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Inhibition of Angiogenesis by High Salt Diet Is Associated with Impaired Muscle Performance Following Chronic Muscle Stimulation

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Abstract

Objective—High dietary salt has been demonstrated to inhibit angiogenesis in skeletal muscle. The purpose of this study was to determine whether high salt impairs steady state muscle performance following a chronic stimulation protocol.

Methods—Sprague-Dawley rats were placed on a control diet (CD, 0.4% NaCl) or high salt diet (HSD, 4.0% NaCl) prior to implantation of an electrical muscle stimulator. In chronically stimulated animals, hind limb muscles were stimulated to contract eight hours daily for seven days. Sham animals received a stimulator that was never activated.

Results—Following chronic stimulation, tibialis anterior (TA) muscles of animals on CD demonstrated an 84.6% increase in force of contraction at the end of an acute stimulation bout relative to sham animals fed CD. Decreased muscle fatigue was associated with an increase in capillaries per TA fiber (C:F). Chronic stimulation in HSD rats induced a smaller improvement (52.2%) in final force compared to HSD sham rats. This impairment of muscle performance in high salt-fed rats correlated with inhibited angiogenesis. Infusion of angiotensin II in HSD animals restored angiogenesis and muscle fatigue to CD levels.

Conclusions—This study suggests that angiogenic inhibition by high salt is associated with impaired skeletal muscle performance following chronic stimulation.

Keywords

hyperemia; fiber type; electrical stimulation

INTRODUCTION

Long-term exercise [35] and chronic electrical stimulation of muscle contraction [23,31] result in a number of physiological adaptations that improve muscle performance and enhance the resistance of the muscle to fatigue [7,11]. These changes include increases in mitochondrial biomass [4], changes in the expression and activity of important metabolic enzymes [28], myofiber phenotype conversion [16], and increased capillary density, or angiogenesis.

The angiogenic response to chronic muscle stimulation is associated with a large increase in capillary surface area [25], presumably improving efficiency of metabolic substrate exchange. A number of studies have indicated that oxygen exchange capacity is improved in endurance-trained muscle relative to muscle of sedentary animals [14,29]. Yang et al. [35] attributed the

increase in oxygen extraction and peak oxygen consumption in trained muscles in part to the increase in capillary density associated with training. However, it is unclear to what extent angiogenesis is directly responsible for increases in muscle function and to what degree other important biochemical and structural changes associated with training may impact muscle performance.

The renin-angiotensin system (RAS) has been demonstrated to play an important role in regulating microvessel density in skeletal muscle. Treatment with pharmacologic agents inhibiting the actions of angiotensin II (ANGII), the primary signaling molecule of the RAS, has been shown to eliminate the angiogenic response to a number of physiological stimuli, including exercise [2], severe limb ischemia [8,32], and electrical stimulation of muscle contraction [27]. Importantly, the effect of the RAS on the skeletal muscle microcirculation may have consequences for muscle function. Minami et al. [24] reported that inhibition of the RAS by pharmacologic treatment or administration of a diet high in salt, which suppresses circulating levels of ANGI, was capable of reducing exercise capacity in rats.

Previous experiments from our laboratory have demonstrated that a high salt diet inhibits angiogenesis in response to chronic muscle stimulation [27]. However, the impact of high salt intake and the associated angiogenic inhibition on muscle performance is unknown. The purpose of the current study was to determine whether high salt intake and inhibition of angiogenesis impact acute skeletal muscle performance following a chronic electrical stimulation period. The results of these studies support the hypothesis that a high salt diet has a deleterious impact on steady state muscle performance and that angiogenesis may be an important determinant of the effect of high salt on the complex process of acute muscle fatigue following a chronic training period.

MATERIALS AND METHODS

Animal Surgery

All animal protocols were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. Animals were housed and cared for in the MCW Animal Resource Center and were given food and water ad libitum. Seventy-four 7-week old male Sprague-Dawley rats were anesthetized with an intramuscular injection of a mixture of ketamine (100 mg/kg), xylazine (50 mg/kg), and acepromazine (2 mg/kg). Under aseptic conditions, a polyethylene catheter was placed in the left jugular vein, tunneled subcutaneously and exteriorized at the back of the neck. The catheters were then passed through a flexible spring that was secured to the rat subcutaneously. The spring was attached to a swivel above the cage, allowing the animal full range of movement in the cage while protecting the catheters. Immediately following cannulation of the vessels, an incision was made over the thoracolumbar region, and a miniature battery-powered stimulator, previously designed and validated for chronic studies by our laboratory [19], was implanted subcutaneously and secured in place. A second incision was made in the skin and fascia covering the lateral side of the knee joint (over the region of the common peroneal nerve) of the right hind limb. A pair of electrodes were guided under the skin from the stimulator and secured to the muscles surrounding the knee in close proximity to the common peroneal nerve with surgical suture. The skin over both incisions was sutured closed and the rats were allowed to recover for 24 hours before the stimulator was activated.

Experimental Protocols

Following the one day recovery period, intravenous infusion was initiated, and the implanted stimulator was activated by momentary closure of the magnetic reed switch using a small magnet. The stimulator produced muscle contractions in the lower limb muscles by indirect

stimulation of the right common peroneal nerve with square-wave impulses of 0.3 ms duration, 10 Hz frequency and 3 volts potential [19]. Contractions of the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were automatically initiated at 8 AM each day and sustained for 8 hours per day over seven consecutive days. The stimulators of animals in sham treatment groups were not activated at any point during the seven day experimental period.

