Oxidant stress-induced increase in myogenic activation of skeletal muscle resistance arteries in obese Zucker rats

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Frisbee, Jefferson C., Kristopher G. Maier, and David W. Stepp. Oxidant stress-induced increase in myogenic activation of skeletal muscle resistance arteries in obese Zucker rats. Am J Physiol Heart Circ Physiol 283: H2160–H2168, 2002. First published July 8, 2002; 10.1152/ajpheart.00379.2002.—This study characterized myogenic activation of skeletal muscle (gracilis) resistance arteries from lean (LZR) and obese Zucker rats (OZR). Arteries from OZR exhibited increased myogenic activation versus LZR; this increase was impaired by endothelium denudation or nitric oxide synthase inhibition. Treatment of vessels with 17-octadecynoic acid impaired responses in both strains by comparable amounts. Dihydroethidine microfluorography indicated elevated vascular superoxide levels in OZR versus LZR; immunohistochemistry demonstrated elevated vascular nitrotyrosine levels in OZR, indicating increased peroxynitrite presence. Vessel treatment with oxidative radical scavengers (polyethylene glycol-superoxide dismutase/cata

THE ABILITY OF SKELETAL MUSCLE resistance microvessels to alter their tone in an appropriate manner in response to changes in intraluminal pressure is of critical importance for regulating blood flow within perfused tissue (13, 22). Investigating signaling mechanisms underlying this response, henceforth referred to as myogenic activation, has long been an area of active investigation (7), and recent studies have indicated that the nature of myogenic activation in resistance vessels can be radically altered by the development of pathological conditions, including hypertension (10, 21, 32) and diabetes mellitus (16, 18, 19, 23, 31, 35).

To date, there has been no attempt to determine either the nature of myogenic activation of skeletal muscle resistance arteries from obese Zucker rats versus their control animal, the lean Zucker rat, or the mechanisms that might contribute to any alteration in this response. This represents an important avenue of investigation, because these vessels lie immediately proximal to the microcirculation and play a critical role in regulating the flow of blood through the downstream arteriolar networks. The purpose of the present study was to determine the characteristics of myogenic activation of isolated skeletal muscle arteries from lean and obese Zucker rats and to elucidate cellular mechanisms that contribute to alterations in the constriction of these vessels between the rat strains in response to increased intraluminal pressure.

MATERIALS AND METHODS

Animals. Male lean (LZR) and obese (OZR) Zucker rats (13 to 15 wk old) maintained on standard rat chow and tap water ad libitum were used for all experiments. Rats were housed in an animal care facility at the Medical College of Wisconsin,
which is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols received prior Institutional Animal Care and Use Committee approval. Rats were anesthetized with an injection of pento-barbital sodium (60 mg/kg ip), and a carotid artery was cannulated for determination of arterial pressure. In addition to being heavier than LZR (mass = 555 ± 9 g; mean arterial pressure (MAP) = 108 ± 5.8 mmHg; blood glucose concentration (\[\text{glucose}_{\text{blood}}\]) = 141 ± 13 mg/dl), OZR (mass = 594 ± 16 g; MAP = 141 ± 6.0 mmHg; \[\text{glucose}_{\text{blood}}\] = 551 ± 37 mg/dl) demonstrated significant hypertension and hyperglycemia.

**Preparation of isolated vessels.** The small muscular branch of the femoral artery supplying the gracilis muscle was removed from the anesthetized rat, with care taken to minimize vessel stretching and to handle arteries by their surrounding connective tissue only. Arteries were placed in a heated chamber (37°C) that allowed the lumen and exterior of the vessel to be perfused and superfused, respectively, with physiological salt solution (PSS) from separate reservoirs. The PSS used in these experiments was equilibrated with CO2 and 74% N2 gas mixture and had the following composition (in mM): 119 NaCl, 4.7 KCl, 1.17 MgSO4, 1.6 CaCl2, 1.18 Na2HPO4, 24 NaHCO3, 0.026 EDTA, and 5.5 glucose. Vessels were cannulated at both ends with glass micropipettes and were secured to the inflow and outflow pipettes with 10-0 nylon suture. Any side branches were ligated with a single strand teased from 6-0 silk suture. The inflow pipette was connected to a reservoir perfusion system that allowed the intraluminal pressure and luminal gas concentrations to be controlled. Vessel diameter was measured using television microscopy and an on-screen video micrometer.

