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Distinct temporal phases of microvascular rarefaction in skeletal muscle of obese Zucker rats

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tion of metabolic syndrome is associated with a progressive reduction in skeletal muscle microvessel density, known as rarefaction. Although contributing to impairments to mass transport and exchange, the temporal development of rarefaction and the contributing mechanisms that lead to microvessel loss are both unclear and critical areas for investigation. Although previous work suggests that rarefraction severity in obese Zucker rats (OZR) is predicted by the chronic loss of vascular nitric oxide (NO) bioavailability, we have determined that this hides a biphasic development of rarefaction, with both early and late components. Although the total extent of rarefaction was well predicted by the loss in NO bioavailability, the early pulse of rarefaction developed before a loss of NO bioavailability and was associated with altered venular function (increased leukocyte adhesion/rolling), and early elevation in oxidant stress, TNF-α levels, and the vascular production of thromboxane A2 (TXA2). Chronic inhibition of TNF-α blunted the severity of rarefaction and also reduced vascular oxidant stress and TXA2 production. Chronic blockade of the actions of TXA2 also blunted rarefaction, but did not impact oxidant stress or inflammation, suggesting that TXA2 is a downstream outcome of elevated reactive oxygen species and inflammation. If chronic blockade of TXA2 is terminated, microvascular rarefaction in OZR skeletal muscle resumes, but at a reduced rate despite low NO bioavailability. These results suggest that therapeutic interventions against inflammation and TXA2 under conditions where metabolic syndrome severity is moderate or mild may prevent the development of a condition of accelerated microvessel loss with metabolic syndrome.

skeletal muscle perfusion; vascular remodeling; vascular reactivity; rodent models of obesity; nitric oxide bioavailability; chronic inflammation

Epidemiological studies have clearly and consistently demonstrated that both the incidence and prevalence of the metabolic syndrome is continuing to increase in Western society and in most developed economies worldwide (4, 9, 24). Although each of the constituent systemic pathologies (e.g., impaired glycemic control, atherogenic dyslipidemia, hypertension, obesity) have been well documented to increase the risk for development of impaired vascular structure/function and peripheral vascular disease (PVD) (10, 11, 13, 14, 16, 25, 29, 45), the significance of this multi-pathology state is that it increases the risk for individuals to develop PVD well beyond that for any single contributing element. Given the impact of PVD on life expectancy, quality of life, depression, and the direct (health care) and indirect (lost productivity) economic costs to society (3, 41, 44), considerable emphasis has been placed on not only the study of PVD and its contributing elements in human subjects but also for the detailed investigation of appropriate animal models of vasculopathy in the metabolic syndrome (1, 49, 51). However, although the existing literature is replete with descriptions of correlations between the systemic pathologies of the metabolic syndrome and specific indexes of vasculopathy, our understanding of these relationships is hampered since insufficient knowledge has been presented, which details the developments of vascular impairments that accompany this constellation of pathologies. The obese Zucker rat is an animal model of the metabolic syndrome that has become important for the study of vascular dysfunction in this setting (7, 20, 31). Due to its characteristic dysfunctional leptin receptor gene, the obese Zucker rat (OZR) experiences chronic hyperphagia and rapidly becomes obese compared with its control strain, the lean Zucker rat (LZR). Stemming from this, the OZR becomes progressively insulin-resistant, dyslipidemic (moderate hypercholesterolemia and severe hypertriglyceridemia), and moderately hypertensive (1, 15, 20, 49). Although considerable evidence has been presented by our laboratory (5, 27) and by numerous others (18, 30, 32, 35, 36, 47, 53) regarding the correlation between indexes of vascular dysfunction with characteristics of the metabolic syndrome in these animals at specific ages, minimal evidence has been produced regarding the temporal development of the vascular impairments in these animals, and how these correlate with the evolution of the metabolic syndrome, and other putative markers of dysfunction (e.g., markers of inflammation).
A consistent observation that has been made in the metabolic syndrome in both OZR and in human subjects (33, 38, 43) is a loss in peripheral microvessel density (rarefaction) that is associated with this disease state. As defined by Goligorsky (26), microvascular rarefaction is a form of microvascular adaptation in response to a challenged or disease state that results in a reduction in arteriolar or capillary density within an affected tissue. In OZR, this process plateaus at an ~25% reduction in microvessel density compared with control levels in LZR (21, 42, 48), with clear implications for mass transport/exchange and muscle fatigue resistance (46). Furthermore, previous results have demonstrated that this response is not dependent on the development of hypertension in OZR (22) and that it is associated with the development of insulin resistance and low bioavailability of nitric oxide (NO) within the microvasculature (23). However, at present we do not have a detailed understanding of either how or why microvessels begin to degrade in skeletal muscle of OZR with the metabolic syndrome, and our understanding of the time course of this evolving rarefaction is superficial at best. The primary purpose of the present study was to address this gap in our understanding of skeletal muscle microvasculopathy in the metabolic syndrome and to determine the initiating factors for and temporal development of the microvascular rarefaction that evolves in this tissue in OZR. Our initial hypothesis was that skeletal muscle microvessel density declines in OZR in parallel to the evolving reduction to vascular NO bioavailability.