The animals were randomly assigned to six groups. All animals were fed normal rat chow until one week before surgical protocols were carried out. The animals were then placed on a control diet (CD, Dyets 0.4% NaCl chow) or high salt diet (HSD, Dyets 4.0% NaCl chow) for the duration of the protocol. Animals in the CD Sham group (n = 15) were fed a control diet and received an implanted stimulator that was never activated. CD Stim animals (n = 14) were fed a control diet and received muscle stimulation for seven days. HSD Sham rats (n = 17) were fed a high salt diet and received a stimulator that was never activated. HSD Stim animals (n = 15) were fed high salt and stimulated for seven days. HSD+ANGII Sham animals (n = 7) were fed a high salt diet, received a sham stimulator, and were infused via the jugular catheter with ANGII (Sigma, St. Louis, Missouri, USA) at a dose of $3 \text{ ng/kg}^{-1}/\text{min}^{-1}$ dissolved in sterile saline. This dose has previously been demonstrated to restore stimulated angiogenesis in rats fed a high salt diet [27]. HSD+ANGII Stim animals (n = 6) received seven days of stimulation along with the ANGII infusion. A number of CD Sham, CD Stim, HSD Sham, and HSD Stim animals received a vehicle saline infusion via the jugular catheter to determine whether vehicle infusion altered muscle function.

Measurements of Steady State Muscle Performance

On the day following the final stimulation period, 42 rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Ovation Pharmaceuticals, Deerfield, Illinois, USA) and prepared for measurement of steady state muscle performance as previously described [26]. Rats were secured to the working surface by padded staples that insured immobility of the hind limb without resulting in significant blood loss. The staples were secured under the isolated muscle to prevent disruption of the circulation to the muscle. The distal tendon of the TA muscle of the right hind limb was isolated near the bony attachment, cut, and attached to a force transducer (model FT-10, Grass Instruments, Quincy, Massachusetts, USA) by a piece of 2.0 surgical suture. The exposed portion of the muscle was kept covered throughout the stimulation period by gauze moistened with warmed saline.

Steady state muscle performance was measured by isometric muscle contractions indirectly stimulated with the use of an external stimulator unit (model SD9, Grass Instruments). Needle electrodes were placed in the muscle on either side of the common peroneal nerve and optimal muscle length and supra-maximal stimulation voltage were determined by twitch contractions. Contractions were stimulated for one hour by pulses of 0.3 ms duration and 5 Hz frequency. The supramaximal voltage was determined in each experiment and varied between 3 and 5 V. The results of preliminary experiments demonstrated that stimulation at a frequency of 5 Hz avoided temporal summation between contractions and allowed accurate measurement of individual muscle twitches. Data was recorded throughout the experiment using the WINDAQ data acquisition program (Dataq, Akron, Ohio, USA). For data analysis, the peak force of individual contractions was averaged over five minute periods throughout stimulations, using the contraction of greatest force as the peak force of the experiment. The peak contraction occurred within 10 seconds of the initiation of acute stimulation in most experiments.

Measurements of Iliac Arterial Blood Flow

On the day following the completion of the seven day chronic stimulation period, an additional group of 32 rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital. The right common carotid artery was cannulated to allow blood pressure

measurement throughout the stimulation period. An incision was also made in the lower abdomen and the right common iliac artery was isolated. A 0.7 V ultrasonic flow probe was placed around the artery, and flow was measured with the use of a Transonics flowmeter (model T106, Transonic Systems Inc., Ithaca, New York, USA). Data was recorded using the WINDAQ program.

A 10 minute baseline period was recorded before activation of the implanted electrical stimulator. Contractions of the TA and EDL muscles were stimulated at a frequency of 10 Hz, as described above. Continuous measurement of blood flow was made during the baseline period, during a one hour stimulation period, and for a five minute recovery period following deactivation of the stimulator. For data analysis, flow was averaged over five minute periods throughout the experiment, with the exception of the first and second minutes of stimulation, which were averaged individually in one minute periods.

Tissue Harvest and Processing

Following measurement of either muscle fatigue or iliac arterial blood flow, the animals were euthanized by an overdose of pentobarbital sodium and the TA muscles from the right hind limb were removed and weighed. TA tissue was imbedded in Tissue-Tek O.C.T. compound, (Sakura Finetek USA, Torrance, Colorado, USA) and frozen in dry ice-cooled 2-methyl butane (Sigma) for immunohistochemical analysis.

Immunohistochemical Determination of Vessel Density and Fiber Type

Frozen muscles were sectioned to 10 μ m with a cryostat. Ten serial cross sections were made from the midbelly of each muscle. Sections were then stained for visualization of capillaries and specific myosin heavy chain (MHC) isoforms for determination of myofiber phenotype. Prior to blocking, sections were incubated with 25 μ g/mL rhodamine-labeled *GS-I* lectin in PBS for one hour. *GS-I* lectin has previously been used in our laboratory for visualization of microvessels [30]. Sections were then blocked for 20 minutes in PBS containing 2% goat serum and 1% BSA.

Following the blocking step, sections were incubated with one of several MHC isoform specific mouse IgG primary antibodies in PBS containing 1% BSA for one hour. The MHC type I specific clone (BA-D5), the MHC type IIa antibody (SC-71), and the MHC type IIb antibody (BF-F3) were generous gifts of the laboratory of Dr. Dan Riley at the Medical College of Wisconsin. The pan-MHC type II antibody MY32 was purchased from Sigma. Sections were rinsed in PBS, and then incubated with fluorescently labeled secondary antibody (Alexa Fluor 488 anti-mouse IgG, Invitrogen, Carlsbad, California, USA) for one hour. After rinsing, sections were mounted with ProLong Antifade mounting medium (Invitrogen).