Arteries were extended to their in situ length and were equilibrated at 80% of the animal’s MAP (86 ± 5 mmHg for LZR; 112 ± 4 mmHg for OZR) to approximate in vivo perfusion pressure (25). Any vessel that did not demonstrate active tone at rest was discarded. Active tone at the equilibrium pressure was calculated as \((\Delta D/D_{\text{max}})\) 100, where \(\Delta D\) is the diameter increase from rest in response to Ca2+-free PSS and \(D_{\text{max}}\) is the maximum diameter measured at the equilibrium pressure in Ca2+-free PSS. Active tone for vessels in the present study averaged 39.2 ± 3.4% in LZR and 32.1 ± 3.7% in OZR.

To determine myogenic activation, the vessel perfusate outflow line was clamped, stopping perfusate flow through the vessel, and the height of the perfusion reservoir was changed to vary intraluminal pressure in 20-mmHg intervals between 40 and 160 mmHg. Vessel diameter was determined after 10–15 min at each pressure, and pressure levels were randomized for each myogenic activation curve. After all procedures were completed, the perfusate and superfusate were replaced with Ca2+-free PSS, and the passive diameter of the fully relaxed vessel was determined over the identical intraluminal pressure range.

**Removal of the vascular endothelium.** The endothelium of isolated microvessels was removed via air bolus perfusion (10–12). Endothelium denudation procedures were deemed successful when all vascular reactivity in response to 10−7 M acetylcholine was eliminated, whereas responses to 10−7 M forskolin were unaltered.

**Inhibition of cytochrome P-450 4A enzymes.** To assess the contribution of cytochrome P-450 4A enzymes to myogenic activation of vessels, these enzymes were inhibited using 17-octadecenoic acid (17-ODYA; 10 μM; Ref. 10). 17-ODYA is a suicide substrate inhibitor of cytochrome P-450 4A enzymes and irreversibly inhibits production of 20-hydroxyeicosatetraenoic acid (20-HETE) via ω-hydroxylation of arachidonic acid and production of epoxyeicostrienoic acids by arachidonic acid epoxidation (33).

**Inhibition of nitric oxide production.** To determine the extent to which endothelial nitric oxide (NO) production contributes to myogenic activation of arteries, vessels were treated with N-nitro-l-arginine methyl ester (L-NAME; 100 μM; Ref. 12). Treatment of the isolated vessels with this concentration of L-NAME abolished the dilator response to acetylcholine (1 μM) in all vessels.

**Inhibition of KCa channels.** Previous studies have determined that 20-HETE inhibits the opening of large conductance Ca2+-activated potassium (KCa) channels, preventing membrane hyperpolarization in the face of elevated intracellular calcium (36). To assess the manner in which KCa channels contribute to the myogenic activation of vessels from LZR and OZR, the selective KCa channel blocker iberiotoxin (100 nM) was added to the vessel chamber (10, 11, 28).

**Scavenging of oxidative free radicals.** Previous studies (12) have demonstrated that with the development of obesity, hypertension, and diabetes, oxidant stress levels in tissues of Zucker obese rats exceed the extent to which increased oxidant stress levels might contribute to alterations in myogenic activation of skeletal muscle microvessels between LZR and OZR, vessels from both groups were treated with the free radical scavengers polyethylene glycol-superoxide dismutase (PEG-SOD; 200 U/ml; Sigma) and catalase (80 U/ml; Sigma) (12, 20, 24).

**Application of peroxynitrite.** Previous studies have suggested that peroxynitrite acts as a constrictor of vascular smooth muscle cells through inhibition of KCa channels (4). To assess the extent to which peroxynitrite influences the vascular tone of isolated gracilis arteries from LZR and OZR, vessels were treated with peroxynitrite (100 nM; Calbiochem) under control conditions and after treatment of vessels with iberiotoxin.

