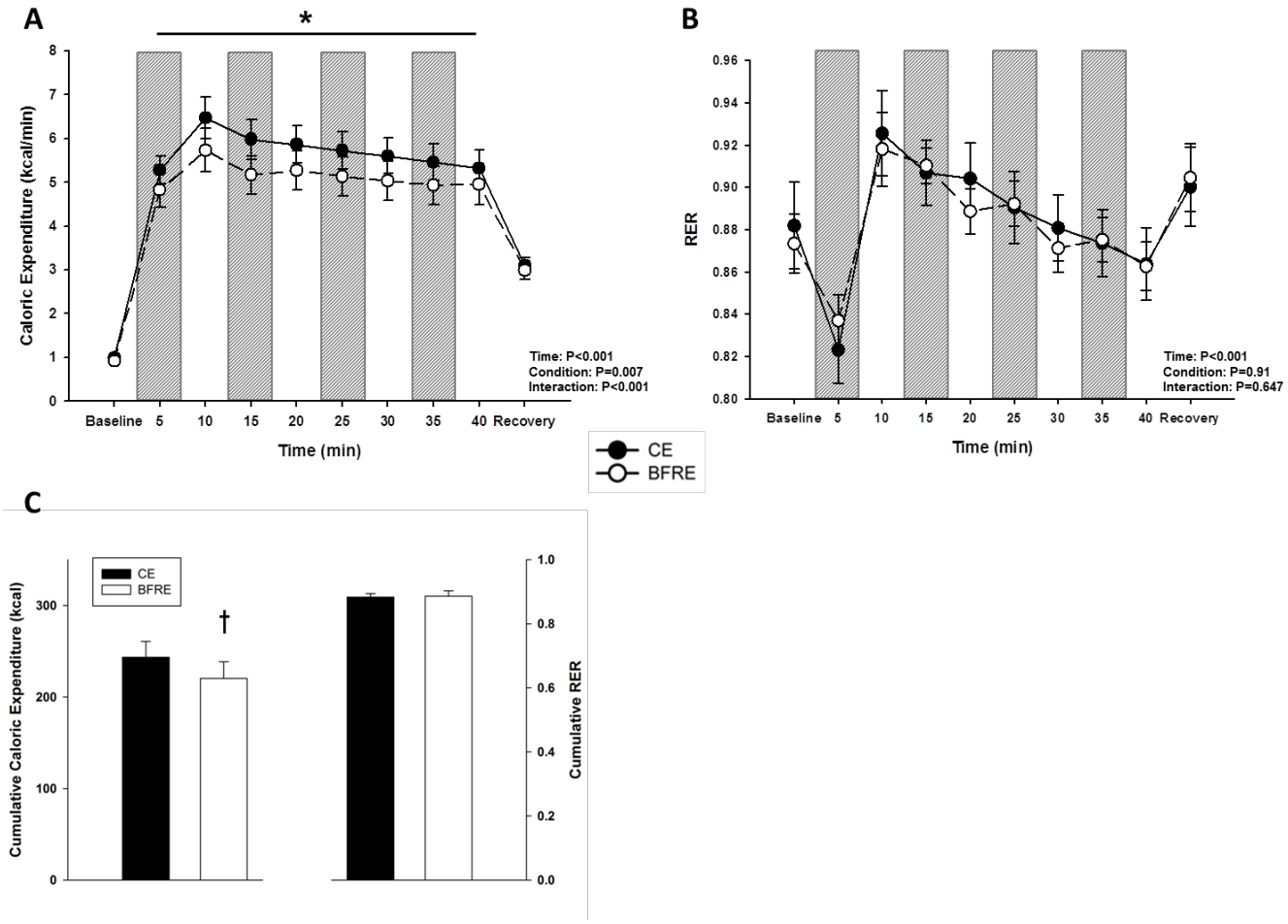
A
*
Caloric Expenditure (kcal/min)
Time (min)
Baseline 5 10 15 20 25 30 35 40 Recovery
Time: P<0.001
Condition: P=0.007
Interaction: P<0.001
B
RER
Time (min)
Time: P<0.001
Condition: P=0.91
Interaction: P=0.647
CE
BFRE
C
CE
BFRE
†
Cumulative Caloric Expenditure (kcal)
Cumulative RER

Figure 2: Caloric expenditure and respiratory exchange ratio responses to conventional exercise (CE; ●, continuous line) and blood flow restriction exercise (BFRE; ○, dashed line). Cuff inflation periods denoted by vertical grey bars. Caloric expenditure (Panel A) increased with exercise ($P \leq 0.001$ for both BFRE and CE) and was higher with CE compared to BFRE (condition effect, $P=0.007$). The Respiratory Exchange Ratio (RER, Panel B) initially decreased at the onset of exercise, then increased in both conditions by the end of the second 5-min period, followed by a progressive fall to baseline values by the end of exercise; there were no differences between conditions (condition effect, $P=0.91$). Cumulative caloric expenditure over the entire exercise bout was greater (†, $P=0.01$) with CE compared to BFRE (Panel C; left). There was no difference ($P=0.84$) in cumulative RER during exercise between conditions (Panel C; right). The data in panels A and B were analyzed via two-way repeated measures ANOVAs to compare differences across time (factor 1) and between conditions (factor 2). Tukey post-hoc tests were performed to assess differences between conditions at specific time points. Paired t-tests were performed to compare cumulative caloric expenditure and RER between conditions (data in Panel C). *, $P \leq 0.07$ for CE vs BFRE.

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CHAPTER V

Cytokine and Nitric Oxide Responses to an Acute Bout of Cyclical Blood Flow Restriction Exercise

Justin D. Sprick, Steven A. Romero, & Caroline A. Rickards

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ABSTRACT

Both remote ischemic preconditioning (RIPC) and exercise have been independently associated with cardio-protection, mediated, in part, by nitric oxide (NO) and the cytokines interleukin-10 (IL-10) and interleukin-6 (IL-6). Based on these similarities, we combined RIPC with exercise in a novel exercise paradigm, cyclical blood flow restriction exercise (BFRE), and hypothesized that cyclical BFRE would augment the release of nitrite (an NO metabolite), IL-10, and IL-6. Thirteen healthy subjects (6M/7F) completed 5 experiments (≥1 month apart): RIPC (4 x 5-min bilateral thigh cuff inflations/deflations to 220 mmHg), conventional aerobic exercise [CE_{AERO}: 40-min treadmill walking at 65-70% maximal heart rate, (HR_{max})], cyclical blood flow restriction aerobic exercise (C-$BFRE_{AERO}$: 40-min treadmill walking at 65-70% HR_{max} with superimposed RIPC), conventional resistance exercise [CE_{RES}: 4 x 3 sets of 10 repetitions at 65% 1-repetition maximum (1RM)], and cyclical blood flow restriction resistance exercise (C-$BFRE_{RES}$: 4 x 3 sets of 10 repetitions at 20% 1RM with superimposed RIPC). Venous blood sampling was performed pre- and post-protocol. IL-6 only increased with CE_{AERO} (Pre: 0.26±0.03 pg/ml, Post: 0.40±0.07 pg/ml, P=0.03), and there were no changes in IL-6, IL-10 or nitrite with any condition (P≥0.75). A sub-study (N=7) indicated that thigh cuff inflation to 220 mmHg at rest elicited a 10.7-70.5% reduction in superficial femoral artery flow (mean=47.2±9.3%). Contrary to our hypothesis, cyclical BFRE did not result in an augmented release of cytokines or NO. This may be due to the

timing of blood sampling, and/or the large variability in degree of blood flow restriction between subjects.

INTRODUCTION

Remote ischemic preconditioning (RIPC) is a nonpharmacological therapy characterized by repeated cycles of limb cuff inflation-deflation (58). This therapy was developed as a cardio-protective strategy to attenuate the damage incurred from ischemia-reperfusion injury (10, 58). Recently, routine performance of RIPC has also been shown to provide cardiovascular benefit even in the absence of injury, such as enhancements in vascular function in healthy humans (30, 31). Exercise is also a commonly used therapy that confers protection from ischemia-reperfusion injury (1, 44, 57), and the long-term cardiovascular benefits of exercise are well established (60, 61). Importantly, RIPC and exercise share many similarities in the signal transduction that mediates these beneficial effects, such as the acute production of both cytokines (7, 25, 34, 44) and nitric oxide (NO) (8, 9, 59). Interestingly, some studies have even suggested that crosstalk exists between cytokine and NO signaling (65). In skeletal muscle, NO has been demonstrated to upregulate the expression of interleukin-6 (IL-6) in response to exercise (65), and in the myocardium, interleukin-10 (IL-10) has been shown to upregulate the expression of endothelial nitric oxide synthase (eNOS) in response to RIPC (7). Taken together, these similarities in signaling provide a molecular link that is common to both RIPC and exercise.

Evidence for the role of cytokines in RIPC-induced cardio-protection comes from animal studies which have demonstrated a reduction in infarct size with RIPC that was accompanied by increased plasma IL-10 concentrations (6, 7). Furthermore, the cardio-protection associated with RIPC is abrogated in IL-10 knock-out mice (7). Similarly, IL-6 has also been implicated as a

mediator of exercise-induced cardio-protection, as this cardio-protection is not present in IL-6 knock-out mice (44). Studies directly linking IL-6 to the cardio-protection associated with RIPC are lacking, but this is certainly possible based on the role of IL-6 in mediating exercise-induced cardio-protection (44). Similarly, while IL-10 has not been directly linked to exercise-induced cardio-protection, plasma IL-10 is acutely elevated in response to exercise (16, 28, 50), thus it is plausible that it could confer cardio-protection, based on the role of IL-10 in RIPC induced-cardio-protection (7). Furthermore, as skeletal muscle has recently been identified as a source of cytokine production (i.e. myokines) (15, 25, 34), it is possible that other cytokines produced in muscle could also be linked to the cardio-protective effects of exercise.

NO has been linked to the cardio-protection induced by RIPC in a number of studies (2, 32, 59). NO release is stimulated by the shear stress that occurs with rapid cuff deflation during the reperfusion periods of RIPC (i.e., reactive hyperemia) (59). Once formed, NO is rapidly oxidized to nitrite, where it circulates systemically (12). Nitrite is then reduced back to NO by cardiac myoglobin, allowing it to subsequently modify complex I of the electron transport chain (11). This modification exerts its protective effects by dampening the production of reactive oxygen species (ROS) during reperfusion (11), and ultimately inhibiting the opening of the mitochondrial permeability transition pore (MPTP), a hallmark of ischemia-reperfusion injury (23, 52). Importantly, NO formation with exercise has also been implicated in exercise-induced cardio-protection (8, 9). Four weeks of voluntary exercise in mice resulted in increased NO metabolites in muscle, plasma, and the myocardium, and a reduction in infarct size during a subsequent ischemia-reperfusion injury (9). When this experiment was subsequently performed in endothelial nitric oxide synthase (eNOS) knock-out mice, the reduction in infarct size was not

present (9). Collectively, these studies suggest that NO mediated signaling plays a critical role in the cardio-protection afforded by both RIPC, and exercise.

