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Increased nitric oxide synthase activity and Hsp90 association in skeletal muscle following chronic exercise

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Abstract

Exercise training results in dynamic changes in skeletal muscle blood flow and metabolism. Nitric oxide (NO) influences blood flow, oxidative stress, and glucose metabolism. Hsp90 interacts directly with nitric oxide synthases (NOS), increasing NOS activity and altering the balance of superoxide versus NO production. In addition, Hsp90 expression increases in various tissues following exercise. Therefore, we tested the hypothesis that exercise training increases Hsp90 expression as well as Hsp90/NOS association and NOS activity in skeletal muscle. Male, Sprague-Dawley rats were assigned to either a sedentary or exercise trained group (n=10/group). Exercise training consisted of running on a motorized treadmill for 10 weeks at 30 m/min, 5% grade for 1 h. Western blotting revealed that exercise training resulted in a 1.9±0.1-fold increase in Hsp90 expression in the soleus muscle but no increase in nNOS, iNOS, or eNOS. Exercise training also resulted in a 3.4±1.0 fold increase in Hsp90 association with nNOS, a 2.3±0.4-fold increase association with eNOS measured by immunoprecipitation as well as a 1.5±0.3-fold increase in eNOS phosphorylation at Ser-1179. Total NOS activity measured by the rate of conversion of [¹⁴C]-L-arginine to [¹⁴C]-L-citrulline was increased by 1.42±0.9-fold in soleus muscle following exercise training compared to controls. In summary, a 10-week treadmill training program in rats results in a significant increase in total NOS activity in the soleus which may be due, in part, to increased NOS interaction with Hsp90 and phosphorylation. This interaction may play a role in altering muscle blood flow and skeletal muscle redox status.

Keywords

nitric oxide synthase; exercise; skeletal muscle; Hsp90

INTRODUCTION

Exercise creates a unique physiologic challenge resulting in large increases in cellular metabolism and stress (Jackson 2005; Ji et al. 2006). Repeated bouts of exercise (or chronic exercise training) result in adaptation of the skeletal muscle that improves the response to the increased metabolic demand and the stress of exercise. However, the mechanisms

involved in skeletal muscle adaptation to exercise are not fully understood. Recently, it has been suggested that nitric oxygen species play an important role in regulating the cellular adaptations to chronic exercise training in skeletal muscles (Jackson 2005). Tidball and colleagues have shown that nitric oxide synthases (NOS) mediate adaptive responses of skeletal muscle to mechanical stimuli by increasing the expression of structural proteins and the addition of sarcomeres (Tidball et al., 1999; Koh and Tidball, 1999). In addition, it has been well established that nitric oxide (NO) plays an important role in regulating blood flow and the growth of new blood vessels (Sessa 2004). More recently, NO has been shown to regulate glucose metabolism (Kingwell 2000) and the cellular redox state in skeletal muscles (Jackson 2005). NO may also influence expression of antioxidant enzymes as well as cellular inflammation in exercised skeletal muscles as suggested by studies in vascular tissues (Fukai 2000; Harrison 2000). Therefore, understanding the regulation of NO production through NOS expression or activity may provide insight into the potential mechanisms by which skeletal muscles adapt to the stress of chronic exercise.

Several previous studies have examined changes NOS protein expression in skeletal muscles in response to endurance exercise training in both humans and rats. Two of these studies in humans demonstrated an increase neuronal nitric oxide synthase (nNOS) (McConell et al. 2007; Rudnick et al. 2004) whereas in rats both nNOS and endothelial nitric oxide synthase (eNOS) have been shown to increase following chronic treadmill or swim training (Balon and Nadler 1997; Tatchum-Talom et al. 2000; Vassilakopoulos et al. 2003). However, since NO production from NOS is tightly controlled by posttranslational modifications and cellular signaling events, it is necessary to not only characterize changes in NOS protein expression but to also examine changes in NOS activity and interaction with regulatory proteins.

