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Untargeted metabolomics analysis of ischemia–reperfusion-injured hearts ex vivo from sedentary and exercise-trained rats

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Abstract

Introduction—The effects of exercise on the heart and its resistance to disease are well-documented. Recent studies have identified that exercise-induced resistance to arrhythmia is due to the preservation of mitochondrial membrane potential.

Objectives—To identify novel metabolic changes that occur parallel to these mitochondrial alterations, we performed non-targeted metabolomics analysis on hearts from sedentary and exercise-trained rats challenged with isolated heart ischemia–reperfusion injury (I/R).

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Traci L. Parry and Joseph W. Starnes have contributed equally to this study.

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Author Contributions TP, JS, and MW conceived and designed the experiments; TP, JS, AH, PH, JB, MM, and AI performed the experiments, SO, JB, MM, and MW were involved in the analysis and interpretation of the data, and JS, TP, and MW wrote the draft manuscript. TP, JS, SO, JB, MM, AH, AI, PC, CP, and MW edited and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This investigation was approved by the UNC-Greensboro’s Animal Care and Use Committee and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Methods—Eight-week old Sprague–Dawley rats were treadmill trained 5 days/week for 6 weeks (exercise duration and intensity progressively increased to 1 h at 30 m/min up a 10.5% incline, 75–80% $\text{VO}_{2\text{max}}$). The recovery of pre-ischemic function for sedentary rat hearts was $28.8 \pm 5.4\%$ ($N = 12$) compared to exercise trained hearts, which recovered $51.9\% \pm 5.7$ ($N = 14$) ($p < 0.001$).

Results—Non-targeted GC–MS metabolomics analysis of (1) sedentary rat hearts; (2) exercise-trained rat hearts; (3) sedentary rat hearts challenged with global ischemia–reperfusion (I/R) injury; and (4) exercise-trained rat hearts challenged with global I/R (10/group) revealed 15 statistically significant metabolites between groups by ANOVA using Metaboanalyst ($p < 0.001$). Enrichment analysis of these metabolites for pathway-associated metabolic sets indicated a > 10 -fold enrichment for ammonia recycling and protein biosynthesis. Subsequent comparison of the sedentary hearts post-I/R and exercise-trained hearts post-I/R further identified significant differences in three metabolites (oleic acid, pantothenic acid, and campesterol) related to pantothenate and CoA biosynthesis ($p = 1.24\text{E}-05$, FDR = $5.07\text{E}-4$).

Conclusions—These studies shed light on novel mechanisms in which exercise-induced cardioprotection occurs in I/R that complement both the mitochondrial stabilization and antioxidant mechanisms recently described. These findings also link protein synthesis and protein degradation (protein quality control mechanisms) with exercise-linked cardioprotection and mitochondrial susceptibility for the first time in cardiac I/R.

Keywords

Exercise; Ischemia/reperfusion injury; Metabolism; Cardioprotection

1 Introduction

Ischemic heart disease, including acute myocardial infarction, is a leading cause of morbidity and mortality in the world (Moran et al. 2014; Finegold et al. 2013; Nowbar et al. 2014). Beyond its benefits of reducing cardiovascular risk factors, exercise has been reported to protect against ischemia/reperfusion injury via direct effects on the heart (Powers et al. 2002, 2008). The specific mechanism involved in this exercise-induced cardiac “pre-conditioning” is not clear. One recent systemic review found evidence for increased heat shock protein production, increased cardiac anti-oxidant capacity, improvement of ATP-dependent K^+ channel function, and opioid system activation (Borges and Lessa 2015). However, no clear conclusions have been drawn as to the mechanisms of exercise-induced cardioprotection (Borges and Lessa 2015).

Exercise-induced cardioprotection from the biochemical point of view has yielded evidence for redox-based mechanisms in producing cross-tolerance induced by endurance training (Ascensao et al. 2007). This includes stabilization of mitochondrial energetics, with exercise training in rats providing sustained respiration with lower H_2O_2 emission rates as compared to sedentary rats (Alleman et al. 2016). Exercise has been found to increase mitochondrial antioxidant enzymes (i.e., glutathione peroxidase, manganese superoxide dismutase, copper–zinc superoxide dismutase) and prevent IR-induced release of pro-apoptotic mitochondrial proteins (Lee et al. 2012).

