Effect of a high-salt diet on oxidant enzyme activity in skeletal muscle microcirculation

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Lenda, Deborah M., and Matthew A. Boegehold. Effect of a high-salt diet on oxidant enzyme activity in skeletal muscle microcirculation. Am J Physiol Heart Circ Physiol 282: H395–H402, 2002.—Increased salt intake attenuates the endothelium-dependent dilation of skeletal muscle arterioles by abolishing local nitric oxide (NO) activity. There is evidence of oxidative stress in arteriolar and venular walls of rats fed a high-salt diet, and depressed arteriolar responses to acetylcholine (ACh) in these rats are reversed by scavengers of reactive oxygen species (ROS). In this study, we tested the hypothesis that this salt-dependent increase in microvascular ROS and the resulting attenuation of endothelium-dependent dilation are due to increased expression and/or activity of oxidant enzymes in the microvascular wall. Resting arteriolar and venular wall oxidant activity, as assessed by tetranitroblue tetrazolium reduction, was consistently higher in the spinotrapezius muscle of rats fed a high-salt diet (7% NaCl, HS) for 4–5 wk than in those fed a normal diet (0.45% NaCl, NS) for the same duration. Western analysis of protein from isolated microvessels showed no difference between HS and NS rats in the expression of NAD(P)H oxidase or xanthine oxidase. Inhibition of NAD(P)H oxidase and/or xanthine oxidase with diphenyleneiodonium chloride and oxyxipurinol, respectively, reduced resting arteriolar wall oxidant activity to normal levels in HS rats but had no effect in NS rats, suggesting that the basal activities of NAD(P)H oxidase and xanthine oxidase are increased in HS microvessels. However, inhibition of these enzymes in HS rats did not restore normal arteriolar responses to ACh, suggesting that this stimulus activates an alternate source of ROS that eliminates the role of NO in the subsequent dilation.

dietary salt; reactive oxygen species; nitric oxide; endothelium-dependent dilation

CONSUMPTION OF A HIGH-SALT DIET can lead to structural and functional changes in the microcirculation that are independent of any change in arterial pressure (4, 5, 17, 18, 24, 30–32). For example, this laboratory has previously reported that in normotensive rats fed a high-salt diet for 2–5 wk, the endothelium-dependent dilation of spinotrapezius muscle arterioles in response to acetylcholine (ACh) or increased shear stress is impaired due a selective loss of nitric oxide (NO) activity (4, 5). Chronic or acute ingestion of high dietary salt also leads to attenuated endothelium-dependent responses in arterioles of the rat cremaster muscle (17, 18) and cerebral cortex (32) and small feed arteries of the rat gracilis muscle (31).

Recently, we have found evidence of oxidative stress in spinotrapezius muscle arterioles and venules of normotensive rats fed a high-salt diet (30). Exposure of the muscle to different scavengers of reactive oxygen species (ROS) for as little as 30 min reduces this oxidative stress and restores normal arteriolar responsiveness to ACh, suggesting that the increased oxidant activity in these salt-fed animals is decreasing the bioavailability of arteriolar NO. Superoxide anion (O2•-) and NO interact at a nearly diffusion-limited rate, resulting in the elimination of generated NO (27). The product of this interaction is peroxynitrite, itself a potent oxidant that may also prevent further NO production (34).

Under normal conditions, NAD(P)H oxidase and xanthine oxidase are considered to be the major enzymatic sources of ROS in the peripheral vasculature (20, 38, 44). An increase in the expression and/or activity of these enzymes has been reported in various conditions that also exhibit increased ROS levels and vascular dysfunction, such as hypertension (10, 49, 51, 56), atherosclerosis (33), diabetes (10), and aging (23). In this study, we tested the hypothesis that increases in the expression or activity of vascular NAD(P)H oxidase and/or xanthine oxidase contribute to the increased levels of ROS and reduced endothelium-dependent dilation in the microcirculation of rats fed a high-salt diet.

