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Short-term treadmill running in the rat: what kind of stressor is it?

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Abstract

The use of short-term (1 to 5 days) treadmill running is becoming increasingly common as a model to study physiological adaptations following the exercise. Although the beneficial effects of acute exercise seem clear, a paucity of data exist describing potential markers of stress in response to forced running. We subjected male and female Sprague-Dawley rats to 0, 1, 2, 5, or 10 days of treadmill running. Twenty-four to thirty-two hours after the last bout of exercise animals were sacrificed and examined for training-induced changes in several physiological variables. No effect of skeletal citrate synthase activity was observed in the male animals after any duration, and only at 10 days of running did females show a significant increase in citrate synthase. Myocardial heat shock protein 72 (HSP72) content was higher in male rats than female rats, and exercise led to increased HSP72 in both sexes, although the time-course was different between males and females. Animals displayed several markers of systemic stress in response to the treadmill running, and in a sex-dependent manner. Serum corticosterone was significantly elevated in both sexes 24 hours after exercise in three of four exercise groups. Corticosterone binding globulin was higher in females, and decreased after running in female rats. Body and spleen weights decreased in males (but not females) in response to the exercise training, and running did not alter adrenal gland weights in either sex. These data indicate that in response to short-term treadmill running both male and female rats show signs of systemic stress, but that the pattern of changes occurs in a sex-specific manner.

Introduction

Short-term (1 to 10 consecutive days) treadmill exercise models are frequently employed to elicit positive phenotypic changes such as improved myocardial tolerance to ischemia/reperfusion injury (7, 12, 18, 33, 42, 43), improved insulin sensitivity (13) and augmented flow-dependent vasodilation (24). Given the potent effects that have been observed across studies, the short-term exercise regimen is credited with the elicited change in phenotype. However, a key limitation of these investigations is that animals are forced to run on a treadmill and often receive external motivation such as a mild electrical shock, blasts of air, or prodding if they do not run. While the efficacy of the exercise intervention to induce physiological changes is clear, a paucity of data exists regarding the adverse effects of acute forced-exercise protocols.

Recent work by Moraska et al. (28) showed that male rats exposed to long-term forced treadmill running exhibited both positive and negative physiological changes following eight weeks of running. Specifically, these authors found that chronic running in male rats elicited positive adaptations such as increases in skeletal muscle oxidative capacity and decreased body weight. However, they noted that these beneficial effects of running were accompanied several markers of chronic stress (37). Interestingly, subsequent experiments exposed female rats to similar long-term running protocols, and animals experienced comparable increases in plantaris citrate synthase activity in the absence of morphological indicators of chronic stress (5, 19). While a sex-specific stress response to chronic treadmill running seems apparent, no study to date has examined similar responses following short-term running protocols. In this study, we postulated that male rats and female rats would experience physiological changes

indicative of activation of the stress response after short-term (1-10 days) treadmill running. We hypothesized that this response would be characterized by decreased body weight, adrenal gland hypertrophy, atrophy of the spleen, and elevated levels of biologically-active corticosterone.

Methods

Experimental Animals

Male (n=42) and female (n=43) adult Sprague-Dawley rats (aged 6-8 months, Harlan) were used in the study. This age of animals is commonly used in exercise studies (6, 10). Animals were exposed to a short-term exercise protocol as previously described (7, 10). Briefly, animals were familiarized for 5 days on a motorized treadmill (0% grade) at 15 meters/min for 5 min (1st day) to 20 min (5th day). Rats then exercised for 1, 2, 5, or 10 days. The exercise protocol began with 10 minutes at 15 meters/min, followed by 40 minutes at 30 meters/minute, and ended with 10 minutes at 15 meters/minute. Animal numbers were n = 8, 9, 8, 10, and 7 for male animals exposed to 0, 1, 2, 5, and 10 days of running, respectively. Animal numbers were n = 7, 10, 8, 9, and 9 for female rats exposed to 0, 1, 2, 5, or 10 days of exercise, respectively. All animals in the sedentary group were placed on the non-moving treadmill for twenty minutes each day to serve as handling controls and were sacrificed intermittently following 1 to 10 days of handling. Twenty-four to thirty-two hours following the exercise (or handling control session), animals were anesthetized with sodium pentobarbital (35 mg/kg; ip injection) and killed by midline thoracotomy and excision of hearts. To decrease environmental stress on animals, animals stayed in home cages and were not exposed to the room where tissue was harvested until immediately before the time of sacrifice. Hearts were immediately frozen for subsequent assay of heat shock protein 72 (HSP72) content. At the time of death, body, left ventricle, left and right adrenal, and spleen weights were obtained, and plantaris muscle was dissected and frozen for citrate synthase content as previously described (6). Trunk blood was obtained and serum

collected and frozen for subsequent corticosterone and corticosterone binding globulin assays. Weights of adrenal glands and spleen were normalized to tibia length to account for differences in animal size. All procedures were carried out with prior approval from the University of Colorado Animal Care and Use Committee consistent with the guidelines established by the American Physiological Society.

Myocardial HSP72

Myocardial tissue from the left ventricle was homogenized as previously described (7). Content of myocardial HSP72 was analyzed using a commercial HSP72 assay kit and expressed as pg per microgram of left ventricle (Stressgen Biotechnology).