As a metabolic omnivore, the heart utilizes fatty acids, glucose, ketone bodies, pyruvate, and lactate, as well as amino acids (in descending preference) to maintain function. In cardiac ischemia reperfusion injury, alterations in metabolism and electrical conduction are well known to occur. The contribution of amino acids in maintaining cardiac stability during ischemia reperfusion stress is considered underappreciated (Drake et al. 2012). Studies to date seeking mechanisms of exercise-induced cardioprotection have primarily relied on reductionist approaches. Advances in technology allow for more integrative, systemic approaches that permit rigorous characterization and mapping of the physiological network and greater insight into mechanisms underlying cardioprotection. Metabolomics is one of these technologies and the present study is the first to apply it to explore mechanisms of exercise-induced cardioprotection. To further understand the changes that occur in exercise-induced cardioprotection in the broader cardiac metabolome, hearts from rats completing a chronic endurance exercise program and those from age-matched sedentary controls were challenged with an ischemia–reperfusion stress ex vivo. Hearts were then analyzed by non-targeted metabolomics to reveal novel molecular changes associated with exercise-associated pre-conditioning functionality.

2 Materials and methods

2.1 Animals and exercise treatment

Male Sprague–Dawley rats were purchased from Charles River and housed in the UNC-Greensboro animal facility in an isolated room maintained at 22 °C with a 12:12-h light–dark cycle. They were singly housed to allow for food consumption monitoring. Rats were fed ad libitum with an AIN-93G based rodent chow (Cat#100700, Dyets, Inc., Bethlehem, PA) composed of 19.3% protein, 64.0% carbohydrates, and 16.7% fat (Reeves et al. 1993). At 8 weeks of age, they were divided into sedentary (S; $n = 26$) and exercise-trained (E; $n = 23$) groups. Exercise was carried out on a motor-driven treadmill, set at a 10.5% incline, 5 days/wk for 6–7 weeks in an adjoining room maintained at 20 °C. Running duration and speed were gradually increased over 22 days to 60 min at 30 m/min, corresponding to 75–80% $\text{VO}_{2\text{max}}$ (Dudley et al. 1982), and then maintained at this level for the remaining 2–3 weeks. Sedentary rats were placed on a stationary treadmill in the same room during the daily exercise bout. This investigation was approved by the UNC-Greensboro's Animal Care and Use Committee and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996).

2.2 Isolated heart perfusions

At least 5 h after the last exercise session, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). Upon reaching the surgical plane of anesthesia, 100 IU heparin was injected into the inferior vena cava and the heart removed, weighed, and its aorta rapidly connected to the perfusion apparatus as previously described (Bowles et al. 1992). The composition of the Krebs–Henseleit buffer was (in mM) 118 NaCl, 4.7 KCl, 1.25 CaCl_2 , 0.6 KH_2PO_4 , 1.2 MgSO_4 , 24.7 NaHCO_3 , 10 glucose, and 6 IU/L insulin; the pH was maintained at 7.4 by equilibration with 95% O_2 :5% CO_2 ; and the temperature was maintained at 37 °C. Hearts were initially perfused in a non-recirculating retrograde, or Langendorff, mode at a perfusion pressure of 65 mmHg, while the left atrium

was cannulated for subsequent measures of performance in the working heart mode. Cardiac function in the working mode was evaluated at 13 cm H₂O (atrial filling pressure) with an 80-cm-high aortic column (ID 3.18 mm). Coronary (CF) and aortic (AF) flows were determined by timed collection of the effluent dripping off the heart and aortic column overflow, respectively. Cardiac output (CO) was determined as the sum of CF and AF. Cardiac external work in the working heart mode is defined as the product of CO and peak aortic systolic pressure (CO × SP). Aortic pressure and heart rate were monitored with a Gould DTX pressure transducer interfaced with an ADInstruments PowerLab system. Pre-ischemic function was evaluated after 25 min of perfusion. Global ischemia was then induced by simultaneously cross-clamping the atrial inflow and the aortic outflow lines for 25 min. During ischemia, hearts were submerged in non-oxygenated perfusion buffer within a water-jacketed chamber maintained at 37 °C. After ischemia, hearts were initially reperfused in the Langendorff mode for 15 min at a perfusion pressure of 65 mmHg and then switched to the working heart mode. Hearts recovered for 30 min after ischemia. After the last functional measurement, hearts were returned to the Langendorff mode, the atrial cannula removed, and the heart freeze-clamped and stored at −80 °C. The total length of time on the perfusion apparatus was 80 min. Preischemic control hearts ($n = 10$ in both groups) were perfused in oxygenated buffer for 10 min to wash out blood, then freeze-clamped and stored at −80 °C. Additional animals from the sedentary group ($n = 3$) were perfused with oxygenated buffer for 80 min to serve as time controls for the ischemia–reperfusion procedure and to verify the stability of the perfusion system.