Blood flow restriction exercise (BFRE) is a novel exercise modality that shares similarities with RIPC. This form of exercise also uses limb occlusion via cuff inflation, and was originally developed to augment the muscle hypertrophic effects of exercise (38). In contrast to RIPC which uses multiple cycles of limb cuff inflation and deflation, BFRE is typically characterized by continuous cuff inflation throughout the entire exercise bout. Importantly, lower loads (i.e., 20-30% of 1 repetition maximum,1RM) are commonly used with BFRE (38), in contrast to the 65-80% 1RM that is typically prescribed with conventional exercise (CE) (53). While the cardio-protective effects of exercise have been almost entirely demonstrated with aerobic training modalities, BFRE is more commonly performed with resistance training. As acute cytokine production has been associated with aerobic (13, 75) and resistance exercise (28), as well as RIPC (7), it is possible that superimposing the RIPC stimulus onto either form of exercise (i.e. aerobic or resistance) could result in an additive release of cytokines. Similarly, as NO release has also been associated with aerobic exercise (20, 49) resistance exercise (35), and RIPC (59), it is also likely that the combination of RIPC and exercise would augment the release of NO. Such an augmented release of these factors could potentially result in an even greater stimulus for cardio-protection. We adapted BFRE to more closely resemble the RIPC paradigm, by using cyclical cuff inflations. We hypothesized that this novel form of cyclical BFRE would augment the release of NO (indexed by nitrite), IL-10, and IL-6 compared to RIPC or exercise alone.

METHODS

This study was part of a larger project in which we comprehensively assessed sympathetic, hemodynamic, and cerebrovascular responses to cyclical BFRE (aerobic and resistance) and RIPC; these data have been published elsewhere (63, 64).

Subjects

Young, healthy volunteers participated in this study conducted at the University of North Texas Health Science Center (UNTHSC) in Fort Worth, TX. All experimental procedures were conducted in accordance with a protocol approved by the UNTHSC Institutional Review Board (IRB # 2014-149). Before participation, all subjects underwent a medical evaluation, including seated and standing 12-lead electrocardiogram (ECG) and blood pressure measurements, and were cleared to participate by a physician. Subjects did not routinely use any nicotine products (including tobacco cigarettes, electronic cigarettes, chewing tobacco). Before each experiment, subjects abstained from caffeine, alcohol, dietary supplements, medications, and exercise for 24 h and fasted for at least 8 h (overnight). Female subjects completed a urine pregnancy test to ensure they were not pregnant. All subjects underwent a familiarization session in which they were shown all equipment and experimental procedures that would be performed in the subsequent experimental sessions. Each subject gave written informed consent to participate in this study.

Maximal Exercise Testing

All subjects underwent a 1-repetition maximum (1 RM) test on a leg press machine (VR3, Cybex, Medway, MA) before a maximal aerobic exercise test [data previously published (63, 64)]. As the maximum load of the leg press machine is 184 kg, individuals who had a 1 RM greater

than 184 kg were excluded from participation in this study. After a 1-h rest period, peak oxygen uptake (VO_{2peak}) was assessed via a treadmill test (TMX428CP, TrackMaster, Newton, KS) in accordance with the Bruce Protocol (53). Testing was terminated when subjects reached volitional fatigue. To recruit a relatively homogenous subject population in regards to cardiovascular fitness, only subjects with a VO_{2peak} between 30 and 50 $ml \cdot kg^{-1} \cdot min^{-1}$ were included for participation in this study.

Experimental Protocols

At least 2 weeks after completion of the maximal exercise testing session, subjects reported back to the laboratory for one of five experimental sessions 1) cyclical blood flow restriction aerobic exercise ($C\text{-}BFRE_{AERO}$); 2) conventional aerobic exercise (CE_{AERO}); 3) cyclical blood flow restriction resistance exercise ($C\text{-}BFRE_{RES}$); 4) conventional resistance exercise (CE_{RES}), and; 5) remote ischemic preconditioning (RIPC). Subjects completed each experimental session in a block-randomized, cross over design, with at least 1 month intervening between sessions. Female subjects were tested in the early follicular phase of their menstrual cycle (first 4 days determined by self-report), and completed a urine pregnancy test at the start of each visit to the laboratory to ensure they were not pregnant. All sessions were performed in a thermo-neutral laboratory (temperature = 23.1 ± 0.1 °C, humidity = 49.7 ± 2 %, barometric pressure = 744.4 ± 0.7 mmHg).

Instrumentation

Upon arrival to the laboratory, subjects were encouraged to empty their bladder to ensure optimal comfort and to limit the potential confounding effects of increased sympathetic nervous

system activation with bladder distension (17). While non-invasive arterial pressure (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands) and ECG (shielded leads, cable, and amplifier, AD Instruments, Bella Vista, NSW, Australia) data were collected continuously, these data have been previously published in related papers (63, 64). A venous catheter was inserted into an antecubital vein of the arm contralateral to the blood pressure measurements for serial collection of venous blood samples. During the blood flow restriction protocols (C-BFRE$_{AERO}$, C-BFRE$_{RES}$, and RIPC), 5-cm wide inflatable cuffs (SC5, D.E. Hokanson, Bellevue, WA) were placed around both upper thighs, secured in place with tape, and connected to an inflation system (E20 Rapid Cuff Inflation System, D.E. Hokanson, Bellevue, WA). During the aerobic exercise protocols (C-BFRE$_{AERO}$ and CE$_{AERO}$), a wireless heart rate monitor (wireless strap; Polar H1 Series, Polar Electro Oy, Kempele, Finland) was used to verify that subjects were within their target heart rate range (65-70% HR_{max}) for the duration of the experiment.

Cyclical Blood Flow Restriction Aerobic Exercise Session (C-BFRE$_{AERO}$)

Following instrumentation, subjects underwent a 15-min seated baseline, after which they were moved onto the treadmill for the 40-min exercise bout. At the start of exercise, the thigh cuffs were rapidly inflated to 220 mmHg and the treadmill speed was adjusted to a target of 4 km/h (2.5 mph). While the treadmill speed remained constant (by design), the incline (% grade) was adjusted throughout the exercise bout to achieve the target HR intensity corresponding to 65-70% of HR_{max}. The cuffs remained inflated at 220 mmHg for 5-min followed by a 5-min deflation and reperfusion period. This cyclical occlusion and reperfusion protocol was repeated for four cycles throughout the 40-min of exercise. After the 40-min of exercise was completed, the subjects immediately commenced a 5-min active recovery period during which the treadmill

speed was reduced to 2.4 km/h (1.5 mph) and the incline was reduced to 0°. Three blood samples (10 ml each) were collected throughout the protocol: 1) 5-min into baseline ("pre"), 2) at the end of the second occlusion period ("mid") and, 3) at the end of the final reperfusion period ("post").

Conventional Aerobic Exercise Session **(CE_{AERO})**

This protocol was performed in exactly the same manner as the BFRE session with the exception that there were no thigh cuffs applied and hence no occlusive stimulus. Exercise intensity was set at 65-70% of HR_{max}, achieved with a treadmill speed of 4 km/h (2.5 mph) and variable incline, as previously described. Blood samples were collected at the same time points as the C-$BFRE_{AERO}$ condition.

Cyclical Blood Flow Restriction Resistance Exercise Session **(C-$BFRE_{RES}$)**

After instrumentation, a 15-min baseline period commenced during which subjects were seated on the leg press machine with their feet placed on the ground. During the final minute of rest, subjects placed their feet onto the leg press platform (knee at a ~90° angle), and 10 s before commencement of exercise, the bilateral thigh cuffs were rapidly inflated to a target pressure of 220 mmHg. The exercise session was 40-min in duration, divided into 4 x 10-min blocks (figure 1). Each 10-min block consisted of a 5-min exercise period with cuffs inflated, and a 5-min recovery/reperfusion periods with cuffs deflated. The exercise period consisted of 3 sets of 10 repetitions with a load corresponding to 20% of 1RM. Each repetition was performed at a pace of 4 s/repetition (2 s concentric contraction, 2 s eccentric contraction; timed via a metronome), and each set was separated by 1-min rest periods. The thigh cuffs remained inflated throughout the 5-min exercise period, including the 1-min rest periods. At the end of the 5-min exercise

period, the thigh cuffs were rapidly deflated, and subjects placed their feet back on the floor and rested quietly for a 5-min reperfusion period. An additional 5-min recovery period followed completion of the 40-min of exercise. Blood samples were collected at the same three time points as the other conditions.

***Conventional Resistance Exercise Session* (CE_{RES})**

This session was performed in exactly the same manner as the C-$BFRE_{RES}$ condition but without use of the inflatable thigh cuffs, and with a higher leg press load of 65% of 1 RM per conventional guidelines (53). The timing of the exercise periods and rest periods were identical to the C-$BFRE_{RES}$ condition, and blood samples were collected at the same time points.

Remote Ischemic Preconditioning Session

This session served as a control condition to isolate the effects of the repeated cuff occlusions and reperfusions, independent of exercise. Following instrumentation, subjects completed a 15-min seated baseline. The thigh cuffs were then rapidly inflated to 220 mmHg for 5 min, followed by rapid deflation and reperfusion for 5 min. This occlusion/reperfusion protocol was repeated four times over 40 min followed by a 5-min recovery period. Blood samples were collected at the same time points as the exercise conditions.

Vascular Ultrasound Assessments

In order to quantify the degree of arterial inflow reduction achieved with the application of 220 mmHg cuff pressure, a subset of subjects (N=7) were brought back into the laboratory for a separate experimental session. This session took place 1-14 months after completion of the

final experimental session (mean = 7 ± 2 months). During this session, subjects were again instrumented with 5 cm inflatable cuffs around both upper thighs (as used in the previous experiments), while blood velocity and diameter of the superficial femoral artery (SFA) were measured via Doppler ultrasound (Vivid T8, GE Healthcare, Milwaukee, WI). Following a 10-min baseline period, both thigh cuffs were rapidly inflated to a cuff pressure of 220 mmHg for 1-min. SFA velocity and diameter were measured during the final 30 s of baseline and the final 30 s of the cuff inflation period. Diameters were subsequently assessed offline via the use of digital calipers on the ultrasound machine. Triplicate measures were performed by an experienced investigator (S.A.R.) during the end-diastolic phase of the cardiac cycle, and the mean diameter measurement was used for subsequent SFA flow calculations. SFA flow was calculated using the equation [time averaged mean velocity x [$(\text{radius})^2 \times \pi$]].

Blood Sample Collection

Whole blood was collected into a chilled syringe before being transferred to individual collection tubes containing either citrate (for nitrate/nitrate analysis) or EDTA (for cytokine analysis). These tubes were immediately centrifuged at 1500 RPM at 4°C for 10-min (nitrate/nitrite) or 15-min (cytokines). Following centrifugation, plasma was separated into micro-centrifuge tubes, and snap frozen in liquid nitrogen prior to being stored at -80 °C until further analysis.

Hematocrit

Hematocrit was assessed from the baseline "pre" blood sample via micro-centrifugation (Clay Adams MHCT II, Becton Dickinson and Company, Parsippany, New Jersey) to ensure

adequate and similar hydration status between each trial. Duplicate measurements were performed, and the mean of these measurements were used for comparison.

Cytokine Analysis

Plasma samples were analyzed for cytokines via multiplex ELISA (Meso Scale Diagnostics, Rockville, MD) at the Translational Aging Research Program Laboratory at The University of North Texas Health Science Center (PI: Dr. Sid O'Bryant). The following markers were analyzed (see table 1 for definitions): IL-6, IL-10, tumor necrosis factor α (TNF-α), C-reactive protein (CRP), serum amyloid-A (SAA), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), and brain derived neurotrophic factor (BDNF). All analyses were performed via an automated workstation (Hamilton STAR Plus, Reno NV) and with automated plate washing between incubations (BioTek 405, Winooski, VT). All samples were measured in duplicate and only samples with a coefficient of variation <20% were included in the final results.