Sections were visualized using a computerized fluorescent microscope (Eclipse 600; Metamorph Imaging System, Molecular Devices, Downingtown, Pennsylvania, USA). The oxidative core of the TA muscle section was defined as the region in which MHC type I positive fibers were located, as previously described [6]. The glycolytic cortex was defined as the area in which only type II fibers were located. The diameter of TA sections was approximately 6 millimeters from the deepest aspect of the core of the muscle to the superficial surface of the cortex. In each section, images for analysis were taken 1 mm from the inner core to represent the oxidative core of the muscle and 5 mm from the core to represent the cortex. For each muscle, the reported capillary: fiber (C:F) ratio was calculated as the mean of two separate fields (one each from the core and cortex) in four serial sections. Fiber type distribution of each muscle was calculated as the mean of two separate fields in two serial sections for each MHC antibody used. Capillary contacts per fiber (CC:F, the mean number of capillaries in contact with the individual fiber types quantified) were calculated for each MHC antibody used.

Data Analysis and Statistics

All values are presented as mean values $\pm$ S.E.M. The significance of differences in capillary: fiber ratio, capillary contacts: fiber, and fiber type distribution was evaluated by a two factor analysis of variance using treatment (diet + infusion) and stimulation protocol as factors. The significance of differences in blood flow and force generation values measured between groups was evaluated by a two factor repeated measures analysis of variance, with time and treatment as factors. Significant differences were further evaluated by Holm-Sidak post hoc analysis. Significance was established at $p < 0.05$.

RESULTS

Table 1 displays rat and TA muscle weights. Body weight increased significantly from the beginning to the end of the treatment period in all groups. There was no difference between groups in body weight at the start or completion of the study. Electrical stimulation did not result in significant hypertrophy of the TA muscle, as determined by TA wet weight at the end of the study.

Figure 1 displays the results of measurements of steady state muscle performance over one hour of muscle contraction in CD- and HSD- fed animals. Acute muscle stimulation resulted in a dramatic fatigue response in the TA muscles of CD Sham animals and HSD Sham animals that was attenuated by seven days of chronic electrical stimulation. At peak force production and throughout the one hour period of acute muscle stimulation, the force produced by the TA muscle of the CD Sham animals was not different than that of the HSD Sham animals. Chronic muscle stimulation did not significantly alter the peak force produced in both the CD Stim and HSD Stim groups. The force produced at all other time points of the experiment was significantly higher in the CD Stim and HSD Stim groups compared to the corresponding sham operated group ($p < 0.05$). Beginning at five minutes of stimulation and proceeding throughout the rest of the experiment, the force produced by the CD Stim group was significantly greater than that of the HSD Stim group ($p < 0.05$).

Figure 2 displays the capillary: fiber (C:F) ratio in all fibers of both the oxidative core and glycolytic cortex of the TA muscle in CD and HSD Sham and Stim groups. Consumption of high salt alone had no effect on C:F in the TA muscle, as there was no difference between CD Sham animals and HSD Sham animals in either the core or the cortex. Chronic electrical stimulation resulted in a significant increase in C:F in CD Stim animals compared to CD Sham animals in both the core (2.70 ± 0.18 capillaries/fiber vs. 2.07 ± 0.19 , $p < 0.05$) and the cortex (3.27 ± 0.33 capillaries/fiber vs. 1.68 ± 0.12 , $p < 0.05$). The angiogenic response to chronic stimulation was dramatically reduced by high salt intake. C:F was not significantly increased in the HSD Stim group compared to the HSD Sham animals in the core (2.15 ± 0.15 capillaries/fiber vs. 1.95 ± 0.16), while in the cortex the angiogenic response (2.26 ± 0.20 vs. 1.73 ± 0.24 , $p < 0.05$) was significantly less than that observed in the CD Stim group.

To determine whether the increase in muscle fatigue observed in the HSD Stim group was due to impaired angiogenesis, ANGII was infused in high salt-fed animals to restore the angiogenic response to chronic stimulation. There was no significant difference in steady state muscle performance of HSD+ANGII Sham animals relative to CD Sham and HSD Sham groups (data not shown). Infusion of saline vehicle alone in CD Sham and HSD Sham animals did not significantly alter force production of muscle (data not shown). Figure 3 demonstrates the results of steady state muscle performance measurements in CD Stim, HSD Stim, and HSD +ANGII Stim, and all combined sham animals normalized to peak force. As presented above, CD Stim animals fatigued to a lesser degree than HSD Stim animals. HSD+ANGII Stim animals demonstrate muscle fatigue similar to CD Stim by 25 minutes of stimulation. Figure 4 displays the C:F ratio of TA muscles from HSD+ANGII Sham and Stim animals. Stimulation

resulted in a significant increase in C:F in HSD+ANGII Stim animals compared to corresponding sham animals in both the core (2.14 ± 0.23 capillaries/fiber vs. 1.46 ± 0.12 , $p < 0.05$) and the cortex (2.86 ± 0.09 capillaries/fiber vs. 1.40 ± 0.07 , $p < 0.05$).