METHODS

Surgical Preparation and Intravital Microscopy

All surgical and experimental procedures for this study were approved by the West Virginia University Animal Care and Use Committee. Weanling male Sprague-Dawley rats (Harlan Sprague Dawley; Indianapolis, IN) were placed on a whole grain diet containing either 0.45% NaCl (normal salt, NS) or 7% NaCl (high salt, HS) by weight (NS, TD88311; HS, TD88313). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and the supernatant was stored at −80°C until use. Three
rata were homogenized and centrifuged at 3,100
35°C and equilibrated with 95% N2:5% CO2. Supersufusate
flow rate was set at 4–6 ml/min to minimize equilibration with atmospheric oxygen (7).

The rat was then transferred to the stage of an Olympus BX50WI intravital microscope fitted with a CCD video cam-
era (Dage-MTI; Michigan City, IN). Video images were dis-
played on a Panasonic high-resolution video monitor and
stored on videotape for offline analysis. Arteriolar inner di-
ameters were measured during videotape replay with a video
caliper (Microcirculation Research Institute, Texas A&M
University).

The blood supply to the spino trapsius muscle is carried by
arteriolar branches of three small feed arteries that intercon-
nect within the muscle to form a structure known as the
“arcade bridge” (43). In protocols 2 and 3, we studied the
arterioles that branch directly from the arcade bridge to
form an extensive anastomosing network.

Experimental Protocols

Protocol 1: Expression of oxidant enzymes. Western blot
analysis was used to assess the expression of NAD(P)H
oxidase (by determining levels of p67phox, one of the enzyme’s
cytosolic subunits) and xanthine oxidase in isolated arte-
rioles and venules from each dietary group. After induction of
thiopental sodium anesthesia, the right and left spino trapsius
muscles were removed, rinsed in 4°C electrolyte solu-
tion, and pinned out in a Silastic-coated petri dish filled with
100% methanol at 4°C. With the use of a dissecting micro-
scope, arcade arterioles and their paired venules were identi-
fied and excised from both muscles. To obtain enough pro-
tein for Western blot analysis, the microvessels from
each sample. The amount of protein needed for reliable
analysis was found to be 3 μg, as determined in an initial
series of trials to find the amount that would consistently
give bands that were sufficiently well defined for densitom-
etry. The isolated vessels were placed in 4°C storage buffer
(pH 7.25, 30% glycerol, containing 100 mM KPO4, 1 mM
dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride)
and centrifuged at 1,370 g for 5 min. Microvessels were then
homogenized and centrifuged at 3,100 g for 15 min at 4°C,
and the supernatant was stored at −70°C until use. Three
microvessels of vessel protein (measured with a Bio-Rad pro-
tein assay kit) from each dietary group were separated on a
NuPage 4–12% Bis-Tris polyacrylamide gel (Novex; San Di-
ego, CA) and transferred onto a nitrocellulose membrane.
The membrane was probed with either goat anti-p67phox
(1:100; Santa Cruz Biotechnology; Santa Cruz, CA) or rabbit
anti-xanthine oxidase (1:1000; Biosdesign; Saco, ME) and
then incubated with horseradish peroxidase-conjugated anti-
 goat IgG antibody fragment [F(ab’2)] (1:12,000; Santa Cruz
Biotechnology) or goat anti-rabbit F(ab’2) (1:15,000 Bio-
design) for 1 h. After treatment with SuperSignal (Pierce;
Rockford, IL), membranes were exposed to autoradiography
film. X-ray images were digitally captured, and optical den-
sity measurements were made from the images using Meta-
Morph 3.5 image analysis software.

Protocol 2: Effect of enzyme inhibition on microvascular
oxidant activity. To assess the effect of a high-salt diet on the
in vivo activities of NAD(P)H oxidase and xanthine oxidase,
we investigated the degree to which microvascular wall ox-
idant activity was decreased in the presence of the NAD(P)H oxidase inhibitor diphenyleneiodonium chloride (DPI; Sigma;
St. Louis, MO) and/or the xanthine oxidase inhibitor oxypuri-
lin (Oxy; Sigma). Oxidant activity was identified by the
reduction of tetranitroblue tetrazolium (TNBT), which forms
dark insoluble formazan deposits when reduced by ROS (50,
51). The exteriorized muscle was continuously superfused
with either a normal superfusate containing 2% TNBT for 60
min or a superfusate containing DPI (2 × 10−5 M), Oxy (3 ×
10−4 M), or DPI + Oxy for 90 min, with 2% TNBT added for
the final 60 min. The muscle was then fixed in situ and
excised. To determine whether these inhibitors were reach-
ing the microvascular endothelium in sufficient concentra-
tions to exert a maximal effect, another group of experiments
was performed in which DPI (0.1 mg/kg), Oxy (40 mg/kg), or
DPI + Oxy were infused as a bolus intraarterially. After
inhibitor infusion and a 15-min equilibration period, the
exteriorized muscle was exposed to TNBT for 60 min, as
described above.

