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Initial signaling response to acute exercise bout is similar in hearts of rats bred for divergent exercise capacities

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1. ABSTRACT

Rats artificially selected as low capacity runners (LCR) exhibit features of the metabolic syndrome, and blunted exercise training-induced cardiac hypertrophy compared with high capacity runners (HCR). We tested the hypothesis that the divergent cardiac phenotypes may be due to diminished activation of signaling proteins in LCR vs HCR rats. LCR (n=18) and HCR (n=18) rats were randomly assigned to acute exercise or control groups. Ten minutes after a 10-min bout of high intensity treadmill exercise, rats were euthanized, and left ventricles (LV) were harvested. LV homogenates were immunoblotted for phosphorylated and total levels of extracellular regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), p38, Akt, S6, and the ribosomal S6 protein kinases S6K and p90RSK. Alterations in protein ubiquitination were examined as an index of protein turnover. In LCR and HCR rats, S6 was activated to a similar extent after exercise (5-fold vs control), as were JNK1/2, p38, and ERK1/2 (each 1.5-fold). Exercise significantly reduced ubiquitination of some proteins, suggesting diminished post-exercise protein degradation. That no significant LCR/HCR differences were observed 10-min post-exercise in the signaling pathways studied herein suggests that the source of the differing cardiac phenotypes in LCR/HCR rats may involve differing activation times and/or other signaling pathways.

2. INTRODUCTION

Physical inactivity is an independent risk factor for cardiovascular disease comparable in relative risk to hypertension, hypercholesterolemia, and smoking, and is a characteristic of 70% of the American population (1). These observations and others that link low oxygen metabolism to many pathologies led to the development of an *in vivo* rat model of complex disease based on intrinsic (untrained) capacity for physical activity (2). By selectively breeding N:NIH rats based on running capacity, two divergent rat lines emerged in which low capacity runners (LCR) were only able to run 29% of the distance achieved by high capacity runners (HCR) after 11 generations (3). More importantly, LCR rats demonstrate a more negative cardiovascular disease risk profile (than HCR) including hyperglycemia, hyperlipidemia, as well as blunted systolic and diastolic indices (3). Additionally, LCR rats demonstrate a smaller cardiac myocyte hypertrophic response to high intensity interval exercise training compared with HCR rats, suggesting that LCR rats have a reduced ability to respond robustly to an imposed physiological hypertrophic stress (3). The distinctive cardiac phenotypes observed in the LCR/HCR model may be due in part to differential activation of signaling proteins, caused by inherited differences that result from selective breeding for intrinsic running capacity.

To explore this hypothesis, one strategy is to focus on a set of signaling pathways that have been shown to be involved in both cardiac hypertrophy and in the response to exercise. Several signaling proteins are involved in cardiac hypertrophy including: Akt (PKB), Mitogen Activated Protein Kinases (c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK), p-38 mitogen activated protein kinase (p38)), and S6 ribosomal protein kinases (S6K, p90RSK) (4-9). Upon activation, MAPKs and Akt can activate transcription factors such as NF-κB and AP-1 that increase transcription and result in protein accumulation (10). In parallel, S6K and p90RSK increase phosphorylation of the S6 ribosomal protein, thereby augmenting the rate of translation and contributing to protein accumulation (11).

Each of the hypertrophic signaling molecules identified above has also been shown to be activated in the heart by various exercise stimuli (12-16). Much less is known, however, regarding the exercise-dependent activation of these signals compared with their activation by other hypertrophic stimuli. Previous exercise studies (12, 17) systematically assessed the temporal activation profile of JNK and S6K and found that their activation peaked 10 min after a high intensity 10-min bout of exercise in naive but not trained Wistar rats. Taken together with the role these proteins play in other models of hypertrophy (4-9), these data suggest that early activation of JNK, S6K, and other signaling intermediates may be a determinant of exercise-induced hypertrophy. It is not currently known, however, whether any signaling pathways are activated by acute exercise in hearts of LCR and HCR rats, nor is it known whether the extent of the activation in the heart differs according to intrinsic exercise capacity. Accordingly, we sought to determine whether the major mitogen activated protein kinase pathways (ERK1/2, JNK1/2, and p38), as well as Akt, p90RSK and S6K were activated by a single 10-min bout of intense exercise in LCR and HCR rats. Furthermore, we examined whether the magnitude of activation of any of these signaling molecules differed at the critical 10-minute post-exercise time point in LCR compared with HCR rats.