Nitrite Analysis

Plasma samples were analyzed via ozone-based chemiluminescence at the NO Metabolomics Facility at the University of Pittsburgh (PI: Dr. Sruti Shiva). This method has been previously described (39, 55), and allows for quantification of plasma nitrite, a biomarker of NO bioavailability. One measurement was made for each sample.

Statistical Analysis

For the cytokine and nitrite analysis, two separate two-way linear mixed model analyses with repeated measures (*factor 1*: time, *factor 2*: condition) were used to compare the effects within and between conditions over time (JMP Pro 10; SAS Institute Inc. Cary, NC, USA). As we were primarily concerned with comparing the effects of cyclical BFRE to CE and RIPC within each exercise modality (aerobic or resistance), one analysis grouped the RIPC condition with the two resistance conditions (CE_{RES} and C-$BFRE_{RES}$), while another analysis grouped RIPC with the two aerobic conditions (CE_{AERO} and $BFRE_{AERO}$). Tukey post hoc tests were performed following the linear mixed model analyses. Prior to these analyses, a Grubbs test for outliers was performed for each analyte at each time point within each condition, and outliers were removed accordingly. Separate one-way linear mixed model analyses with repeated measures (condition only) were used to compare baseline hematocrit between conditions. Conditions were grouped in the same way for these comparisons (analysis 1: RIPC, CE_{RES}, C-$BFRE_{RES}$; analysis 2: RIPC, $BFRE_{AERO}$ and CE_{AERO}). Paired two-tailed t-tests were used to compare SFA flow in response to 220 mmHg cuff inflation during the vascular ultrasound experiment. Linear regression analysis was used to assess the relationships between the reductions in SFA flow during the vascular ultrasound assessment to the change in cytokines at the "post" time point during the RIPC experiment, and Pearson correlations (r-values), *P*-values, and slopes were determined for each comparison (SigmaPlot 11.0, Systat Software, Inc. San Jose CA, USA). Exact *P* values are reported for all comparisons. Unless otherwise stated, all data are presented as means ± SE.

RESULTS

Twenty-four subjects were originally recruited to participate in this study. Of these 24 subjects, 2 were excluded due to VO_{2peak} <30 ml·kg^{-1}·min^{-1}, 3 were excluded due to 1RM > 184kg, 2 were excluded due to medication use, and 2 withdrew due to scheduling or personal reasons. Of the remaining 15 subjects, 14 completed all 5 experiments, and 1 subject only completed 3 of the experimental conditions (RIPC, CE_{RES}, C-$BFRE_{RES}$). Of these 14 subjects, analysis for cytokine and inflammatory markers were only measured on 13 subjects (6M/7F, age 28 ± 2 y, height 169 ± 2 cm, weight 71 ± 4 kg, body mass index (BMI) 24.5 ± 0.8 kg/m^2) due to issues associated with the optimization of the assays used for these analyses. Specifically, we initially planned to analyze samples for only IL-10 using a standard commercially available ELISA. However, due to difficulties in optimizing this approach, and the superiority of the multiplex ELISA method once for analyzing multiple cytokines, we chose to analyze our remaining samples with this more comprehensive approach. As some samples had already been analyzed with the IL-10 ELISA kit, the overall N is reduced from 14 to 13 for the final cytokine analysis with the multiplex assays. Similarly, nitrite analysis was initially performed with a commercially available colorimetric kit. Due to concerns about the sensitivity of this method, we opted to utilize a more sensitive approach for the remaining samples to ensure that the sensitivity of the assay was appropriate for the anticipated nitrite concentrations in our samples. As a result, this final analysis was only performed on a subset of 7 subjects. There were no differences in hematocrit between conditions for either of the two comparisons (P=0.53 for RIPC, CE_{RES}, C-$BFRE_{RES}$; P=0.31 for RIPC, $BFRE_{AERO}$ and CE_{AERO}). Additionally, all hematocrit values were within the normal range for all conditions (C-$BFRE_{AERO}$: 39.9 ± 0.8 %, CE_{AERO}: 40.3 ± 1.1 %, C-$BFRE_{RES}$: 41.8 ± 1.0 %, CE_{RES}: 41.9 ± 1.1 %, RIPC: 40.8 ± 1.3 %).

Of all the cytokines measured, only IL-6 changed from baseline, with an increase during the CE_{AERO} condition only (P=0.03; table 2). IL-6 did not change from baseline under any other condition (P≥0.75). During the vascular ultrasound assessment, rapid cuff inflation to 220 mmHg elicited a 47.2 ± 9.3% reduction in SFA flow (P=0.01; figure 2). We also observed high variability between subjects when assessing the individual responses; the percent reduction in SFA flow ranged from 10.7-70.5 %. As such, the relationships between the change in cytokine concentrations at the "post" time point during the RIPC experiment and the change in SFA flow with cuff inflation during the vascular ultrasound experiment were assessed. The data in table 3 shows statistically meaningful associations for the reductions in SFA flow and the increases in TNF-α and sICAM-1.

There were no changes in plasma nitrite under any of the experimental conditions (table 2). Unfortunately, the limited sample size for the nitrite analysis did not allow for comparisons between the change in SFA flow and the change plasma nitrite (N=2 for both nitrite and SFA flow data).

DISCUSSION

In the current investigation, we tested the hypothesis that cyclical BFRE would result in an augmented production of NO (measured via plasma nitrite) and the cytokines IL-10 and IL-6 compared to RIPC or conventional exercise alone. We also conducted an exploratory assessment of the systemic release of several other cytokines in response to an acute bout of cyclical BFRE. Furthermore, in a subset of subjects, we quantified the degree of reduction in SFA flow in response to a standard thigh cuff pressure of 220 mmHg. Contrary to our hypothesis, cyclical BFRE did not result in an augmented release of circulating IL-10, IL-6, or nitrite. We also

observed no changes in any of the other cytokines that were explored (TNF-α, CRP, SAA, sICAM-1, sVCAM-1, and BDNF). Lastly, we report that thigh cuff inflation to 220 mmHg resulted in a ~47% reduction in SFA flow in our subjects, but there was large inter-subject variability in the degree of blood flow restriction achieved with this standardized restrictive stimulus.

The fact that we observed no change in the release of cytokines with cyclical BFRE is surprising, given that cytokine release has previously been demonstrated in response to an acute bout of either RIPC (7), or exercise (67, 73), when performed independently. In fact, we did observe an increase in plasma IL-6 concentrations in response to CE_{AERO} (~50 ± 18%), which is consistent with previous reports assessing IL-6 responses to aerobic exercise (67, 73). While we observed no increase in plasma IL-10 in response to RIPC or exercise, this may be related to the timing of blood sample collection. Previous studies exploring the role of IL-10 with RIPC have waited extended periods of time (24 hours) after performing the RIPC stimulus before collecting blood samples. Thus, it is possible that changes may have been observed had the timing of blood sampling been extended beyond the immediate "post" intervention time point.

When examining the relationship between the changes in cytokines during the RIPC condition and the change in SFA flow in response to cuff inflation, we observed statistically meaningful relationships for TNF-α and sICAM-1, where greater reductions in blood flow were associated with higher circulating concentrations of these inflammatory mediators. To our knowledge, this is the first time that the relationship between limb blood flow restriction and increases in these circulating cytokines has been assessed. This finding is intriguing based on the role of these factors in mediating inflammation (5, 33), and the notion that ischemia is generally associated with a pro-inflammatory state. These responses could suggest that an acute bout of

RIPC may be detrimental to cardiovascular health. This is unlikely, however, given that previous work has demonstrated enhancements in endothelial function (one aspect of vascular health) in response to repeated bouts of RIPC over an extended period of time (30, 31). An alternative interpretation is that an acute increase in these inflammatory mediators in response to RIPC could signal an adaptive response that actually enhances vascular function, although this remains to be tested experimentally. Future work should continue to explore the relationship between blood flow restriction and acute increases in inflammatory mediators as they relate to vascular health in the context of RIPC and C-BFRE. Additionally, the use of a more individualized blood flow restriction stimulus should be explored as a way to optimize the release of these signaling molecules with C-BFRE. As C-BFRE continues to be optimized, it is possible that a relative pressure stimulus could be identified that would favor positive, rather than maladaptive responses in regards to the signaling molecules that are released.

Contrary to our hypothesis, we observed no change in plasma nitrite with any of the experimental interventions. Although NO signaling has been implicated in mediating the cardio-protection associated with RIPC in a number of studies (2, 32, 59), other more recent work in healthy human subjects has demonstrated no change in plasma nitrite in response to an acute bout of RIPC (48). One potential explanation for why plasma nitrite did not change is that the restrictive stimulus applied in our RIPC condition may not have been of sufficient magnitude to induce an increase in NO. This is supported by the high variability in SFA flow reduction that we observed during the vascular ultrasound experiment with our standardized cuff pressure of 220 mmHg. Future work should seek to assess the increase in NO metabolite formation in response to RIPC when individualized cuff pressures are used for each subject, based on a percentage of their limb occlusive pressure (i.e. the cuff pressure that results in complete cessation of arterial

inflow). While it is also surprising that we observed no change in plasma nitrite with any of the exercise interventions, much of the previous work relating NO signaling to exercise has used other metrics of NO bioavailability. These include the use of eNOS inhibitors during steady state whole body exercise in humans (22), or the utilization of eNOS knock out models in mice (9). Differences in the sensitivity of different analytical techniques, the timing of blood samples, and the nature of the exercise modalities employed may also contribute to the disparity between studies regarding the role of NO signaling with exercise.

The high variability in the degree of blood flow restriction observed in response to a standardized 220 mmHg cuff pressure is an important finding as it relates to the practical application of both RIPC and BFRE. Much of the work with BFRE (40-42, 66), and virtually every RIPC study, has used a standard cuff pressure across all subjects. Importantly, recent work has highlighted that physical and physiological characteristics of the subject (i.e. limb size, blood pressure, limb adiposity) can influence the degree of blood flow restriction attained when the same cuff pressure is used for all subjects (26, 36). These findings are supported by the data obtained in the present investigation, and suggest that while the same absolute cuff pressure was standardized across all subjects, the degree of blood flow restriction achieved by this stimulus for each subject was not. As such, a more individualized approach should be adopted. Future work in our laboratory will be utilizing an approach that allows for matching of the occlusive pressure for each subject based on a percentage of the total occlusive pressure of the limb (i.e. the minimum pressure required to completely restricts arterial inflow). This approach will potentially overcome the limitations discovered over the course of the present investigation.

Methodological Considerations

In addition to the use of a standard occlusive pressure stimulus, there are a number of other methodological considerations that must be mentioned in relation to these findings. First, all experimental conditions were separated by at least 1 month. The purposes of this were two-fold: 1) to allow adequate wash-out of any blood-borne factors that may have been released in response to the experimental interventions, and; 2) to allow for control of menstrual cycle phase in our female subjects. Previous work has demonstrated that improvements in vascular function following RIPC are still present up to 8 days later (30). Although blood samples were not collected in this previous study, it is likely that one or more humoral circulating factors contributed to these improvements in vascular function, as improvements were also present on the contralateral arm that received no RIPC treatment (30). As such, allowing adequate wash-out between experiments was essential to prevent the potential confounding effects of repeated exposures to the RIPC stimulus. Since our female subjects were already undergoing a 1 month wash-out period between experiments to control for menstrual cycle, we opted to implement the same approach with our male subjects to minimize variability between subjects. It is possible, however, due to the prolonged length of time between experiments that this approach could have actually increased the variability of responses due to seasonal variations or lifestyle changes of our subject population over the course of the study. This possibility is minimized, however, through the use of our randomized, counter-balanced design. Furthermore, subjects reported their exercise habits through the use of a physical activity questionnaire during their familiarization session, and were instructed to inform us of any major changes in their exercise habits or diet over the course of their participation.