Using immunohistochemical techniques, the distribution and content of specific muscle fiber types were quantified to determine whether high salt diet had non-angiogenic effects on muscle phenotype. Analysis of fiber type revealed that seven days of chronic muscle stimulation did not result in any change in the distribution of type I, type IIa, or type IIb fibers in any group (Table 2). Type IIx fibers were identified as fibers stained by the non-specific MHC type II antibody, but not stained by either the type IIa- or type IIb- specific antibodies. The number of total muscle fibers per microscope field was similar among treatment groups, providing indirect evidence that mean cross sectional area was unaltered by diet or stimulation. By co-staining muscle sections with both antibodies against specific myosin heavy chain isoforms and *GS-I* lectin to identify capillaries, the number of capillary contacts per fiber (CC:F) for each fiber type was assessed (Table 3). The CC:F ratio of type I and type IIa fiber types was not altered by chronic stimulation, but the CC:F of type IIb fibers was significantly increased in CD Stim animals relative to the CD Sham group in both the oxidative core and glycolytic cortex, suggesting that the initial angiogenic response in the first week of chronic stimulation is specific to type IIb glycolytic fibers. HSD Stim animals demonstrated a weak but insignificant angiogenic response in type IIb fibers relative to the HSD Sham group. Figure 5 displays representative micrographs of muscle cross sections stained with MHC-specific antibodies (green) and *GS-I* lectin (red) to identify capillaries.

Iliac arterial blood flow in the stimulated limb was also measured during acute stimulation to determine if high salt diet was associated with changes in gross limb hemodynamic variables. As presented in Fig. 6, acute muscle stimulation resulted in a hyperemic response in the stimulated hind limb, as measured by changes in iliac arterial flow. Baseline blood flow was not significantly different in any treatment group. Blood flow during chronic muscle stimulation was similar at all time points between CD Sham and HSD Sham animals. Chronic muscle stimulation resulted in an augmented hyperemic response to muscle contraction in the CD Stim group relative to the CD Sham group at time points from 25 minutes to 60 minutes of acute stimulation ($p < 0.05$). The hyperemic response was significantly augmented in the HSD Stim animals compared to the HSD Sham group at all time points of acute stimulation ($p < 0.05$). In addition, iliac arterial flow of the HSD Stim animals was greater than that of the CD Stim animals at all points from two minutes through 40 minutes of stimulation ($p < 0.05$). Recovery blood flow following the stimulation period was not different in any groups. Figure 7 displays this data as a percent of baseline blood flow, with similar trends observed. Differences in limb blood flow were not due to changes in mean arterial pressure (MAP), as the MAP throughout the course of the experiment was not different between animals fed control diet (118 ± 4.0 mm Hg) and high salt diet (121 ± 2.7 mm Hg). We have previously demonstrated that chronic stimulation does not change MAP [27].

DISCUSSION

The purpose of the current study was to assess the functional consequences of high salt intake and inhibition of angiogenesis on acute skeletal muscle performance following a chronic stimulation protocol. The results described in this study support an important role for dietary salt intake in regulation of microvascular structure. In addition, this study indicates that inhibition of angiogenesis by consumption of a high salt diet is associated with impaired muscle function, suggesting a possible direct role for angiogenesis as an important adaptation made by muscle to develop fatigue resistance.

Previous studies have demonstrated that angiogenesis occurs in skeletal muscle in response to long term elevations in muscle activity, such as chronic exercise [20,34] or muscle stimulation [1,23]. A number of these reports have indicated that improvement in muscle function and resistance to muscle fatigue occurs in parallel with angiogenesis [7,11,35]. However, these increases in muscle function often occur in the presence of a number of important metabolic adaptations, making a direct assessment of the relative importance of angiogenesis difficult. The results displayed in Figure 1 of the current study demonstrate that the inhibition of angiogenesis by a high salt diet occurs in conjunction with a reduction in the development of fatigue resistance in response to chronic electrical stimulation of muscle. To our knowledge, these studies are the first to directly demonstrate an impairment of muscle performance following an inhibition of the angiogenic response associated with chronic muscle activity. While this deficit in muscle performance may not be manifested in normal activities of a rat in caged environment, it is possible that the increased rate of fatigue would become apparent in situations of more strenuous use, for example sustained running. Further, in tissues with sustained high rates of metabolism, such as the heart, this high salt diet induced reduction in function could result in additional pathologies related to impaired delivery of oxygen.

The enhancement in fatigue resistance in response to muscle stimulation observed in this study supports previous studies indicating improvements in muscle performance following chronic stimulation [7,11]. While this robust increase in fatigue resistance in a period of seven days of stimulation is likely the result of a complex interaction between biochemical, physiological, and structural changes, angiogenesis appears to be among the earliest adaptations observed in response to training and muscle stimulation. Increases in capillary density have been reported to occur following three days of exercise training [2] and four days of muscle stimulation [16] in the rat. The rapid nature of the angiogenic response is in contrast to other important adaptations made to muscle stimulation, including increased expression and activity of enzymes involved in oxidative metabolism [31] and fiber type transition [5], which have not been consistently reported to occur within the first week of stimulation and, therefore, may not be necessary for the initial protection from muscle fatigue described in this and other studies.

Green and Pette [11] reported that the reduction in muscle fatigue following eight days of chronic stimulation was associated with improved maintenance of levels of the metabolic substrates ATP and glycogen and reduced accumulation of the metabolic byproduct lactate, despite the absence of pronounced increases in oxidative enzyme activity. Given that this duration of muscle stimulation is known to induce angiogenesis, this finding suggests a possible critical role for angiogenesis in improving fatigue resistance by increasing the delivery of oxygen and nutrient molecules to the muscle and removing metabolic waste products. Henderson et al. [13] demonstrated the critical nature of the relationship between capillary-tissue exchange and muscle performance in rats selectively bred for endurance exercise, as the primary difference between rats of high and low running capacity was capillary-tissue oxygen exchange. Therefore, the reduction in muscle performance in high salt-fed animals in the current study is likely due at least in part to an inhibition of angiogenesis, resulting in an inadequate increase in delivery of nutrients as compared to control animals undergoing angiogenesis in response to stimulation.