One of the proposed mechanisms regulating the activity of NOS (Garcia-Cardena et al. 1999; Song et al. 2001) as well as the balance of NO formation and superoxide formation from NOS is the interaction with Hsp90 (Pritchard et al. 2001; Song et al. 2002). Changes in Hsp90 expression have been shown to alter oxidative stress under pathological conditions such as persistent pulmonary hypertension (Konduri et al. 2003; Konduri et al. 2007), preeclampsia (Gu et al. 2006), and hypoxia (Shi et al. 2002). However, it is unknown and whether exercise-induced changes in Hsp90 expression and/or NOS expression alters the interaction between these two proteins and whether this results in a change in NOS activity. In addition, few studies have examined changes in Hsp90 expression in skeletal muscle following exercise training. Locke et al. (1990) demonstrated an increase in Hsp90 expression skeletal muscle following an acute bout of exercise (Locke et al. 1990), however, it appears that only one study has examined the effects of chronic exercise training on skeletal muscle Hsp90 expression. Atalay et al. (2004) observed no changes in Hsp90 protein expression in the red gastrocnemius and vastus lateralis of normal diabetic rats following chronic treadmill training for 8 weeks. However, these authors did not evaluate Hsp90 in primarily slow-twitch, oxidative muscles such as the soleus which would be expected to be highly active in this type of long-duration treadmill training.

These observations are of particular interest because an increase in NOS expression as observed in previous exercise studies (Balon and Nadler 1997; McConell et al. 2007; Rudnick et al. 2004; Tatchum-Talom et al. 2000; Vassilakopoulos et al. 2003) without an increase in Hsp90 expression and association may result in “uncoupling” shifting the balance of NO formation by NOS toward the production of superoxide. This may be an important part of the cellular adaptation response to exercise as both NO and reactive oxygen species have been suggested to be important signaling molecules initiating these events (Ji et al. 2006). In light of these observations, we hypothesized that chronic treadmill exercise training would increase both NOS and Hsp90 protein expression in soleus muscle

of rats. Furthermore, we hypothesized that chronic exercise training would increase Hsp90 association with NOS resulting in an increase in NOS activity.

METHODS

Animals and training protocol

Experiments and procedures were approved by the Institutional Committee for Animal Use in Research and Education at the Medical College of Georgia and adhered to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). 8 wk old male, Sprague-Dawley rats were obtained from Harlan. Animals were kept on a 12-h:12-h light-dark cycle and fed ad libitum. Rats were randomly divided into two groups, sedentary (SED) and exercise (EX) following 1 week of habituation on the treadmill (see Table 1). Following the week of treadmill habituation animals in the EX group began a 10 wk exercise training program which progressed from 10 min of treadmill running at 20 m/min and a 5% grade to 60 min of treadmill running at 30 m/min and a 5% grade for by the end of 3 weeks. Animals continued to train 5d/wk at this intensity from week four to week 10 (Table 1).

Tissue preparation

24 h following the last exercise bout, animals were anesthetized with pentobarbital sodium (40 mg/kg ip) and sacrificed by the removal of the heart. Soleus muscles were immediately dissected and snap frozen in liquid nitrogen. The soleus was then pulverized and homogenized 1:20 in tissue lysis buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 1mM PMSF, 1 mM Na₃VO₄) using a glass-glass homogenizer. Samples were centrifuged at 10,000 g for 10 min at 4°C and divided for analysis.

Immunoblotting

Immunoblotting of tissue samples was carried out as previously described (Harris et al. 2003). Protein concentrations on tissue lysates were determined using the Bio-Rad detergent compatible protein assay and volumes were adjusted to equalize the protein concentrations of the samples. 100 µg of each sample suspended in SDS-sample buffer and boiled for 5 min. Samples were then loaded and run on a 10% polyacrylamide gel and transferred to nitrocellulose membrane, which was subsequently immunoblotted with anti-eNOS (610297, BD Transduction Laboratories), anti-nNOS (610309, BD Transduction Laboratories), or anti-Hsp90 (610419, BD Transduction Laboratories) antibodies. In order to detect inducible nitric oxide synthase (iNOS), iNOS was first affinity purified by adding 80 µl of 2',5'-ADP Sepharose slurry (50%) to 1 ml of the cleared homogenate (equalized for protein concentration) and incubated at 4°C for 2 h. The beads were then washed twice with tissue lysis buffer, resuspended in 100 µl of SDS-sample buffer and boiled for 5 min to elute the proteins from the beads. The samples were centrifuged the supernatant was used for immunoblotting as described above with an anti-iNOS (610333, BD Transduction Laboratories) antibody.