It is possible that differential activation of pro-hypertrophic signaling pathways is not the mechanism of the divergent hypertrophic phenotypes observed by Wisloff and coworkers (3). An alternative mechanism for the differential cardiac hypertrophic response to exercise training is decreased protein degradation. The ubiquitin-proteasome system (UPS) is a non-lysosomal proteolytic system that plays a pivotal role in normal protein turnover (18). More importantly, recent evidence has implicated a reduction in proteasome activity as a contributing factor in the progression to heart failure in pressure-overloaded mouse hearts (19). Although the UPS has been shown to play a role in exercise-induced remodeling of skeletal muscles (20-23), how it is affected in the heart by exercise has not been determined. Furthermore, differential activation of the UPS in hearts of LCR/HCR rats may represent a potential mechanism of the reduced exercise training-induced hypertrophy observed in LCR rats (3).

The primary goal of this study was to first determine whether an acute bout of exercise would activate the pro-hypertrophic signaling proteins JNK1/2, ERK1/2, p38, Akt, p90RSK, S6K, and S6 in HCR and LCR rats, and whether activation of these pathways was less pronounced in the LCR compared with the HCR rat line. An additional goal was to determine whether protein ubiquitination was diminished by acute exercise, and whether this diminution was less pronounced in LCR compared with HCR rats. Data presented here demonstrate that S6, JNK1/2, p38, and ERK1/2 are activated by acute exercise in both rat lines whereas Akt, S6K, and p90RSK are not activated by exercise at the time point studied. Furthermore, ubiquitination of selected proteins was significantly decreased by exercise in both LCR and HCR rats by a similar magnitude.

3. MATERIALS AND METHODS

3.1. Materials

Antibodies against p-p38 (Thr^{180}/Tyr^{182}), p38, p-ERK1/2 (Thr^{202}/Tyr^{204}), pS6 ($Ser^{235/236}$), S6, p-Akt (Ser^{473}), p-JNK1/2 (Thr^{183}/Tyr^{185}), p-p90RSK (Ser^{380}), p-FOXO1 (Ser^{256}) were from Cell Signaling (Beverly, MA); antibodies against ERK2, p-p70 S6k (Ser^{411}), p70S6k, JNK1, and Akt1 were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibodies detecting GAPDH and ubiquitinated proteins were purchased from Chemicon International (Temecula, CA), and Biomol International (Plymouth Meeting, PA), respectively. Horseradish peroxidase-linked anti-mouse and anti-rabbit antibodies were from GE Healthcare (Piscataway, NJ). Enhanced chemiluminescence (ECL) kits were from Pierce Biotechnology (Rockford, IL). Bradford reagent was purchased from BioRad (Hercules, CA). Polyvinylidine difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). All other chemical reagents were ordered from Sigma-Aldrich (St. Louis, MO).

3.2. Animal models

A previous report gives a detailed description on the development of the rat models for aerobic exercise capacity (2). In summary, low and high lines were generated by artificial selected breeding from a founder population of heterogeneous NIH stock rats based on level of intrinsic aerobic exercise capacity. At each generation young adult rats (11 weeks of age) are tested for their inherent ability to perform speed-ramped treadmill running until exhausted. The highest scored female and male are selected as breeders for the next generation of high capacity runners (HCR). The same process is used with lowest scored females and males to generate low capacity runners (LCR).

The rats used in the current study were four- month old females derived from generation 16. For this experimental population, the mean distance run to exhaustion was 1,738 ± 39 m for HCR rats (n=18) compared to 308 ± 13 m for LCR rats (n=18), a 5.6-fold difference. Rats were housed 2-3/cage with food and water *ad libitum*, and maintained on a 12:12-h light-dark cycle. All animal protocols were approved by the University of Michigan's Committee on the Use and Care of Animals.

3.3. Experimental protocol

LCR and HCR rats were randomly assigned to exercise or control groups (n=9/group). The initial test of intrinsic running capacity done at 11 weeks of age showed that high capacity runners (HCR) were capable of running at maximal speeds 2.1-fold faster than low capacity runners

Table 1. Running protocol for LCR/ HCR exercise

	50% of Max Speed (m/min)	80% of Max Speed (m/min)	90% of Max Speed (m/min)	Max Speed (m/min)
LCR	10.3 +/- 0.2	16.5 +/- 0.4	18.6 +/- 0.4	20.7 +/- 0.5
HCR	21.6 +/- 0.4	34.6 +/- 0.6	38.9 +/- 0.7	43.2 +/- 0.8
Duration	1 min	4 min	4 min	1 min

Rats were run progressively from 50% of maximum (Max) speed to 100% of Max speed for the durations indicated. Running speeds are presented as mean ± SEM but were individualized to each rat's maximum speed.