The inhibitory effect of high salt diet on physiological angiogenesis in muscle (Figure 2) and the restoration of angiogenesis by infusion of ANGII (Figure 4) is supported by previous experiments from our laboratory [27], although the current study is the first to report capillary supply relative to muscle fiber number and specific fiber type. However, the effects of high salt diet and resultant ANGII suppression on vascular structure are not limited to inhibition of capillary growth. Administration of a high salt diet for four weeks has been shown to induce rarefaction, or a reduction capillary density, in skeletal muscle [12,15]. The rarefaction induced by high salt diet may have important consequences on muscle function. Long term high salt

treatment leading to rarefaction has been shown to reduce tissue oxygen levels in resting skeletal muscle [21]. However, given that the animals in the current study do not demonstrate rarefaction in the TA muscle following a shorter duration of high salt diet (two weeks), as has been previously demonstrated [27], and that muscle performance and fatigue resistance are identical between sham animals fed control or high salt diets, it is unlikely that the reduction in muscle function in chronically stimulated high salt animals observed in this study is due to changes in resting tissue oxygen.

High salt consumption has also been reported to directly impact arteriolar function, as arterioles harvested from rats fed a long term high salt diet demonstrate a blunted dilatory response to a number of vasoactive stimuli [9]. This reduction in vascular reactivity following elevations in salt intake has also been reported in acutely stimulated skeletal muscle, as Marvar et al. [22] reported that the increase in arteriolar diameter in response to muscle contraction is blunted in high salt-fed animals; however, this blunting of dilation did not result in a reduction of hyperemia or the increase in blood flow to the stimulated muscle. This observation by Marvar et al. supports the data displayed in Fig. 6 of the current study in which the initial hyperemic response to muscle contraction in high salt sham animals was not different than that of sham animals on a control diet.

The finding that chronically stimulated animals demonstrate an augmentation of the hyperemic response to acute stimulation compared to sham animals supports the findings of Egginton and Hudlicka [7]. This augmentation may be explained by the chronic nature of the hyperemic response, which could result in remodeling of the large arteries upstream from the stimulated limb [17], allowing a greater bulk blood flow to the stimulated limb. Tulis et al. [33] demonstrated that remodeling of arteries in response to elevations in flow is directly dependent on the degree of flow increase. The measurements of bulk blood flow in the current study were made in the common iliac artery, a vessel that provides flow to a number of structures in addition to the hind limb musculature. Therefore, the relative flow increase to the contracting muscles is likely underestimated in the current study, as only a fraction of the baseline blood flow measured in these experiments reaches the hind limb muscles. The additional increase in stimulated hyperemia observed in the chronically stimulated high salt animals as opposed to the stimulated animals on control diet may demonstrate an important compensatory response that in part attenuates the increase in muscle fatigue as a result of high salt intake. The current study cannot preclude the presence of these or other compensatory adaptations that may be occurring in response to high salt diet and the associated angiogenic inhibition. Future studies will be required to determine the nature and importance of the augmented hyperemic response in high salt-fed animals.

While the current study demonstrates that the inhibition of angiogenesis by high salt is associated with an impairment of muscle function, we cannot rule out the possibility that the impairment observed in the chronically stimulated animals fed a high salt diet is independent of effects related to inhibition of angiogenesis. While O'Drobinak and Greene [26] noted reductions in muscle performance in high salt-fed animals made hypertensive by surgical reduction of renal mass, these effects appeared to be due to hypertension rather than high salt. Minami et al. [24] reported that a four week treatment with high salt diet resulted in a lower exercise capacity in rats compared to control animals. The authors attributed this response in part to the rarefaction response discussed above [15] but could not rule out non-angiogenic effects of high salt intake. A direct effect of high salt diet on muscle fatigue is supported by the results of this study, as displayed in Figure 3. In the initial stages of the one-hour acute stimulation period, HSD and HSD+ANGII Stim demonstrated more rapid fatigue than CD Stim counterparts; however, fatigue during the latter stages of the acute stimulation period appears to be independent of high salt intake and correlates with angiogenesis, as HSD+ANGII

and CD Stim animals demonstrate similar muscle fatigue and HSD Stim animals fatigue to a greater degree.

It is also possible that high salt diet may have detrimental effects on muscle function through suppression of ANGII independent of vascular changes. It has been reported that ANGII may have a direct impact on the hypertrophic response [10] and addition of myonuclei in overloaded skeletal muscle. These reports leave open the possibility that the findings in the current study may be explained by vascular-independent effects of ANGII suppression on muscle. The results of the current study do not suggest any effect of ANGII suppression on muscle hypertrophy or muscle performance, as HSD Sham animals produced similar peak force and fatigued to a similar level as HSD+ANGII Sham animals, but direct effects of ANGII on muscle force following chronic stimulation cannot be strictly ruled out in these experiments. Further experiments are also necessary to determine whether important age- or strain-specific effects exist.