**Measurement of superoxide production.** Vascular superoxide levels in LZR and OZR were assessed by using dihydroethidine (DHE) microfluorography (3, 17, 26). For the present studies, femoral arteries were removed from LZR and OZR, cleared of connective tissue, and immersed in warm PSS (37°C) for 30 min. Subsequently, segments were incubated for 30 min in control PSS or in PSS containing PEG-SOD and catalase. DHE (10 μM; Molecular Probes) was then added to the PSS for 30 min followed by an additional 30 min in control PSS. Segments were split longitudinally and placed endothelium-side down on a PSS-moistened coverslip. The medial smooth muscle layer was visualized by using confocal laser microscopy (Odyssy Systems) and acquired by using Metamorph Image Acquisition software (Universal Imaging). Acquired images were analyzed for fluorescent intensity using National Institutes of Health Image software.

**Nitrotyrosine assay.** To determine whether peroxynitrite levels were chronically elevated in the vasculature, we examined nitrotyrosine levels in the medial layer of aortic segments of LZR and OZR. Aortas were rapidly dissected free of connective tissue and rapidly frozen in liquid nitrogen. Frozen tissues were embedded in OCT compound (VWR Scientific Products), and 5-μm-thick sections were generated from each tissue. Sections were washed 0.1% Triton X-100 in PBS and then incubated in anti-nitrotyrosine antibody (1:50, rabbit, Molecular Probes) for 56 h at 4°C. After incubation in the primary antibody, sections were washed for 1 h in PBS and then blocked in 10% goat serum for an additional hour. After three 10-min washes with PBS, sections were incubated in Alexa 594 goat anti-rabbit secondary antibody (Molecular Probes) for 2 h at room temperature. All sections
were shielded from light during the secondary incubation. Sections were then washed five times with PBS, stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes), and mounted with VectaShield (Vector Labs).

Nitrotyrosine staining was assessed with MagnaFIRE (Optronics) imaging software and a fluorescent microscope. Elastin autofluorescence was used to delineate the boundary of individual smooth muscle layers, and the nuclear stain DAPI was used to identify individual cells. Fluorescence was measured using FITC and DAPI filters, respectively. Alexa 594 fluorescence was assessed with a Cy3 filter. All filters were purchased from Chroma Technologies Alexxa 594. Fluorescence intensity was quantified with Scion Image (Scion) and expressed in arbitrary fluorescence units.

Data and statistical analyses. For the present study, myogenic activation was defined as the ability of the isolated vessel to maintain diameter over the intraluminal pressure range. From each experiment, data describing the change in vessel diameter with increasing intraluminal pressure were fit with a linear regression equation (OLS analysis; $r^2 > 0.85$); $y = \alpha + \beta(x)$, where $y$ represents arterial diameter at a specific intraluminal pressure, $\alpha$ is an intercept term, and $x$ represents intraluminal pressure). $\beta$ represents the slope of the pressure versus diameter curve (i.e., the rate of change in arterial diameter for an incremental change in intraluminal pressure). Differences between $\beta$-coefficients for the curves, as well as differences in resting vessel diameter, were determined using ANOVA (Tukey’s test post hoc) or Student’s $t$-test where appropriate. Throughout all analyses, a probability level of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

Data describing the diameter of isolated gracilis arteries of LZR and OZR at their respective equilibration pressure under the conditions of the present study are presented in Table 1. Under control conditions, the diameter of isolated arteries was not different between LZR and OZR. Treatment of arteries of OZR with...
PEG-SOD and catalase increased vessel diameter over the control value. Interestingly, the passive diameter of arteries from OZR was reduced compared with that for LZR; this was responsible for the reduced level of calculated active tone in vessels from OZR compared with LZR.

Figure 1 presents data describing the change in diameter of arteries from LZR and OZR with alterations in intraluminal pressure. In response to increased pressure, myogenic activation of gracilis arteries from OZR was significantly greater than that from LZR. Whereas removal of the arterial endothelium had no significant effect on myogenic activation of vessels from LZR, this procedure significantly increased the slope coefficient describing this response in vessels of OZR (thus impairing the enhanced response of the vessel to elevated intraluminal pressure).