2.3 Training status

Plantaris muscles from eight animals in each group were used for measurement of cytochrome *c* oxidase activity, a marker of mitochondria content. Briefly, muscles were homogenized in 20 volumes of ice-cold 50 mM KH₂PO₄, 0.1 mM EDTA, and 0.1% Triton X-100 (pH 7.4) and centrifuged at 10,000×*g* for 5 min at 4 °C. The supernatants were analyzed for cytochrome *c* oxidase activity polarographically at 37 °C using a Clark-type oxygen electrode and done in triplicate as described previously (Mitchell et al. 2002).

2.4 Non-targeted metabolomics determination by GC–MS instrumentation

Left ventricular tissue was flash frozen in liquid nitrogen, weighed (25–50 mg wet wt), then placed in buffer (50% acetonitrile, 50% water, 0.3% formic acid) at a standard concentration of 25 mg/475 µl buffer and fully homogenized on ice for 20–25 s. Tissues were then placed on dry ice and stored at −80 °C. Samples were analyzed by GC/MS, as previously described (Banerjee et al. 2015). The raw, transformed, and sorted data used is found in Supplemental Table 1. Four groups with ten biological replicate samples were analyzed (40 total). If more than three individuals did not have a metabolite detected in a group (of 10 total), they were excluded from further analysis for that metabolite. In groups missing values, the lowest value of that group was used to impute those values. The data obtained in this study is accessible at the NIH Common Fund's Data Repository and Coordinating Center (supported by NIH Grant, U01-DK097430) website, <http://www.metabolomicsworkbench.org> (Project ID PR000623) (Sud et al. 2016).

2.5 Metabolomic statistical analyses

Metaboanalyst (v3.0) run on the statistical package R (v2.14.0) used metabolite peak areas (as representative of concentration) (Xia et al. 2009, 2015). These data were scaled using Pareto scaling feature. A one-way analysis of variance (ANOVA) and Fisher LSD post-hoc test across the groups (hearts from sedentary animals, sedentary hearts challenged with I/R, hearts from exercise-trained animals, and exercise-trained hearts challenged with I/R) were performed using Metaboanalyst v3.0. ANOVA-significant metabolites ($FDR < 0.05$) were matched to metabolomics pathways using the Pathway Analysis and Enrichment Analysis features in Metaboanalyst 3.0. Only metabolites identified and detected in all groups were included in the one-way ANOVA. All data from this study are available in Supplemental Table 1. Data are presented as mean $\pm$ SEM, unless otherwise indicated.

2.6 Other statistical analyses

Differences between sedentary and exercise-trained groups were compared using an independent t test (2-tailed); comparisons of increases after exercise training between muscle types were analyzed using a 2-tailed t test followed by a one-way ANOVA (Fig. 1) and plotted in Prism 7.0 (GraphPad Software, Inc., La Jolla, CA).

3 Results

After exercise training 5 days a week on a motor-driven treadmill for 6 weeks, exercised rat body weight was significantly less (-6.6% , $p < 0.05$) than that of sedentary animals (Table 1). Exercise training resulted in significant increases in heart weight as compared to sedentary controls, with an increase in $+9.2\%$ ($p < 0.05$) heart weight to body weight ratio (Table 1). In addition to physiological increases in heart size, significant increases in plantaris cytochrome oxidase activity was identified, consistent with exercise conditioning (Table 1). Hearts were assayed ex vivo for function using an isolated working heart preparation, where ischemia and reperfusion were applied to determine their effects on function. Hemodynamic parameters were recorded after 25 min of normoxic perfusion (pre-ischemia). Global no-flow ischemia was then applied for 25 min, followed by 30 min of reperfusion (I/R). Pre-ischemic function did not differ between sedentary and exercise-trained rat hearts (Table 2). Global ischemia induced significant mechanical dysfunction (both CO and systolic pressure) in both groups, which was attenuated ($54.9 \pm 5.3\%$ recovery) in exercise-trained hearts, compared to sedentary hearts, which recovered only $27.5 \pm 5\%$ of their function (Table 2). In time-control hearts, no decline in cardiac function occurred, with $100.3 \pm 2.9\%$ of their function present at 80 minutes compared to their function 25 min pre-ischemia. Taken together, these changes verified a high state of exercise training and identified that the exercise-trained hearts were significantly protected from I/R injury, indicative of the exercise pre-conditioned cardioprotection known to be induced with exercise training.

