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WHY DOES EXERCISE "TRIGGER" ADAPTIVE PROTECTIVE RESPONSES IN THE HEART?

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□ Numerous epidemiological studies suggest that individuals who exercise have decreased cardiac morbidity and mortality. Pre-clinical studies in animal models also find clear cardioprotective phenotypes in animals that exercise, specifically characterized by lower myocardial infarction and arrhythmia. Despite the clear benefits, the underlying cellular and molecular mechanisms that are responsible for exercise preconditioning are not fully understood. In particular, the adaptive signaling events that occur during exercise to "trigger" cardioprotection represent emerging paradigms. In this review, we discuss recent studies that have identified several different factors that appear to initiate exercise preconditioning. We summarize the evidence for and against specific cellular factors in triggering exercise adaptations and identify areas for future study.

Key words Preconditioning, cardiac, mitochondria, exercise, heart

INTRODUCTION

The beneficial effects of exercise on the cardiovascular system have been well characterized over the last several decades and it is now accepted that exercise can be used as primary prevention for cardiovascular disease (Oberman 1985). Manifestations of cardiovascular disease are blunted with exercise in experimental animal models, and epidemiological data in humans further support these findings (Wang *et al.* 1993; Hamalainen *et al.* 1995). Exercise-induced protection against acute coronary syndromes encompasses a reduction in myocardial infarction (Brown *et al.* 2005b; Lee *et al.* 2012; Frasier *et al.* 2013), arrhythmia (Frasier *et al.* 2011b; Frasier *et al.* 2013), and stunning (Bowles *et al.* 1992; Taylor *et al.* 1999; Lennon *et al.* 2004; Taylor *et al.* 2007). While there is an abundance of literature on proposed mechanisms that seek to explain the protective effects of exercise (Starnes and Taylor 2007; Frasier *et al.* 2011a; Lee *et al.* 2012), a large portion of this research focuses on end points of protection as well as the downstream signaling events that protect the myocardium.

During exercise, an increase in cardiac output is warranted so that the heart can meet the demands of exercising muscles. Aside from matching cardiac output with peripheral demand, exercise also induces precondi-

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tioning whereby the heart is more resistant to injury even long after the exercise has ceased. The proverbial "triggers" that induce cardioprotective signaling are clearly multi-factorial, and include neural, endocrine, and paracrine factors, as well as autocrine signaling and adaptations that arise from within the heart itself.

Exercise can be thought of as eustress; positive stress that a cell responds to in a way that allows it to better cope with that stressor. The adaptive mechanisms associated with exercise ultimately induce a cardioprotective phenotype, resulting in increased tolerance to metabolic stressors (i.e. ischemia). Proposed triggers of exercise cardioprotection include: adenosine, opioids, adenosine monophosphate-activated protein kinase (AMPK), cytokines, mitochondrial and cytosolic derived reactive oxygen species (ROS), nitric oxide (NO), and adrenergic signaling. This review will focus on studies investigating cardioprotection induced by acute aerobic exercise regimens (i.e. days, weeks, and months of training) at moderate to high intensity. The windows of protection include an early window that occurs within the first hour after exercise, and a late window that typically lasts from 24 to 72 hours (Yamashita *et al.* 1999; Brown *et al.* 2003). Studies that utilize different exercise regimens or include protection outside of these time points will be described in detail. We will start by briefly discussing epidemiological findings in humans pertaining to exercise duration and disease risk prevention, and then shift the focus to the various biological compounds that are responsible for cardioprotection. The main objectives are: 1. To review the literature addressing the different factors that induce cardioprotective adaptive responses during/after exercise, and 2. To shed light on gaps in the literature where future studies will advance the field. The first half of our review will focus on circulating factors released during exercise that converge on the heart, with the latter portion focusing on adaptations that occur within the heart during exercise.

What dose of exercise is needed for cardioprotection?

Although there are benefits of exercise across intensities, both epidemiological and animal studies suggest that moderate to high-intensity exercise is best for the heart. The dose-response aspect relating the quantity of exercise that results in a reduction in cardiovascular risk has been extensively investigated across a number of human epidemiological studies. In a longitudinal study Lee *et al.* (2000) tracked physical activity in 482 males (average 66 years of age) over a five year period and showed that energy expenditure was the key variable in reducing coronary heart disease risk. They found shorter intervals of exercise at a higher intensity provides the same protective benefit as longer intervals of exercise at a lower intensity, as long as the overall energy expenditures were equal. The study also supports the idea that exercise intensity is an important