Serum Corticosterone and Corticosterone Binding Globulin

Total corticosterone was assayed using an ELISA (Assay Designs) and concentrations expressed as μg per dL of serum. Serum corticosterone binding globulin (CBG) levels were assessed using a competitive binding assay adapted from that of Chader and Westphal (9). The samples were initially diluted 1:150 in buffer consisting of 10mM Trizma base, 1.0 mM EDTA, 10% glycerol (v/v), and 1.0 mM diothiothreitol, pH 8.0. Samples were then stripped of endogenous corticosterone by placing 500 μl of diluted sample in a microfuge tube containing a dry dextran (MW ~ 70,000) coated charcoal pellet. The charcoal pellet was resuspended in the diluted sample with vigorous vortexing. After sitting 10 min at room temperature the charcoal-sample suspension was centrifuged and the supernatant (corticosterone-stripped diluted

sample) carefully removed from the charcoal pellet. The stripped diluted samples were then mixed with a saturating concentration of ^{3}H-CORT (15 nM) ± unlabelled CORT (10 μM) and allowed to incubate overnight at 4^{o} C. Bound and unbound steroids were separated using activated charcoal (performed in triplicate). The bound fraction was mixed with scintillation cocktail and counted with a liquid scintillation counter (TriCarb 1600TR, Packard, Meriden, CT). Data were expressed as nmol specific ^{3}H-CORT binding/ L serum.

Statistical Analyses

Data are expressed as mean ± standard error. Changes in each dependent variable were analyzed for each sex using a one-way ANOVA. When the ANOVA revealed significant between group differences, post-hoc analysis was performed using Dunnett's method of multiple comparisons since our interest was to compare each exercise group with the sedentary controls. Comparisons of simple effects between sedentary male and female were made using a Student's t-test. Statistical significance was determined with an alpha level of 0.05.

Results

Citrate Synthase Content

Plantaris citrate synthase activity data are presented in Figure 1. There were no differences between the sexes before the onset of training ($P = 0.21$, Student's t-test), and short-term treadmill running significantly increased plantaris citrate synthase levels only in females exposed to 10 days of running ($P < 0.05$, Dunnett method).

Heat Shock Protein

Myocardial heat shock protein 72 (HSP72) data are presented in Figure 2. Male rats had significantly higher baseline myocardial HSP72 content than female rats ($P < 0.05$, Student's t-test). Increased myocardial HSP72 was observed following 1 day of running in female rats and after 10 days of running in male rats ($P < 0.05$, Dunnett method). No other statistically significant increases in myocardial HSP72 were observed in the study.

Corticosterone and Corticosterone Binding Globulin

Serum corticosterone levels are presented in Figure 3A. Corticosterone levels were elevated in both sexes after three of the four exercise protocols. Female rats showed elevated corticosterone when compared to sedentary after 1, 2, and 10 days of exercise ($P < 0.05$ for each comparison, Dunnett method). Total serum corticosterone in males was elevated following 1 and 10 days of exercise compared sedentary animals

($P < 0.05$, Dunnett Method), and there was a trend towards a significant increase in the 5 day group ($P = 0.09$, Dunnett Method).

Corticosterone binding globulin (CBG) data are presented in Figure 3B. Sedentary females had significantly higher serum CBG levels than sedentary males ($P < 0.05$, Student's t-test). Exposure to treadmill running did not alter serum CBG content in any group of male rats ($P > 0.05$, ANOVA). CBG levels were significantly decreased in female rats that exercised for 10 days ($P < 0.05$, Dunnett method), and females that were exposed to treadmill running for 2 and 5 days displayed statistical trends towards decreased CBG ($P = 0.14$ and 0.09, respectively; Dunnet method). There were no differences in either corticosterone or CBG as a function of the time of day that animals were sacrificed post-exercise (since the time varied from 24 to 32 hours post-exercise; data not shown).

Body Weight

Body weights for the experimental animals are presented in Figure 4. There were no differences in body weight in any of the female groups after exposure to treadmill running ($P > 0.05$, ANOVA). Male rats that ran for 10 days were significantly leaner than their sedentary counterparts ($P < 0.05$, Dunnett method), with trends towards decreased body weights in the 1 and 2 day groups ($P = 0.09$ and 0.16, respectively; Dunnett Method).

Adrenal Gland Weights

Left and right adrenal gland weights (normalized to tibia length) are presented in Figure 5. Baseline adrenal gland weights were significantly greater in sedentary female

rats than sedentary male rats ($P < 0.05$, Student's t-test). Short-term exercise did not lead to significant adrenal gland hypertrophy in either sex ($P > 0.05$, ANOVA).

Spleen Weights

Spleen weights are presented in Figure 6. Male rats had significantly larger spleens than female rats ($P < 0.05$, Student's t-test). Treadmill running did not alter the size of the spleen in female rats at any time point ($P > 0.05$, ANOVA). In contrast, treadmill running elicited significant decreases in spleen size in males exposed to 2, 5, or 10 days of running ($P < 0.05$, Dunnett method), and a trend towards splenic atrophy in the 1 day group ($P = 0.10$, Dunnett method).

Discussion

The major objective of this study was to characterize if male and female rats show signs indicative of activation of the stress response after 1 to 10 days of treadmill running. Such protocols have previously been used by our laboratory (7, 10, 41) and others (12, 13, 17, 24, 33, 42, 43) as models of exercise. We first characterized how two common markers of exercise training, skeletal muscle citrate synthase activity and myocardial heat shock protein 72 content, changed twenty-four hours after short-term treadmill running in male and female rats. Secondly, and more importantly, we also set out to measure systemic changes related to stress after the onset of short-term running. Despite the fact that animals receive adverse treatment (mild shock, prodding, or blasts of air) when forced to run, a thorough investigation of the time- or gender-dependent impact of this procedure on markers of the stress response has not been done. We hypothesized that exercised animals would experience a stress response characterized by altered body, spleen, and adrenal weights, and that the bioavailability of the stress hormone corticosterone would be increased.