Additionally, it is important to be cognizant of the differences in the occlusive stimulus adopted by most RIPC studies versus what is utilized in implementation of BFRE. Typically, RIPC protocols apply the restrictive stimulus to the upper arm, and use a cuff size and pressure that likely results in complete cessation of blood flow to the occluded limb (31, 45, 46). Conversely, the goal of BFRE is to apply a stimulus that minimizes restriction of arterial inflow, but completely occludes venous return (37, 56). As we are the first, to our knowledge, to combine the cyclical nature of RIPC with BFRE, future work should seek to optimize the dose of the occlusive stimulus applied so that the benefits related to both RIPC and BFRE can be achieved.

In conclusion, we demonstrate that cyclical BFRE does not augment the release of cytokines or NO in young healthy subjects compared with conventional exercise, when a standard occlusive pressure is used for all subjects. We also report that there is high variability in the degree of blood flow restriction achieved between subjects when a standard cuff pressure of 220 mmHg is applied. Based on these findings, future work should delineate the inflammatory and NO responses induced by cyclical BFRE with the use of individualized occlusive pressures, and at more extended recovery time points in both healthy subjects, and patients participating in cardiac- and stroke-rehabilitation.

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FIGURES

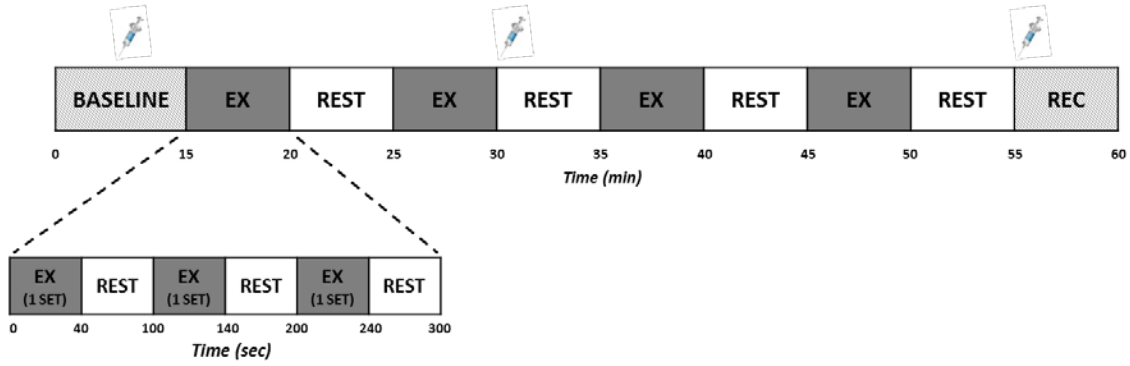

Figure 1: Experimental timeline consisting of a 15-min baseline, 40-min of exercise (4 sets of exercise (EX) and 4 rest/reperfusion periods), and a 5-min recovery period (REC). One 5-min exercise period consisted of 3 sets of exercise with a 1-min rest period between each set; each set consisted of 10 repetitions performed at a load corresponding to 20% (BFRE) or 65% (CE) of 1RM. For the BFRE condition, the occlusive stimulus (cuff inflation) was maintained throughout the entire 5-min of the exercise period. Blood sampling is denoted by syringes. This figure and figure legend was previously published in a related manuscript (64) and is reproduced with permission.

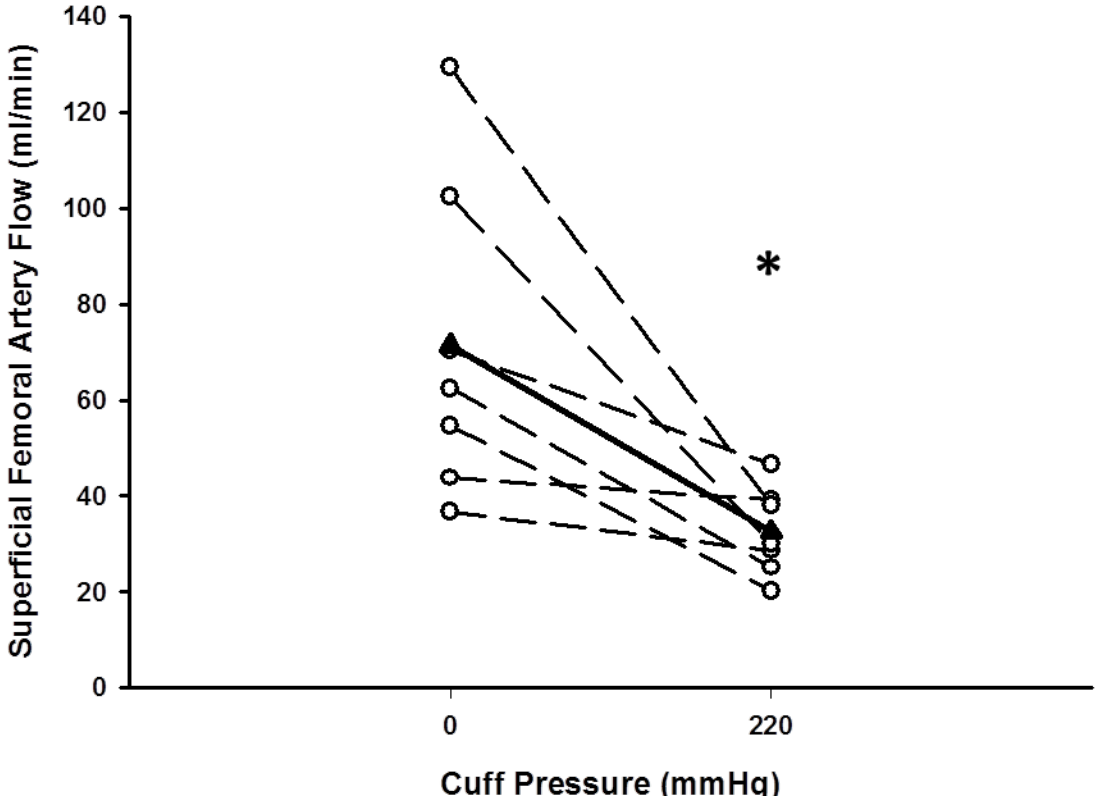

Figure 2: Individual subject (○, dashed line) and group mean (▲, solid line) superficial femoral artery flow responses to thigh cuff inflation of 220 mmHg (N=7). *, P=0.01 vs. baseline (0 mmHg cuff pressure).

Table 1. Descriptive information for measured cytokines

Parameter	Abbreviation	General Functions	References
Tumor Necrosis Factor α	TNF-α	Mediator of systemic inflammation.	Wajant et al. 2003 (70) Bradley 2008 (5)
Serum amyloid A	SAA	Acute phase protein associated with mediating inflammation.	McAdam et al. 1982 (43) Ye & Sun 2015 (74)
Interleukin-6	IL-6	Primary "myokine" secreted from muscle during exercise. Mobilizes substrates for fuel use during exercise. Mediates inflammation. May also exhibit an anti-inflammatory effect through inhibiting the release of pro-inflammatory cytokines.	Pederson 2012 (51) Febbrario et al. 2004 (18) Wolsk et al. 2010 (72) Petersen & Pedersen 2006 (54)
Brain Derived Neurotrophic Factor	BDNF	Associated with neurogenesis, synaptic plasticity, and cell survival. May be involved in exercise-induced improvements in cognition.	Binder & Scharfman 2004 (4) Barnabe-Heider & Miller 2003 (3) Hall et al. 2000 (24) Gomez-Pinnela et al. 2008 (21) Ferris et al. 2007 (19) Vaynam et al. 2004 (68)
Interleukin-10	IL-10	Primary anti-inflammatory cytokine. Suppresses secretion of pro-inflammatory cytokines.	De Waal Malefyt et al. 1991 (14) Murray 2005 (47)
Soluble Intercellular Adhesion Molecule-1	sICAM-1	Promotes leukocyte adhesion and trans-endothelial migration. Associated with endothelial dysfunction and cardiovascular disease.	Witkowska & Borawska 2004 (71) Lawson & Wolf 2009 (33)
Soluble Vascular Adhesion Molecule-1	sVCAM-1	Promotes leukocyte adhesion and trans-endothelial migration. Associated with endothelial dysfunction and cardiovascular disease.	Jenny et al. 2006 (29) Shai et al. 2006 (62) Hwang et al. 1997 (27)
C-Reactive Protein	CRP	Acute phase protein associated with mediating inflammation.	Volankis 2004 (69)

Table 2. Cytokine and nitrite responses to Remote Ischemic Preconditioning (RIPC), Conventional Aerobic Exercise (CE_{AERO}), Cyclical Blood Flow Restriction Aerobic Exercise ($C\text{-}BFRE_{AERO}$), Conventional Resistance Exercise (CE_{RES}), and Cyclical Blood Flow Restriction Resistance Exercise ($C\text{-}BFRE_{RES}$).

	RIPC			**CE_{AERO}**			**$C\text{-}BFRE_{AERO}$**			**CE_{RES}**			**$C\text{-}BFRE_{RES}$**		
Time	**Pre**	**Post**	**P**	**Pre**	**Post**	**P**	**Pre**	**Post**	**P**	**Pre**	**Post**	**P**	**Pre**	**Post**	**P**
IL-6 (pg/ml) N=13	0.31±0.04	0.32±0.03	1.0	0.26±0.03	0.40±0.07	**0.03**	0.28±0.05	0.34±0.07	0.75	0.29±0.05	0.33±0.08	1.0	0.53±0.16	0.48±0.14	1.0
IL-10 (pg/ml) N=12	0.21±0.02	0.21±0.02	1.0	0.16±0.02	0.17±0.02	1.0	0.17±0.02	0.14±0.01	0.80	0.18±0.02	0.18±0.02	1.0	0.22±0.04	0.21±0.04	1.0
TNF-α (pg/ml) N=12	1.3±0.1	1.3±0.1	1.0	1.2±0.1	1.3±0.1	0.52	1.2±0.1	1.3±0.1	0.94	1.3±0.1	1.30 ± 0.1	1.0	1.4±0.1	1.3±0.1	0.99
CRP (mg/L) N=12	5.4±2.4	5.6±2.6	1.0	4.5±1.6	4.5±1.6	1.0	3.1±1.0	3.3±1.1	1.0	2.4±0.5	2.4±0.6	1.0	9.3±5.2	10.9±6.3	1.0
SAA (µg/ml) N=12	11.7±2.4	11.8±2.2	1.0	10.1±3.0	9.8±2.3	1.0	8.9±2.3	10.0±3.5	1.0	10.2±1.8	12.3±3.4	1.0	24.6±11.4	28.9±15.0	1.0
sICAM-1 (ng/ml) N=13	851±80	820±77	1.0	785±68	804±63	1.0	735±47	773± 60	0.99	821±45	805±50	0.99	832±91	867±85	0.99
sVCAM-1 (mg/L) N=13	1.3±0.1	1.3±0.1	1.0	1.1±0.1	1.1 ± 0.1	1.0	1.1±0.1	1.1±0.1	1.0	1.2±0.1	1.3±0.1	1.0	1.2±0.1	1.3±0.1	0.90
BDNF (pg/ml) N=10	2107±248	1612±274	0.90	2657±570	2633±399	1.0	2056±316	2800±748	0.56	2376±639	2855±435	0.83	2489±526	2291± 474	1.0
Nitrite (µM) N=7	0.14±0.02	0.13±0.03	1.0	0.26±0.03	0.25±0.03	1.0	0.24±0.03	0.23±0.03	1.0	0.28±0.04	0.29±0.03	1.0	0.27±0.1	0.25±0.01	0.99

IL-6: Interleukin-6, IL-10: Interleukin-10, TNF-α: Tumor Necrosis Factor-α, CRP: C-Reactive Protein, SAA: Serum Amyloid A, sICAM-1: Soluble Intercellular Adhesion Molecule-1, sVCAM1-1: Soluble Vascular Adhesion Molecule-1, BDNF: Brain Derived Neurotrophic Facto

Table 3. Associations between the percent reduction in superficial femoral artery (SFA) flow during the vascular ultrasound experiment and the percent change in plasma concentrations of cytokines during the RIPC experiment.