Image analysis was used to determine microvessel wall
formazan levels after TNBT exposure from calculated light
absorption values (30, 50, 51). Briefly, a series of gray-value
measurements was made along the vessel wall and paired
with a corresponding series of measurements from the avas-
cular region immediately adjacent to the wall. Microvascular
light absorption (A) was calculated as: A = −ln(I/I0), where
I0 is the vessel wall gray value and I is the gray value for
the paired avascular region. Control studies have verified
that the amount of formazan formed from TNBT is directly
proportional to the level of oxidant activity and that there is
also a similar linear relationship between tissue TNBT levels
and light absorption (2, 29).

Protocol 3: Effect of oxidant enzyme inhibition on endothe-
lium-dependent responses. In these experiments, we first
evaluated the in vivo responsiveness of arcade arterioles to
tiosthetically applied ACh (Sigma) under the normal
superfusate. For ACh application, a micropipette (2–3 μm
outer tip diameter) was filled with 0.025 M ACh in distilled
water and placed in light contact with the arteriolar wall.
Ejection currents of 5, 20, and 40 nA (delivered in random
order) were used to apply ACh. For each current dose of ACh,
the vessel was continuously observed during a 2-min control
period, a 2-min application period, and a 2-min recovery
period. The series of ACh applications was then repeated
after 30 min of exposure to a superfusate containing DPI,
Oxy, or DPI + Oxy, or 15 min after arterial infusion of DPI +
Oxy as described above. Passive arteriolar diameters were
then measured after addition of adenosine (Sigma, 10−4 M)
to the superfusate.

Data and Statistical Analysis

For protocol 1, the effect of high salt intake on microvessel
protein expression was evaluated by comparing protein band

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optical densities between the two dietary groups. Each gel contained two lanes of protein from HS rats and two lanes of protein from NS rats. The optical densities for the two bands representing each diet were averaged, and the ratio of HS to NS density was calculated for each gel to control for any gel-to-gel variation. These individual ratios for a given enzyme were then combined and averaged.

For protocol 3, arteriolar diameter and arteriolar pressure measurements were digitized, stored, and analyzed using Polyview Lab Manager (Grass Instrument Division/Astro-Med; West Warwick, RI). Data were collected at 100 samples/s during control, ACh application, and recovery periods. Resting vascular tone (T) was calculated as $T = (D_{\text{pass}} - D_{\text{rest}})/D_{\text{pass}} \times 100$, where $D_{\text{pass}}$ is the passive vessel diameter in the presence of $10^{-4} \text{ M}$ adenosine and $D_{\text{rest}}$ is the average resting diameter measured during the 2-min control period. A tone value of 0% represents a vessel that is completely passive, whereas a value of 100% represents a vessel that is constricted to the point of closure. Arteriolar responses to vasoactive agents were determined by comparing the steady-state diameter reached during the application period to the immediately preceding control diameter.

All data are expressed as means ± SE, and statistical analysis was carried out using commercially available software (SigmaStat, SPSS; Chicago, IL). Comparisons between groups were made using two-way analysis of variance (ANOVA) in combination with Student-Newman-Keuls post hoc analysis to isolate pairwise differences. One-way ANOVA was used to determine differences within a group subjected to repeated measures. $P$ values ≤0.05 were considered significant for all tests.

**RESULTS**

General characteristics of the rats used to assess enzyme activities and endothelium-dependent responses in vivo (protocols 2 and 3) and the characteristics of the arterioles studied in vivo (protocol 3) are shown in Table 1. The HS rats were slightly older than the NS rats, but there was no significant difference in either body weight or mean arterial pressure between the two groups. The high-salt diet did not affect resting or passive arteriolar diameters, and therefore the calculated resting tone of the vessels was unchanged.