Elevated basal citrate synthase activity is widely used as an indicator of peripheral adaptation to exercise training in both sexes (5, 6, 19, 28), but to the best of our knowledge no studies have examined the time-course for basal citrate-synthase elevations days after the onset of a training regimen. Our data indicate that the basal (as opposed to increases immediately after the exercise) increase in peripheral muscle citrate synthase requires 10 days or more of continuous running. That citrate synthase is not elevated 24 hours following short-term treadmill running corroborates previous work (30), with our findings providing new insight regarding the time-course of training-

induced elevations in tonic citrate synthase. Our observation that skeletal muscle citrate synthase is increased at 10 days in female animals compliments previous observations by Baldwin et al. (2), who showed that this training adaptation after two weeks of treadmill running is sustained following several months of exercise.

We found a baseline sex-difference in myocardial heat shock protein 72 (HSP72) as noted in Figure 2. That sedentary males had greater myocardial HSP72 than sedentary females is in contrast to previous findings in the literature where there was no sex difference (1, 29, 33) or greater HSP72 in female hearts (40). While the discrepancy among these studies is not clear, one putative explanation may be that the age of animals used in this study (6-8 months of age) was approximately twice the age of animals used in previous studies.

Myocardial HSP72 content is known to increase following chronic exercise in both male (30) and female (20) rats, but little is known about the time-course for the exercise-related increase in this stress protein. Previous experiments have shown that acute treadmill running can increase basal HSP72 in male (11, 25, 26, 33) and female (12, 17, 18, 38) hearts. Paroo and colleagues (33) reported that female rats had reduced cardiac HSP72 responses following exercise. In contrast, Nickerson et al., 2006 (29) reported an equal increase in cardiac, and a blunted increase in liver, pituitary and lymph node HSP72 after exposure to tail-shock stressor in female compared to male rats. Thus, another objective of this study was to directly compare the cardiac HSP72 response in both sexes following short-term treadmill running. An important finding of our study was that the HSP72 response following the onset of an exercise regimen was sex-specific (Figure 2). In both sexes, a statistically significant increase in

myocardial HSP72 was observed following exercise, with females displaying a transient increase at a much earlier time-point than males (1 day females versus 10 days in the males). In earlier work it was demonstrated that the effect of exercise on myocardial HSP72 induction is more profound in male rats compared to females (33). Our data are qualitatively similar to those findings insofar as we see a more sustained increase in HSP72 protein expression in males compared to females, especially after multiple days of running. Our data in males are also in agreement with the findings of Locke et al. (25), who demonstrated that more than one day of running is required to observe a significant increase in myocardial HSP72. Our HSP72 results in female rats that have undergone one bout of exercise are, in general, also consistent with other studies that have shown short-term exercise can increase HSP72 in hearts from females (12, 17, 18, 38). Nevertheless, there are subtle differences in the temporal onset of elevated HSP levels in hearts among these studies. Most striking is that HSP72 levels in females increased significantly after one day of treadmill running, yet returned to baseline levels after two or more consecutive days of exercise stress. The observation in female rats that a stress protein such as HSP72 was not sustained in our study, despite repeated delivery of the stressor, represents an intriguing finding whose underlying causes are not readily apparent. Regarding this point, it may be important to consider is that previous studies examining female myocardial HSP72 and acute exercise all used Western blots (12, 17, 18, 38), where we used an ELISA herein. This distinction is noteworthy in light of the findings of Milne and Noble (26). These authors found that observable elevations in myocardial HSP72 after exercise (using a treadmill speed identical to that used herein), depended on whether the HSP72 was measured

by Western blot or ELISA (26). While this is the first study to directly examine the onset of HSP72 elevations in both sexes after 1-10 days of running, further experiments are needed to properly address the transient response in female rats.

While many studies have attributed the increase in myocardial HSP72 to the short-term treadmill exercise (11, 12, 18, 25, 33) , it is noteworthy that several other potentially negative stressors such as heat stress (22, 27) and tail shock (8) elicit elevated myocardial HSP72 hours after the stimulus. Because animals that are forced to run experience mild electrical shocks, blasts of air, or prodding during treadmill running, we probed further to determine if the protocol may have also elicited a stress response in the rats by examining the bioavailability of a key stress hormone, corticosterone.

Secreted by the adrenal glands, corticosterone is the major glucocorticoid in rat and basal elevations in corticosterone is a typical sign of chronic stress (14, 23, 32, 35). In both males and females, total serum corticosterone was significantly elevated in rats 24 hours after 3 of the 4 exercise regimens (Figure 3A). Corticosterone can exist in blood either free or bound to corticosterone binding globulin (CBG), with the free form of corticosterone denoting biologically active corticosterone. Produced in the liver, CBG is a serum protein that buffers corticosterone, with a stress-induced decrease in CBG rapidly increasing biologically active corticosterone (14). While the response of CBG following chronic treadmill running has been previously investigated (28), no study to date has examined CBG in response to a short-term treadmill running protocol in both male and female rats.

Greater content of CBG in females, with respect to males, was observed in this study and corroborates data from several other laboratories (15, 16, 21, 31, 39). An intriguing finding of this study was that male and female rats exposed to short-term treadmill running exhibited a sex-specific difference in the CBG response. While there was no change in serum CBG content in male rats following 1 to 10 days of running, female rats displayed a marked decrease in CBG levels following 2 and 10 days of running (Figure 2). Our observation that CBG was decreased in female (and not males) following acute exposure to treadmill running is consistent with literature where stress caused by restraint (16, 21) or swimming (39) elicited CBG decreases in female, but not male rodents. It is important to note that male rats are not resistant to stress-induced modulation of CBG. Fleshner et al. (14) reported, for example, a long-lasting (48h) decrease in CBG in male rats after exposure to a 90 min intermittent tail-shock stressor.