Table 2. Morphological characteristics of LCR and HCR rats

	LCR Control	LCR Exercised	HCR Control	HCR Exercised
Body weight (g)	279.7 +/- 6.7	291.8 +/- 12.1	226 +/- 5.7 [1]	219.9 +/- 6.8 [1]
RV (mg)	198 +/- 10	185 +/- 5	155 +/- 8 [1]	168 +/- 11 [1]
LV (mg)	628 +/- 27	634 +/- 11	545 +/- 25 [1]	549 +/- 27 [1]
LV/BW (mg/g)	2.25 +/- 0.09	2.19 +/- 0.06	2.41 +/- 0.09 [1]	2.50 +/- 0.09 [1]
RV/BW (mg/g)	0.708 +/- 0.033	0.640 +/- 0.031	0.687 +/- 0.020	0.767 +/- 0.051 [2]

Abbreviations: LCR, Low Capacity Runner; HCR, High Capacity Runner; RV, Right Ventricle; LV, Left Ventricle; BW, Body Weight. [1] $p<0.01$ main effect of Rat Line; [2] $p<0.05$ Rat Line x Exercise interaction.

(LCR) at exhaustion. This estimate of maximal running speed was used to adjust running speeds, such that LCR and HCR exercise groups ran at comparable relative intensities equivalent to 50%, 80%, 90% and 100% (Table 1). The duration for the exercise protocol was based on previous studies (12, 17) that demonstrate maximal activation of JNK and S6 10 min after a 10-min high-intensity exercise. Therefore, the treadmill exercise proceeded as follows: 1 min at 50% maximum speed, 4 min at 80% maximum speed, 4 min at 90% maximum speed, 1 min at 100% maximum speed (Table 1). After 10 min of exercise, or 10 min placement on the non-moving treadmill (control), animals were allowed to recover for 10 min in their cage prior to euthanasia by decapitation.

3.4. Tissue preparation

After euthanasia, left ventricles (LV) were isolated, frozen in liquid nitrogen and stored at -80°C until fractionation. Subcellular fractionation was performed as described previously with minor modifications (24). Briefly, LV were minced and homogenized by glass-glass tissue grinder in 10 vol of buffer A containing (in mM): 250 sucrose; 10 Tris-HCl, pH 7.4; 1 EDTA, pH 7-8; 1 ortho-vanadate; 1 NaF; 0.3 PMSF; 5 μg/ml each of leupeptin and aprotinin; and 0.5 μg/ml pepstatin A. To obtain a total homogenate, an aliquot of homogenate had NP-40 added to a final concentration of 1% and was centrifuged at 16,000 x *g* (4°C) for 10 min. The remainder of the homogenate was centrifuged at 1,000 x *g* to isolate the nuclear fraction. The resultant supernatant was centrifuged at 10,000 x *g* to pellet the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 x *g* to separate the cytosolic fraction (supernatant) from the particulate fraction (pellet). Protein concentrations were determined according to the method of Bradford (25).

3.5. Western blotting

Western blotting was performed as described previously (17). Briefly, equal amounts of total or cytosolic protein (20-30μg) were electrophoresed through 4-15% SDS-PAGE gels, transferred to PVDF membranes overnight, and blocked with 1% BSA in TBS-Tween. Membranes were then incubated for 1h (JNK1/2, p-p38, p38, Akt, ERK2, p-S6, p-S6K, S6K, GAPDH) or overnight at 4°C (p-JNK1/2, p-ERK1/2, p-Akt, S6, p-p90RSK) at a dilution of 1:1,000 except for p-JNK (1:500) and ERK2 (1:2,000). After appropriate washes, membranes were incubated with HRP-linked anti-rabbit (for S6, p-S6, p-JNK1/2, p-Akt, ERK2, p-p90RSK; 1:20,000), anti-mouse (for p-p38, p38, p-ERK1/2, p-S6K, GAPDH, ubiquitination; 1:2,500), or anti-goat (for Akt, S6K; 1:10,000) antibodies. Antibodies were detected using ECL and densitometry performed using Scion Image software. To correct for potential differences in levels of target proteins loaded, membranes were first probed with phosphospecific antibodies, then stripped for 30 min (70°C) in stripping buffer (62.5 mM Tris-HCl pH 6.80, 7% 2-mercaptoethanol, 2% SDS), reblocked and probed with antibodies that detect the target proteins in both phosphorylated and unphosphorylated states. All data are expressed relative to the levels of target protein per lane.