Parameter (vs. %Δ SFA Flow)	N	r	r^2	Slope (%/%)	P-Value
%Δ IL-6	6	0.25	0.06	-24.1	0.64
%Δ IL-10	6	0.60	0.35	-26.7	0.22
%Δ TNF-α	6	0.82	0.68	-13.1	**0.05**
%Δ CRP	6	0.006	0.00003	-1.64	0.99
%Δ SAA	6	0.20	0.04	-10.8	0.71
%Δ sICAM-1	6	0.79	0.62	-23.4	**0.06**
%Δ sVCAM-1	6	0.14	0.02	1.21	0.79
%Δ BDNF	5	0.63	0.39	-214.3	0.26

IL-6: Interleukin-6, IL-10: Interleukin-10, TNF-α: Tumor Necrosis Factor-α, CRP: C-Reactive Protein, SAA: Serum Amyloid A, sICAM-1: Soluble Intercellular Adhesion Molecule-1, sVCAM1-1: Soluble Vascular Adhesion Molecule-1, BDNF: Brain Derived Neurotrophic Factor

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CHAPTER VI

Development of an Assay for Measurement of Endogenous Opioid Activity in Plasma

INTRODUCTION

One category of humoral factors that have been implicated in mediating the cardio-protection associated with both RIPC (5, 10, 11) and exercise (2, 4-6) are the endogenous opioids. When either an acute bout of RIPC or exercise is performed prior to experimentally-induced myocardial infarction, endogenous opioids have been shown to mediate the observed reduction in infarct size (5). This effect has been demonstrated through the use of bioassays in which venous blood was initially collected from humans following RIPC or exercise (5, 10). Plasma samples were then transferred to isolated heart preparations (i.e. Langendorff preparations) (5) or isolated cardiac myocytes (10) prior to induction of ischemia-reperfusion injury. A reduction in infarct size in the isolated hearts or cardiac myocytes was demonstrated following perfusion with plasma collected from both of these interventions. This protection was not present, however, if the preparations were co-treated with naloxone, a nonspecific opioid receptor antagonist. These observations suggest that an opioid dependent signaling pathway is responsible for the observed cardio-protective effects.

While the aforementioned studies did not address which class of endogenous opioids was responsible for mediating the cardio-protection, other work using selective δ-opioid receptor antagonists has implicated the δ-opioid receptor and its ligands (i.e., the enkephalins) as the primary mediators (2, 6, 12). Virtually all of the evidence supporting the δ-opioid receptor pathway in mediating cardio-protection comes from studies using antagonists to block the δ-

opioid receptor (5, 6, 10), studies measuring pro-enkephalin mRNA (6), or direct application of enkephalins to cardiac myocytes *in vitro* (12). Surprisingly, very few studies have directly quantified plasma concentrations of enkephalins in response to an acute bout of RIPC or exercise. From a methodological perspective, quantification of enkephalins in plasma is difficult as these peptides circulate at very low concentrations (3, 9), and are subject to rapid degradation due to the presence of proteases in the blood (7). While high-performance liquid chromatography and mass spectrometry (HPLC-MS) is a potential option for quantification, this equipment is very expensive and requires advanced technical expertise to operate. Additionally, the equipment must be configured in a way that is specific to the measurement of this analyte, which limits the opportunities for collaboration unless a laboratory is already set up for measuring enkephalins. Furthermore, even when configured appropriately, this technique may be unable to detect enkephalins at very low concentrations. ELISAs are also an option for analysis, but require either the use of solid phase extraction, or radioactive ligand binding, both of which are also limited by access to equipment, technical expertise, and the sensitivity of their detection limits. As such, we decided to explore a novel alternative technique for assessment of plasma enkephalins in our human blood samples - *sniffer cells*.

The *sniffer cell* approach has been previously used to study neurotransmitter release in the brain (8, 15). Sniffer cells are Chinese Hamster Ovary (CHO) cells that can be transfected with a receptor of interest, along with a fluorescent calcium reporter (GCaMP). CHO cells are especially suitable for this purpose as they are devoid of plasma membrane receptors but contain the 2nd messenger proteins that are required to elicit G-protein mediated signaling. As many signaling pathways (including those utilized by the δ-opioid receptor) rely on calcium signaling to mediate signal transduction (13), co-transfecting the cells with this reporter and a receptor of

interest may allow for the detection and/or quantification of ligands for that specific receptor. Specifically, an agonist in a solution (e.g., plasma, cerebrospinal fluid, tissue homogenate) will bind to the transfected receptor and trigger an increase in intracellular calcium through influx via plasma membrane bound calcium channels as well as release from the endoplasmic reticulum. This increase in intracellular calcium will induce an increase in fluorescence of the cell, through the binding of calcium to the calcium reporter, GCaMP. While there is some basal fluorescence due to the presence of intracellular calcium within the cytoplasm, this fluorescence increases substantially with an influx of calcium. Specialized software can be used to identify the transfected cell as a region of interest, and quantify the fluorescence within that specific region (i.e. the cell). This process can be performed on multiple cells, to account for the inherent variability of a single cell recording. Thus the presence of the agonist can be detected by measuring the increase in fluorescence of these cells. As the intensity of fluorescence within the cells should be directly proportional to the concentration of ligand in solution, the concentration of the ligand of interest could potentially be quantified. To achieve this, a dose response curve could be constructed by treating the cells with a series of known concentrations of agonist and measuring the increase in fluorescent intensity in response to each dose (figure 1).

The following chapter describes our progress in the development of this novel approach to quantify δ-opioid receptor agonist concentrations in human plasma in response to cyclical BFRE, CE, and RIPC alone; this work directly relates to Specific Aim 1 of my dissertation studies.

METHODS & PRELIMINARY RESULTS

This work is part of a collaborative effort between the laboratories of Dr. Rickards, Dr. Yuan, and Dr. T. Cunningham (including Dr. Farmer). Some figures presented within this chapter are reproduced with permission from these collaborators.

Summary of Proposed Approach

For our purposes, the CHO cells will be transfected with the δ-opioid receptor and the GCaMP calcium reporter. They will then be treated with known concentrations of enkephalins so that a standard curve can be constructed in which the increase in fluorescent intensity (y-axis; dependent variable) is plotted against the known concentration (x-axis; independent variable) in a step-wise fashion. We will then apply human plasma containing unknown concentrations of enkephalins, and the concentrations can be derived with the equation describing the standard curve. We anticipate that this strategy will provide a novel and reliable approach to measure endogenous opioid concentrations in a variety of biological fluids, including human plasma.

Creation of Sniffer Cells

CHO cells were transfected with plasmids (Lipofectamine 3000, Thermo Fisher Scientific) to express the δ-opioid receptor (NM000911, Origene) and the calcium indicator GCaMP (Plasmid # 40754, Addgene). To allow for selection of cells that were successfully transfected, cells were also transfected with a gene conferring protection to the antibiotic G418 (Geneticin, Thermo Fisher Scientific, Waltham, MA). Cells were then cultured in media containing G418, so that only cells that were successfully transfected were able to proliferate. Cells were cultured to confluence and cloned using Trypsin-EDTA cloning cylinders. Lastly,

individual colonies were frozen in liquid nitrogen and stored for subsequent use in fluorescent calcium imaging experiments. Western blotting was then performed to confirm expression of both the δ-opioid receptor and GCaMP (figure 2).

Initial Pilot Experiments with Enkephalin Application

Cells were plated on a coverslip and continuously bathed in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3.0 mM KCL, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, 1.25 NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-Glucose). Osmolality of the solution was 300 mOsm, and pH was 7.4. Calcium imaging was performed using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Measurements of fluorescent intensity were taken at 2 s intervals and normalized to baseline fluorescence of individual cells. Pure met-enkephalin (500 nM, Sigma Aldrich) and leu-enkephalin (500 nM, Sigma Aldrich) were focally applied for 10 s onto cells using a micropipette (1-3 uM tip diameter) connected to a pneumatic picopump (WPI, Sarasota, Fl) set to 10 psi. Fluorescent regions of interest (i.e. sniffer cells) were identified prior to drug application, as well as an additional background region (to be used for background subtraction) for subsequent analysis using specialized software (MetaFluor, Molecular Devices).

Response of a single cell evoked by focal application of leu-enkephalin (500 nM) is depicted in figure 3.While some cells were responsive to application of met- and leu-enkephalin, other cells were not. Furthermore, there was high variability in the responsiveness between cells. We speculated that the variability in responsiveness was due to differences in the expression of GCaMP between cells. To overcome this variability, new cells were created via the same methods previously described; the Western blot indicating successful transfection is depicted in figure 4. The new cells then underwent Fluorescence Activated Cell Sorting (FACS; SH800,

Sony, San Jose, CA) to ensure consistent expression of GCaMP among cells (figure 5). Specifically, the indicator fluorescein isothiocyanate (FITC) was used to isolate cells that had medium or high expression of GCaMP. As the fluorescent intensity of FITC is directly proportional to GCaMP expression, the cells with highest GCaMP expression can be identified and separated out. This sorting allowed for selection and isolation of cells that had medium expression only. These cells were cultured to confluence and cloned as previously described. Cells were then frozen in liquid nitrogen to be stored for subsequent use in fluorescent calcium imaging experiments.

Dose Response Curve with FACS Cells

FACS cells were plated on a coverslip and continuously bathed in aCSF as previously described. Met-enkephalin was dissolved in dimethyl sulfoxide and pilot studies were initially performed with doses ranging from 100 pM to 500 nM. The purpose of these initial experiments was to determine the optimal range for subsequent dose response curves. Based on the cells responsiveness, doses of 1, 10, and 100 nM were used for the subsequent dose response curve recordings. Met-enkephalin at each concentration was applied via bath application, and was separated by wash out with aCSF. First, drugs were applied to the same group of cells at varying concentrations. Initial observations from this pilot study indicated that the cells had not fully recovered from the first dose, however, as they exhibited an attenuated response to all subsequent doses. Based on this finding, different groups of cells were then selected for each drug application.

A separate issue is that many of the cells exhibit decay in fluorescent intensity at the start of the recording (i.e. photobleaching). As a result, individual baselines differ between cells depending on the extent of this decay. Figure 6 shows the initial decay in fluorescence of sniffer cells at the start of the recording period followed by a uniform spike in fluorescent intensity in response to bath application of met-enkephalin (100 nM). While the uniform responsiveness to drug application is an improvement from the previously non-FACS cells, the differences in fluorescence at baseline could be a potentially confounding factor for ultimate quantification of concentration.