determinant of cardioprotection following an acute exercise regimen (e.g. days to weeks), and that multiple small bouts of intense exercise may have the same net result as one extended bout of exercise. Mora *et al.* (2007) investigated differing levels of physical activity in a group of 27,055 healthy women, determined by kcal/wk expended. They showed a dose-dependent relationship with 200-599, 600-1499 and >1500 kcal/wk groups having a 27%, 32% and 41% reduction in cardiovascular disease risk, respectively, compared to the baseline group which expended less than 200 kcal/wk. Although the authors acknowledged more research was necessary to determine the exact biological mechanisms that resulted in this protection, they found that the reduction in risk seen with increasing levels of physical activity can be explained in large part by a reduction in inflammatory/hemostatic biomarkers.

In animal studies, cardioprotection from I/R injury has been shown to occur after only a single bout of exercise and is sustainable if the exercise continues for many months (reviewed in (Frasier *et al.* 2011a; Quindry and Hamilton 2013)). The majority of our focus herein is on factors released during exercise itself. Long-term chronic exercise is likely a combination of acute factors (reaping the benefits of each individual exercise session) and additional adaptations that include shifted autonomic nervous system tone, heightened levels of cardioprotective proteins (described below), and beneficial hypertrophy. In terms of acute exercise, cardioprotection (reductions in myocardial infarction) is observed after moderately high-intensity exercise (>70% VO_2 max) (Yamashita *et al.* 1999; Hoshida *et al.* 2002; Brown *et al.* 2003; French *et al.* 2008; Quindry *et al.* 2010b), consistent with the notion that higher intensity appears to be the most beneficial for the heart. In the following sections, we will describe the different factors released during exercise that initiate the protective phenotypic shift.

ADENOSINE

Adenosine is a purine nucleoside molecule that has been identified as a trigger of exercise-induced adaptations within the myocardium. Signaling occurs through four cell-surface receptors distributed heterogeneously throughout regions of the myocardium: adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm *et al.* 2011). Adenosine receptor activation signals through G-protein coupled receptors (G_i, G_s, G_o, and G_q) leading to the targeting of various downstream effectors and divergent regulation of cardiac function (Chandrasekera *et al.* 2010). During exercise cardiac adenosine levels rise proportional to increasing heart rate (Watkinson *et al.* 1979). A potential interplay between heart rate and adenosine release in exercise cardioprotection was demonstrated in dogs where the infarct salvage observed following intermittent bouts of tachycardia was abolished with administration of an adenosine receptor blocker (Domenech

et al. 1998). Support for the cardioprotective effect of adenosine is also provided in non-exercise, non-I/R studies whereby treatment with adenosine leads to the activation of endogenous antioxidant defense systems, and the adenosine receptor antagonist theophylline abolishes this effect (Maggirwar *et al.* 1994; Husain and Somani 2005). Similarly, adenosine receptor blockade during exercise exacerbates post-exercise oxidative stress biomarkers (Husain and Somani 2005). Taken together, these findings indicate that the increase in heart rate during exercise leads to a transient oxidative stress which is blunted through adenosine-induced upregulation of the antioxidant defense system. However, the intermediate signaling of adenosine that may be responsible for triggering exercise cardioprotection is less well defined. Non-exercise studies suggests that A_1 receptor activation reduces infarct size by priming the opening of mitochondrial potassium adenosine triphosphate (mitoK_{ATP}) channels, presumably through a PKC mediated mechanism (Sato *et al.* 2000). One study demonstrated that opening of mitoK_{ATP} channels may play a role in the early phase of exercise cardioprotection, as the early window of protection was abolished with channel blockade during exercise (Domenech *et al.* 2002). However, exercise-induced mitoK_{ATP} channel activity has not been linked to adenosine signaling and merits further research before conclusions can be drawn. Therefore, these results suggest that transient increases in adenosine levels are important for ROS buffering during acute exercise, but whether or not this is due to opening of mitoK_{ATP} channels is not known. While cardiac adenosine signaling following exercise seems to be important for the activation of redox networks, adenosine has not been established as being solely responsible for the increase in antioxidant capacity. In addition, it is also unknown if adenosine receptor blockade during consecutive exercise bouts would mitigate the upregulation in antioxidant defense systems.