Ten heart samples from exercise-trained, exercise-trained I/R, sedentary, and sedentary I/R rats were analyzed by non-targeted metabolomics, which identified forty-one named metabolites (Supplemental Fig. 1, Supplemental Table 1). Principal component analysis (PCA) of the results identified a clear separation of exercised and sedentary hearts (PC1

25.8%) and of the sedentary and exercise-trained hearts along PC2 (13.7%) (Fig. 1). Fifteen (15) metabolites were significantly different by ANOVA comparison of the four groups (Fig. 2a). Notably, metabolites were significantly altered after I/R in both exercise-trained and sedentary hearts (yellow boxes, Fig. 2a), with seven increased (top, e.g. lactic acid) and eight decreased (bottom, e.g. glutamine). Pathway analysis of the 15 ANOVA-significant metabolites identified multiple pathways involved, including nitrogen metabolism, aminoacyl-tRNA, and arginine and proline metabolism, with p values $< 1.9 \times 10^{-4}$ and false discovery rates = 5×10^{-3} (Fig. 2b). Pathway enrichment analysis using metabolic sets identified a nearly 10-fold enrichment in ammonia recycling and protein biosynthesis pathways with p values $\sim 5 \times 10^{-5}$ (Fig. 2c).

The three metabolites related to nitrogen metabolism were significantly decreased after I/R injury in both sedentary and exercise hearts, including histidine, glutamine, and glutamic acid (Fig. 3). Exercise hearts had increases in histidine, glutamine, and glutamic acid, which were not significantly different from sedentary hearts (Fig. 3a–c). Of the remaining metabolites in this pathway, glycine also followed similar trends as the other three metabolites (Fig. 3d) but did not reach significance by ANOVA analysis. Specifically, glycine was elevated in exercise-trained hearts compared to sedentary hearts, which decreased in both groups challenged with I/R (Fig. 3d, inset).

The aminoacyl-tRNA biosynthesis pathway was also identified with six ANOVA-significant metabolites (of 69 possible), including aspartic acid, serine, and proline (Fig. 4a–c) along with histidine, glutamine, and glutamic acid (as shown in Fig. 3a–c), also involved in nitrogen metabolism. All six of these metabolites were elevated in exercise-trained hearts compared to sedentary hearts. Like histidine, glutamine, and glutamic acid discussed above, aspartic acid and serine were significantly decreased in both exercise-trained and sedentary hearts after I/R injury (Fig. 4a, b), with aspartic acid concentrations significantly higher in exercise-trained I/R hearts compared to sedentary I/R hearts as well (Fig. 4a).

Of the remaining ANOVA-significant metabolites, six were involved in the citric acid cycle (Fig. 5). These included glucose (Supplemental Fig. 3a), glucose-6-phosphate (Supplemental Fig. 3b), glycerol-1-phosphate, lactic acid, citric acid/isocitric acid, and fumaric acid (Fig. 5a–e). Differences in sedentary and exercise-trained hearts after I/R injury were seen in glucose, glycerol-1-phosphate and lactic acid, which were significantly decreased in exercise I/R versus sedentary I/R hearts (Supplemental Fig. 3a, Fig. 5b, c). Of the remaining ANOVA-significant metabolites (Fig. 5f–h), heptadecanoic acid/octadecanol was also significantly decreased in exercise I/R versus sedentary I/R hearts (Fig. 5g).