In addition to inducing angiogenesis, chronic exercise and muscle stimulation have been shown to induce changes in skeletal muscle myofiber phenotype. Both chronic electrical stimulation of muscle in rat [5,16] and long-term voluntary running in mouse [18,34] have been shown to involve conversion of glycolytic fibers, usually type IIb or IId/x, to a phenotype more appropriate for oxidative metabolism (type I or IIa). The duration of training or stimulation necessary for fiber type transition is somewhat controversial and may depend on methods used to differentiate between fiber phenotypes. Conversion has been reported to occur most extensively following four weeks of chronic activity [5,18,34]. Hudlicka et al. [16] reported a mild muscle-specific conversion of type IIb glycolytic to IIa intermediate fibers following four days of stimulation using enzyme activities as indicators of phenotype. The findings of the current study suggest that seven days of stimulation does not result in significant fiber type conversion as measured by immunohistochemical changes in myosin heavy chain isoforms in rat TA muscle. Importantly, these results also do not suggest any effect of high salt diet or ANGII suppression on muscle fiber type.

The current study also supports previous studies regarding the existence of region- and fiber type-specific angiogenesis. Angiogenesis was observed in both the oxidative core and glycolytic cortex in chronically stimulated tibialis anterior muscles of animals fed a control diet, but the degree of angiogenesis was greater in the cortex, the site of high numbers of glycolytic fibers, as reported previously [3]. The results of the current study also suggest that the increase in capillary density observed with stimulation was specific to glycolytic type IIb fibers, as the capillary: fiber ratio was not significantly increased in type I and type IIa fibers following stimulation. Waters et al. [34] reported a similar enhancement of angiogenesis in IIb fibers relative to oxidative fibers in the initial training response in mice. These results suggest that the initial angiogenic response observed in stimulated muscle is targeted to fibers that rely heavily on glycolysis for metabolism, perhaps in an attempt to enhance the oxidative capacity of the fibers by increased delivery of oxygen. These results are intriguing as a possible explanation for the improvement in muscle performance observed in the chronically stimulated CD animals compared to HSD counterparts.

The results of the current study suggest that a high salt diet is associated with an inhibition of stimulated angiogenesis in skeletal muscle and a reduction in the ability of the muscle to resist fatigue. This increased fatigue response of muscle is reversed by restoration of angiogenesis by ANGII infusion. Impairment of muscle function in high salt-fed animals occurs despite an increase in bulk blood flow to the stimulated limb compared to chronically stimulated animals receiving a control diet. This study supports the hypothesis that angiogenesis may be a critical adaptation made in response to electrical stimulation and suggests the existence of

compensatory mechanisms aimed at partially maintaining muscle function during high salt intake.

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References

1. Amaral SL, Linderman JR, Morse MM, Greene AS. Angiogenesis induced by electrical stimulation is mediated by angiotensin II and VEGF. *Microcirculation* 2001;8:57–67. [PubMed: 11296854]
2. Amaral SL, Papanek PE, Greene AS. Angiotensin II and VEGF are involved in angiogenesis induced by short-term exercise training. *Am J Physiol Heart Circ Physiol* 2001;281:H1163–1169. [PubMed: 11514283]
3. Badr I, Brown MD, Egginton S, Hudlicka O, Milkiewicz M, Verhaeg J. Differences in local environment determine the site of physiological angiogenesis in rat skeletal muscle. *Exp Physiol* 2003;88:565–568. [PubMed: 12955155]
4. Constable SH, Favier RJ, McLane JA, Fell RD, Chen M, Holloszy JO. Energy metabolism in contracting rat skeletal muscle: adaptation to exercise training. *Am J Physiol* 1987;253:C316–322. [PubMed: 3618765]
5. Delp MD, Pette D. Morphological changes during fiber type transitions in low-frequency-stimulated rat fast-twitch muscle. *Cell Tissue Res* 1994;277:363–371. [PubMed: 7521794]
6. Deveci D, Egginton S. Differing mechanisms of cold-induced changes in capillary supply in m. tibialis anterior of rats and hamsters. *J Exp Biol* 2002;205:829–840. [PubMed: 11914391]
7. Egginton S, Hudlicka O. Early changes in performance, blood flow and capillary fine structure in rat fast muscles induced by electrical stimulation. *J Physiol* 1999;515:265–275. [PubMed: 9925896]
8. Emanuelli C, Salis MB, Stacca T, Pinna A, Gaspa L, Madeddu P. Angiotensin AT(1) receptor signalling modulates reparative angiogenesis induced by limb ischaemia. *Br J Pharmacol* 2002;135:87–92. [PubMed: 11786483]
9. Frisbee JC, Lombard JH. Chronic elevations in salt intake and reduced renal mass hypertension compromise mechanisms of arteriolar dilation. *Microvasc Res* 1998;56:218–227. [PubMed: 9828160]
10. Gordon SE, Davis BS, Carlson CJ, Booth FW. ANG II is required for optimal overload-induced skeletal muscle hypertrophy. *Am J Physiol Endocrinol Metab* 2001;280:E150–159. [PubMed: 11120669]
11. Green HJ, Pette D. Early metabolic adaptations of rabbit fast-twitch muscle to chronic low-frequency stimulation. *Eur J Appl Physiol Occup Physiol* 1997;75:418–424. [PubMed: 9189729]
12. Hansen-Smith FM, Morris LW, Greene AS, Lombard JH. Rapid microvessel rarefaction with elevated salt intake and reduced renal mass hypertension in rats. *Circ Res* 1996;79:324–330. [PubMed: 8756011]
13. Henderson KK, Wagner H, Favret F, Britton SL, Koch LG, Wagner PD, Gonzalez NC. Determinants of maximal O₂ uptake in rats selectively bred for endurance running capacity. *J Appl Physiol* 2002;93:1265–1274. [PubMed: 12235024]
14. Hepple RT, Hogan MC, Sary C, Bebout DE, Mathieu-Costello O, Wagner PD. Structural basis of muscle O₂ diffusing capacity: evidence from muscle function in situ. *J Appl Physiol* 2000;88:560–566. [PubMed: 10658023]
15. Hernandez I, Cowley AW Jr, Lombard JH, Greene AS. Salt intake and angiotensin II alter microvessel density in the cremaster muscle of normal rats. *Am J Physiol* 1992;263:H664–667. [PubMed: 1415589]
16. Hudlicka O, Dodd L, Renkin EM, Gray SD. Early changes in fiber profile and capillary density in long-term stimulated muscles. *Am J Physiol* 1982;243:H528–535. [PubMed: 6214958]