The effects of treatment of isolated arteries of LZR and OZR with 17-ODYA on myogenic activation are presented in Fig. 2. In vessels from both LZR (Fig. 2A) and OZR (Fig. 2B), inhibition of cytochrome P-450 enzymes impaired myogenic activation of arteries during alterations in intraluminal pressure. However, the effect of 17-ODYA on myogenic activation of vessels from both strains was comparable, because the change in the slope coefficient describing this response from the respective control values was not different between the two groups (ΔβLZR = 0.110 ± 0.025 μm/mmHg; ΔβOZR = 0.131 ± 0.029 μm/mmHg).

Figure 3 presents data describing oxidant stress levels in femoral arteries of LZR and OZR. As determined with the use of the DHE microfluorography assay, vascular superoxide levels were significantly lower in vessels of LZR (Fig. 3A) versus vascular superoxide levels in OZR (Fig. 3B). After application of PEG-SOD and catalase to arteries from both rat strains, oxidant tone in OZR was attenuated (Fig. 3D), such that it was not distinguishable from that determined in LZR. Application of PEG-SOD and catalase had no significant effect on oxidant stress levels in vessels from LZR (Fig. 3C). These data are presented graphically as arbitrary units of fluorescence in Fig. 3F.

Figure 4 presents the effects of treatment of gracilis arteries from LZR (Fig. 4A) and OZR (Fig. 4B) with PEG-SOD and catalase on myogenic activation. Application of the oxidative radical scavengers had no effect on myogenic activation of vessels from LZR. In contrast, treatment of vessels of OZR with PEG-SOD and catalase impaired myogenic activation from levels determined under control conditions (Fig. 4B). Treatment of vessels with catalase alone had no effect on myogenic activation versus control responses in either LZR or OZR (βLZR = 0.042 ± 0.039 μm/mmHg; βOZR = −0.175 ± 0.032 μm/mmHg). Finally, addition of 17-ODYA to vessels from OZR and LZR that had been treated with PEG-SOD and catalase caused a comparable impairment of myogenic activation in vessels from both groups, such that arteries responded similarly to elevated intraluminal pressure (βLZR = 0.119 ± 0.029 μm/mmHg; βOZR = 0.109 ± 0.034 μm/mmHg).

Figure 5 presents data describing myogenic activation of gracilis arteries from LZR (Fig. 5A) and OZR (Fig. 5B) under control conditions and after inhibition of NO synthase with L-NAME. Treatment of vessels of LZR with L-NAME had no effect on the response of vessels to elevated intraluminal pressure versus the control condition. In contrast, NO synthase inhibition
significantly impaired myogenic activation of vessels from OZR.

Levels of N-tyrosine staining in LZR and OZR are depicted in Fig. 6. Aortic segments from OZR (Fig. 6B) demonstrated significantly more staining compared with those from LZR (Fig. 6A). Fluorescence intensity of N-tyrosine staining was 51% greater in OZR relative to LZR (Fig. 6C). No significant Alexa 594 staining was observed in aortic segments treated with no primary antibody (data not shown).

Figure 7 shows data describing myogenic activation of isolated gracilis arteries from LZR (Fig. 7A) and OZR (Fig. 7B) after application of iberiotoxin. Application of the KCa channel antagonist caused a significant constriction of isolated vessels from LZR, although treatment of arteries from OZR with iberiotoxin did not significantly constrict the vessel (Table 1). After application of iberiotoxin, myogenic activation of skeletal muscle resistance arteries of LZR was not altered in response to elevated intraluminal pressure (Fig. 7A). In contrast, blockade of KCa channels significantly impaired the pressure-induced constriction of skeletal muscle arteries from OZR (Fig. 7B). The decrease in vessel diameter following application of iberiotoxin did not cause a maximal constriction of vessels in either LZR or OZR, because subsequent addition of 10^{-7} M norepinephrine to iberiotoxin-treated vessels resulted in a significant additional vasoconstriction in vessels from both rat strains (data not shown).