Immunoprecipitation

Immunoprecipitation was carried out as described previously (Harris et al. 2003). Briefly, 1 ml of cleared tissue lysates, equalized for protein concentrations, were precleared by the addition of protein A/G agarose (sc-2003, Santa Cruz Biotechnology). Anti-eNOS, anti-nNOS or anti-Hsp90 antibody was then added to the supernatant and incubated overnight at 4°C. Protein A/G agarose was then added, and samples were incubated for an additional 4 h at 4°C. The agarose beads were then centrifuged and washed twice with tissue lysis buffer. Immunoprecipitated proteins were then eluted from the beads by adding SDS-Sample buffer

and boiling for 5 min. The agarose beads were pelleted by centrifugation and the supernatants were used for immunoblotting as described using either anti-eNOS, anti-nNOS or anti-Hsp90 antibodies.

NOS activity

Nitric oxide synthase activity was determined by measuring the rate of conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline in tissue lysates under the conditions previously described (Resta et al. 1999; Venema et al. 1995). Briefly, ~150 µg of tissue lysates were incubated for 15 min at 37°C in buffer containing excess cofactors including 2 mM Ca²⁺, 200 units CaM, 1 mM NADPH, 4 µM FAD, 4 µM FMN, and 40 µM tetrahydrobiopterin in the presence or absence of 1 mM L-NAME. The reaction was stopped by the addition of equal volume of ice-cold buffer containing 20 mM HEPES, 2 mM EDTA and 2 mM EGTA. Samples were run on AG 50W-X8 cation exchange columns (BioRad) and the flow through collected in vials and counted on a scintillation counter.

Statistics

Descriptive data (means ± SD and SE) were calculated for each dependent variable. And independent t-test was used to determine differences between means. A probability level of $P < 0.05$ was used as the decision rule for significance testing.

RESULTS

NOS expression

Protein levels for eNOS, iNOS and nNOS were determined in soleus muscles harvested and frozen 24 h following the last exercise bout using Western blot analysis. The results are shown in Fig 1 (Panel A, B, and C) and are expressed as arbitrary densitometric units. No significant differences ($P > 0.05$) were observed in protein levels of eNOS, iNOS, or nNOS in the soleus muscles from chronically exercise trained rats compared to sedentary controls.

Hsp90 expression and association with nNOS and eNOS

Hsp90 protein levels were determined in soleus muscles harvested and frozen 24 h following the last exercise bout using Western blot analysis with anti-nNOS, anti-eNOS and anti-Hsp90 antibodies. The results are shown in Figs 2 and are expressed as arbitrary densitometric units. As in Fig. 1 (Panel A) no significant difference in eNOS protein levels were observed while a significant ($P < 0.05$) 1.9±0.1-fold increase in Hsp90 protein levels were observed in soleus muscles from chronically exercise trained rats compared to sedentary controls.

Hsp90 association with nNOS and eNOS was determined in cleared lysate from freshly harvested and homogenized soleus muscles by immunoprecipitation of anti-nNOS or anti-eNOS antibody and subsequent immunoblotting with anti-Hsp90 antibody. The results shown in Fig 2 (Panel B and C) reveal a significant ($P < 0.05$) 3.4±1.0-fold increase in Hsp90 immunoprecipitated with nNOS and a significant ($P < 0.05$) 2.3±0.4-fold increase in Hsp90 immunoprecipitated with the eNOS in soleus muscles from chronically exercise trained rats compared to sedentary controls. Similar results were obtained from immunoprecipitation of Hsp90 and subsequent immunoblotting with anti-nNOS or anti-eNOS antibody (data not shown).

eNOS phosphorylation

Phosphorylation of eNOS at Ser-1179 were determined in soleus muscles harvested and frozen 24 h following the last exercise bout using Western blot analysis with both anti-

phospho-1179-eNOS antibody. The results are shown in Fig 3 and are expressed as arbitrary densitometric units. A significant ($P < 0.05$) 1.5 ± 0.3 -fold increase in eNOS phosphorylation at Ser-1179 was observed in soleus muscles from chronically exercise trained rats compared to sedentary controls.