3.6. 2D Gel electrophoresis

2D gel electrophoresis was performed as described previously with minor modifications (26). Briefly, 200ug of total LV free wall homogenate was subjected to isoelectric focusing for 150,000 Vhrs using ReadyStrip IPG strips (pH 3-10; BioRad) according to the following protocol: 300V for 1h, 600V for 1h, 1,500V for 3h, 9,000 V until 150,000 Vh. IPG strips were then loaded into the 4% stacking gel of a 8-12% polyacrylamide gradient gel and proteins were separated by SDS-PAGE. Gels were run in parallel and subjected to western blotting (as described above) for the detection of ubiquitinated proteins, or to Sypro-Ruby staining for spot identification by MS/MS as described (27). Ubiquitin-immunoreactive spots were matched to corresponding spots on the Sypro-Ruby-stained gels. Gel spots of interest were excised, trypsinized, and subjected to MS/MS as described previously (26).

3.7. Statistics

Data are presented as mean ± SEM and analyzed using Sigmastat. A two-way ANOVA (Exercise x Rat Line) was used to detect group differences. An alpha level of $p \leq 0.05$ was defined as statistically significant.

4. RESULTS

Table 2 shows morphological characteristic differences between LCR and HCR rats separated by groups, control versus exercise. HCR rats were significantly lighter in body weight compared to LCR for both control and exercise groups. Consistent with earlier studies of these rat models (3), left ventricular weights

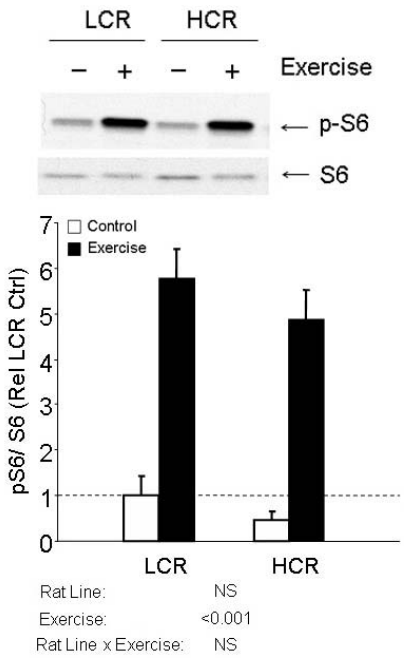

Figure 1. S6 phosphorylation after 10 min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of total protein were separated by SDS-PAGE. Phosphorylated S6 was corrected for total S6 levels and expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box; NS, not significant.

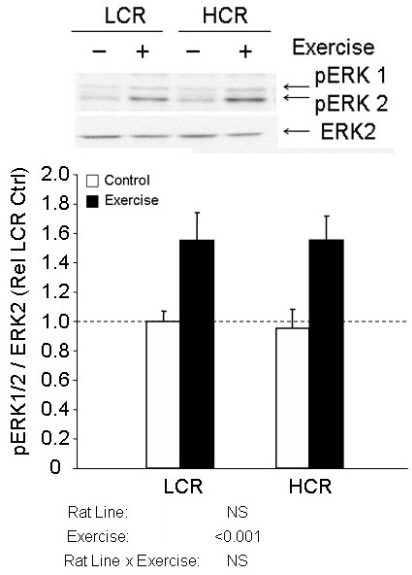

Figure 2. Extracellular-Regulated Kinase (ERK1/2) activation after 10 min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of cytosolic protein were separated by SDS-PAGE. Phosphorylated ERK1/2 was corrected for total ERK1/2 levels and expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box; NS, not significant.

(adjusted for body weight) of HCR were significantly greater (10%) than those of LCR. Body weight-corrected right ventricular wet weights were also significantly greater in HCR exercise group vs LCR.