One of the limitations in our understanding of adenosine as a trigger for exercise cardioprotection is the lack of knowledge pertaining to the specificity of adenosine receptor activation following exercise. As mentioned previously, there are four different adenosine receptors, and the specific subtypes activated following exercise has not been well characterized. For example, pharmacological blockade of adenosine receptors with theophylline is thought to inhibit signaling through A_1 and A_{2A} receptors (Jacobson *et al.* 2012). Theophylline is commonly used as an adenosine receptor blocker (Domenech *et al.* 1998; Husain and Somani 2005), but the specificity of its action and the downstream signaling events has not been tested in exercise-preconditioning studies. The use of non-specific pharmacological compounds is problematic from a mechanistic standpoint because adenosine receptor activation elicits divergent effects depending on the subtype of receptors activated. Further, adenosine receptors possess the ability to dimerize with other subtypes (Fredholm

et al. 2011), leading to greater complexity in the biological actions of adenosine. Nonetheless, adenosine appears to exert a substantial effect on cardiac physiology and pathophysiology, but more research is needed to solidify adenosine as a required trigger for exercise cardioprotection.

OPIOIDS

Opioids are another cell-surface signaling molecule that can trigger a protective phenotype. Endorphins, enkephalins, and dynorphins predominately signal through μ-, δ-, and κ-opioid receptors respectively, each with various subtypes distributed centrally and peripherally (van den Brink *et al.* 2003). Pharmacological activation of κ- and δ-opioid receptors reduces infarct size, with a 'second window of preconditioning' similar to what is seen with exercise (Schultz *et al.* 1998; Fryer *et al.* 1999). Opioid-mediated signaling occurs throughout the nervous system, and there is evidence that striated muscle can produce preproenkephalin mRNA and peptide products (Springhorn and Claycomb 1989; Weil *et al.* 2006).

Several studies have examined opioids following exercise. A ten-fold increase in overall serum opioid activity immediately following exhaustive exercise has been observed in human (Rahkila *et al.* 1988; Schwarz and Kindermann 1992) and rodent models (Debruille *et al.* 1999), with release of various endorphins being most prominent following high-intensity exercise (>90% $VO2_{max}$). These data are particularly interesting from a dose-response standpoint, as the opioid release occurred following near-maximal exercise, and many studies find benefit after sub-maximal exercise regimens (Brown *et al.* 2005a; Brown and Moore 2007; Calvert *et al.* 2011; Frasier *et al.* 2011a; Taylor and Starnes 2012; Quindry and Hamilton 2013; Powers *et al.* 2014).

Further support for the role of opioids in exercise-induced cardioprotection comes from studies examining blood-borne factors. Michelsen *et al.* (2012) recently observed infarct size reductions in isolated rabbit hearts that were perfused with human plasma dialysates conditioned by acute high-intensity exercise. Co-perfusion with a non-specific opioid antagonist reduced the infarct sparing effect (Michelsen *et al.* 2012), which is consistent with other studies where pre-exercise administration of the non-specific opioid antagonist naloxone/naltrexone abolished protection afforded by a 12-week exercise regimen (Dickson *et al.* 2008; Galvao *et al.* 2011).

Like adenosine mediated protection, there is evidence that opioid signaling also acts through the $mitoK_{ATP}$ channel. In non-exercise studies, protection observed following opioid receptor activation is abolished with the $mitoK_{ATP}$ blocker 5-HD (Fryer et al. 1999). Administration of 5-HD prior to I/R also abolishes the anti-arrhythmic effect of exercise, but opioid levels were not measured (Quindry *et al.* 2010b). However, unlike adenosine-mediated protection, opioid signaling may not exert its protective

effects through the upregulation of antioxidant defense systems. Twenty-four hours after a five-day exercise regimen, mRNA levels of opioid precursors and receptors increased in unstressed hearts while there was no change in superoxide dismutase, HSPs, or catalase (Dickson *et al.* 2008). Even though these specific antioxidant gene transcripts did not change, enhanced ROS buffering cannot be ruled out because antioxidant capacity was not comprehensively analyzed. Although we are still early in our understanding of how opioids are influencing exercise cardioprotection, these preliminary studies provide rationale for their release and biological activity following exercise.

CYTOKINES

Cytokine production during exercise is another putative triggering mechanism of exercise cardioprotection that has received less attention from the scientific community. During exercise contracting muscle acts as an endocrine organ by secreting various cytokines that can facilitate downstream biological actions (Donges *et al.* 2014). In non-exercise studies, early work demonstrated a cardioprotective role for cytokines in I/R injury that involved lower oxidative stress during the reperfusion period (Brown *et al.* 1990; Eddy *et al.* 1992). Subsequent studies by Yamashita *et al.* (1999) sought to determine how exercise-induced cytokine production influenced infarct salvage. They demonstrated that administration of TNF-α and IL-1 antibodies prior to a single exercise bout abolished the early- and late-windows of cardioprotection. However, aside from the cardioprotective effects that cytokines can exert on the myocardium, there are deleterious effects as well. The discrepant findings in the literature regarding adverse and cardioprotective actions of cytokines on I/R has been reviewed (Saini *et al.* 2005). The cardioprotective action of cytokines appears to occur at lower concentrations, whereas higher concentrations may exert harmful effects. Moving forward more research is needed to uncover the divergent roles of cytokines on myocardial physiology before they can be implemented as therapeutic agents for I/R injury.