![Western Blot Images](http://ajpheart.physiology.org/)

Figure 2 shows some representative images of arterioles and venules exposed to 2% TNBT in NS rats (left) and HS rats (right). The microvessels in HS rats typically had a darker appearance due to deposition of the TNBT reduction product formazan in the microvascular walls.

![Figure 2](http://ajpheart.physiology.org/)

Figure 3 shows the average level of oxidant activity under control conditions in microvessels of TNBT-exposed NS and HS rats, as assessed by the effect of formazan on vascular wall light absorption. The mean absorption value for arterioles was significantly greater in HS rats ($0.100 \pm 0.004$) than in NS rats ($0.066 \pm 0.004$). A similar difference was found in the venules, with the value in HS rats ($0.129 \pm 0.004$) significantly greater than that in NS rats ($0.090 \pm 0.004$). These findings, which are consistent with a previous report by this laboratory (30), indicate that arteriolar and venular ROS activity is greater in HS rats than in NS rats. This interpretation is supported by our recent finding that ROS scavengers abolish these differences in microvascular wall light absorption between HS and NS rats (30).

As shown in Fig. 4, exposure to Oxy, DPI, or Oxy + DPI via either the superfusate (left) or intraarterial infusion (right) had no effect on arterioles in NS rats.

[In contrast, these inhibitors significantly reduced arteriolar oxidant activity in HS rats (respective de-]
creases in absorption to 0.0835 ± 0.005, 0.079 ± 0.006, and 0.077 ± 0.005 with superfusion of Oxy, DPI, and Oxy + DPI, and to 0.071 ± 0.004, 0.072 ± 0.004 and 0.062 ± 0.003 with infusion of Oxy, DPI, and Oxy + DPI). In the presence of Oxy and/or DPI, arteriolar values in HS rats were not significantly different from those in NS rats.

The effects of these inhibitors on venules in each group are shown in Fig. 5. In the NS rats, superfusion of Oxy, DPI, or Oxy + DPI had no effect on the venules (left), but infusion of these inhibitors significantly decreased absorption values from 0.0898 ± 0.004 to 0.063 ± 0.003, 0.069 ± 0.003 and 0.071 ± 0.004, respectively (right). In the HS rats, venular oxidant activity was significantly decreased after either superfusion or infusion of the inhibitors (from 0.129 ± 0.004 to 0.115 ± 0.006, 0.103 ± 0.006, and 0.083 ± 0.005 with superfusion and to 0.107 ± 0.004, 0.097 ± 0.005, and 0.085 ± 0.004 with infusion).

Figure 6 shows the mean arteriolar responses to ACh under the normal superfusate for all vessels studied in protocol 3. Arterioles in NS rats dilated in a dose-dependent manner to ACh applied at currents of 5, 20, and 40 nA (dilations of 2.7 ± 0.2, 4.8 ± 0.3, and 7.1 ± 0.4 μm, respectively). At all three doses, arteriolar dilation in HS rats was significantly less than that in NS rats (0.8 ± 0.1, 1.7 ± 0.2, and 2.6 ± 0.2 μm, respectively). There was no difference between groups in maximal arteriolar dilation in response to adenosine (NS: 26.1 ± 1.4 μm; HS: 23.4 ± 1.1 μm).
The effect of inhibiting NAD(P)H oxidase and/or xanthine oxidase on arteriolar responsiveness to ACh in NS and HS rats is shown in Fig. 7. Inhibiting NAD(P)H oxidase with DPI had no effect on the arteriolar dilation to any level of ACh in either group (Fig. 7A). Inhibition of xanthine oxidase with Oxy also did not affect the responsiveness to ACh in either group (Fig. 7B). Finally, exposure to DPI + Oxy either via the superfusate (Fig. 7C) or infusion (Fig. 8) also did not affect arteriolar responses to ACh in either group.

