Impaired NO-dependent dilation of skeletal muscle arterioles in hypertensive diabetic obese Zucker rats

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Frisbee, Jefferson C., and David W. Stepp. Impaired NO-dependent dilation of skeletal muscle arterioles in hypertensive diabetic obese Zucker rats. Am J Physiol Heart Circ Physiol 281: H1304–H1311, 2001.—This study determined alterations to nitric oxide (NO)-dependent dilation of skeletal muscle arterioles from obese (OZR) versus lean Zucker rats (LZR). In situ cremaster muscle arterioles from both groups were viewed via television microscopy, and vessel dilation was measured with a video micrometer. Arteriolar dilation to acetylcholine and sodium nitroprusside was reduced in OZR versus LZR, although dilation to aprikalim was unaltered. NO-dependent flow-induced arteriolar dilation (via parallel microvessel occlusion) was attenuated in OZR, impairing arteriolar ability to regulate wall shear rate. Vascular superoxide levels, as assessed by dihydroethidine fluorescence, were elevated in OZR versus LZR. Treatment of cremaster muscles of OZR with the superoxide scavengers polyethylene glycol-superoxide dismutase and catalase improved arteriolar dilation to acetylcholine and sodium nitroprusside and restored flow-induced dilation and microvascular ability to regulate wall shear rate. These results suggest that NO-dependent dilation of skeletal muscle microvessels in OZR is impaired due to increased levels of superoxide. Taken together, these data suggest that the development of diabetes and hypertension in OZR may be associated with an impaired skeletal muscle perfusion via an elevated vascular oxidant stress.

skeletal muscle microcirculation; type II diabetes; hypertension; flow-induced dilation; superoxide

TYPE II DIABETES MELLITUS impacts ~11 million Americans and is a potent risk factor for the development of peripheral vascular disease, a debilitating condition impacting nearly 60 million Americans (1). Over the last 10 years, the incidence of type II diabetes mellitus has increased more than 70% in 30- to 39-yr-old Americans and has become increasingly common among American children (25). Due to a nonfunctional leptin receptor gene (11), the obese Zucker rat (OZR) provides a valuable animal model for examining the effects of type II (non-insulin-dependent) diabetes. As a result of an extreme increase in food consumption, OZR rapidly develop numerous pathological conditions that are highly relevant to public health issues challenging Western society; among these conditions are type II diabetes, moderate hypertension, and obesity (15, 26, 28). Although the progression of these pathologies has been well documented, the effects of these conditions developing concurrently on peripheral vascular function remain unknown.

In OZR, previous studies (14, 29, 32) have indicated that intestinal and mesenteric microvessel dilation to nitric oxide (NO)-dependent stimuli was significantly reduced compared with responses in lean Zucker rat (LZR) control animals. This impaired dilation may have been related to either a reduced sensitivity of vessels to NO (31) or to a reduction in the bioavailability of NO in OZR compared with LZR. Reduction in the bioavailability of NO in OZR may be due to an increase in vascular superoxide levels (2, 21). The goal of the present study was to test the hypothesis that NO-mediated dilation is impaired in skeletal muscle distal arterioles of diabetic OZR compared with responses determined in LZR. Furthermore, we also hypothesized that an increased level of vascular oxidant stress underlies this impaired NO-dependent dilator reactivity of skeletal muscle microvessels through reductions in the bioavailability of NO.

MATERIALS AND METHODS

Animals. All experiments used 13- to 15-wk-old male LZR (Harlan; body weight, 358 ± 12 g; mean arterial pressure, 125 ± 4.1 mmHg; [glucose] in blood, 138 ± 15 mg/dl) and OZR (Harlan; body weight, 604 ± 16 g; mean arterial pressure, 159 ± 5.8 mmHg; [glucose] in blood, 486 ± 48 mg/dl) fed standard rat chow and tap water ad libitum. Rats (n = 16 per group) were housed in an animal care facility at the Medical College of Wisconsin that was approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Rats were anesthetized with an injection of pentobarbital sodium (60 mg/kg ip), and a carotid artery was cannulated for determination of arterial pressure.