Figure 8 presents data describing the constriction of isolated arteries from LZR and OZR in response to direct application of peroxynitrite. In vessels of LZR, application of peroxynitrite caused a small vasoconstriction, which was not impacted by elevated intraluminal pressure. In contrast, challenge with peroxynitrite caused a significantly greater constriction of vessels from OZR versus responses in LZR at 80 and
100 mmHg of intraluminal pressure. With increased intraluminal pressure, the response of vessels of OZR to peroxynitrite was significantly reduced, such that at 120 mmHg, there was no difference in peroxynitrite-induced vasoconstriction between LZR and OZR. In both LZR and OZR, pretreatment of vessels with iberiotoxin abolished arterial reactivity in response to peroxynitrite.

**DISCUSSION**

Ongoing epidemiological studies (1, 2) has indicated that the occurrence of obesity, hypertension, and Type II diabetes mellitus is increasing dramatically in the Western society and that each of these represents major risk factors for the development of peripheral vascular disease. As such, it is vitally important to determine the effects of these pathologies on the nature of vascular responses to fundamental physiological stimuli (e.g., intravascular pressure). The Zucker rat provides a valuable animal model for this effort, because this animal develops Type II diabetes mellitus as a consequence of the evolving obesity and progres-
The fundamental observation from the present study is that myogenic activation of skeletal muscle resistance arteries from OZR is strongly enhanced compared with responses determined in vessels from LZR (i.e., a decreased slope coefficient describing the data; Fig. 1). Whereas hypertension-induced enhancement of myogenic activation of vessels has been demonstrated previously (10, 21, 32), previous studies examining alterations to this response in models of diabetes have produced contrasting data (16, 18, 19, 23, 31, 35). Ungvari et al. (31) and Zimmerman et al. (35) demonstrated that streptozotocin-induced diabetes resulted in increased myogenic reactivity of the skeletal muscle and cerebral arterioles, respectively. An increased myogenic activation of mesenteric arteries has also recently been reported in a mouse model of Type II diabetes (23). In contrast, other investigators have found diminished myogenic activation of renal afferent arterioles (16), skeletal muscle resistance (18), and distal arterioles (19) in streptozotocin-induced diabetic rats. Additionally, Cipolla et al. (5) demonstrated that acute elevations in superfusate glucose concentration impaired myogenic tone of posterior cerebral arteries. The results of the present study contrast with these latter findings and indicate that the Zucker rat model of obesity-induced Type II diabetes is associated with an increased myogenic activation of skeletal muscle resistance arteries, a result that may have been partially due to the concurrent development of arterial hypertension. The myogenic activation in arteries from LZR was independent of the vascular endothelium, because denudation procedures (air bolus perfusion) did not alter vessel reactivity to increased intraluminal pressure (Fig. 1). This lack of dependence for myogenic activation on the vessel endothelium has been demonstrated previously in numerous strains of rats, both in our laboratory (10) and by many others (7). In contrast, myogenic activation of vessels from OZR was partially dependent on the presence of an intact endothelium, because removal of this cell layer caused a small, but significant, reduction in the strength of the vessel constriction with elevated intraluminal pressure (Fig. 1). The partial dependence of myogenic activation on the vascular endothelium has previously been identified in cerebral vessels (15). Furthermore, the results of the present study suggest that the production and release of nitric oxide by the microvessel endothelium may contribute to this partial endothelium dependence, because blockade of nitric oxide synthase with L-NAME impaired myogenic activation of skeletal muscle resistance arteries from OZR (Fig. 5). A growing number of previous studies have demonstrated that full manifestation of myogenic activation is partially dependent on the generation of 20-HETE (via cytochrome P-450 ω-hydroxylase (7, 10, 36). The results of the present study support these previous observations and extend them to skeletal muscle arteries of Zucker rats in that application of 17-ODYA impaired myogenic activation of these vessels in both LZR and OZR (Fig. 2). Comparable to previous studies (10), our results also indicate that production of 20-HETE only contributes to myogenic activation, because under no level of intraluminal pressure did the diameter of 17-ODYA-treated vessels ever approach that for vessels in a Ca^{2+}-free environment. Interestingly, the application of 17-ODYA to vessels from either LZR or OZR caused a comparable impairment in myogenic activation because evidenced by a similar difference in slope (β) coefficients describing this response between control and 17-ODYA-treated vessels in both rat groups. These data suggest that, although production of 20-HETE is necessary for the full development of myogenic activation of gracilis arteries in both LZR and OZR, the relative contribution of 20-HETE to this response is not altered with the development of hypertension and diabetes in Zucker rats.