Activation of the ribosomal protein S6 is thought to enhance overall rates of translation (11) and has been shown to be increased by hypertrophic stimuli (9, 28, 29). Furthermore, our previous observation that S6 phosphorylation is increased following acute exercise (12) led us to examine whether S6 was differentially activated in LCR and HCR rats. As an index of S6 activation, phosphorylation of S6 was assessed in LCR and HCR rats following an intense bout of acute exercise. Phosphorylation of S6 was increased by exercise 5-fold ($p<0.001$ main effect of exercise; Figure 1) in both LCR and HCR rats.

One mechanism by which S6 is activated involves Akt/PKB signaling through S6K (p70s6k), two proteins that have been implicated in skeletal and cardiac muscle hypertrophy (5, 7, 28, 29). Accordingly, we examined exercise-induced phosphorylation of Akt at Ser^{473} and found that Akt was not activated by exercise in either LCR or HCR rats at this time point (data not shown). In agreement with this observation, no differences in S6K phosphorylation were observed (data not shown).

Although research into the mechanisms of cardiac hypertrophy usually focuses on S6K-dependent activation of S6, S6 can also be activated by ERK1/2 signaling through p90 ribosomal S6 protein kinase (p90RSK) (8, 16, 29). We sought to determine whether this pathway was differentially activated by exercise in LCR/ HCR rats. ERK1/2 phosphorylation was increased after exercise to a similar extent (1.6-fold) in both LCR and HCR rats ($p<0.001$ main effect of exercise) (Figure 2), but downstream activation of p90RSK remained unchanged by exercise or rat line (data not shown).

In addition to ERK1/2, other members of the MAPK superfamily have been shown to be involved in cardiac hypertrophy (4, 7, 30, 31) and are activated during acute exercise in Sprague-Dawley rats (14). Accordingly, we studied whether JNK1/2 and/or p38 were differentially activated in hearts of LCR and HCR rats following exercise (Figures 6, 7). Ten minutes after cessation of exercise, activation of JNK1/2 was significantly increased (1.5-fold) in both LCR and HCR rats (Figure 3). Similarly, exercise increased p38 phosphorylation 1.6-fold in both rat lines (Figure 4; $p<0.001$ main effect of exercise). We noted that there was a trend ($p = 0.14$) for HCR rats to have a lower level of p38 phosphorylation compared to LCR both in control and exercised animals; the magnitude of the mean differences between LCR and HCR was 18%.

Because the enhanced exercise training-induced cardiac hypertrophy observed in HCR rats (3) could potentially be due, in part, to decreased protein degradation, we investigated whether levels of ubiquitinated proteins were decreased in HCR (vs LCR) following acute exercise. Although no significant

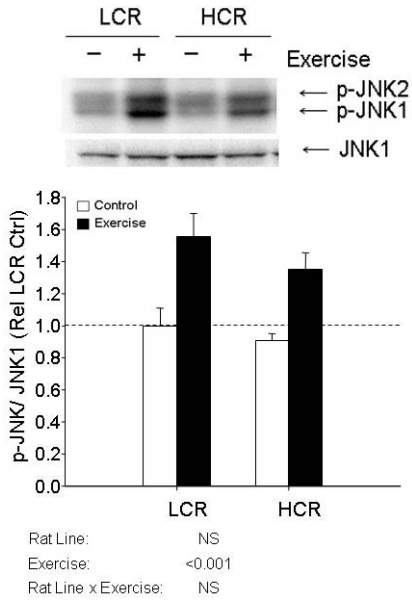

Figure 3. c-Jun N-Terminal Kinase (JNK1/2) activation after 10 min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of cytosolic protein were separated by SDS-PAGE. Phosphorylated JNK1/2 was corrected for total JNK levels and expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box; NS, not significant.

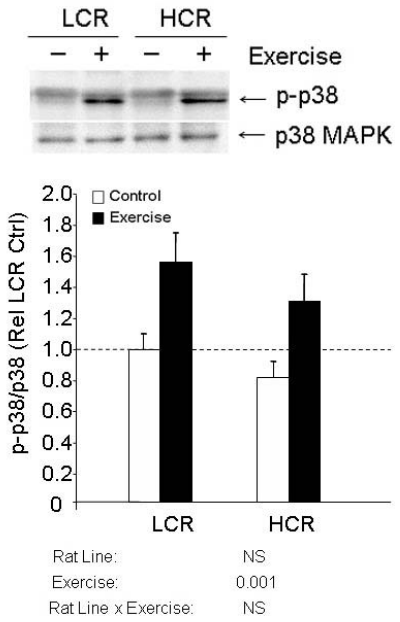

Figure 4. p38 activation after 10 min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of cytosolic protein were separated by SDS-PAGE. Phosphorylated p38 was corrected for total p38 levels and expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box; NS, not significant.