ADRENERGIC SIGNALING

The role of adrenergic receptor stimulation during exercise has become recognized as a part of exercise-induced cardioprotection. In response to systemic demand, β-adrenergic stimulation increases cardiac chronotropy, inotropy and lusitropy (reviewed in (Steinberg 1999)). These effects are mainly attributed to the β_1-adrenergic receptor which is the predominant isoform in the heart, but β_3-adrenergic receptors appear to play a contradictory role, as stimulation leads to a negative inotropic response (Tavernier *et al.* 2003; Napp *et al.* 2009). The negative inotropic effect is mediated through downstream activation of eNOS (Gauthier *et*

al. 1998). However, the existence of a functional β_3-adrenergic receptor in the human heart has recently been called into question due to the lack of selectivity of pharmacological tools used to study its function and expression (reviewed in (Michel *et al.* 2011)). Nonetheless, it has been postulated that β-adrenergic stimulation may trigger exercise cardioprotection by increasing NO bioavailability. β-adrenergic stimulation of cardiac tissue via the sympathetic nervous system has been shown to be important in triggering the protective phenotype, as ablation of the cardiac sympathetic nerve with topical application of phenol abolishes the infarct salvage afforded by seven days of exercise in mice (Akita *et al.* 2007). The authors attributed these effects to a decrease in eNOS activity because an increase in eNOS phosphorylation was not observed in mice with cardiac sympathetic nerve ablation, but was increased with exercise alone. Interestingly, the transient oxidative stress observed with exercise was also absent with cardiac sympathetic nerve ablation, indicating interplay between adrenergic stimulation, NO, and ROS in exercise cardioprotection. In another study Calvert *et al.* (2011) also demonstrated that adrenergic receptors play an important role in exercise cardioprotection via interaction with the NOS isoforms. Plasma catecholamine and β_3-adrenergic receptor density increased following four weeks of voluntary wheel running, with no changes in the β_1 and β_2 isoforms. The cardioprotection against myocardial infarction observed in the voluntary wheel running mice was abolished in β_3-adrenergic receptor deficient mice. Similar to the previous study that linked adrenergic signaling to increased eNOS phosphorylation, eNOS phosphorylation as well as cardiac NO metabolites were depressed in β_3-adrenergic receptor deficient mice exposed to voluntary wheel running. These findings implicate adrenergic signaling as a triggering mechanism during exercise. In this regard, Calvert *et al.* (2011) demonstrated that a single epinephrine bolus increased eNOS phosphorylation and heart NO metabolites. Importantly, infarct salvage following voluntary wheel running was lost when NO metabolites returned to normal levels after four weeks of exercise cessation. Taken together, there is strong evidence for a role of adrenergic signaling in the triggering phase of exercise cardioprotection and the subsequent upregulation of NO bioavailability. However, more research is needed to fully characterize the specific role of β_3-adrenergic receptor stimulation in NOS activation, especially in light of the fact that β_2-adrenergic receptor activation has also been shown to be cardioprotective and can increase eNOS activity and NO metabolites (Bhushan *et al.* 2012).

NITRIC OXIDE

NO was initially thought to act only through local mediation of vasodilation due to its short half-life and high reactivity with biological substrate (Liu *et al.* 1998). However, more recent work implicates NO in

downstream mechanisms distant from the site of production (Calvert *et al.* 2011; Chouchani *et al.* 2013; Farah *et al.* 2013), as well as in cardiac myocytes themselves (reviewed in (Bloch *et al.* 2001)). During exercise, blood flow and vascular shear stress are elevated in tissue beds with high metabolic activity, which leads to the activation of endothelial nitric oxide synthase (eNOS) and heightened release of NO (Wang *et al.* 1993; Sessa *et al.* 1994; Zhang *et al.* 2009; Barbosa *et al.* 2013). NO metabolites such as nitrite, nitrate, and nitrosothiols were once thought of as inert, but are now widely accepted as storage forms of NO that undergo inter-conversion to exert biological effects (Zweier *et al.* 1995; Webb *et al.* 2004; Bryan *et al.* 2005; Calvert *et al.* 2011). In non-exercise studies, the molecular reduction of nitrite to NO and nitrosothiols during I/R is cardioprotective (Webb *et al.* 2004; Bryan *et al.* 2007; Chouchani *et al.* 2013), which indicates that an increase in NO bioavailability may be an important determinant of exercise-induced cardioprotection.