To account for the differences in baseline between cells, the final 10 s of baseline for each cell was averaged before drug application. The peak response evoked by the drug application was then divided by this 10 s baseline period for each individual cell and a one-way ANOVA (factor: dose) was used to compare the ratio of peak response to baseline in response to increasing concentrations of met-enkephalin (figure 7). Unfortunately, the cells did not respond with a dose-dependent increase in the ratio of peak response/baseline as there were no differences in the cells responsiveness between doses ($P=0.92$). This dose response series was repeated with a new cover slip of cells and the variability in responses was still present, as there was no clear pattern of increasing fluorescence in response to increasing concentrations of met-enkephalin (data not shown).

DISCUSSION

Summary of Progress and Future Directions

This project is still ongoing, as this method is yet to be optimized for quantification of enkephalins in our human plasma samples. We have been successful in creating sniffer cells that

express the δ-opioid receptor and GCaMP. Additionally, these cells are responsive to the application of the δ-opioid receptor agonists met- and leu-enkephalin. Unfortunately, we have not yet been able to create a dose-response curve with varying doses of enkephalins. Despite having undergone FACS, the cells still demonstrate high variability in their responses to enkephalin application. Our inability to reliably and repeatedly provoke a dose-dependent increase in fluorescence suggests that this assay is not yet ready for use with our experimental human plasma samples. Future work may include 1) another round of FACS to isolate cells that have consistent δ-opioid receptor expression, in addition to the consistent GCaMP expression that we have already achieved; 2) the utilization of different methods of applying the enkephalins (micropipette "puff" rather than bath application), and; 3) adopting strategies to ensure that all cells are in the same developmental stage for each drug application.

Once we are able to reliably and repeatedly construct a dose-response curve for the cells, there are still other issues that we anticipate having to resolve. One limitation is that δ-opioid receptor will bind both met- and leu-enkephalin with similar affinity (13). As our plasma samples presumably contain both enkephalins, we will not be able to discriminate the specific concentrations of each ligand independently, but rather, we will evaluate the collective response of the cells to both ligands. This is not necessarily a limitation for our specific purposes, as both enkephalins play a role in mediating cardio-protection. In fact, this could even be viewed as astrength; if the assay was specifically targeted towards one of the two enkephalins only (met- or leu-enkephalin), we could potentially fail to detect a response, even if the other, unmeasured enkephalin increased with our experimental interventions. As virtually all of the evidence supporting the role of enkephalins in cardio-protection is derived from blockade studies (5, 6,

10), we are unsure of the independent changes in each of these enkephalins, so this is an important issue to consider in development of this assay.

A separate issue is that the cells are also responsive to other endogenous opioids that circulate in human plasma. Specifically, preliminary testing with pure β-endorphin (500 nM, Genscript) and Dynorphin-B (500 nM, Genscript) were also able to elicit a fluorescent response (data not shown). This pilot testing was performed with the first batch of cells that had not undergone FACS. Based on the varying expression between those cells, we have yet to quantify if the magnitude of response to these other ligands is attenuated compared to the response elicited by the enkephalins. When we repeat these experiments with the FACS cells, however, we anticipate that these other ligands will also still provoke a response. Since exercise also increases plasma concentrations of other endogenous opioids (1, 14), this lack of specificity for the enkephalins will be a limitation of the assay with our experiments using samples collected after cyclical BFRE and conventional exercise. However, since the enkephalins should exhibit a higher binding affinity for the δ-opioid receptor than the endorphins and dynorphins, the responses elicited by these other agonists may only account for a small percentage of the overall increase in fluorescence we observe with plasma application.

The variability of responses could potentially be due to differences in δ-opioid receptor expression between cells or differences in receptor sensitivity between cells. Although the FACS analysis allowed us to separate the cells based on overall expression of GCaMP, we have not yet sorted cells by δ-opioid receptor expression. Thus, even though all cells may contain the same amount of GCaMP, it is possible that one cell may have a greater δ-opioid receptor density compared to an adjacent cell, and thus would exhibit a greater fluorescence with enkephalin application simply because there are more cell membrane binding sites.

Additionally, there still could be differences present in regards to the calcium handling of individual cells. For example, a cell with greater receptor sensitivity would exhibit a greater increase in intracellular calcium with enkephalin binding. This would cause an amplified fluorescent intensity relative to cells with lower receptor sensitivity, independent of the total number of binding sites (receptor density). Differences in sensitivity could result from differences in expression of some of the other molecular signaling components of the δ-opioid receptor pathway that were not accounted for with the FACS sorting (i.e. protein kinases, 2^{nd} messengers, and calcium channels). As these components of the signaling cascade are inherently present in the cell (rather than transfected), it is difficult to account for varying expression between cells.

It is also possible that the inherent ability of the cells to regulate calcium varies with the age of the cell. CHO cells typically tend to become more elongated through development. Anecdotal observations of elongation of some cells compared to the more oval shape of others, suggest that cells on some cover slips may be more mature than cells on other coverslips. This is another factor that could potentially be targeted as this method continues to be developed and optimized.

In summary, there are multiple avenues that should be explored as this assay continues to be developed. Further testing is required before this assay will be ready for use. Despite these shortcomings, however, we have still made progress in the development of this novel analytical technique. We have been successful in developing cells that express the δ-opioid receptor and GCaMP, and these cells respond to application of enkephalins. We have further refined these cells through the use of FACS, allowing us to elicit a more uniform response to enekphalins between cells. Ultimately, it is envisioned that this novel technique may be used to measure

unknown enkephalin concentrations in biological fluids, including the human plasma samples collected as part of this dissertation project.

ACKNOWLEDGEMENTS

I would like to acknowledge our collaborators, Dr. Joseph Yuan, Dr. Gef Farmer, and Dr. Tom Cunningham, whose involvement has been essential in the development of this project. Dr. Yuan transfected and cultured cells, performed western blots, and FACS. Dr. Farmer performed calcium imaging and dose response curves. Dr. Cunningham conceived this idea and provided essential insight into the approach throughout this process.

FIGURES

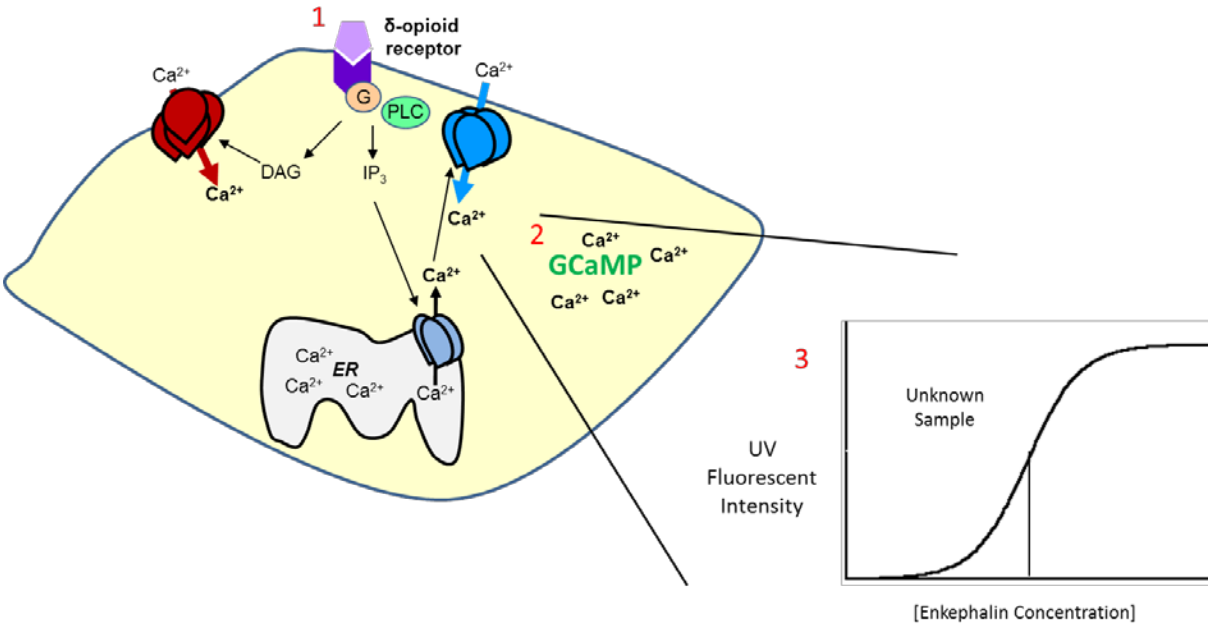

Figure 1: Schematic representation of enkephalin-induced calcium spike and quantification in sniffer cell. 1) Enkephalin in solution binds to δ-opioid receptor and initiates G-protein-mediated activation of Phospholipase C (PLC). PLC activates DAG and IP_3 to induce an increase in intracellular calcium; 2) calcium binds to GCaMP, causing an increase in fluorescent intensity of the cell; 3) this fluorescent intensity can be used to quantify enkephalin concentration in solution through a dose response curve constructed based on known concentrations of enkephalins. G: G-protein coupled receptor DAG: Diacylglycerol IP3: Inositol triphosphate ER: Endoplasmic Reticulum. This figure was adapted, with permission, from an original figure provided by Dr. Joseph Yuan.

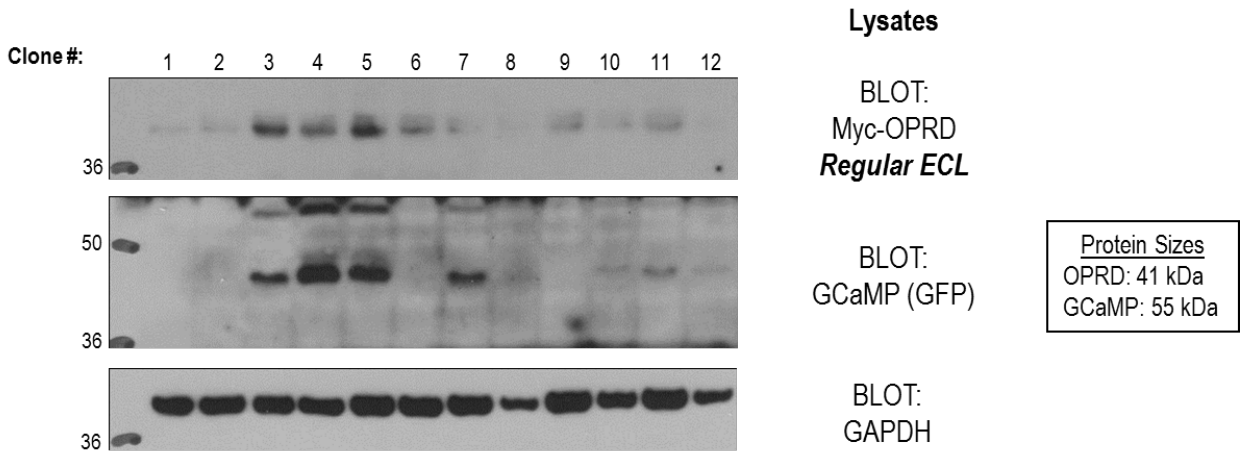

Figure 2: Initial western blot demonstrating successful transfection of δ-opioid receptor (OPRD) and GCaMP in CHO cells. Myc-OPRD: Myc-tagged opioid receptor-δ. ECL: Enhanced Chemiluminescent (control). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase (control).

This figure was provided by Dr. Yuan and is used with permission.