For all experiments, a cremaster muscle in each rat was prepared for television microscopy as described previously (7, 8). After the muscle preparation was completed, the tissue was continuously superfused with physiological saline solution (PSS), equilibrated with a gas mixture containing 5% CO2-95% N2, and maintained at 35°C as it flowed over the muscle. The ionic composition of the PSS was as follows (in mM): 119.0 NaCl, 4.7 KCl, 1.6 CaCl2, 1.18 NaH2PO4, 1.17

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MgSO₄, and 24.0 NaHCO₃. Arteriolar diameter was determined with a video micrometer accurate to ±1 μm (23). Centerline erythrocyte velocity (in mm/s) was measured with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX).

After an initial postsurgical equilibration period of 30 min, a second-order arteriole (60 μm diameter) was identified in a clearly visible region of the muscle. This vessel was examined along its length until a suitable bifurcation was identified in which one third-order branch of the vessel served as the occluded daughter vessel and the other branch (20 μm diameter) as the perfused daughter arteriole of interest. Arterioles chosen for study had walls that were clearly visible, a brisk flow velocity, and active tone, as indicated by the occurrence of significant dilation in response to topical application of 10⁻⁶ M adenosine. All arterioles that were studied were located in a region of the muscle that was away from any incision.

The methods used for parallel arteriolar occlusion followed those originally described by Koller and Kaley (16–18). Briefly, blood flow in one perfused daughter branch (third-order arteriole) from a parent arteriole (second-order arteriole) was physically impeded by lowering a microoccluder along its length until a suitable bifurcation was identified in a region of the muscle that was away from any incision. The methods used for parallel arteriolar occlusion followed those originally described by Koller and Kaley (16–18). Briefly, blood flow in one perfused daughter branch (third-order arteriole) from a parent arteriole (second-order arteriole) was physically impeded by lowering a microoccluder onto the perfused daughter branch at a site located 500–2,000 μm from the point of observation. Physical occlusion of this arteriole, hereafter referred to as “parallel occlusion,” increases blood flow and wall shear rate in a parallel (non-occluded) daughter branch (third-order arteriole) from the parent vessel, leading to a flow- or shear-induced dilation of the nonoccluded daughter arteriole. The microoccluder used in these studies was a glass micropipette with a blunt rounded end (~50 μm diameter) to prevent vessel damage during occlusion. A Leitz micromanipulator was used to position the occluder over the arteriole.

Microvessel diameter of, and erythrocyte velocity within, the perfused daughter arteriole were recorded at rest and at 20-s intervals after occlusion of the parallel microvessel. After 120 s, the microoccluder was lifted from the cremaster muscle, and flow through the network was allowed to recover for 25 min before subsequent procedures were performed.

Inhibition of prostanoïd and NO production. To assess the role of prostanoïd or NO release from the endothelium in regulating flow-induced dilation of in situ arterioles, the cremaster muscle was treated with the cyclooxygenase inhibitor indomethacin (10⁻⁶ M) or the NO synthase inhibitor N°-nitro-L-arginine methyl ester (l-NAME; 10⁻⁴ M), respectively (9). Both inhibitors were added to the PSS superfusate.

Dilator agonists. In addition, the dilation of in situ cremasteric arterioles from LZR and OZR were assessed in response to 1) acetylcholine (10⁻⁹–10⁻⁶ M), a receptor-mediated endothelium-dependent dilator agonist; 2) sodium nitroprusside (10⁻⁹–10⁻⁶ M), an endothelium-independent NO donor; 3) aprakilim (10⁻⁹–10⁻⁶ M), an activator of ATP-sensitive potassium (K ATP) channels; and 4) Ca²⁺-free PSS containing 10⁻³ M adenosine, to produce complete relaxation of the microvessel and determine the maximum (passive) arteriolar diameter (10).

Scavenging of oxidative free radicals. To determine the extent to which increased oxidant stress levels might underlie alterations to NO-dependent dilation of in situ skeletal muscle arterioles between LZR and OZR, the cremaster muscle from both groups was treated with the oxidative free radical scavengers polyethylene glycol-superoxide dismutase (PEG-SOD; 200 U/ml, Sigma) and catalase (80 U/ml, Sigma). Treatment of tissues with these substances has been previously demonstrated to significantly lower oxidant stress levels within skeletal muscle and vascular tissues (13, 22).