Following exercise, there is an increase in eNOS activation and NO metabolites (Akita *et al.* 2007; Calvert *et al.* 2011), and when eNOS is genetically knocked out, the infarct sparing effects after seven days of exercise is abolished (Akita *et al.* 2007). The study also demonstrated that upregulation of eNOS during exercise was necessary for the subsequent increased activity of inducible NOS (iNOS) and the downstream infarct sparing effect of exercise (Akita *et al.* 2007). Others have observed an increase in iNOS activity following an acute bout of exercise, and when an iNOS inhibitor was administered prior to I/R the antiarrhythmic effect of exercise was abolished (Babai *et al.* 2002). However, the role of iNOS in exercise cardioprotection has been called into question due to the interspecies variability in expression patterns and a lack of increase following various exercise regimens (Quindry *et al.* 2010a; Calvert *et al.* 2011). More recently Farah *et al.* (2013) demonstrated a role for eNOS in exercise cardioprotection in rats after five weeks of training. Following the exercise regimen, phosphorylation of eNOS and levels of s-nitrosylated proteins and nitrite were increased in the exercise group. Perfusion with a global NOS inhibitor prior to and immediately after I/R abolished the infarct sparing and mechanical recovery observed with exercise. They also demonstrated that eNOS uncoupling during the reperfusion period was required for the cardioprotection. However, not all groups demonstrate an essential role for NO in exercise cardioprotection. Taylor *et al.* (2007) administered a global NOS inhibitor prior to two days of exercise with the idea that cardioprotection would be lost. However, the beneficial effects of exercise on mechanical recovery and LDH release after I/R in rats were not different than with exercise alone (Taylor *et al.* 2007). The main difference in these studies is the timing of NOS inhibition (before exercise vs before I/R), and the duration of the exercise regimen. The study by Taylor *et al.* (2007) provides evidence against a role for NO production

during exercise as a triggering mechanism for cardioprotection. However, NO production during exercise may not be responsible for cardioprotection per se, rather the increase in NO bioavailability and increase in eNOS activation (phosphorylation) seems to be more important in the cardioprotective phenotype. A mechanism whereby NO production can increase after exercise has been demonstrated. Following acute exercise, circulating bradykinin levels increase (Blais *et al.* 1999), stimulating the production of NO and NO metabolites (Zhou *et al.* 2010). Furthermore, bradykinin has been demonstrated to mediate its anti-arrhythmic effects through liberation of NO during I/R (Vegh and Parratt 2005). Given the discrepant findings, a few questions are left that need to be addressed moving forward: What is the locus of NO production that leads to an increase in NO metabolites (endothelium vs cardiac myocytes)? What are the temporal characteristics of NO production during and/or following exercise? When precisely does the cardioprotective phenotype become evident? In response to the latter, most studies indicate that storage forms of NO precipitate their cardioprotective effects during reperfusion. Clearly more work is needed to definitively determine the role of NO production during/after exercise and how this affects NO metabolite accumulation en route to cardioprotection.

ADENOSINE MONOPHOSPHATE-ACTIVATED PROTEIN KINASE

Cardiac myocytes are densely packed with mitochondria in order to support cellular energetic requirements. In the healthy heart, the heightened rate of ATP hydrolysis during exercise increases mitochondrial respiration, ultimately allowing healthy myocytes to efficiently match ATP generation to cardiac workload. While cellular ATP:ADP ratios remain constant, AMP levels are thought to rise with increasing exercise intensity, leading to the activation of AMPK in cardiac muscle (Frederich and Balschi 2002; Coven *et al.* 2003). In this context, the activation of AMPK stimulates catabolic processes and down regulates anabolism allowing the cell to regulate metabolism for the production of ATP (Coven *et al.* 2003). AMPK has been deemed as one of the energy sensors of the cell and its activity increases by phosphorylation within 10 minutes of the onset of moderate and high intensity exercise (Coven *et al.* 2003). AMPK has also been shown to be important in post-ischemic cardiac injury, with exacerbated injury in transgenic mice expressing a dominant negative kinase dead α subunit of AMPK (Russell *et al.* 2004). Canonical AMPK signaling increases glucose and lipid oxidation, which is essential for replenishing ATP following an ischemic period.