To identify further differences between the sedentary and exercise-trained responses to I/R, we next performed a *t* test analysis between these two groups directly. Using Principal Components Analysis (Fig. 6a), the forty-four identified metabolites (Supplemental Fig. 2) showed remarkable similarity between the sedentary and exercised-trained metabolites. The first principal component (PC1), accounting for 42.5% of the differences between groups, did not delineate the two groups (Fig. 6a). It was PC2, accounting for 16.8% of the differences, that distinguished the two groups. Three metabolites were significantly different in the sedentary versus exercise-trained I/R groups, namely oleic acid, pantothenic acid, and

campesterol (Fig. 6b). Pathway analysis of these three significant metabolites indicated significance in the pantothenate and CoA biosynthesis pathway ($p = 0.03$) (Fig. 7a). Pantothenic acid was the single metabolite elevated in the pantothenate and CoA biosynthesis pathway (Fig. 7b), increased ~ 1.8-fold (Fig. 7c). When oleic acid, pantothenic acid, and campesterol were plotted for all groups (Supplemental Fig. 4), a striking loss of oleic acid occurred in exercise training after I/R, which did not occur in the sedentary hearts (Supplemental Fig. 4a). In contrast, pantothenic acid significantly decreased with I/R in the sedentary hearts, but did not decrease in the exercise-trained hearts (Supplemental Fig. 4b). Lastly, campesterol was significantly higher in exercise I/R compared to sedentary I/R hearts, but exercised hearts did not have significantly more campesterol before I/R (Supplemental Fig. 4c). Further enrichment analysis of these three metabolites identified their enrichment in beta-alanine metabolism (20+ fold, Supplemental Fig. 5a), diseases associated with enzymes in the pantothenate metabolic pathway (Supplemental Fig. 5b), and localization in muscle, skeletal muscle, and mitochondria (Supplemental Fig. 5c), as would be expected.

In summary, we identified significant alterations in exercise-trained hearts post-I/R compared to sedentary hearts post-I/R associated with cardioprotection, including increases in aspartic acid (Fig. 4a), and significant decreases in glycerol-1-phosphate (Fig. 5b), lactic acid (Fig. 5c), and heptadecanoic acid/octadecanol (Fig. 5g) compared to sedentary hearts post-MI by ANOVA. Moreover, *t* test analysis of exercise heart post-I/R compared to sedentary hearts identified a significant decrease in oleic acid and significant increases in pantothenic acid and campesterol (Fig. 6b).

4 Discussion

In cardiac ischemia and reperfusion injury, aspartic acid (and glutamic acid) become preferential energy substrates (Arsenian 1998). In hypoxia, the consumption of cardiac aspartic acid (and glutamic acid) increases, resulting in decreases in tissue levels (Taegtmeyer et al. 1977; Gailis and Benmouyal 1973), as seen in the present study (Fig. 4a). The formation of acetyl-CoA by malonyl-CoA decarboxylase, followed by the conversion of acetyl-CoA to citric acid in the presence of oxaloacetate involves transamination of oxaloacetate, which utilizes aspartate aminotransferase and aspartic acid (Constantin-Teodosiu et al. 1991). In the present study exercise significantly attenuated the aspartic acid loss post-I/R (Fig. 4a), which has not previously been reported to our knowledge. Previous studies have found that giving the heart aspartic acid prior to ischemia reperfusion challenge is cardioprotective (Rau et al. 1979). The improvement in contractile function post-MI observed with aspartic acid supplementation is thought to work by decreasing the tissue/cytosolic NADH/NAD⁺ ratio (i.e., the amount of oxidized NADH) in the myocardium in a dose-dependent manner (Park et al. 1998; Saggerson and Greenbaum 1970). Cardiac I/R induces a shift to anaerobic metabolism in the absence of oxygen, resulting in the accumulation of glycerol-1-phosphate, and lactic acid (Jennings and Reimer 1991) as observed in the present study (Fig. 5b–c). During prolonged ischemia, ATP levels and intracellular pH decrease as a result of anaerobic metabolism and lactic acid accumulation (Dennis et al. 1991). The I/R-induced accumulation of these metabolites is significantly attenuated in hearts from rats that were exercise trained (Fig. 5b–c) reflecting a nearly

complete attenuation (esp. in lactic acid) in the shift to anaerobic metabolism. Challenging sedentary hearts to I/R induced a significant increase in heptadecanoic acid/octadecanol levels (Fig. 5g), which has not been previously reported. With our present studies, we cannot differentiate heptadecanoic acid from octadecanol levels. Octadecanol is a fatty alcohol present in human tissue membrane (plasmalogen) glycerophospholipids. Defects in plasmalogen lipids underlie the peroxisomal disorder Zellweger syndrome (Rizzo et al. 1993) and Sjogren–Larsson syndrome characterized by the accumulation of fatty alcohols and reduced activity of the NAD⁺ oxidoreductase complex (Koone et al. 1990). Heptadecanoic acid is a fatty acid of exogenous (primarily ruminant) origin found in human adipose tissue. It has been reported to be a good biological marker of long-term fat intake in individuals that consume dairy products (Wolk et al. 1998). The significant attenuation of heptadecanoic acid/octadecanol in exercise hearts post-MI (Fig. 5g) may reflect differences in the NAD⁺ oxidoreductase complex (if octadecanol).