differences were noted between HCR and LCR, there was a trend (p=0.18) for exercise to reduce the overall level of ubiquitinated proteins in rat myocardium after 10 min of exercise (Figure 5A). When individual protein bands were analyzed, a statistically significant decrease in ubiquitination in response to exercise was observed (p=0.05) for the protein band indicated by the arrow in Figure 5A. Mean data for the indicated protein band are displayed in the bar graph (Figure 5B). To identify this band, tissue homogenates were separated by 2-D electrophoresis and subjected to immunoblotting or Sypro-Ruby staining (not shown). An unidentified spot of the appropriate molecular weight and pI (~80 kDa; pI = 8) was excised from Sypro-Ruby stained gels and identified by MS/MS as the alpha subunit of the trifunctional mitochondrial protein (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase; HADHA) necessary for beta-oxidation of fatty acids. Subsequent one-dimensional immunoblots of protein samples from hearts of each of the four study groups confirmed that the 80 kDa HADHA protein was expressed at similar levels in each of the four groups (data not shown).

To investigate other aspects of the UPS pathway and how it might be affected by exercise, we studied the phosphorylation (inactivation) of FOXO1 (forkhead box, subgroup O transcription factor 1). FOXO transcription factors have been shown to increase abundance of E3-ubiquitin ligases and inhibit calcineurin signaling (32). Therefore we sought to determine whether FOXO1 was inactivated (phosphorylated) by exercise, as a potential contributor to exercise training-induced hypertrophy. Interestingly, FOXO1 phosphorylation trended to be increased (~65%) by exercise in both LCR and HCR rats (p=0.06; Figure 6).

5. DISCUSSION

Physical inactivity can result in a host of pathological phenotypes, many of which are displayed by LCR rats, including the finding that LCR rats do not exhibit the same degree of exercise training-induced cardiac hypertrophy as HCR rats (3). It was hypothesized that the blunted hypertrophic response to exercise training may be due to differential recruitment of pro-hypertrophic signaling pathways. To test this hypothesis, two main questions were addressed simultaneously: 1) Which of the known pro-hypertrophic signaling pathways are activated in the heart by an acute bout of exercise? and 2) Does the magnitude of activation of any of the known pro-hypertrophic signaling pathways differ between LCR and HCR? This investigation examined initial acute exercise-induced activation of signaling pathways that have been shown to be activated in the heart by exercise and by hypertrophic stimuli. The key findings of the present study are that a single 10 minute bout of high intensity exercise: 1) increased the phosphorylation of S6 similarly in hearts of untrained HCR and LCR rats in the absence of Akt, S6K, or p90RSK activation; 2) increased the activation of p38, JNK1/2, ERK1/2 to a similar extent in untrained HCR and LCR rats; and 3) decreased ubiquitination of the HADHA protein and increased FOXO1 phosphorylation in hearts of untrained HCR and LCR rats to similar extents.

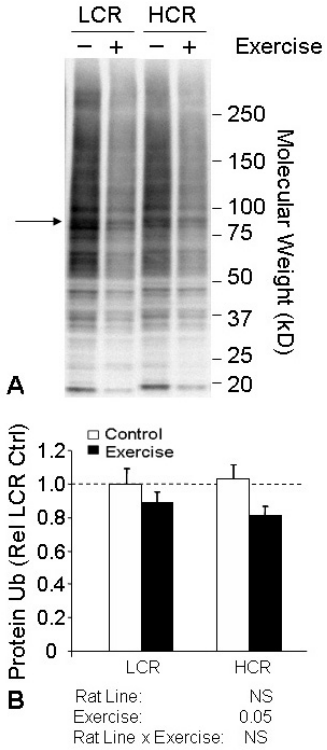

Figure 5. Protein ubiquitination (Ub) after 10 min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of total protein were separated by SDS-PAGE. A representative blot is shown in Panel A. The degree of protein ubiquitination was assessed over the entire molecular weight range studied (data not shown) and for a distinct band (arrow, and Panel B). Data are expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box. Ub, ubiquitination; NS, not significant.