Changes in CBG are most commonly associated with chronic stress protocols. In contrast, corticosterone rapidly responds to acute stressors. Here we report that treadmill running increases total corticosterone in both sexes. Combined with a sharp decrease in CBG in the females, it is clear that short-term running markedly increased biologically active corticosterone, and was likely a significant stimulus to robustly activate the stress response in both sexes. While exercise elicited a reduction in CBG content in females, it is relevant to note that total CBG levels were still ~2 times higher than that observed in males, suggesting that there was still a greater corticosterone buffering capacity that might have the effect of mitigating the overall systemic stress response. This idea is consistent with the observed sex-dependent effects of exercise on body and spleen weights (see below).

Classic studies by Hans Selye indicated that male rats acutely exposed to various noxious stimuli exhibited an alarm reaction characterized by many factors including decreased body weight, splenic atrophy, and adrenal gland hypertrophy hours after the delivery of the stressor (37). Thus, in order to gauge other markers of stress, we measured changes in body, adrenal, and spleen weights. In response to acute exercise, male rats exhibited a significant decrease in body weight after 1, 2, and 10 days of running. To the contrary, body weight in female rats was not altered at any point following the initiation of treadmill running. The observation that short-term exercise leads to a reduced body weight in males only is consistent with previously published data from chronic exercise studies where males (28) but not females (4-6) lost weight in response to treadmill running. Although there is a paucity of data regarding the effects of short-term exercise on body weight, recent evidence using a swimming training model also supports our finding that body weight decreases rapidly and significantly in response to days of exercise for male animals (13).

Altered spleen and adrenal weights are indicative of increased activity of the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, which facilitates hyperplasia of adrenocortical cells and atrophy/contraction of the spleen (36, 37). In our study, adrenal gland weights did not change in either sex following the onset of exercise (Figure 5A). Interestingly, significant atrophy of the spleen was found in every exercise group in male (but not female) rats exposed to short-term treadmill running (Figure 6).

One potential limitation to our study is that animals were run at the same treadmill speed, yet body weights for males were higher than females. While we cannot rule out that animals may have been working at different relative workloads, previous

studies have indicated that when exposed to the same treadmill speeds, male and female rats demonstrate comparable adaptations in enhanced $VO_{2\ max}$ (3) and acquired cardioprotection (7) after treadmill running.

Our findings indicate that short-term treadmill running protocols are likely confounded by a stress response in both male and female rats. While the objective of our study was to determine if classic markers of stress were elevated following 1-10 days of treadmill running, we will briefly comment on three considerations with animal exercise models. First regards the type of exercise. The two common models of exercise training in animals are treadmill running and voluntary free wheel running. Free wheel running has the advantage that, to date, no signs of systemic stress have been observed. However, upregulation of a number of cardioprotective proteins occurs at intensities that are not obtained by free wheel running (26, 30, 34), giving advantage to treadmill protocols.

Second is the duration (number of days) of exercise. While many studies have used short-term (1 to 5 days) of exercise (7, 10, 12, 13, 17, 24, 33, 41-43), it is clear from our results that negative adaptations due to forced running are also present during this period. Studies where animals undergo months of exercise are likely more relevant to the human population, as changes that are beneficial following exercise must also be shown to be sustainable. While chronic exercise has also been shown to elicit negative adaptations in male rats (28), chronic treadmill running in female rats does not. Previous experiments from our laboratory have indicated that chronic exercise in females results in beneficial adaptations to training including increased HSP72 (20), up-regulation of superoxide dismutase (both isoforms) (6), increased KATP channel

expression (5), and higher basal citrate synthase activity (5, 6, 19). These changes were associated with improved coronary flow and protection against myocardial infarction and stunning after ischemia and reperfusion. Importantly, in these same studies when female rats ran on the treadmill daily for several months, they did not display markers of stress such as adrenal hypertrophy, splenic atrophy, or decreased body weight (5, 6, 19). In addition, females who are exercised for >8 weeks also show no signs of elevated serum corticosterone (unpublished results).

One final consideration with exercise protocols is the sex of animal used. Despite the fact that cardiovascular disease remains the #1 killer of both men and women in the industrialized world, and that females make up roughly the same proportion of taxpayers that fund federally-supported research, the overwhelming majority of animal studies looking at beneficial effects of exercise are done using male animals. Our hope is that more attention will be devoted to the sex of animal used, the type of exercise, and the duration of the protocol employed.

In conclusion, this study provides novel insight into the peripheral adaptations in response to the onset of a treadmill running protocol. Our data clearly indicate that markers of systemic stress are observed in males and females after acute bouts of forced running. As it cannot be ascertained if changes in rat phenotype after short-term treadmill running are due to the exercise itself, or the strain put on the animals by forced running, caution should be used when interpreting results from short-term exercise studies.

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Figure Legends

Figure 1: Plantaris citrate synthase activity for male (closed bars) and female (open bars) animals 24 hours after the last exercise bout. *, signifies a significant ($P < 0.05$) training effect compared to sedentary females.

Figure 2: Myocardial heat shock protein 72 (HSP72) content in males (closed bars) and females (open bars) 24 hours following 0, 1, 2, 5, or 10 days of treadmill running. +, $P < 0.05$ sex-effect versus sedentary male; #, denotes a significant ($P < 0.05$) training effect compared to sedentary males. *, signifies a significant ($P < 0.05$) training effect compared to sedentary females.

Figure 3: (A) Serum levels of total corticosterone for male (closed bars) and female (open bars). (B) Serum corticosterone binding globulin (CBG) content in males (closed bars) and females (open bars) 24 hours following 0, 1, 2, 5, or 10 days of treadmill running. + denotes significant ($P < 0.05$) sex-difference compared to sedentary male; # represents significant ($P < 0.05$) training effect versus sedentary male; ##, $P < 0.1$ training effect compared to sedentary male; *, $P < 0.05$ training effect compared to sedentary females; **, $P < 0.1$ training effect compared to sedentary females.