In addition to increasing catabolism, AMPK has been shown to play a role in ischemic preconditioning by regulating sarcolemmal K_{ATP} channel trafficking and activity (Sukhodub *et al.* 2007). These studies suggest an important role for AMPK activity following ischemia/reperfusion (I/R),

but the extent to which AMPK influences exercise cardioprotection has received less attention. Although studies have consistently shown that exercise increases the phosphorylation of AMPK (Coven *et al.* 2003; Ogura *et al.* 2011), AMPK has not been shown to be crucial for exercise adaptations. Similar levels of exercise can be attained in transgenic mice expressing a cardiac-specific dominant-negative AMPKα2 subunit (Musi *et al.* 2005). Following 30 minutes of exercise, transgenic mice had similar cardiac glycogen and ATP levels as wild-type controls. A similar metabolic profile between the wild type and transgenic mice indicates that AMPK may not be crucial for enhanced cardiac metabolism, and that other overlapping pathways can help meet energy requirements during increased demand. Although AMPK is an attractive target for the cell to regulate its energy needs during metabolic stress, there is a gap in the literature linking exercise-induced AMPK activation with cardioprotection. More research is required to definitively determine if/how exercise influences AMPK activity in the heart, and whether or not these changes modify cardioprotection.

REACTIVE OXYGEN SPECIES

Cardiac ROS are another potential candidate involved in exercise cardioprotection, as well as other preconditioning stimuli such as ischemic and pharmacological preconditioning (Garlid *et al.* 2013). A large body of literature suggests that exercise induces a transient oxidative stress that leads to upregulation in antioxidant defense systems; however the locus of ROS production and downstream effectors during exercise remains unclear. In this section we will focus on evidence for the role of mitochondrial ROS in exercise cardioprotection, and cytosolic ROS in the following section.

ROS have received considerable attention in the cardiac literature due to their role in pathologies like I/R injury, heart failure, and cardiomyopathies. However, a growing body of literature suggests that ROS exert hormesis, where transient bursts of ROS leads to favorable adaptive redox signaling. Cellular ROS can act as second messengers in downstream signaling by altering the activity of redox sensitive enzymes throughout the cytosol and/or mitochondria of cardiac myocytes (Sadoshima 2006). Similar mechanisms may occur when transient bursts of ROS are generated during exercise (Bolli 1988; Hamilton *et al.* 2004; Dabkowski *et al.* 2008). Following acute exercise there is an alteration in cellular redox status towards a more oxidized environment which may act as a signal to activate endogenous protective mechanisms (Nelson *et al.* 2011; Frasier *et al.* 2013).

There is general consensus that an increase in antioxidant enzymes is responsible for a large portion of exercise cardioprotection, and transient oxidative stress with exercise may play a role in this adaptation. Evidence

for this has been provided by several groups who have observed increases in key antioxidant enzymes following exercise (Yamashita *et al.* 1999; Demirel *et al.* 2001; Hamilton *et al.* 2001; Quindry *et al.* 2010b; Frasier *et al.* 2011b; Lee *et al.* 2012; Frasier *et al.* 2013). Studies in favor of this hypothesis have shown that administration of antioxidants prior to exercise abolishes infarct salvage (Yamashita *et al.* 1999; Akita *et al.* 2007) and prevents exercise-induced improvements in cardiac performance (Nelson *et al.* 2011). However, another study indicated that ROS generated during exercise were not required for functional recovery following I/R (Taylor and Starnes 2012). The antioxidant frequently used in these studies was N-(2-mercaptopropionyl)glycine) (MPG), which was administered intraperitoneally 10-30 minutes prior to exercise. An important note to consider is that MPG has been shown to have higher specificity for hydroxyl radicals rather than hydrogen peroxide (H_2O_2) and superoxide (Bolli *et al.* 1989), indicating that not all ROS signaling is abolished with treatment. In addition, MPG has a plasma half-life of approximately 7 minutes (Horwitz *et al.* 1994), making it difficult to interpret how effective the treatment was at scavenging ROS during hour-long exercise bouts. These methodological differences make it difficult to directly compare their results (i.e. differences in species, duration of I/R, duration of exercise, measurement of injury, the timing of the administration of antioxidants, and in vivo versus ex vivo experiments).