With the use of DHE microfluorography, the results of the present study indicated that vascular oxidant stress in OZR is substantially elevated compared with levels determined in LZR (Fig. 3). Furthermore, the present results also demonstrated that treatment of vessels with the oxidative radical scavengers PEG-SOD and catalase was associated with an attenuation of the enhanced myogenic activation of vessels to levels that were not different from those determined in arteries of...
LZR (Fig. 4). These data suggest that the elevated vascular oxidative stress in OZR could be responsible for differences in the patterns of myogenic activation between OZR and LZR.

A recent study by Huang et al. (20) suggested that elevated intravascular pressure per se increases production of superoxide anion in gracilis muscle arterioles; this process clearly has the potential to significantly impact the regulation of skeletal muscle vascular resistance. However, the majority of existing studies suggest that superoxide anion can activate KCa channels in numerous tissues, thus increasing the K+ current leading to membrane hyperpolarization (6, 9, 29, 30). These previous observations can be difficult to reconcile with the present data, where elevated oxidant tone is associated with increased myogenic activation. To address this, we hypothesized that it was not the production of superoxide that necessarily impacted patterns of myogenic activation, rather the generation of peroxynitrite as a result of the breakdown of superoxide anion by endothelium-derived NO may contribute to alterations in myogenic activation. This hypothesis was developed as a result of three lines of evidence: 1) treatment of vessels of OZR with PEG-SOD and catalase normalized vascular oxidant stress and myogenic activation to levels determined in vessels of LZR (Figs. 3 and 4), 2) treatment of vessels of OZR with l-NAME reduced myogenic activation of vessels significantly from control levels (Fig. 5), and 3) the previous study by Brzezinska et al. (4) indicated that peroxynitrite can act as a constrictor of cerebral arteries through decreasing the open-state probability and mean open time of KCa channels. Under these conditions, myogenic activation of vessels from OZR was normalized to levels determined in vessels from LZR (an index of the presence of peroxynitrite; Fig. 6), we tested our hypothesis in two final series of experiments. First, gracilis arteries from LZR and OZR were pretreated with iberiotoxin to inhibit vascular smooth muscle membrane KCa channels. Under these conditions, myogenic activation of vessels from OZR was normalized to levels determined in vessels from LZR (Fig. 7). These observations suggest that progressive inhibition of KCa channels by peroxynitrite with increased intraluminal pressure may contribute to differences in myogenic activation between arteries of LZR and OZR. Second, our results also indicate that peroxynitrite itself is a constrictor of skeletal muscle resistance arteries, with this effect being exacerbated in OZR compared with LZR, and that this vasoconstrictor influence of peroxynitrite was abolished by blockade of KCa channels with iberiotoxin (Fig. 8). These final results support two preliminary conclusions: 1) that peroxynitrite exerts its effects via KCa channels because prior blockade of these channels in both LZR and OZR abolished vascular responses to peroxynitrite, and 2) that increased myogenic activation of arteries from OZR results in a reduced vascular sensitivity to peroxynitrite because the constriction of arteries in response to 100 nM peroxynitrite diminished as intraluminal pressure was elevated from 80 to 120 mmHg.

Taken together, results from the present study and from previous investigations suggest the development of a fundamental alteration in OZR compared with LZR for reactivity of skeletal muscle resistance arteries in response to increased intraluminal pressure. We hypothesize that in skeletal muscle resistance, arteries of OZR increased intraluminal pressure causes an elevated vascular superoxide generation (20) that (in combination with endothelium-derived NO) is converted to peroxynitrite (8, 34). This generated peroxynitrite can inhibit the opening of KCa channels (4), resulting in a greater vasoconstrictor response to a given level of intraluminal pressure compared with responses in LZR. Whereas alterations to intravascular-pressure induced generation of superoxide anion (20) may be an initial signaling process that underlies the difference in myogenic activation of skeletal muscle resistance arteries between LZR and OZR, further study will be needed to more fully test these hypotheses and to determine the mechanisms and relationships involved.

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