17. Korshunov VA, Berk BC. Flow-induced vascular remodeling in the mouse: a model for carotid intima-media thickening. *Arterioscler Thromb Vasc Biol* 2003;23:2185–2191. [PubMed: 14576075]
18. Li P, Akimoto T, Zhang M, Williams RS, Yan Z. Resident stem cells are not required for exercise-induced fiber type-switching and angiogenesis, but required for activity-dependent muscle growth. *Am J Physiol Cell Physiol* 2006;290:C1461–1468. [PubMed: 16407413]
19. Linderman JR, Kloehn MR, Greene AS. Development of an implantable muscle stimulator: measurement of stimulated angiogenesis and poststimulus vessel regression. *Microcirculation* 2000;7:119–128. [PubMed: 10802854]
20. Lloyd PG, Prior BM, Li H, Yang HT, Terjung RL. VEGF receptor antagonism blocks arteriogenesis, but only partially inhibits angiogenesis, in skeletal muscle of exercise-trained rats. *Am J Physiol Heart Circ Physiol* 2005;288:H759–768. [PubMed: 15471974]
21. Lombard JH, Frisbee JC, Greene AS, Hudetz AG, Roman RJ, Tonellato PJ. Microvascular flow and tissue PO₂ in skeletal muscle of chronic reduced renal mass hypertensive rats. *Am J Physiol Heart Circ Physiol* 2000;279:H2295–2302. [PubMed: 11045965]
22. Marvar PJ, Nurkiewicz TR, Boegehold MA. Reduced arteriolar responses to skeletal muscle contraction after ingestion of a high salt diet. *J Vasc Res* 2005;42:226–236. [PubMed: 15855795]
23. Milkiewicz M, Hudlicka O, Brown MD, Silgram H. Nitric oxide, VEGF, and VEGFR-2: interactions in activity-induced angiogenesis in rat skeletal muscle. *Am J Physiol Heart Circ Physiol* 2005;289:H336–343. [PubMed: 15734877]
24. Minami N, Mori N, Nagasaka M, Harada T, Kurosawa H, Kanazawa M, Kohzuki M. Effect of high-salt diet or chronic captopril treatment on exercise capacity in normotensive rats. *Clin Exp Pharmacol Physiol* 2004;31:197–201. [PubMed: 15053813]
25. Myrhaug R, Hudlicka O. Capillary growth in chronically stimulated adult skeletal muscle as studied by intravital microscopy and histological methods in rabbits and rats. *Microvasc Res* 1978;16:73–90. [PubMed: 692460]
26. O'Drobinak DM, Greene AS. Decreases in steady-state muscle performance and vessel density in reduced renal mass hypertensive rats. *Am J Physiol* 1996;270:H661–667. [PubMed: 8779843]
27. Petersen MC, Munzenmaier DH, Greene AS. Angiotensin II Infusion Restores Stimulated Angiogenesis in the Skeletal Muscle of Rats on a High Salt Diet. *Am J Physiol Heart Circ Physiol* 2006;291:H114–120. [PubMed: 16461372]
28. Reichmann H, Hoppeler H, Mathieu-Costello O, von Bergen F, Pette D. Biochemical and ultrastructural changes of skeletal muscle mitochondria after chronic electrical stimulation in rabbits. *Pflugers Arch* 1985;404:1–9. [PubMed: 4011395]
29. Richardson RS, Grassi B, Gavin TP, Haseler LJ, Tagore K, Roca J, Wagner PD. Evidence of O₂ supply-dependent VO₂ max in the exercise-trained human quadriceps. *J Appl Physiol* 1999;86:1048–1053. [PubMed: 10066722]
30. Rieder MJ, O'Drobinak DM, Greene AS. A computerized method for determination of microvascular density. *Microvasc Res* 1995;49:180–189. [PubMed: 7603355]
31. Simoneau JA, Kaufmann M, Pette D. Asynchronous increases in oxidative capacity and resistance to fatigue of electrostimulated muscles of rat and rabbit. *J Physiol* 1993;460:573–580. [PubMed: 8487208]
32. Tamarat R, Silvestre JS, Kubis N, Benessiano J, Duriez M, deGasparo M, Henrion D, Levy BI. Endothelial nitric oxide synthase lies downstream from angiotensin II-induced angiogenesis in ischemic hindlimb. *Hypertension* 2002;39:830–835. [PubMed: 11897773]
33. Tulis DA, Unthank JL, Prewitt RL. Flow-induced arterial remodeling in rat mesenteric vasculature. *Am J Physiol* 1998;274:H874–882. [PubMed: 9530199]
34. Waters RE, Rotevatn S, Li P, Annex BH, Yan Z. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. *Am J Physiol Cell Physiol* 2004;287:C1342–1348. [PubMed: 15253894]
35. Yang HT, Ogilvie RW, Terjung RL. Peripheral adaptations in trained aged rats with femoral artery stenosis. *Circ Res* 1994;74:235–243. [PubMed: 8293562]

Figure 1.