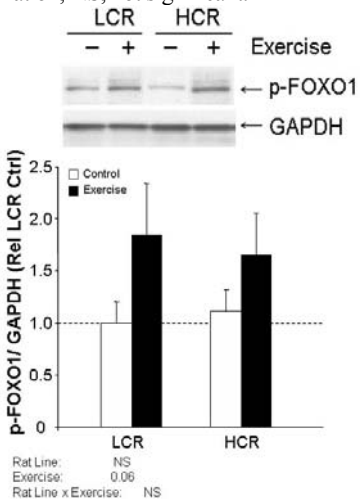

Figure 6. FOXO1 phosphorylation after 10min acute treadmill exercise. LV were isolated from exercised or sedentary LCR or HCR rats (n=9/group) and equal amounts of total protein were separated by SDS-PAGE. Phosphorylated FOXO1 was corrected to levels of GAPDH and expressed as mean ± SEM relative LCR control. p-values for 2-factor ANOVA are displayed in the inset box; NS, not significant.

5.1. S6 activation

Previous research has shown that the ribosomal S6 protein is activated in the heart by pressure overload (28, 33), and by an acute bout of exercise (12). Upon activation, S6 (located in the 40S ribosome proximal to the mRNA-binding site) appears to play a role in increasing the rate of translation of polypyrimidine tract mRNAs, thereby contributing to increased protein accumulation (11, 34). The current study demonstrates an exercise-induced activation of S6 in hearts of LCR and HCR 10 minutes after a brief, high-intensity bout of exercise. These findings are consistent with our previous studies that show S6 activation peaks at 10 min post-exercise in hearts of Wistar rats (12), and those of Williamson *et al* (16), who reported a 2-fold increase in S6 phosphorylation in mouse gastrocnemius muscles immediately following acute exercise.

The upstream signaling events most commonly associated with S6 activation involve Akt-dependent activation of mTOR which in turn leads to the activation of S6K. Since we and others have previously reported S6K activation in response to exercise (12, 15), it was surprising that in the present study we did not observe activation of either Akt or S6K 10 min after cessation of high intensity treadmill running. It is important to note that no time course studies to date have been conducted on cardiac signaling in the HCR/LCR rat lines and the temporal pattern of activation of these pathways may be different than the temporal pattern observed in Wistar rats (12). Thus it is possible that Akt and S6K may have been transiently activated during exercise or early in recovery in the LCR/HCR model, but had returned to baseline by 10 min after exercise. The possibility that Akt was activated transiently before the study termination is supported by the observation that FOXO1 phosphorylation (a downstream target of Akt) tended to be increased after acute exercise. Although previous studies in Wistar rats had identified 10 min post-exercise as the time point at which activation of cardiac S6 and JNK peak (12, 17), multiple time point studies will be needed to understand the temporal nature of pro-hypertrophic molecular signaling in the HCR/LCR heart.

An alternative interpretation of the present results is that S6 is not activated by exercise via Akt/ S6K which is suggested by several studies (15, 16). Konhilas *et al* (15) found that voluntary exercise-dependent activation of Akt and S6K in mice depended on the loading status of the exercise wheel. Although Akt and S6K were activated when exercise wheels were resistance-free, their activation was absent when even slight resistance (<5 g) was applied. Although it is unclear how the exercise wheel resistance reported by Konhilas and colleagues (15) relates to the motor-driven treadmill exercise used in the present study, it is possible that our treadmill exercise more closely resembles the loaded wheel condition. In addition, studies of acute exercise by Williamson *et al* (16) found that exercise-induced activation of skeletal muscle S6 was not associated with Akt-S6K activation but rather activation of the ERK1/2-p90RSK pathway.

5.2. MAPK activation

In agreement with these previous studies (16), we found that acute exercise significantly increased ERK1/2 phosphorylation in both LCR and HCR rats; however, this activation was not reflected in p90RSK phosphorylation. This apparent discrepancy is possibly due to the single time point studied here. That all MAPKs studied herein were activated 10 min after an acute bout of high intensity exercise confirms and extends the findings of Iemitsu *et al* (14). These researchers found that cardiac ERK1/2, JNK1/2, and p38 were activated after 15 or 30 min of exercise in untrained Sprague-Dawley rats with JNK1/2 and p38 phosphorylation returning to baseline 30 min after cessation of exercise (14). In the present study, we show that exercise for only 10 min with a 10 min recovery period is sufficient to activate p38, JNK1/2, and ERK1/2. Although a similar finding has previously been reported for JNK1/2 in Wistar rats (17), this is, to our knowledge, the first report of exercise-induced activation of MAPKs in the hearts of LCR or HCR rats.