Measurement of superoxide production. Vascular superoxide levels in LZR and OZR were assessed using the dihydroethidium (DHE) microfluorography assay as described previously (5, 12, 24). Briefly, DHE is a lipophilic cell-permeable dye that is rapidly oxidized to ethidium in the presence of free radical superoxide. The produced ethidium is fixed by intercalation into nuclear DNA, thus giving an indication of oxidant stress within cells under investigation. In LZR and OZR, the aortas were removed from all animals, cleared of surrounding connective tissue, cut into segments, and placed in warm PSS (37°C) for 30 min. After this period, segments were incubated for 30 min in either control PSS or in PSS containing PEG-SOD (200 U/ml) and catalase (80 U/ml). DHE (Molecular Probes) was then added to the PSS for 30 min, followed by a final 30-min wash in DHE-free PSS. Segments were split longitudinally and placed endothelium side down on a PSS-moistened coverslip. The medial smooth muscle layer was visualized using confocal laser microscopy (Odyssey Systems) and acquired using Metamorph Image Acquisition software (Universal Imaging; West Chester, PA). Acquired images were analyzed for fluorescent intensity using National Institutes of Health Image software.

Experimental procedures. In all preparations, dilator reactivity of in situ cremaster muscle arterioles to acetylcholine, sodium nitroprusside, and aprakilim was evaluated as described above. Subsequently, experiments were completed to determine whether a difference in the dilation of arterioles to elevated wall shear rate exists between LZR and OZR and to determine the mediators of this response. As such, cremasteric arteriolar dilation in response to parallel occlusion was assessed in both rat groups under control conditions, after treatment of muscle with l-NAME or indomethacin, and after simultaneous application of both inhibitors.

In additional experiments, the response of in situ cremasteric arterioles from LZR and OZR to acetylcholine and sodium nitroprusside and in response to parallel microvessel occlusion was assessed using the oxidative free radical scavengers polyethylene glycol-superoxide dismutase (PEG-SOD; 200 U/ml, Sigma) and catalase (80 U/ml, Sigma).

### Table 1. Resting diameter and WSR of in situ cremasteric arterioles of LZR and OZR under experimental conditions

<table>
<thead>
<tr>
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<th>LZR</th>
<th>OZR</th>
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<tr>
<td>Diameter</td>
<td>WSR, ×10⁶</td>
<td>Diameter</td>
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<tr>
<td>μm</td>
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<td>μm</td>
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<tr>
<td>Control</td>
<td>19.0 ± 1.2</td>
<td>1.34 ± 0.21</td>
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<tr>
<td>+l-NAME</td>
<td>15.4 ± 1.0*</td>
<td>1.55 ± 0.23</td>
</tr>
<tr>
<td>+Indomethacin</td>
<td>17.8 ± 1.8</td>
<td>1.57 ± 0.43</td>
</tr>
<tr>
<td>+PEG-SOD</td>
<td>18.5 ± 1.5</td>
<td>1.41 ± 0.21</td>
</tr>
<tr>
<td>Maximum diameter</td>
<td>31.4 ± 1.8*</td>
<td>0.97 ± 0.17</td>
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<tr>
<td>Active tone</td>
<td>48.6 ± 3.8</td>
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Values are means ± SE. LZR, lean Zucker rats; OZR, obese Zucker rats; WSR, wall shear rate; l-NAME, N°-nitro-L-arginine methyl ester; PEG-SOD, polyethylene glycol-superoxide dismutase. *P < 0.05 versus control; †P < 0.05 versus LZR.
occlusion was determined before and after treatment of the cremaster muscle with the oxidative free radical scavengers PEG-SOD and catalase as described above.