NOS activity

Total NOS activity was determined in homogenates from freshly harvested soleus muscles 24 h following the last exercise bout by measuring the conversion of L-[^{14}C] arginine to L-[^{14}C]citrulline. Results are shown in Fig. 4 and are expressed as cpm. A significant ($P < 0.05$) 1.42 ± 0.9 -fold increase in total NOS activity was observed in soleus muscles from chronically exercise trained rats compared to sedentary controls.

DISCUSSION

Previous studies have reported an increase in NOS expression and activity in various skeletal muscles following chronic exercise training in humans and rats (Balon and Nadler 1997; Bradley et al. 2007; McConell et al. 2007; Rudnick et al. 2004; Tatchum-Talom et al. 2000; Vassilakopoulos et al. 2003). Despite the important regulatory role of Hsp90 on NOS activity, only one study has reported changes in Hsp90 in skeletal muscles following chronic exercise training (Atalay et al. 2004) and no previous studies have examined changes in Hsp90/NOS association in skeletal muscle following chronic exercise training. The results of this study show that there is a significant increase in NOS activity in the soleus muscle of rats subjected to a chronic (8 wk) treadmill training program. The increase in NOS activity does not appear to be due to an increase NOS expression but rather due to an increase in Hsp90 expression and Hsp90 association with both nNOS and eNOS.

In the first series of experiments, we compared the protein expression of three NOS isoforms in the soleus muscle of rats following 8 weeks of chronic treadmill training. In Figure 1, we demonstrate that there was no significant increase in any of the three NOS isoforms iNOS (Panel A), nNOS (Panel B), or eNOS (Panel C) failing to confirm our hypothesis that chronic training would increase NOS protein expression. These results are in contrast to a number of studies evaluating NOS protein levels in skeletal muscle following exercise training. Three previous studies evaluating chronic exercise training in rats demonstrated an increase nNOS protein expression in skeletal muscles (Balon and Nadler 1997; Tatchum-Talom et al. 2000; Vassilakopoulos et al. 2003) and one of these studies also reported an increase in eNOS protein expression (Vassilakopoulos et al. 2003).

Two potential explanations for the differences between the prior studies and the present study include: 1) differences in the intensity and duration of training programs and 2) differences in the fiber types of the muscle evaluated. In all three previous studies, the exercise was considerably more strenuous than the present study with a much shorter time period for adaptation. For instance, Balon and Nadler (1997) ran rats 36 m/min, 90 min/day, 5 d/wk including 1-min sprints at 42 m/min every 10 min. This is in contrast to the more moderate protocol used in our study (30 m/min, 5% grade, 60 min/day, 5 d/wk) which was maintained for 6 wks. The protocol used by Vassilakopoulos et al (Vassilakopoulos et al. 2003) ended with rats running on a treadmill at 30 m/min, up a steep 15% grade, 60 min/day for 4 weeks. Finally, Tatchum-Talom et al (Tatchum-Talom et al. 2000) used swim training as their exercise protocol and doubled the daily duration of exercise during the last week of a 3–4 week program from one to two 60 min bouts per day. These differences in training protocols are important to point out because it is possible that the initial adaptive response to exercise training involves an increase in NOS protein expression. Indeed a previous study of acute exercise demonstrates that NOS activity in skeletal muscle is increased after an exhaustive bout of exercise (Roberts et al. 1999). Furthermore, Kojda and Hambrecht (2005)

have reported that eNOS protein expression is elevated in the aorta and heart following 3 weeks of exercise training in mice but returns to normal levels following 9 weeks of training. Based on these findings and the results of the present study, we speculate suggest that following a prolonged moderate intensity exercise program with an adequate period of time for adaptation, NOS protein levels may return to normal in skeletal muscles while NOS activity remains elevated.