Force generated by one hour of acute stimulation of the TA muscle. ◇ Control diet (CD) Sham (n = 7) □ CD Stim (n = 10) ▲ High salt diet (HSD) Sham (n = 7) • HSD Stim (n = 9). (*) Significant difference vs. CD Stim, $p < 0.05$. (#) Significant difference at all points of stimulation except peak vs. corresponding sham group, $p < 0.05$.

Figure 2.

Capillary: fiber ratio in all fiber types of the TA muscle in CD and HSD Sham and Stim groups ($n = 5/\text{group}$) following the seven day stimulation period. (*) Significant difference vs. corresponding sham group, $p < 0.05$. (#) Significant difference vs. HSD Stim group, $p < 0.05$.

Figure 3.

Force generated by one hour of acute stimulation of the TA muscle expressed as a percent of peak force. □ CD Stim (n = 10) • HSD Stim (n = 9) ▲ HSD+ANGII Stim (n = 6) × Combined Sham groups (n = 21).

Figure 4. Capillary: fiber ratio in all fiber types of the TA muscle in HSD+ANGII Sham (n = 4) and Stim (n = 5) groups following the seven day stimulation period. (*) Significant difference vs. HSD +ANGII sham group, $p < 0.05$.

Figure 5.

Representative micrographs of muscle cross sections stained with MHC-specific antibodies (green) and *GS-I* lectin (red) to identify capillaries. Images a and b display MHC type I specific staining in the core and cortex, respectively. Images c and d display MHC type IIa staining, and images e and f demonstrate MHC type IIb staining.

Figure 6. Iliac arterial blood flow in the stimulated hind limb during one hour of acute stimulation. ◇ Control diet (CD) Sham (n = 9) □ CD Stim (n = 5) ▲ High salt diet (HSD) Sham (n = 11) • HSD Stim (n = 7). (*) Significant difference vs. CD Stim, $p < 0.05$.

Figure 7.

Iliac arterial blood flow in the stimulated hind limb during one hour of acute stimulation, expressed as a percent of baseline flow. ◇ Control diet (CD) Sham (n = 9) □ CD Stim (n = 5) ▲ High salt diet (HSD) Sham (n = 11) ● HSD Stim (n = 7).

Table 1

Body and muscle weights

	Body Weight (BW, grams)		TA Weight (mg)
	Start weight	End weight	
Control Diet Sham (n = 15)	218 ± 6	262 ± 6 [*]	409 ± 10
Control Diet Stim (n = 14)	212 ± 4	260 ± 5 [*]	419 ± 20
High Salt Diet Sham (n = 17)	215 ± 4	261 ± 5 [*]	433 ± 10
High Salt Diet Stim (n = 15)	208 ± 5	263 ± 5 [*]	410 ± 17
High Salt + ANGII Sham (n = 7)	208 ± 2	258 ± 7 [*]	420 ± 13
High Salt + ANGII Stim (n = 6)	207 ± 3	258 ± 8 [*]	416 ± 10

Values are presented as mean ± SEM.

* Significant difference vs. corresponding starting body weight.

Table 2
Muscle fiber type distribution in the oxidative core and glycolytic cortex of the TA muscle following the seven day stimulation period

	CD Sham	CD Stim	HSD Sham	HSD Stim
Type I (n = 8)				
Core	13.4 ± 2.6	17.3 ± 2.1	12.8 ± 1.2	14.3 ± 2.4
Cortex	—	—	—	—
Type IIa (n = 7)				
Core	43.3 ± 0.9	39.5 ± 2.6	42.5 ± 1.8	41.0 ± 3.1
Cortex	3.4 ± 2.2	4.0 ± 1.6	1.7 ± 0.9	1.3 ± 0.8
Type IIb (n = 4)				
Core	22.8 ± 1.7	23.8 ± 2.7	32.0 ± 7.2	25.1 ± 6.4
Cortex	75.0 ± 9.4	83.0 ± 4.8	86.4 ± 5.9	84.8 ± 3.8
Type IIx (n = 4)				
Core	22.3 ± 4.1	21.3 ± 8.1	13.2 ± 4.3	23.8 ± 10
Cortex	20.6 ± 5.7	16.3 ± 4.4	13.2 ± 10	13.1 ± 3.0

All values are expressed as a percentage of total fibers in a given microscope field.

Table 3
Capillary contacts per fiber (CC:F) of specific fiber types in the oxidative core and glycolytic cortex of the TA muscle following the seven day stimulation period.

	CD Sham	CD Stim	HSD Sham	HSD Stim
Type I (n = 8)				
Core	4.26 ± 0.13	4.67 ± 0.27	4.21 ± 0.45	4.22 ± 0.17
Cortex	—	—	—	—
Type IIa (n = 7)				
Core	3.36 ± 0.23	3.78 ± 0.12	3.36 ± 0.26	2.99 ± 0.14
Cortex [#]	4.50	4.67	4.00	3.75
Type IIb (n = 4)				
Core	3.40 ± 0.26	4.82 ± 0.55 [*]	3.19 ± 0.36	3.85 ± 0.31
Cortex	1.93 ± 0.15	3.82 ± 0.29 [*]	1.92 ± 0.46	2.44 ± 0.13

* Significant difference vs. corresponding sham group, $p < 0.05$.

[#] CC:F ratios from type IIa fibers in the cortex are included for completeness, although each value represents the mean of only two measurements due to the infrequent appearance of these fibers.