Figure 4: Body weights of male (closed bars) and female (open bars) animals exercised for 0, 1, 2, 5, or 10 days on a motorized treadmill. +, $P < 0.05$ sex effect versus

sedentary male; #, represents a significant ($P < 0.05$) training effect compared to sedentary males; ##, $P < 0.1$ training effect versus sedentary male.

Figure 5: Left (A) and right (B) adrenal gland weights for male (closed bars) and female (open bars) animals in the study; +, denotes a significant ($P < 0.05$) sex effect versus sedentary male.

Figure 6: Spleen weights for male (closed bars) and female (open bars) animals used in the study; + indicates a significant ($P < 0.05$) sex effect versus sedentary male; #, $P < 0.05$ training effect compared to sedentary males; ##, $P < 0.1$ training effect versus sedentary males.

Figure 1

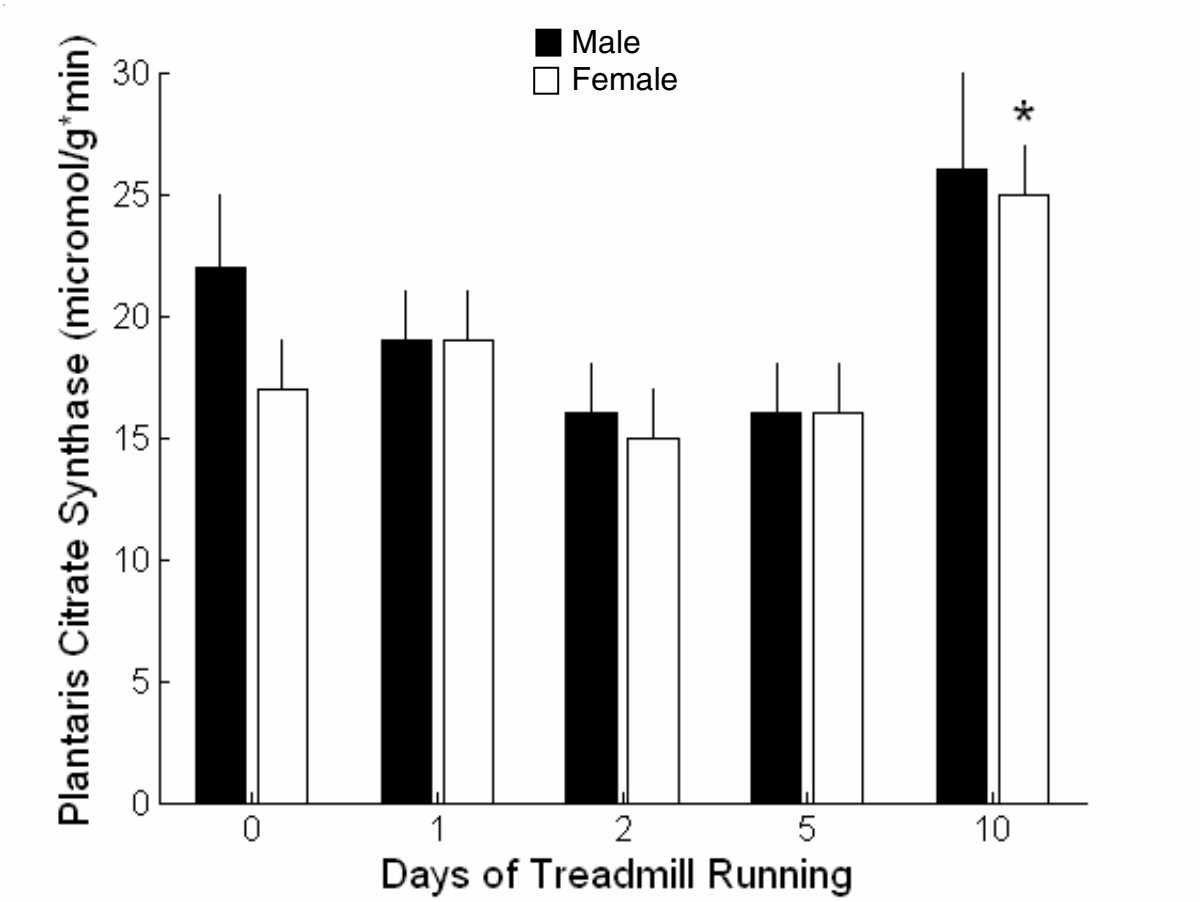

Male
Female
Plantaris Citrate Synthase (micromol/g*min)
30
25
20
15
10
5
0
*
0
1
2
5
10
Days of Treadmill Running