DISCUSSION

Consumption of a diet high in salt leads to functional changes in the microcirculation that may compromise the ability of these vessels to respond to various physiological stimuli (5, 17, 18, 31, 32). Therefore, in addition to its potential importance for blood pressure control in hypertensive patients, lowering salt intake may have benefits for normotensive individuals as well. In the spinotrapezius muscle of normotensive rats fed a high-salt diet, this laboratory has consistently found reduced arteriolar responsiveness to endothelium-dependent stimuli (shear stress and exogenous ACh) due to an apparent loss of NO activity in these vessels (4, 5, 30). A salt-induced decrease in endothelium-dependent responses has also been observed in rat cremaster muscle and cerebral arterioles (17, 18, 32) and in rat gracilis muscle feed arteries (31). Consistent with our previous findings (29), the current findings indicate that under resting conditions, there is increased arteriolar and venular oxidant activity in the spinotrapezius muscle of normotensive rats fed a high-salt diet (Figs. 2 and 3). We have previously reported that exposure of the muscle to ROS scavengers abolishes this oxidative stress and restores normal arteriolar responsiveness to ACh, suggesting that the presence of vascular ROS is either directly or indirectly respon-
ROS may influence vascular tone by reducing the bioavailability of NO. The half-life of NO is decreased when O$_2$ is present and increased in the presence of superoxide dismutase (SOD) (21, 42). In canine coronary arteries, O$_2$ generated by xanthine oxidase inhibits vascular responsiveness to ACh, and this effect is readily reversed by SOD (41). Studies in which NO has been directly measured with porphyrinic microsensors have also demonstrated that increased levels of O$_2$ are responsible for decreased vascular NO levels in hypertension, endotoxemia, and ischemia-reperfusion (9). Xanthine oxidase and NAD(P)H oxidase are important sources of O$_2$, and ultimately other ROS, in the vessel wall (20, 38, 44), and an increase in the expression and/or activity of these enzymes can lead to excessive ROS formation and vascular dysfunction. For example, in cholesterol-fed rabbits, increased aortic O$_2$ production and the accompanying decrease in endothelium-dependent dilation are both ameliorated by xanthine oxidase inhibition (36, 53). Similar findings have been reported in the mesenteric microcirculation of spontaneously hypertensive rats (SHR) (35, 49, 51), in the rat mesentery after ischemia-reperfusion (16), and in the forearm of patients with type 2 diabetes and mild hypertension (10) and hypercholesterolemia (11). Decreased oxygen availability also increases xanthine dehydrogenase/xanthine oxidase gene expression in endothelial and vascular smooth muscle cells (25). Increased NAD(P)H oxidase activity has been reported in the aorta of rats with renovascular hypertension (26) and spontaneous hypertension (56) and in the saphenous veins of patients with increased risk of developing atherosclerosis (22).

In this study, a high-salt diet did not alter the expression of NAD(P)H oxidase or xanthine oxidase in spinotrapezius muscle microvessels (Fig. 1). However, our findings do suggest that the tonic in vivo activity of these enzymes is increased in the HS rats. Exposing the unstimulated vascular bed of HS rats to the NAD(P)H oxidase inhibitor DPI and/or the xanthine oxidase inhibitor Oxy reduced arteriolar and venular light absorption to levels not different from those in NS rats (Figs. 4 and 5), suggesting that under resting conditions, the increased ROS present in these vessels is at least partially due to increased activity of oxidant enzymes. Although DPI reduced arteriolar wall light absorption by the same amount as Oxy, we were surprised to find no additive effect of these two inhibitors when they were applied together (Fig. 4). There is evidence to suggest that under some conditions, DPI can inhibit xanthine oxidase as well as NAD(P)H oxidase (13), and such a lack of specificity could explain our findings in the arterioles. However, we did observe the expected additive effect of DPI and Oxy in the venules (Fig. 5), raising the possibility that DPI may have a more specific effect on NAD(P)H oxidase in the venules than in the arterioles.

The finding of increased oxidant activity in the venules as well as the arterioles of HS rats is in itself an important aspect of this study. Increased arteriolar and venular oxidant activity has also been documented in the mesentery of SHR (47) and hypertensive Dahl rats (50, 51). In those studies, increased levels of ROS in the postcapillary vessels indicated that the development of this oxidant stress is unrelated to increased vascular pressure. Instead, increased microvascular oxidant activity in these models of hypertension may be related to elevated circulating glucocorticoid levels (47) and/or the interaction of activated neutrophils with the endothelium (51). In the normotensive salt-fed rats studied here, the mechanism leading to increased ROS in both the arterioles and venules is open to speculation (see below).