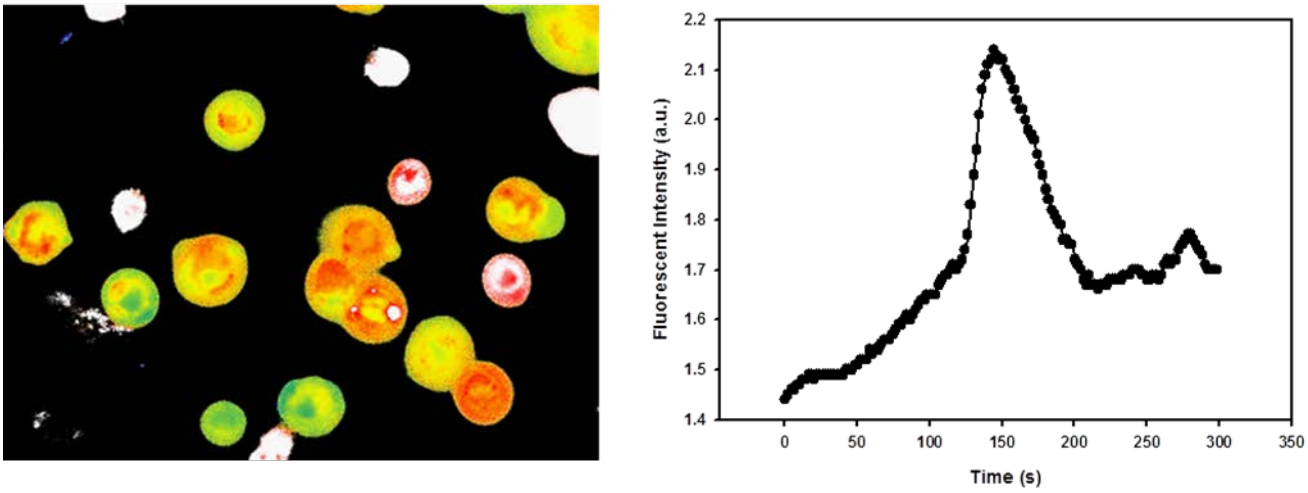

Figure 3: Pilot work with leu-enkephalin demonstrating responsiveness of sniffer cell. Left: image of fluorescing sniffer cells on cover slip; Right: increased fluorescence of individual sniffer cell in response to 500 nM leu-enkephalin. Drug was applied after a 1-min baseline. This image (left) was provided by Dr. Farmer and is used with permission.

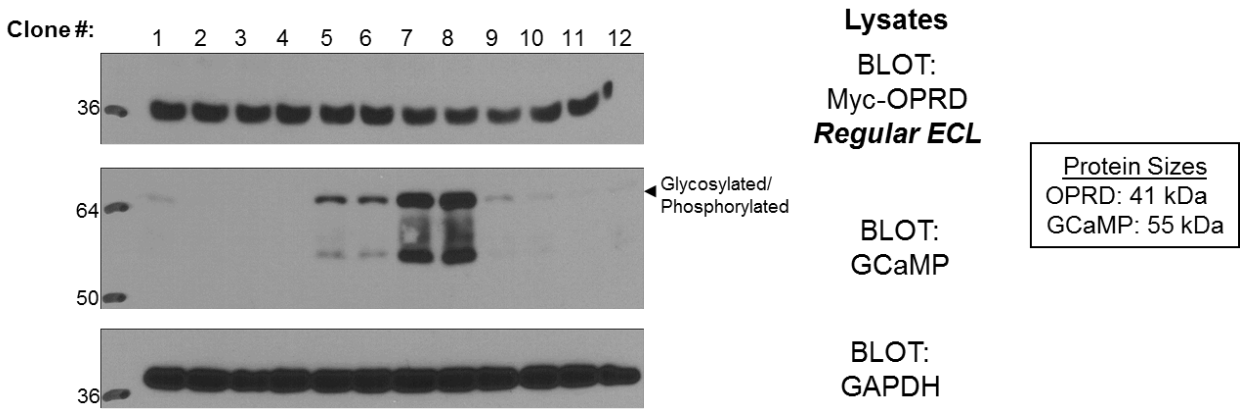

Figure 4: Second western blot demonstrating successful transfection of δ-opioid receptor and GCaMP in CHO cells. Clones # 7 and # 8 were subsequently used for Fluoresence Activated Cell Sorting (FACS). Myc-OPRD: Myc-tagged opioid receptor-δ. ECL: Enhanced Chemiluminescent (control). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase (control).

This figure was provided by Dr. Yuan and is used with permission.

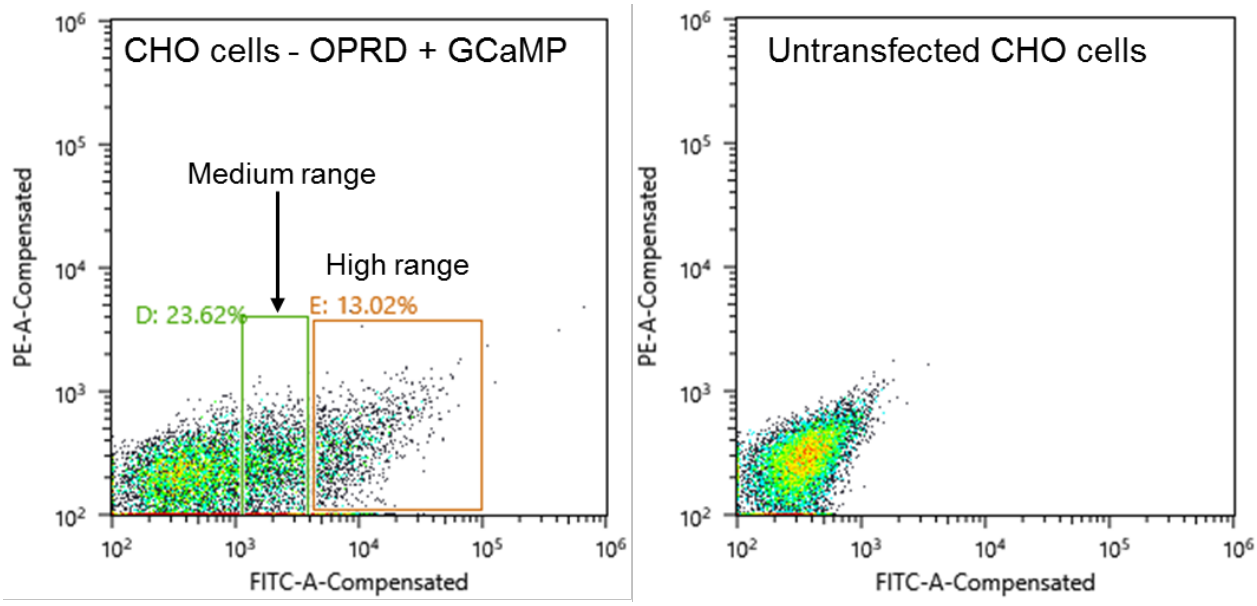

Figure 5: Fluoresence Activated Cell Sorting (FACS) results demonstrating the isolation of cells with medium and high ranges of GCaMP transfection. PE: Phycoerythrin. FITC: Fluorescein isothiocyanate. This figure was provided by Dr. Yuan and is used with permission.

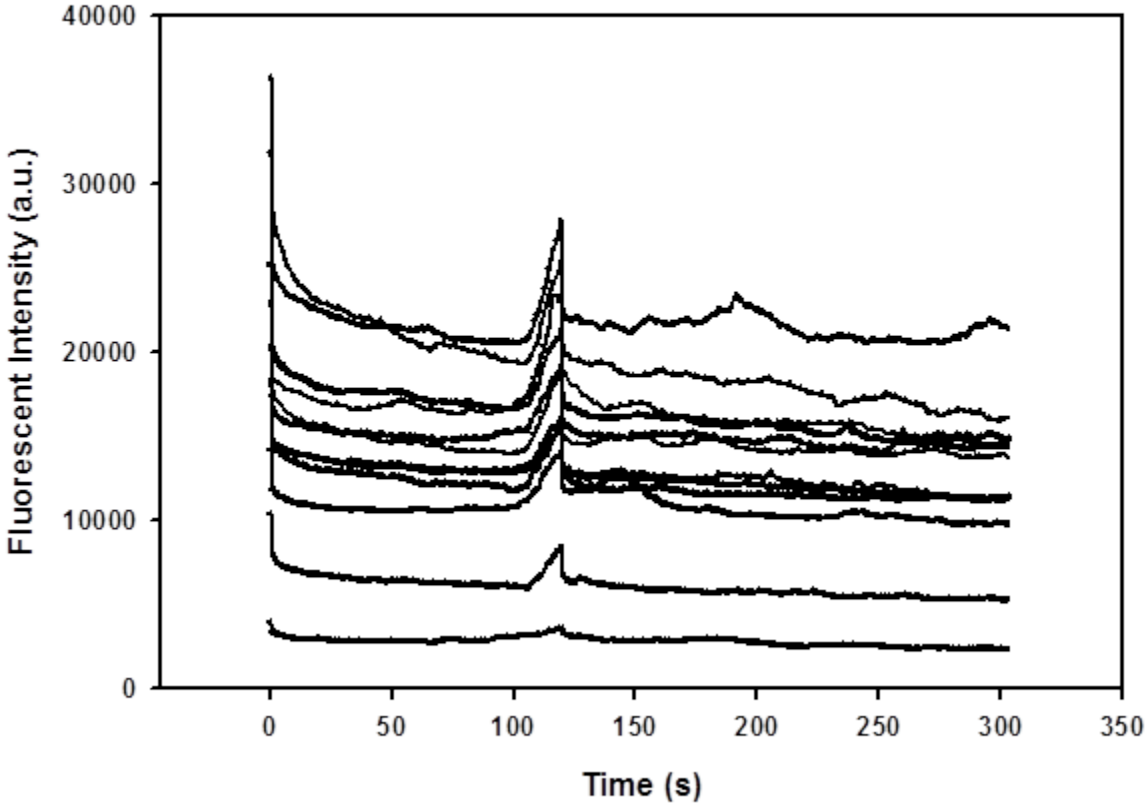

Figure 6: Sniffer cell responses to 100 nM met-enkephalin. This recording was 5 min in duration, and the drug was applied at the 1-min time point. Individual responses to 12 cells are depicted after subtraction of background fluorescence.