5.3. Protein ubiquitination

Cardiac hypertrophy can occur by combinations of changes in the rates of protein synthesis and degradation that tilts the balance in favor of synthesis and results in protein accumulation. Therefore, in addition to pro-hypertrophic signaling events that increase the rate of protein synthesis, it is important to consider factors that regulate protein degradation. Of interest, recent studies have implicated dysfunctions in various aspects of the UPS in the progression of congestive heart failure due to the accumulation of damaged proteins (19). Furthermore, it has been noted that transcripts of several components of the UPS are induced with hypertrophic stimuli such as aortic constriction (19, 21).

That protein ubiquitination tended to be decreased following exercise suggests that a reduction in protein degradation may account (in part) for exercise training-induced cardiac hypertrophy in the LCR/HCR model (3). To our knowledge no studies have examined the ubiquitin pathway in cardiac muscle following exercise, but data from skeletal muscle research suggests that the ubiquitin pathway is upregulated with inactivity and downregulated when activity is resumed (20, 23). Consistent with the studies of skeletal muscle, we identified a cardiac protein (HADHA) that exhibited reduced ubiquitination after an acute bout of exercise. The time course in the present study is likely far too short for UPS-dependent protein downregulation to occur (HADHA levels were unchanged by exercise as assessed by western blotting, data not shown). Whether or not studies at later time points or following exercise training demonstrate higher levels of HADHA (due to decreased protein degradation) remains to be determined. Further studies are required to test this hypothesis and to explore the implications of increased HADHA levels.

5.4. FOXO1 phosphorylation

Tentative support for the notion that a diminished rate of degradation of selected proteins may contribute to cardiac hypertrophy is provided by the finding reported here that FOXO1 tended to be phosphorylated by acute exercise. FOXO proteins can transcriptionally upregulate E3 ubiquitin ligases, as well as pro-apoptotic proteins such as TNF-α and FAS ligand (18, 36, 37). The novel observation that exercise dependent-FOXO1 inactivation was coincident with decreased protein ubiquitination is intriguing and suggests that the FOXO family of transcription factors may play a non-genomic role in regulating protein ubiquitination.

5.5. Summary and conclusions

The data reported here did not support the hypotheses that exercise would differentially activate or differentially ubiquitinate selected proteins in hearts of LCR compared with HCR rats. Because our study was sufficiently powered to detect differences with effect sizes of 15%, these results suggest that the sources of the divergent cardiac phenotypes observed in the LCR/HCR model reside elsewhere. The possibility cannot be eliminated that very small differences or differences in the time course of the activation of some of the studied proteins may contribute to the phenotypes reported previously. The weak trends for the means of S6, Akt, p38, and JNK to be slightly less phosphorylated in the HCR, however, all run counter to this argument. Future studies could utilize systematic proteome mapping to investigate a broader range of signaling proteins to uncover potential differences in the LCR/HCR responses to exercise.

In summary, the data presented here are the first to demonstrate activation of S6, ERK1/2, JNK1/2, and p38 in the hearts of untrained LCR and HCR rats by a single bout of exercise. That activation of these pathways was not different between LCR and HCR rats reduces the likelihood that differences in the activation of the pathways studied explain the diminished hypertrophic response of LCR compared with HCR rats noted after 8 weeks of high intensity interval training (3). The novel observation of exercise-induced reduction in ubiquitination of HADHA protein highlights the need for further research into mechanisms that regulate protein degradation and how acute and chronic exercise influences protein turnover in the heart.

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Abbreviations: ERK1/2: Extracellular Regulated Kinase, GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase, HCR: High Capacity Runners, JNK1/2- c-Jun N-terminal Kinase, LCR: Low Capacity Runners, LV: Left Ventricle, p90RSK: p90 Ribosomal S6 Kinase, PVDF: Polyvinylidine Difluoride, RV: Right Ventricle, S6K: p70 Ribosomal S6 Kinase, UPS: Ubiquitin-Proteasome System

Key Words: Hypertrophy, Exercise, Ubiquitination, S6, MAPK, Akt, ERK, JNK, LCR, HCR

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