In NS rats, inhibiting NAD(P)H oxidase and/or xanthine oxidase had no effect on resting arteriolar light absorption (Fig. 4). Therefore, in the normal state, the scavenging activities of antioxidant enzymes such as SOD and catalase are apparently sufficient to prevent the accumulation of ROS generated by more modest NAD(P)H oxidase or xanthine oxidase activity (54). Consequently, inhibition of NAD(P)H oxidase and xanthine oxidase would not be expected to have any effect on arteriolar wall redox state, and light absorption would not change. In contrast, we found that oxidant enzyme inhibitors did reduce resting venular light absorption in NS rats, but only when delivered by intraarterial infusion (Fig. 5). This method of administration presumably produced higher inhibitor levels in the vicinity of the venular endothelium than could be attained by superfusate delivery. This finding suggests that there is a prevailing and detectable level of oxidant activity in the venular walls of NS rats and is consistent with our earlier findings (30). The normal presence of ROS in the venular walls may reflect a physiological role for these molecules in various cell signaling pathways, as has been documented in other vascular beds (48, 54).

Although our findings suggest that inhibition of NAD(P)H oxidase and/or xanthine oxidase abolishes...
the oxidant stress in unstimulated arterioles of HS rats, normal endothelium-dependent responses to exogenous ACh were not restored by this inhibition (Figs. 7 and 8). Because exposure to ROS scavengers does restore the normal endothelium-dependent dilation of these vessels in HS rats (30), we speculate that there may be another source of microvascular ROS in these animals that is only triggered when the endothelium is stimulated. A plausible candidate for this source is endothelial NO synthase (eNOS), which can generate O$_2^–$ if there is a shortage of the enzymatic cofactor tetrahydrobiopterin (BH$_4^-$) (52, 55), and BH$_4^-$ is rapidly oxidized by peroxynitrite, the product of O$_2^–$ and NO (34). Supplemental BH$_4^-$ reverses the vascular dysfunction associated with hypercholesterolemia (45) and diabetes (39) and significantly reduces eNOS-derived O$_2^–$ in vitro (46, 52). Although purely speculative at this point, it is possible that a high-salt diet may lead to decreased BH$_4^-$ levels, either through peroxynitrite oxidation or some other mechanism, thereby converting eNOS into a O$_2^–$-generating enzyme when its activity is increased above resting levels.

The current findings suggest that increased dietary salt affects the redox state of the resting microvascular wall by increasing the activity of NAD(P)H oxidase and xanthine oxidase. The mechanistic link between high salt intake and these changes in enzyme activity has yet to be identified. Angiotensin II is known to stimulate NAD(P)H oxidase (19), but circulating angiotensin II levels are decreased in rats fed a high-salt diet (24). Other changes in the vascular microenvironment, such as increased sodium accumulation within the vessel wall (3), could alter endothelial cell membrane potential or glycocalyx proteins and lead to enzymatic changes on the surface of or within the endothelium.

Although the amount of ROS generated by NAD(P)H oxidase and/or xanthine oxidase may be insufficient to limit at least some forms of endothelium-dependent dilation in HS rats (Figs. 7 and 8), the oxidative stress that is attributable to these enzymes under resting conditions could influence other NO-dependent processes at the microvascular level. For example, the venules have been shown to release NO under resting conditions (6, 37), and this NO can have a profound influence on the tone of nearby arterioles (6, 8, 14, 15). Continuous NO release from the venular endothelium also contributes to the low venular resistance that helps to shield capillaries from high hydrostatic pressures (6, 8, 37), and the presence of postcapillary NO is also necessary for the regulation of venular wall permeability (1) and for the inhibition of leukocyte adhesion and aggregation in a low-shear environment (12). The increased venular oxidative stress in rats fed a high-salt diet (Fig. 3) may reduce the availability of venular NO and therefore affect one or more of these processes. It is therefore important to further investigate the mechanisms and functional consequences of elevated NAD(P)H oxidase and xanthine oxidase activity associated with high salt intake.

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