Figure 2

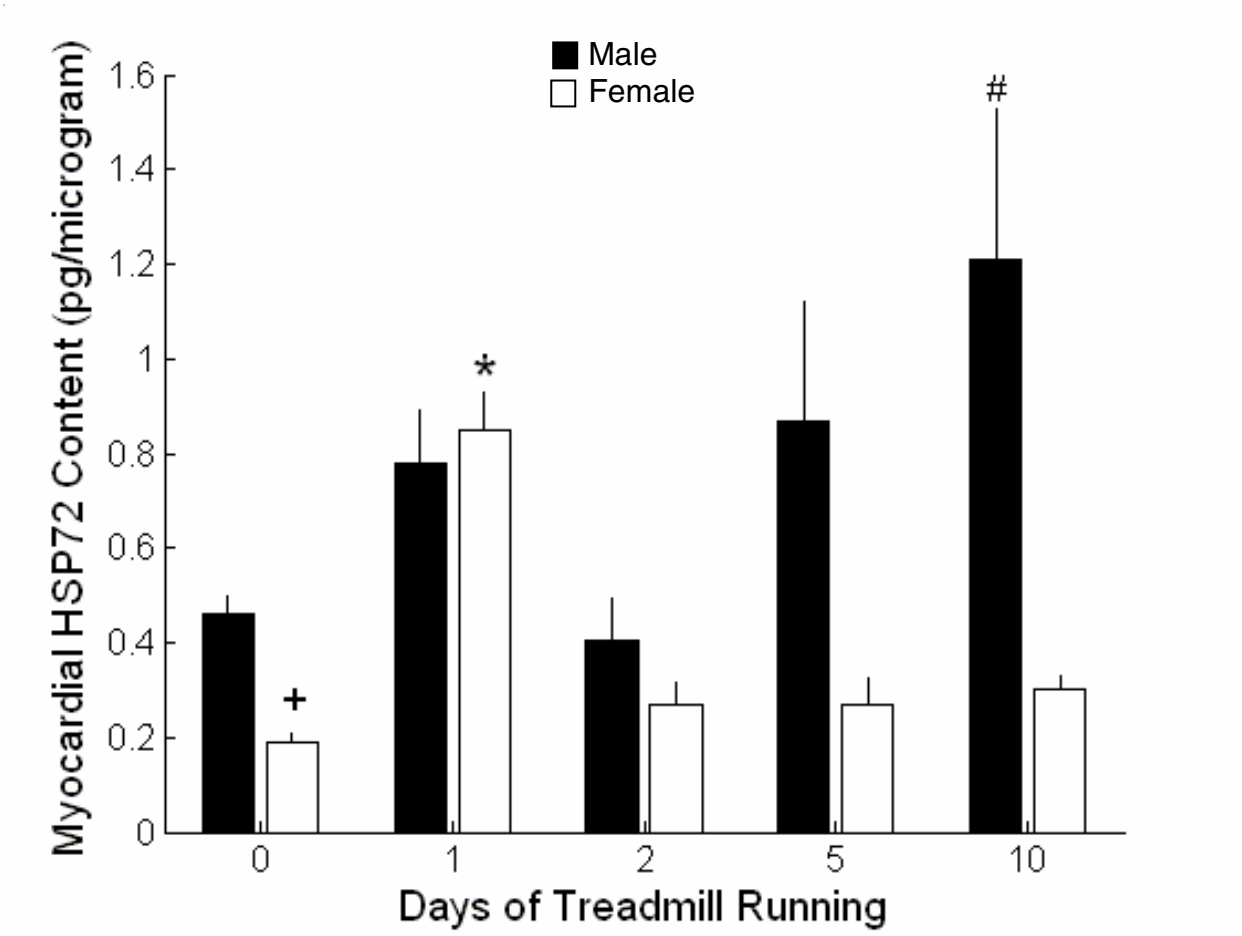
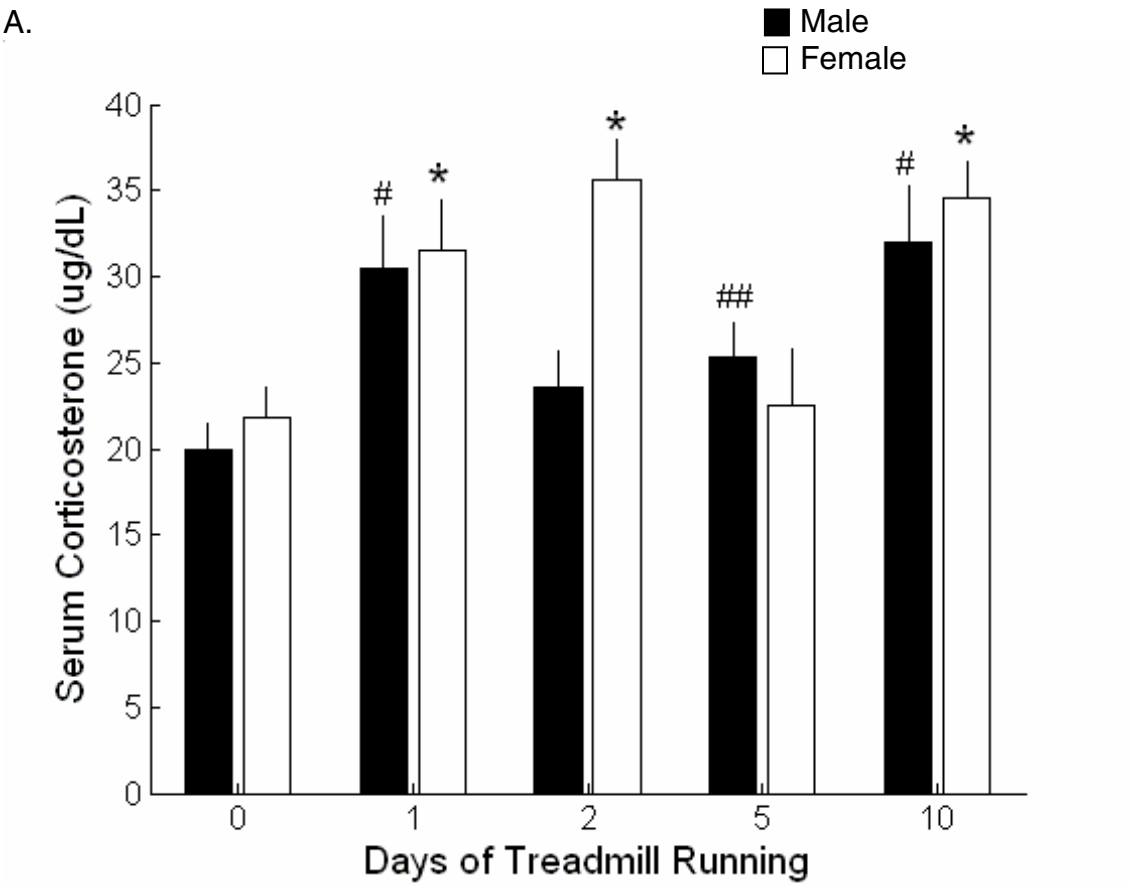

Male
Female
Myocardial HSP72 Content (pg/microgram)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
+
*
#
0
1
2
5
10
Days of Treadmill Running

Figure 3

A.