Mathematical and statistical analyses. Blood flow volume through the daughter vessel was calculated from erythrocyte velocity and vessel radius (4) as follows

\[ F = \left( V \times 1.6^{-1} \right) \times (\pi r^2) \times 0.001 \]

where \( F \) is the volume of blood flow (in nl/s), \( V \) is the centerline erythrocyte velocity (in mm/s), and \( r \) is the vessel radius (in \( \mu m \)). Wall shear rate (WSR; in \( s^{-1} \)) was calculated as outlined by Koller and Kaley (17) as follows

\[ WSR = 8 \times (V_M \times D^{-1}) \]

where \( V_M \) is the mean flow velocity in the daughter vessel (calculated by dividing the optical Doppler measurement of centerline velocity by 1.6 to correct for the parabolic flow pattern in the vessel) and \( D \) is the vessel diameter.

Fig. 1. The response of in situ cremaster muscle distal arterioles of lean Zucker rats (LZR) and obese Zucker rats (OZR) after application of increasing concentrations of acetylcholine (A), sodium nitroprusside (B), and aprikalim (C). \( n = 1 \) arteriole/rat from 6 animals. *\( P < 0.05 \) vs. LZR. \( \beta \), slope coefficient.

Fig. 2. The change in arteriolar diameter (A) and wall shear rate (B) for in situ cremasteric arterioles of LZR and OZR after 120 s of physical occlusion of a parallel arteriole. \( n = 1 \) arteriole/rat from 6 animals. *\( P < 0.05 \) vs. LZR.
All data are presented as means ± SE. Significant differences between experimental conditions for all data collected before parallel occlusion were determined using ANOVA or Student’s t-test where appropriate. For all data collected during parallel occlusion and all agonist-induced responses, repeated-measures ANOVA was employed. All post hoc analyses were done using Tukey’s test. In all cases, a probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Table 1 presents data describing the diameter and wall shear rate of in situ cremasteric arterioles from LZR and OZR under the experimental conditions of the present study. There was no difference in resting diameter of skeletal muscle arterioles of LZR and OZR under control conditions. Treatment of the cremaster muscle with l-NAME reduced arteriolar diameter in LZR only. Application of PEG-SOD and catalase increased diameter of arterioles in OZR only, with no effect in LZR. Furthermore, the passive diameter of vessels in OZR was significantly less than that in LZR, an effect that caused a significantly decreased level of calculated active tone in arterioles of OZR. Calculated wall shear rate in arterioles of OZR was not different from that in LZR under any conditions in the present study.

Data describing the dilation of cremasteric arterioles from LZR and OZR in response to challenge with acetylcholine, sodium nitroprusside, and aprikalim are presented in Fig. 1. In cremasteric arterioles of OZR, dilator responses to acetylcholine (Fig. 1A) and sodium nitroprusside (Fig. 1B) were reduced across the agonist concentration range compared with responses determined in LZR. In contrast, dilator responses to the $K_{ATP}$ channel agonist aprikalim were not different between microvessels of LZR and OZR (Fig. 1C).

The dilation of cremasteric arterioles (Fig. 2A) and the changes in microvessel wall shear rate (Fig. 2B) in LZR and OZR during parallel arteriolar occlusion are presented in Fig. 2. In response to elevated wall shear rate, cremasteric arterioles of LZR underwent a significant dilation, rapidly restoring wall shear rate to precondition values. In contrast, arterioles of OZR did not dilate in response to elevated wall shear rate, thus impairing the ability of the microvessel to regulate wall shear rate.

Figure 3 presents data describing the effects of treatment of in situ cremaster muscles of LZR and OZR with l-NAME or indomethacin on flow-induced dilation and the ability of the arteriole to regulate wall shear rate. In LZR, treatment of vessels with l-NAME significantly impaired arteriolar dilation after parallel occlusion (Fig. 3A), compromising the ability of the vessel to regulate shear levels (Fig. 3B). Treatment of the cremaster muscle of LZR with indomethacin had no effect on arteriolar responses to parallel occlusion. Furthermore, treatment of vessels of OZR with either l-NAME or indomethacin had no discernible effect on either flow-induced dilation (Fig. 3C) or the ability of the vessel to regulate wall shear rate (Fig. 3D).

Figure 4 presents data describing vascular oxidant stress levels in LZR and OZR. As determined using DHE microfluorography, baseline levels of superoxide anion in aortic smooth muscle LZR (Fig. 4A) were substantially less than those in OZR (Fig. 4B). Treatment of aortas of OZR with PEG-SOD and catalase lowered superoxide levels to values that were not distinguishable from those determined in LZR under control conditions (Fig. 4D), although this procedure had no discernible effect on vascular oxidant stress levels in LZR (Fig. 4C). These data are presented graphically as arbitrary units in Fig. 4F.