When we focused our analysis on the differences between sedentary and exercise-trained hearts directly in our *t* test analysis (Fig. 6b), we identified significant reductions in the long-chain fatty acid oleic acid in the present study (Fig. 6b). Recent studies have reported that regular treadmill exercise inhibits mitochondrial accumulation of cholesterol during myocardial ischemia–reperfusion (Musman et al. 2016). The heart is a metabolic omnivore, capable of utilizing fatty acids, glucose, ketone bodies, pyruvate, lactate, and amino acids (in decreasing order). While increases in fatty acid utilization can make the heart more prone to lipotoxic cardiac dysfunction (Palomer et al. 2016), so can decreased fatty acid utilization, making the effects of reduced fatty acids in the present study difficult to interpret.

In addition to decreases in oleic acid, hearts challenged with I/R from sedentary and exercise-trained rats demonstrated elevated pantothenic acid and campesterol levels (Fig. 6b). Pantothenic acid is a precursor of coenzyme A (CoA), which has roles in lipid metabolism and as a prosthetic group in the Krebs cycle (Tuunanen and Knuuti 2011). CoA is critical in transferring fatty acids from the cytoplasm to mitochondria. Decreases in pantothenic acid have been previously identified in metabolomics analysis of isoproterenol-induced myocardial infarction in rats, which was proposed as a biomarker of disturbed lipid/energy metabolism (Liu et al. 2013). Pantothenic acid has been reported to serve as a cardioprotectant in experimental models of I/R in the isolated heart (Kumerova et al. 1992). The other elevated metabolite in exercise trained hearts post-I/R, campesterol, is a phytosterol which competes with cholesterol and reduces cholesterol absorption in the intestine (Fassbender et al. 2008). In vitro, campesterol decreases vascular smooth muscle cholesterol content while increasing prostacyclin release (a vasodilator, of the eicosanoid family of lipid molecules) (Awad et al. 2001). While campesterol has not been tested directly, phytosterols have been reported to protect against cardiac ischemia/reperfusion injury (Wong et al. 2014; Zhang et al. 2010).

Routine exercise has been a practical countermeasure to protect against cardiac injury, with endurance exercise the most protective in young and old animals. The mechanisms that are involved with this cardioprotective effects have included redox changes and the induction of myocardial heat shock proteins, improved antioxidant capacity, and an increase in cardioprotective proteins (Ascensao et al. 2007). Increased levels of HSP70 mRNA occur

after treadmill running in rat myocardium (Paroo et al. 2002) (Locke et al. 1995). Increases in HSP1 occur when hyperphosphorylation of the protein kinase A (PKA), which occurs in many phosphorylative events such as ischemia (Robinet et al. 2005). Administration of PKA inhibitor has been shown to suppress HSP70 mRNA and protein synthesis in rats after a single bout of exercise (Melling et al. 2004). Similarly, exercise training increases the abundance of the heat shock protein 20 (HSP20) (Boluyt et al. 2006), which is phosphorylated by PKA is cardioprotective in ischemic injury (Edwards et al. 2012). Exercise has been found to increase mitochondrial antioxidant enzymes (i.e., glutathione peroxidase, manganese superoxide dismutase, copper-zinc superoxide dismutase) and prevent IR-induced release of pro-apoptotic mitochondrial proteins (Lee et al. 2012). In addition to an increase in cardiac anti-oxidant capacity, studies have found that exercise induces improvement of ATP-dependent K⁺ channel function, and activation of the cardiac opioid system (Borges and Lessa 2015). However, no clear conclusions have been drawn as to the mechanisms of exercise-induced cardioprotection (Borges and Lessa 2015). Exercise-induced cardioprotection from the biochemical point of view has yielded evidence for redox-based mechanisms in producing cross-tolerance induced by endurance training (Ascensao et al. 2007). This includes stabilization of mitochondrial energetics, with exercise training in rats providing sustained respiration with lower H₂O₂ emission rates as compared to sedentary rats (Alleman et al. 2016). Together, these findings demonstrate exercise training induces heat shock proteins, increases antioxidant activity, and increases biochemical changes making cardiomyocytes resistant to cell death.