Male
Female
Serum Corticosterone (ug/dL)
40
35
30
25
20
15
10
5
0
#
*
*
##
#
*
0
1
2
5
10
Days of Treadmill Running

B.

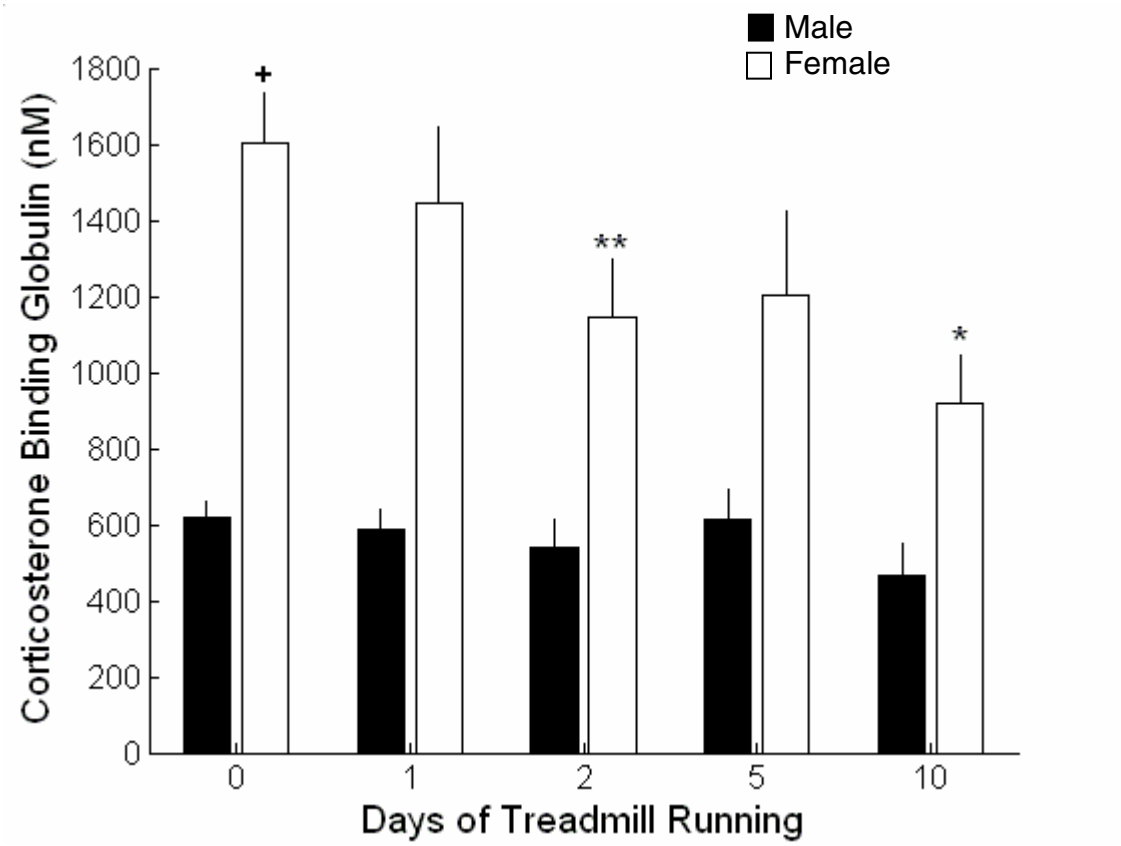

Male
Female
Corticosterone Binding Globulin (nM)
1800
1600
1400
1200
1000
800
600
400
200
0
+
**
*
0
1
2
5
10
Days of Treadmill Running