Data describing the dilation of cremasteric arterioles from OZR after challenge with acetylcholine and sodium nitroprusside under control conditions and in...
Fig. 4. Vascular oxidant stress levels in aortas from LZR and OZR assessed using dihydroethidine microfluorography. Representative data are presented for LZR and OZR under control conditions (A and B, respectively) and after treatment of vessels from LZR and OZR with polyethylene glycol-superoxide dismutase (PEG-SOD) and catalase (C and D, respectively). All images are color matched (color standard; E). Fluorescent intensities in A–D are presented as arbitrary units (F). *P < 0.05 vs. LZR (control); †P < 0.05 vs. OZR (control).

Fig. 5. The dilation of in situ cremaster muscle distal arterioles of OZR under control conditions and after treatment of the cremaster muscle with the oxidative free radical scavengers PEG-SOD and catalase in response to challenge with increasing concentrations of acetylcholine or sodium nitroprusside. The dilation of arterioles to the agonists are normalized to either the control arteriolar diameter (A: acetylcholine; B: sodium nitroprusside) or to the maximum possible dilation of the arteriole, determined during superfusion of the cremaster muscle with Ca^{2+}-free physiological saline solution (C: acetylcholine; D: sodium nitroprusside). n = 1 arteriole/rat from 5 animals. *P < 0.05 vs. control responses. No significant differences were determined in the response of cremasteric arterioles of LZR to the agonists between control conditions and after treatment of the cremaster muscle with PEG-SOD and catalase.
response to treatment of the muscle with PEG-SOD and catalase are presented in Fig. 5. Application of oxidative free radical scavengers significantly increased dilator responses of distal arterioles of OZR to acetylcholine [Fig. 5, A (percent rest diameter) and C (percent maximum possible dilation)] and sodium nitroprusside [Fig. 5, B (percent rest diameter) and D (percent maximum possible dilation)] compared with responses determined under control conditions. In contrast, dilation of cremasteric arterioles of LZR to these agonists after treatment with PEG-SOD and catalase was not altered from responses determined under control untreated conditions (data not shown).

The effects of treatment of the cremaster muscle with oxidative free radical scavengers on flow-induced dilation and wall shear rate after parallel occlusion are presented in Fig. 6. The flow-induced dilation of cremasteric arterioles of LZR after treatment with PEG-SOD and catalase was not altered from responses determined under control conditions (data not shown). In contrast, treatment of the cremaster muscle of OZR with free radical scavengers significantly increased skeletal muscle arteriolar dilation in response to elevated wall shear rate (Fig. 6A), thus enhancing the ability of the microvessel to restore wall shear rate toward normal preocclusion levels (Fig. 6B).

DISCUSSION

Given that a recent study (25) has demonstrated that the incidence of type II diabetes mellitus has increased dramatically in 30- to 39-yr-old Americans and has become increasingly common among American children, ongoing investigation into clinically relevant animal models is of paramount importance. As such, OZR, with a nonfunctional leptin receptor gene (11) and ensuing elevations in caloric intake, provide a critically important animal model for study owing to the rapid development of type II diabetes mellitus and hypertension in parallel with obesity (15, 26, 28). However, as yet there has been little attempt to determine basic characteristics of skeletal muscle microvessels of OZR. To address this paucity in the literature, the present study was designed to determine whether alterations to NO-dependent dilator reactivity of skeletal muscle distal arterioles of OZR exist compared with responses in LZR and to determine the mechanisms that contribute to any alterations in these responses.