Mitochondrial ROS

ROS signaling is a highly regulated and localized process, implying that the origin of ROS generated during exercise may be extremely important. Although mitochondrial ROS are thought to play a central role in ischemic preconditioning (Baines *et al.* 1997; Pain *et al.* 2000; Kloner and Jennings 2001), there is a paucity of evidence suggesting a role in exercise preconditioning. Frasier *et al.* (2013) recently found that the locus of ROS production during exercise is not mitochondrial in origin. As shown in Figure!1, exercise cardioprotection was not lost when administering agents that reduce mitochondrial ROS prior to exercise (mito TEMPO and Bendavia). This indicates that extramitochondrial-derived ROS may be responsible for redox signaling following exercise.

Monoamine oxidase-A (MAO-A) is another potential site for mitochondrial ROS production. MAO-A is located on the outer mitochondrial membrane and catalyzes the oxidative deamination of neurotransmitters such as norepinephrine and serotonin while generating H_2O_2 as a byproduct in the reaction. A recent review highlights the importance of MAO-A in pathological states such as heart failure and I/R (Kaludercic *et al.* 2011). Accumulation of serotonin released by platelets during I/R can lead to the production of H_2O_2 and subsequent apoptotic signaling cascades (Bianchi *et al.* 2005). Recent findings indicate that exercise leads to

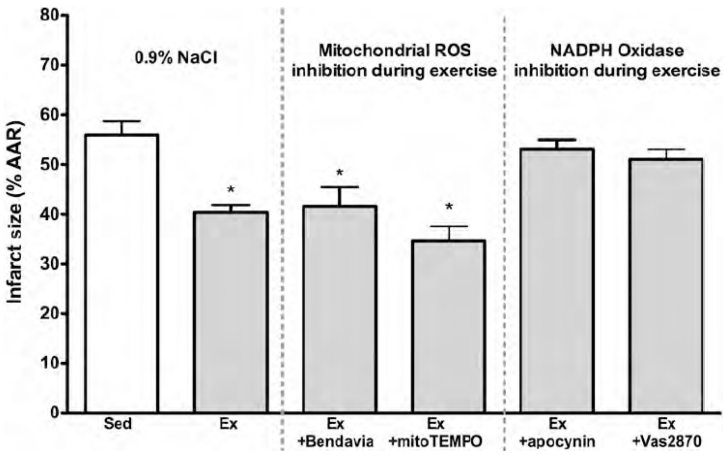

FIGURE 1. Reductions in infarct size are abolished by inhibiting NADPH Oxidase (with pre-exercise treatment of apocynin or Vas2870) during exercise. Inhibition of mitochondrial ROS during exercise (with pre-exercise administration of TEMPO or the mitochondria-targeting peptide Bendavia) had no effect on exercise cardioprotection. Figure reproduced from Frasier et al., *Cardiovascular Research* 2013, with permission (pending).

down-regulation of MAO-A (Kavazis *et al.* 2009), which may play a role in the attenuation of I/R damage associated with exercise cardioprotection. Moreover, these findings indicate that there is likely a *reduction* in mitochondrial ROS production with exercise, given the decrease in MAO-A expression and increases in the activity of key antioxidants such as MnSOD and glutathione reductase (GR) (Quindry *et al.* 2010b; Frasier *et al.* 2013). A decrease in cardiac mitochondrial MAO-A would theoretically dampen the oxidative burden imposed on the cell, not only during exercise, but also during thrombus formation and subsequent I/R injury. A mechanism for the decrease in MAO-A expression following exercise has not been investigated and therefore the triggering event for this adaptation is purely speculative. Perhaps acute increases in cardiac sympathetic nerve stimulation and increasing norepinephrine levels during exercise play a role in downstream silencing of MAO-A through non-canonical adrenergic pathways (Vidal *et al.* 2012). Cardiac sympathetic stimulation increases contractility and myocardial stretch during exercise, which in and of itself may trigger a cardioprotective phenotype through elevated cytosolic ROS production (Frasier *et al.* 2013; Ward *et al.* 2014). Furthermore, cardiac sympathetic nerve ablation has been shown to abolish the infarct sparing effect of exercise, but this was not linked to silencing of MAO-A expression (Akita *et al.* 2007). A hypothetical adrenergic/MAO-A axis scenario opens up an exciting area of research to explore mechanisms controlling MAO-A expression in cardiac tissue during normal physiological as well as pathophysiological states. We will further expand on the topic of stretch-induced activation of cardioprotection in the next section.