Figure 4

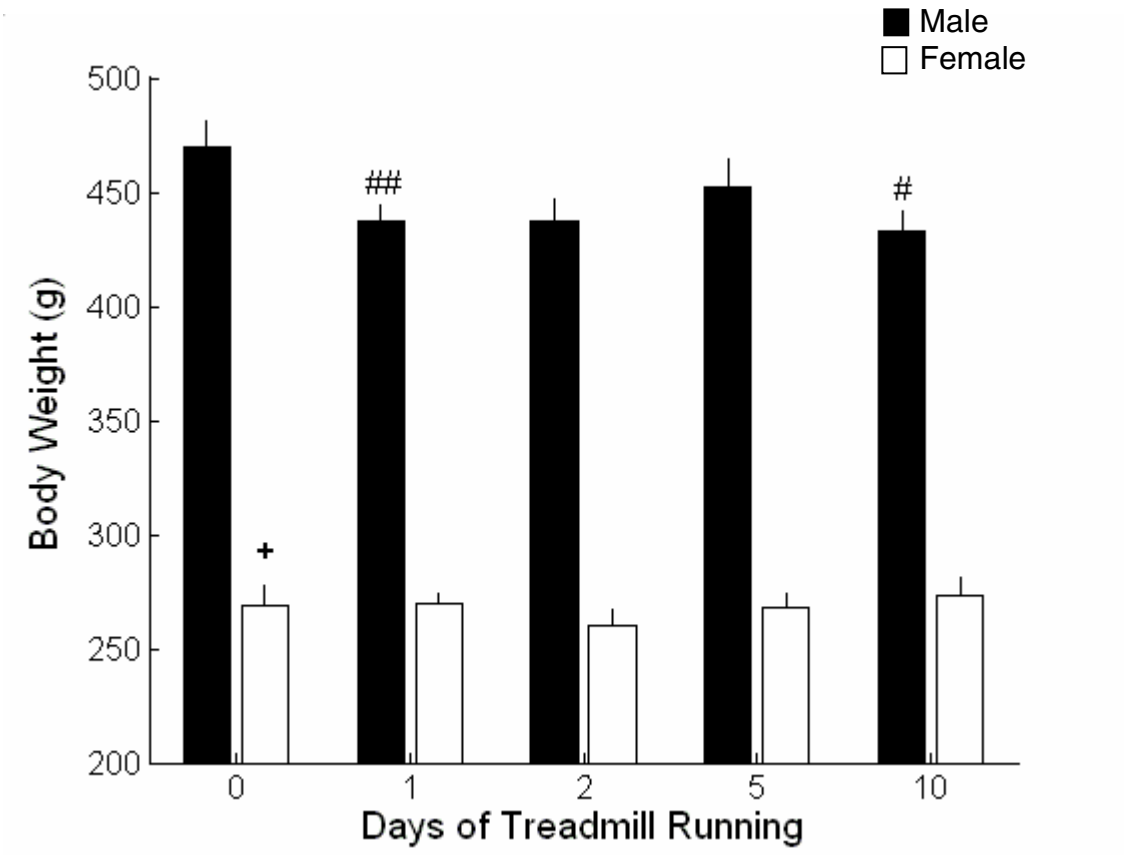
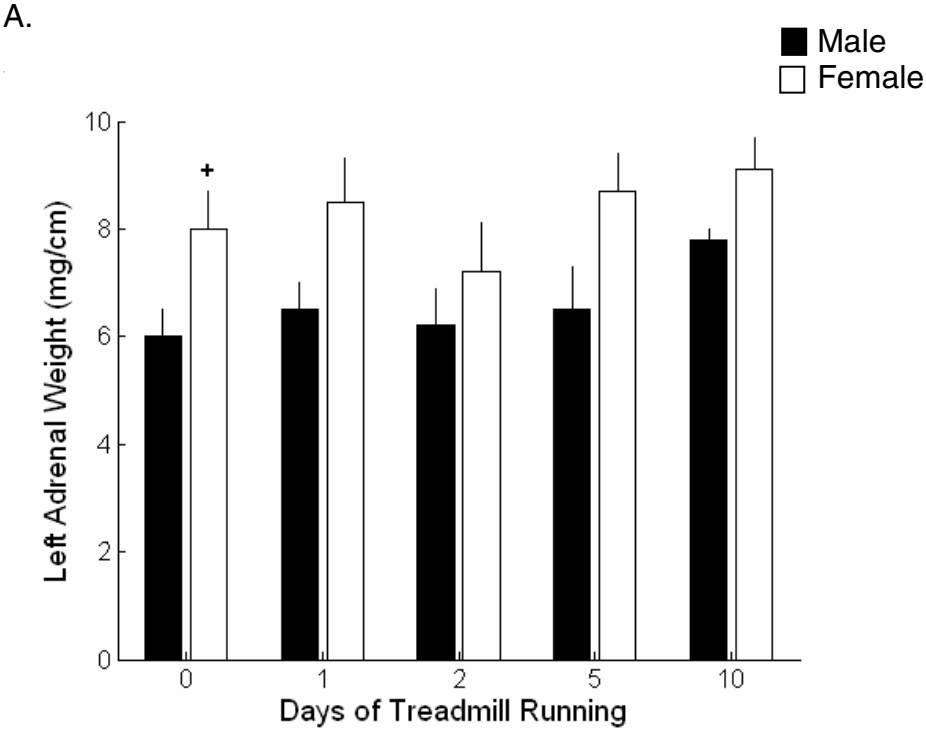

Male
Female
Body Weight (g)
500
450
400
350
300
250
200
##
#
+
0
1
2
5
10
Days of Treadmill Running

Figure 5

A.


Male
Female
Left Adrenal Weight (mg/cm)
0
2
4
6
8
10
+
0
1
2
5
10
Days of Treadmill Running

B.

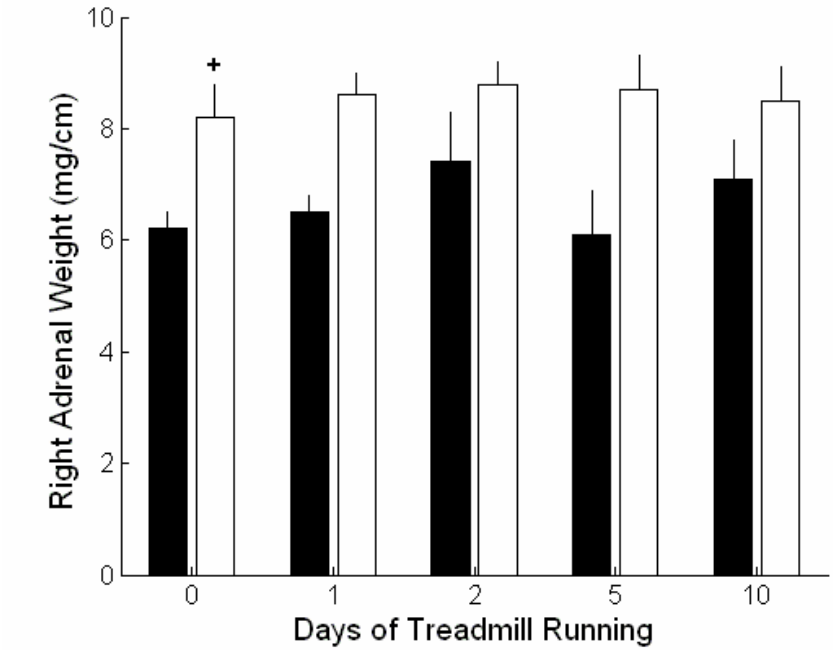

Right Adrenal Weight (mg/cm)
0
2
4
6
8
10
+
0
1
2
5
10
Days of Treadmill Running

Figure 6