Previous reports have identified the critical role of glutamic acid as a rate-limiting component of glutathione, which is created from glutamic acid, cysteine, and glycine (Huster et al. 2000). Others have reported that supplementing cells with glutamine and glutamic acid in anoxia results in further benefits, as cells can easily convert these compounds to α -ketoglutarate for rapid utilization as a substrate in the Krebs cycle for production of ATP (Liu et al. 2007; Stottrup et al. 2006; Wischmeyer et al. 2003). Therefore, it was unexpected to find that exercise-trained hearts with significant protection against I/R-induced dysfunction had similar significant decreases in histidine, glutamine, and glutamic acid as compared to sedentary hearts (Fig. 3a–c), as well as decreased glycine (Fig. 3d). In studies of perfused working rat hearts, substantial decreases in intramuscular glutamine and α -ketoglutarate have been reported; supplementation of glutamine rescued cardiac performance (Rennie et al. 2001; Bolotin et al. 2007). The role of ammonia metabolism within an exercising organism is dynamic (Miller-Graber et al. 1991) and may reflect more complex interactions as well as compensatory mechanisms (i.e., upregulation of non-glutathione antioxidants such as superoxide dismutase [SOD] 1 and SOD 2) in cardiac mitochondria [as recently reviewed (Powers et al. 2014)]. The significant decreases in histamine, glutamine, and glutamic acid may also have other implications, since decreases in cardiac aspartic acid, serine, and proline were also identified with exercise training (Fig. 4a–c). All six of these metabolites are pre-cursors of aminoacyl-tRNAs, which could indicate alterations in protein synthesis and/or clearance.

In summary, hearts of rats completing a chronic, moderate-intensity endurance program displayed cardioprotection against I/R injury, and non-targeted metabolomics revealed that the protection was associated with significant changes in metabolites associated with

ammonia recycling, protein biosynthesis and pantothenate and CoA biosynthesis. The changes discussed above provide insight into novel mechanisms of exercise-induced cardioprotection that complement both the mitochondrial stabilization and antioxidant mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AF	Aortic flow
CF	Coronary flow
CoA	Coenzyme A
CO	Cardiac output
I/R	Ischemia/reperfusion
PC	Principal component
PCA	Principal components analysis
SOD	Superoxide dismutase
SP	Peak systolic pressure

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Fig. 1.
Principal components analysis of metabolites from hearts after ischemia reperfusion injury
from exercise trained and sedentary rats. N = 10 biological replicates/group

Fig. 2. Non-targeted metabolomics analysis of hearts after ischemia reperfusion injury from exercise trained and sedentary rats. **a** Heatmap of ANOVA significant metabolites from exercise trained hearts, exercise trained hearts after ischemia reperfusion injury, sedentary hearts, and sedentary hearts after ischemia reperfusion injury (ex vivo). **b** Pathway analysis of ANOVA significant metabolites. **c** Pathway enrichment of ANOVA significant metabolites using metabolic datasets

Nitrogen Metabolism

ANOVA Significant Metabolites

Fig. 3.

Significantly altered metabolites in the nitrogen metabolic pathway in sedentary and exercise trained hearts $\pm$ ischemia reperfusion injury. Peak values of **a** histidine, **b** glutamine, **c** glutamic acid, and **d** glycine from rat hearts after sedentary, exercise trained, sedentary ischemia reperfusion injury, and exercise trained reperfusion injury. An ANOVA was run to determine significance, followed by a post-hoc Fisher's least significant difference (LSD) multiple comparison between groups. $N = 10$ biological replicates/group. * $p < 0.05$ versus to I/R, ** $p < 0.05$ versus exercise, # $p < 0.05$ versus exercise I/R. Data is presented as the mean $\pm$ SEM

Aminoacyl-tRNA biosynthesis

ANOVA Significant Metabolites

Fig. 4. Significantly altered metabolites in the aminoacyl-tRNA biosynthesis pathway in sedentary and exercise trained hearts $\pm$ ischemia reperfusion injury. Peak values of **a** aspartic acid, **b** serine, and **c** proline are presented from rat hearts after sedentary, exercise trained, sedentary ischemia reperfusion injury, and exercise trained reperfusion injury (histidine, glutamine, and glutamic acid were significant and shown in Fig. 3). An ANOVA was run to determine significance, followed by a post-hoc Fisher's LSD multiple comparison between groups. $N = 10$ biological replicates/group. * $p < 0.05$ versus to I/R, ** $p < 0.05$ versus exercise, # $p < 0.05$ versus exercise I/R. Data is presented as the mean $\pm$ SEM

Fig. 5.