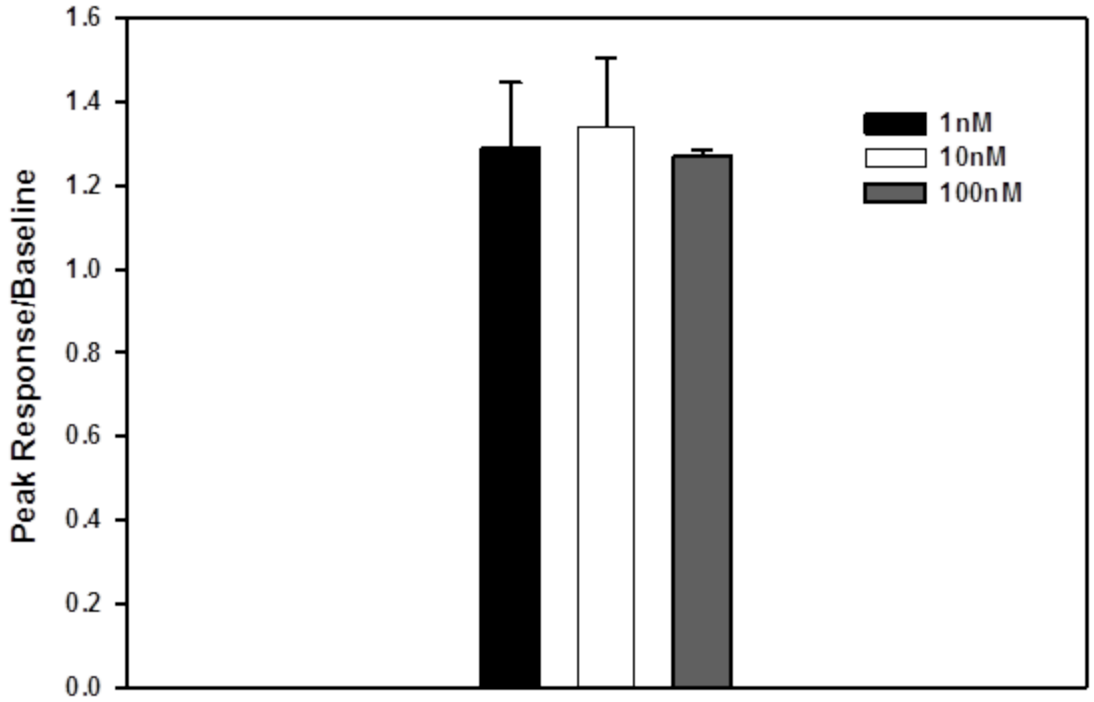

Figure 7: Ratio of peak response to baseline (final 10 s) for three different doses of met-enkephalin via bath-application. Twelve different cells were used for each of the three recordings. Each recording was 5-min in duration and the drug was applied after a 1-min baseline. There were no differences observed in the peak response/baseline between doses (P=0.92). Data is represented as Mean ± SE.

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CHAPTER VII

Discussion

The overall goal of this dissertation project was to investigate a novel exercise intervention we term "cyclical BFRE". In *Specific Aim 1* we sought to compare the effects of cyclical BFRE, CE, and RIPC alone, on the release of circulating factors previously demonstrated to be protective in the face of ischemia-reperfusion injury. In *Specific Aim 2 we* sought to compare the hemodynamic and cerebrovascular responses between cyclical BFRE, CE, and RIPC. These aims were addressed through the completion of four studies reported in this dissertation, with the following key findings:

Specific Aim 1

An acute bout of cyclical BFRE (aerobic or resistance) did not induce an augmented production of some key circulating factors previously demonstrated to be cardio- and cerebro-protective (i.e. cytokines and nitrite). This is potentially due to the high inter-subject variability in the degree of blood flow restriction achieved with our standardized cuff pressure of 220 mmHg (10.7-70.5% reduction in arterial inflow in a sub-set of 7 subjects). It is also possible that the timing of blood sampling may have limited our ability to detect a change in these factors. Importantly, the results of this study have highlighted the need for individualized occlusive cuff pressures based on the degree of limb blood flow reduction rather than a standard stimulus of 220 mmHg for each individual subject.

Specific Aim 2

Aerobic Exercise

Cyclical blood flow restriction aerobic exercise elicits an exaggerated sympatho-excitatory response compared to conventional aerobic exercise, as evidenced by a greater increase in plasma norepinephrine. This increased sympathetic activity, however, was not accompanied by greater increases in arterial pressure, likely due to the cyclical nature of the reperfusion periods that is unique to this novel adaptation of BFRE. Specifically, the rapid thigh cuff deflation during the reperfusion periods caused a reactive hyperemia that decreased arterial pressure, and opposed the effects of increased sympathetic activity. This finding is important as it relates to the safety of cyclical BFRE in clinical settings where an augmented increase in arterial pressure would be undesirable. Additionally, when cyclical BFRE and CE are performed at the same heart rate intensity, subjects can achieve this intensity with a lower mechanical workload (i.e. lower treadmill incline) during the cyclical BFRE condition. This finding is clinically relevant, as a lower mechanical workload would be desirable for patient populations with musculoskeletal limitations. As these patients may be unable to participate in conventional exercise at higher workload intensities, cyclical BFRE could be used to reach targeted heart rate intensities, but at a lower workload. Such an approach could potentially reduce the total volume of work required to achieve the beneficial effects of exercise, or provide additional physiological benefits that are not present with only low to moderate exercise intensities. These adaptations to repeated BFRE will be explored in future training studies.

Lastly, cyclical BFRE and CE elicit similar cerebral blood flow and oxygenation responses during steady-state treadmill walking when both conditions are matched for heart rate. This finding is important as it is envisioned that cyclical BFRE could be implemented in a

stroke-rehabilitation setting, where cerebral hyper-perfusion due to the elevated sympathetic activity and arterial pressure could further damage already fragile cerebral tissue. Importantly, however, cerebral autoregulation may be compromised in an aged or stroke population, and thus the cerebrovascular responses to cyclical BFRE in more clinically relevant patient groups still needs to be explored before implementing BFRE into the clinical setting.

Resistance Exercise

Cyclical blood flow restriction resistance exercise elicits an attenuated increase in sympathetic activity compared to conventional resistance exercise, as evidenced by the lower plasma norepinephrine responses. As a result of the decreased sympathetic activity, hemodynamic responses are also attenuated with cyclical BFRE compared to CE. These attenuated responses are likely due to the use of a lower workload with cyclical BFRE (20% 1RM versus 65% 1RM for CE) and suggests that the decrease in workload is able to offset the increased stimulation of the exercise pressor reflex that occurs with thigh cuff inflation. Lastly, there are no differences in cerebro-metabolic demand between cyclical BFRE and CE, as reflected by comparable cerebral blood flow and oxygenation responses in both conditions. As resistance exercise is a key aspect of stroke rehabilitation, the similarity in cerebrovascular responses between cyclical BFRE and CE suggests that the use of cyclical BFRE should continue to be explored in this setting. As with the aerobic exercise modality, future work in clinical models is required before this can be accomplished.

Limitations and Future Directions

There are a number of general limitations associated with these studies that should be mentioned as they relate to the overall interpretation of our findings. First, we only compared the

acute responses to a single bout of cyclical BFRE and CE. While this information is informative and relevant to the safety concerns associated with BFRE, it does not provide insights into the adaptations that would result from the regular performance of cyclical BFRE over time. The purpose of these initial acute cyclical BFRE studies, however, was to provide the framework for future studies that will be performed in the Rickards' laboratory. First, based on the safety and feasibility demonstrated in response to a single bout of cyclical BFRE, the next step will be to perform a training study in which individuals perform cyclical BFRE on a regular basis for an extended length of time (i.e. 3 days a week for 6 weeks). The purpose of this study will be to determine if the positive health benefits associated with CE can also be extended to cyclical BFRE. These data would support the implementation of cyclical BFRE as a viable exercise alternative for individuals who cannot tolerate the higher mechanical workloads associated with conventional exercise.

Second, all studies were performed in young healthy subjects. While this is an important first step to characterize the responses to this novel exercise paradigm, these responses may be different in more clinically relevant or aged subjects (e.g. cardiac or stroke rehabilitation patients). Since it is conceptualized that cyclical BFRE could ultimately be applied in a cardiac and stroke rehabilitative setting, studies comparing the acute and chronic responses to cyclical BFRE in aged and other "at-risk" subject groups are essential.

Third, we used a standardized cuff pressure of 220 mmHg to elicit the occlusive stimulus in all of our blood flow restriction conditions. While the absolute pressure applied to each subject was the same, the degree of blood flow restriction achieved with this pressure was vastly different between subjects. These differences may have increased the variability of our results and may explain why we were unable to detect differences in the mediators of cardio- and neuro-

protection related to *Specific Aim 1*. To overcome the limitations associated with applying the same standardized cuff pressure to all individuals, in future studies we will aim to prescribe the pressure stimulus based on a percentage of the pressure required to completely stop arterial inflow (i.e., limb occlusive pressure, LOP) for each individual subject. Recent technological developments have enhanced the feasibility of such an approach through the commercialization of specialized cuffs that are equipped with a circumferential ultrasound (PTS, Delfi Medical Innovations Inc., Vancouver BC, Canada). These devices can be used to determine the LOP for each individual subject and then apply a pressure stimulus that is set as a relative percentage of the LOP during the exercise bout (e.g., 60% or 80% of LOP). This method will ensure that all subjects are receiving the same stimulus in regards to the degree of blood flow restriction, and will facilitate a more individualized approach compared to the approach used in these dissertation studies.

Fourth, in *Specific Aim 1* we sought to compare the release of δ-receptor agonists (met- and leu-enkephalin) in response to cyclical BFRE, CE, and RIPC. As previously mentioned, quantification of enkephalins in human plasma is technically challenging, with very few tools available for this purpose. An ongoing collaboration between multiple laboratories within the department will aim to continue developing and refining the *sniffer cells* for this purpose. Plasma samples have been collected and stored from all five of the experimental conditions described in this dissertation. These samples will be analyzed for δ-opioid receptor agonist activity once this assay has been fully developed and validated.

Finally, we did not assess for sex differences in any of our investigations as we were underpowered for accurate statistical evaluation of this effect. This limitation is important as it relates to our ultimate goal of implementing cyclical BFRE within a cardiac- or stroke-

rehabilitative setting. Given the disparities between sexes in the prognosis of patients who have experienced ischemic insults [women tend to exhibit poorer functional outcomes relative to men (2)], it is possible that there will also be sex differences in regards to the the effectiveness of cyclical BFRE. Specifically within the context of my studies, it is possible that some of the variability observed in the degree of blood flow restriction between subjects was influenced by differences in body composition and limb adiposity between sexes; limb adiposity is a factor that influences the degree of blood flow restriction (1). As females exhibit a greater percentage of body fat and greater limb adiposity (3), these factors could have influenced the degree of blood flow restriction that was achieved with our standardized cuff pressure of 220 mmHg. This limitation will be minimized in our future studies, however, as we will be prescribing an occlusive stimulus based on a relative percentage of limb occlusive pressure. In future work we will seek to compare responses based on sex by increasing the sample size of both males and females to allow for these comparisons. Intriguingly there is a paucity of literature regarding the role of sex differences in relation to the cardio- and neuro-protective effects of RIPC, thus it is imperative to address this important gap in our knowledge.

Perspectives

In conclusion, cyclical BFRE as applied in the studies described in this dissertation does not result in an augmented release of cytokines or nitric oxide metabolites. These findings suggest that the current BFRE model may not result in additive cardio- or cerebro-protection as initially hypothesized. In regards to the safety concerns associated with BFRE, the hemodynamic responses elicited by cyclical BFRE are not exaggerated compared to CE, with an attenuation of arterial pressures during cyclical BFRE for both aerobic and resistance training modalities.

Importantly, however, these findings only apply to the model of cyclical BFRE used in these investigations which utilized a standardized cuff pressure. As we move forward with the use of individualized cuff pressures during BFRE, however, it is possible that while this may result in an augmented release of cardio- and cerebro-protective mediators, this may come at the expense of an amplified exercise pressor reflex, and an exaggerated increase in arterial pressure. Ideally, an optimal pressure stimulus could be identified based on individual LOP that is high enough to induce the benefits of RIPC and exercise, but still remain lower than the threshold that would amplify the exercise pressor reflex.

These findings support the safety of cyclical BFRE in a young healthy population, and provide the groundwork for future studies in more clinically relevant populations with the use of individualized cuff pressures. Future studies will expand on the findings presented in this dissertation as the application of cyclical BFRE continues to be optimized.

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