Cytosolic ROS

Free-radical generating enzyme systems outside of the mitochondria have also received considerable interest in normal physiology as well as in pathological states such as I/R injury (McCord 1985; Bolli 1988; Misra *et al.* 2009) and heart failure (Tsutsui *et al.* 2011; Harzand *et al.* 2012). Sources of extramitochondrial-derived ROS in cardiac myocytes include xanthine oxidase, NADPH oxidase, and uncoupled nitric oxide synthase. Of these, the NADPH oxidase (NOX2 in particular) complex generates ROS in a highly localized manner in the sarcolemmal and t-tubule membranes during physiological stretch (Sanchez *et al.* 2008; Prosser *et al.* 2011). Myocardial contraction and wall stress increases during exercise as a function of heart rate and adrenergic signaling. The increased inotropic and chronotropic state is an autoregulatory mechanism that allows for tight regulation of blood pressure and delivery of nutrients to metabolically active tissue. Recent work indicates that the sarcolemmal NOX2-generated ROS system plays a central role in this phenomenon. NOX2-generated ROS imposes redox signaling through ryanodine receptors leading to increased calcium release and subsequent contractile activity (Sanchez *et al.* 2008; Donoso *et al.* 2014). Stretch induction through the microtubule network and NOX2 activation has been termed X-ROS signaling (Prosser *et al.* 2011). X-ROS signaling describes the transfer of a mechanical to a chemical signal throughout the heart via the microtubule system, leading to assembly of the NOX2 ROS generating complex. Recently, several independent groups have established a role for NOX2 as a potential trigger for the cardioprotective phenotype associated with exercise (Sanchez *et al.* 2008; Frasier *et al.* 2013).

As mentioned previously, a critical threshold of exercise intensity appears to be important for cardioprotection, and at higher exercise intensities myocardial contraction increases in conjunction. In line with the X-ROS signaling hypothesis, increased inotropy and myocardial stretch during exercise may lead to activation of NOX2 and perhaps downstream adaptations. We and others have demonstrated that inhibition of NOX2 prior to exercise abolishes the infarct salvage of early and late phases of exercise cardioprotection (Sanchez *et al.* 2008; Frasier *et al.* 2013). Furthermore, the upregulation of GR activity that is typically observed following exercise (Venditti and Di Meo 1996; Ramires and Ji 2001; Kakarla *et al.* 2005; Frasier *et al.* 2011b) is also abolished immediately and 24 hours after the exercise bout when NOX2 is inhibited during exercise (Frasier *et al.* 2013). GR is a central enzyme involved in cellular redox control by utilizing NADPH to convert oxidized glutathione to the reduced form. Therefore, increasing GR activity allows the cell to maintain the glutathione pool in the reduced state, thus providing a greater buffering power during oxidative insults. During an exercise bout mechanical stretch of the myocardium increases, leading to NOX2-generated ROS and activa-

tion of GR (Frasier *et al.* 2013). ROS signaling through GR may be a mechanism where GR acts as a sensor during oxidative shifts of the redox environment, leading to upregulation of endogenous defense systems. Future studies examining the time frame of GR activation and sustainability of protection will shed light on signaling between NOX2 and GR during the cardioprotective window of exercise. Also, studies that determine the importance of GR compartmentalization are warranted, particularly with regard to cytosolic and/or mitochondrial GR pools involved in this adaptive signaling network (Kang *et al.* 2014). While it seems apparent that exercise upregulates redox buffering capacity, more research is needed to definitively determine if transient bursts of ROS during exercise act as a signal to trigger downstream cardioprotection.

CONCLUSIONS

We have described a number of circulating and intrinsic factors postulated to induce cardioprotective signaling with exercise. These factors converge on the myocardium, and result in downstream adaptations that characterize the protective phenotype. Subsequent investigation into these downstream effects using novel approaches will greatly advance the field. For example, ROS production during exercise is an intriguing factor that leads to both post-translational modifications to existing proteins in the short-term, as well as altered protein expression on a longer time-scale. Given that 21,000 to 42,000 thiols in the proteome can contribute to the integration of metabolic function through redox signaling (Jones 2008), further exploration of the redox hypothesis in the context of exercise adaptations is warranted. The convergent effects of cellular ROS production and elevated levels of cell-signaling molecules such as adenosine, NO, cytokines, and catecholamines during elevated workloads transduce the exercise stimulus that culminates into a hormetic cardiac response. Inhibition of any one of these putative triggers can dampen the cardioprotective phenotypic switch observed with exercise, but ultimately these adaptations lead to tolerance to I/R injury characterized by lower arrhythmia and decreased myocardial infarction. Given that exercise is known to confer protection in humans, future studies that continue to advance our understanding of the intrinsic factors responsible for evoking this protective phenotype may ultimately pave the way for novel therapies to reduce the burden of acute coronary syndromes.

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