Significantly altered metabolites in the citric acid cycle in sedentary and exercise trained hearts $\pm$ ischemia reperfusion injury. Peak values of **a** glucose-6-phosphate (shown in Supplemental Fig. 4b), **b** glycerol-1-Phosphate, **c** lactic acid, **d** citric acid/isocitric acid, **e** fumaric acid, **f** dehydroascorbic acid, **g** heptadecanoic acid/Octadecanol, and **h** creatine are presented from rat hearts after sedentary, exercise trained, sedentary ischemia reperfusion injury, and exercise trained reperfusion injury. An ANOVA was run to determine significance, followed by a post-hoc Fisher's LSD multiple comparison between groups. N =

10 biological replicates/group. * $p < 0.05$ versus to I/R, ** $p < 0.05$ versus exercise, # $p < 0.05$ versus exercise I/R. Data is presented as the mean $\pm$ SEM

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Heart T-test Significant Metabolites Sedentary vs. Exercise-trained Hearts Post-IR

Fig. 6. Significantly altered metabolites comparing sedentary and exercise-trained hearts after ischemia reperfusion injury by *t* test analysis. **a** PCA analysis of sedentary and exercise-trained hearts after I/R injury. **b** Heatmap of *t* test significant metabolites from sedentary and exercise-trained hearts after IR injury. N = 10 biological replicates/group. A student's *t* test was performed to determine significance. **p* < 0.05. Data is presented as the mean ± SEM

Heart T-test Significant Metabolites

Sedentary vs. Exercise-trained Hearts Post-I/R

Pathway Analysis

Fig. 7. Identification of pantothenic acid and CoA biosynthetic pathway by pathway analysis of *t* test significant metabolites in sedentary versus exercise-trained hearts after ischemia reperfusion injury. **a** Pathway analysis of *t* test significant metabolites comparing sedentary and exercise-trained hearts after I/R injury. **b** Pantothenate and CoA biosynthetic pathway

Table 1

Animal characteristics at harvest

Group	Body wt (g)	Heart wt (mg)	Heart wt/body wt (mg/g)	Cyto oxidase plantaris ($\mu\text{Mol O}_2/\text{min/g}$)
Sedentary	403.7 $\pm$ 9.8 (26)	1207 $\pm$ 31 (26)	3.01 $\pm$ 0.06 (26)	47.7 $\pm$ 2.7 (8)
Exercise	377.0 $\pm$ 6.8* (23)	1234 $\pm$ 22 (23)	3.28 $\pm$ 0.04* (23)	83.6 $\pm$ 5.5* (8)
p value	0.0429	0.4917	0.00096	0.000043

Values are expressed as mean values $\pm$ standard error of the mean (number of subjects in parentheses). An independent *t* test (2-tailed) was used to determine differences between exercise and sedentary groups

Table 2

Cardiac function pre- and post-ischemia ex vivo post-exercise

Group	CO (ml × /min)	SP (mm Hg)	CO × SP (ml × /min × mmHg)	% Recovery of CO × SP
Pre-Ischemia				
Sedentary	56.9 ± 2.2	108.6 ± 2.6	6208.7 ± 331.0	N/A
Exercise	56.2 ± 1.6	110.2 ± 1.9	6191.1 ± 229.3	N/A
30-min post-Ischemia (I/R)				
Sedentary	19.3 ± 2.9	76.5 ± 3.4	1579.0 ± 279.4	27.5 ± 5.1
Exercise	35.0 ± 3.0 *	94.5 ± 2.6 *	3365.4 ± 330.4 *	54.9 ± 5.3 *
p value	0.0008	0.0003	0.0004	0.00104
Sedentary versus exercise trained				
Post-ischemia				

CO cardiac output, SP systolic aortic pressure, N/A not applicable

Values are expressed as mean values ± SE for $n = 13$ in both groups. The far-right column represents percent recovery of external work and was calculated for each heart as CO × SP at 30-min post-ischemia compared to CO × SP just before ischemia in the same heart. An independent t test (2-tailed) was used to determine differences between E and S.

*
p < 0.05 compared to S at 30-min post-ischemia