Another difference between the present study and previous studies using exercise trained rats is the fiber type of the skeletal muscles that were evaluated. The present study focused on the soleus muscle because it is predominantly a slow-twitch muscle fiber type (Armstrong and Phelps 1984) and would be highly recruited in this type of exercise training. In contrast, both Vassilakopoulos et al (Vassilakopoulos et al. 2003) and Tatchum-Talom et al (Tatchum-Talom et al. 2000) evaluated NOS expression in skeletal muscles that were primarily fast-twitch (gastrocnemius) or more mixed (quadriceps) (Armstrong and Phelps 1984) which would be recruited and stressed in a different manner than the soleus. Indeed, in a study by Rudnick et al (Rudnick et al. 2004) differential expression of NOS isoforms was observed in different fiber types of humans following bed rest and exercise. A decrease in nNOS was seen primarily in the mixed-fiber type vastus lateralis and not in the primarily slow-twitch soleus muscle following bed rest. Furthermore, subjects on bed rest who were allowed to exercise had a significant increase nNOS expression in the vastus lateralis and not the soleus. In addition, eNOS was increased in the vastus lateralis following exercise training primarily in the type II myofibers. These data suggest that NOS expression in type II myofibers is more susceptible to changes in muscular activity than the type I myofibers. However, similar to our study Balon and Nadler (2007) did evaluate NOS protein expression in the soleus muscle and observed significant increases in nNOS and eNOS but as mentioned earlier this may have been due to the differences in the intensity of the training protocol.

Despite the lack of change in NOS protein expression, we did, however, observe an increase in total NOS activity suggesting a change in the posttranslational regulation of NOS in the trained muscle. As mentioned previously, exercise is considered a significant stress to the exercising muscle and typically results in changes in cellular stress proteins. Hsp90 is part of the family of stress proteins and has also been shown to increase the activity of both nNOS (Song et al. 2001) and eNOS (Garcia-Cardena et al. 1998; Harris et al. 2000). In the present study, we are the first to demonstrate an increase in Hsp90 expression as well as increase in the association of Hsp90 with both nNOS and eNOS. The increased Hsp90 association with both nNOS and eNOS could explain, in part, the increase in NOS activity observed in our study despite the lack of change in NOS expression. Interestingly, Hsp90 has also been shown to regulate the balance of superoxide and nitric oxide production from both eNOS (Pritchard et al. 2001) and nNOS (Song et al. 2002). Thus, the observed changes in Hsp90/NOS association in the present study could play a role in modulating the redox balance in trained skeletal muscle and account, in part, for the adaptive antioxidant state of trained skeletal muscle.

The observed increase in phosphorylation of eNOS at Ser1179 in this study is additional evidence that the training induced change in total NOS activity is dependent on post-translational modifications mediated by Hsp90. Previous studies have indicated that chronic exercise training results in an increase in eNOS phosphorylation in vascular tissues (Kojda and Hambrecht 2005). In addition, it has been shown that one way in which Hsp90 increases eNOS activity is by acting as a protein scaffold through which Akt association with eNOS is enhanced and results in a chronic or increased state of phosphorylation of eNOS (Fontana et al. 2002). To our knowledge, however, no previous studies have examined changes in eNOS phosphorylation in skeletal muscle following exercise training. Lastly, it is unclear as to

precisely how Hsp90 increases nNOS activity, but it is evident from this study that Hsp90 association is increased following chronic exercise. These post-translational modifications may be an important component of the long-term adaptation to exercise training where increased NOS activity is maintained, but not by an increase in expression as seen following short-duration exercise training.