The initial observation of the present study was that dilator responses of in situ cremaster muscle distal arterioles of OZR to endothelium-dependent (acetylcholine; Fig. 1A) or endothelium-independent (sodium nitroprusside; Fig. 1B) NO-mediated dilator agonists or stimuli (elevated wall shear rate; Figs. 2 and 3) were severely impaired compared with responses determined in LZR control animals. In contrast, dilator responses to direct activation of K\(_{ATP}\) channels, the ion channel previously suggested as mediating NO-induced dilation in skeletal muscle microvessels (3, 6, 27), produced comparable dilator responses of these vessels between OZR and LZR (Fig. 1C). While our observation of an impaired acetylcholine-induced dilator reactivity of the skeletal muscle vasculature is in agreement with previous studies (2, 20, 21) using in situ skeletal muscle preparations in OZR and our observation of an impaired flow-induced microvessel dilation is in agreement with a previous study (14) in intestinal arterioles, to our knowledge these are the first observations of an impaired response to either challenge with NO or of a preserved dilator reactivity of skeletal muscle arterioles to direct activation of K\(_{ATP}\) channels mediating NO-dependent vasodilation.

At least three primary mechanisms could potentially contribute to the reduced NO-dependent dilation of
skeletal muscle arterioles, each of which having been previously hypothesized as occurring in tissues of diabetic hypertensive OZR: 1) impaired production and/or release of NO by the microvessel endothelium in response to an appropriate agonist and/or stimuli (19); 2) an impaired vascular smooth muscle responsiveness to NO released from the endothelium (31); and/or 3) a reduced bioavailability of NO, potentially due to increased scavenging by oxidative free radicals, thus preventing the full manifestation of the vasodilator effects of released NO (2, 20, 21). The results of the current study support the third mechanism, elevation in superoxide reduced the bioavailability of NO.

On the basis of the results shown in Fig. 4, vascular oxidant stress levels in OZR, assessed using DHE microfluorography (5, 12, 24), were elevated compared with levels determined in LZR (Fig. 4). These observations are in agreement with those of previous studies (2, 19, 20) and support hypotheses recently suggested for the skeletal muscle circulation (21). The dramatic increase in vascular oxidant stress levels in OZR provides compelling evidence in support of the hypothesis that a reduced bioavailability of NO through scavenging by oxidative free radicals may represent the predominant mechanism underlying the impaired dilation of skeletal muscle microvessels to the challenges in the present study. Also evident in Fig. 4 was that treatment of aortas with the oxidative free radical scavengers PEG-SOD and catalase, while having no discernible effect on oxidant stress levels in LZR, reduced these levels in OZR to such an extent that they were not distinguishable from LZR.

After normalization of vascular oxidant stress levels subsequent to treatment of tissues with PEG-SOD and catalase, the dilator reactivity of in situ cremaster muscle arterioles of OZR to acetylcholine (Fig. 5A) and sodium nitroprusside (Fig. 5B) was increased to levels that were comparable with those determined in LZR under control conditions. In addition, the NO-dependent dilator stimulus of elevated wall shear rate was also enhanced in OZR after application of PEG-SOD and catalase, such that the ability of the microvessel to regulate wall shear rate was restored to levels that were comparable with those of LZR under control conditions (Fig. 6). These observations suggest that neither impaired agonist- or stimulus-induced endothelial production of NO nor an impaired vascular smooth muscle sensitivity to NO may be a dominant mechanism underlying the impaired dilation of cremasteric arterioles of OZR to the NO-dependent agonists or stimuli of the present study. Rather, these data suggest that scavenging of endothelium-derived NO by superoxide anion may be occurring, with the potential conversion to the much less biologically active substance peroxynitrate before the NO can effect a response in the vascular smooth muscle (29). However, it should be noted that these data do not rule out a potential contribution of impaired endothelial release of, or vascular smooth muscle sensitivity to, NO that was due to elevated oxidant stress levels that may have also been alleviated after treatment of the cremaster muscle with PEG-SOD and catalase.

In conclusion, the results of the current study describe a significant impairment in NO-mediated dilation of skeletal muscle arterioles in diabetic OZR. The compromised ability of these microvessels to dilate to NO occurred concomitantly with increased vascular oxidant tone. Furthermore, concentrations of free radical scavengers that ameliorated the increased superoxide levels also restored reactivity of microvessels to NO-dependent stimuli. We conclude that skeletal muscle perfusion may be impaired in obese diabetic patients via elevations in tissue oxidant stress. Targeting oxidant pathways therapeutically may by of substantial benefit to populations at risk for peripheral vascular disease.

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