The results of this study shed additional light on the complex series of cellular adaptations to chronic exercise. As suggested in the study by Kojda and Hambrecht (2005) examining changes in NOS expression in the heart and blood vessels during exercise training, it appears that an increase in NOS expression may be part of an early adaptive response to the stress of exercise and that over time as chronic, moderate-intensity exercise continues the early adaptive response (increased NOS expression) may be replaced by more tightly controlled regulatory mechanisms including increased Hsp90 association and phosphorylation. These changes make sense as part of the adaptive response to exercise in which muscular antioxidant defense systems are upregulated (Ji et al. 2006; Fukai et al 2000). An increase in NOS expression without a concomitant increase in Hsp90 association could lead to increased NOS uncoupling and increased oxidative stress as seen in various pathological models (Gu et al. 2006; Konduri et al. 2003; Konduri et al. 2007; Shi et al. 2002). The adaptive response to exercise may transition from an early to late response as Hsp90 association with NOS further increases NOS activity, decreasing superoxide production and potentially reducing the necessity for continued elevation of NOS expression. This model of the adaptive response of NOS could allow for increase NO production improving blood flow and glucose metabolism in the trained muscle without contributing to further to oxidative stress.

In conclusion, this is the first study to examine changes in post-translational modifications of NOS in slow-twitch skeletal muscle following chronic exercise training. In addition, this is the first study to report changes in Hsp90 protein expression in slow-twitch skeletal muscle following chronic exercise training. The data show that NOS activity is increased in the soleus muscle of rats following 10 weeks of treadmill exercise training. It appears that the increase in NOS activity was not due to an increase in NOS expression but rather due, in part, to other regulatory mechanism including an increase in Hsp90 interaction and phosphorylation. However, a limitation of this study and previous studies is the lack of various time points to determine the temporal changes in NOS expression during and following chronic and acute exercise training. Future studies should be conducted to determine the time course of changes in NOS expression following chronic exercise training and the precise role NOS play in regulating the skeletal muscle adaptation to exercise training.

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Fig. 1. NOS protein content in soleus muscles of sedentary (SED) and exercise trained (EX) rats. A: eNOS content, B: iNOS content, C: nNOS content. A representative Western blot for each figure is shown immediately below histogram. Values are means $\pm$ SE, $n = 6$. No significant ($P > 0.05$) differences were observed.

Fig. 2. Hsp90 protein content and immunoprecipitation with nNOS and eNOS in soleus muscles of sedentary (SED) and exercise trained (EX) rats. A: Hsp90 content, B: Hsp90 immunoprecipitated by anti-nNOS antibody, C: Hsp90 immunoprecipitated by anti-eNOS antibody. A representative Western blot for each figure is shown immediately below histogram. Values are means $\pm$ SE, $n = 8$ (Panel A and C), $n = 6$ (Panel B). * Significantly different ($P < 0.05$) from SED.

Fig. 3. eNOS phosphorylation at Ser-1179 in soleus muscles of sedentary (SED) and exercise trained (EX) rats. A representative Western blot is shown immediately below histogram. Values are means $\pm$ SE, $n = 7$. * Significantly different ($P < 0.05$) from SED.

Fig. 4. Total NOS activity in soleus muscles of sedentary (SED) and exercise trained (EX) rats. Values are means $\pm$ SE, $n = 10$. * Significantly different ($P < 0.05$) from SED.

Treadmill exercise training protocol. Time in minutes (min), treadmill belt speed in meters/minute (m/min) and treadmill percent gradient (%).

Table 1

Week #	Mon	Tues	Wed	Thurs	Fri	Sat	Sun
Habit. 1	5 min 5 m/min-5%	5 min 7 m/min-5%	5 min 8 m/min-5%	5 min 9 m/min-5%	5 min 10 m/min-5%		
1	10 min 20 m/min-5%	15 min 20 m/min-5%	20 min 20 m/min-5%	25 min 20 m/min-5%	30 min 20 m/min-5%		
2	35 min 20 m/min-5%	40 min 20 m/min-5%	45 min 20 m/min-5%	50 min 20 m/min-5%	55 min 20 m/min-5%		
3	60 min 20 m/min-5%	60 min 20 m/min-5%	60 min 25 m/min-5%	60 min 25 m/min-5%	60 min 30 m/min-5%		
4-10	60 min 30 m/min-5%	60 min 30 m/min-5%	60 min 30 m/min-5%	60 min 30 m/min-5%	60 min 30 m/min-5%		