### MATERIALS AND METHODS

**Animals.** Male LZR and OZR (Harlan) were fed standard chow and drinking water ad libitum, unless otherwise indicated, and were housed in the animal care facility at the West Virginia University Health Sciences Center. All protocols received prior Institutional Animal Care and Use Committee approval. Animals were used for terminal experiments at 7, 10, 13, or 20 wk of age (n = 8–10 animals in each age group for OZR; n = 6–8 animals in each age group for LZR).

At each age, after an overnight fast, rats were anesthetized with injections of pentobarbital sodium (50 mg/kg ip) and received tracheal intubation to facilitate maintenance of a patent airway. In all rats, a carotid artery and an external jugular vein were cannulated for determination of arterial pressure and for infusion of supplemental anesthetic or pharmacological agents, as necessary. Blood samples were drawn from the venous cannula within ~20 min of implantation for determination of glucose (Freestyle; Abbott Diabetes Care, Alameda, CA) and insulin concentrations (Cayman Chemical, Ann Arbor, MI). Plasma nitrotyrosine levels and markers of inflammation were determined using commercially available ELISA systems (Luminex 100 PS; EMD Millipore, Billerica, MA). Glucose levels were determined at the time of the blood draw. All other samples were spun to remove the plasma and snap frozen in liquid N₂ until they could be analyzed as groups.

**Preparation of isolated skeletal muscle resistance arterioles.** In all rats, the intramuscular continuation of the gracilis artery was removed and cannulated (19). Within each arteriole, vessel reactivity was evaluated in response to application of ACh (10⁻⁹ M - 10⁻⁶ M), arachidonic acid (10⁻⁹ M - 10⁻⁶ M), hypoxia (reduction in PO2 from ~135 mmHg to ~50 mmHg), and sodium nitroprusside (10⁻⁹ M - 10⁻⁶ M). Subsequently, vessels were treated with TEMPO (10⁻⁴ M) or Nα-nitro-L-arginine methyl ester (L-NAME; 10⁻⁴ M) to assess the contribution of vascular oxidant stress and endothelium-dependent NO production to these mechanical responses.

### Table 1. Characteristics of animal groups within the present study

<table>
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<th>Age, weeks</th>
<th>LZR</th>
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<td>20</td>
<td>375 ± 6</td>
<td>706 ± 14*</td>
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**Values are means ± SE. OZR, obese Zucker rat. *P < 0.05 vs. lean Zucker rat (LZR).**

Fig. 1. The change in skeletal muscle microvessel density (MVD) in lean Zucker rat (LZR) and obese Zucker rat (OZR) between 7 and 20 wk of age. Data are presented as means ± SE; n = 8–10 animals in each age group for OZR and n = 6–8 animals in each age group for LZR. A: raw changes in skeletal muscle microvessel density. B: slope of the changes in microvessels density over the 4 age ranges of the present study (7–10 wk, 10–13 wk, 13–17 wk, and 17–20 wk) for the 2 groups. *P < 0.05 vs. LZR at that age.
Measurement of vascular NO bioavailability. From each rat, the abdominal aorta was removed and vascular NO production was assessed using amperometric sensors (World Precision Instruments, Sarasota, FL). Briefly, aortae were isolated, sectioned longitudinally, pinned in a silastic-coated dish, and superfused with warmed (37°C) physiological salt solution equilibrated with 95% O2 and 5% CO2. An NO sensor (ISO-NOPF 100) was placed in close apposition to the endothelial surface, and a baseline level of current was obtained. Subsequently, increasing concentrations of methacholine (10^-10, 10^-6 M) were added to the bath and the changes in current were determined to verify that responses represented NO release, these procedures were repeated following pretreatment of the aortic strip with L-NAME (10^-4 M).

Determination of vascular metabolites of arachidonic acid. Vascular production of 6-keto-prostaglandin F1α (6-keto-PGF1α), the stable breakdown product of PGI2 (34, 39), and 11-dehydro-thromboxane B2 (11-dehydro-TxB2; the stable plasma breakdown product of TxA2) (6) in response to challenge with reduced PO2 was assessed using amperometric sensors (World Precision Instruments, Sarasota, FL). Briefly, aortae were isolated, sectioned longitudinally, pinned in a silastic-coated dish, and superfused with warmed (37°C) physiological salt solution equilibrated with 95% O2 and 5% CO2. After this time, the superfusate was removed, stored in a new microcentrifuge tube, and frozen in liquid N2, while a new aliquot of PSS was added to the vessels and the equilibration gas was switched to 0% O2 for the subsequent 30 min. After the second 30-min period, this new PSS was transferred to a fresh tube, frozen in liquid N2, and stored at −80°C. Metabolite release by the vessels was determined using commercially available EIA kits for 6-keto-PGF1α and 11-dehydro-TxB2 (Cayman).

Histological determination of microvessel density. From each rat, the gastrocnemius muscle from the left leg was removed, rinsed in PSS, and fixed in 0.25% formalin. Muscles were embedded in paraffin and cut into 5 μm cross sections. Sections were incubated with Griffonia simplicifolia I lectin (GS-1), alkaline phosphatase (AlkPhos), and dipeptidylpeptidase IV (DPEP IV), for subsequent determination of microvessel density. GS-1 is a general stain that labels all microvessels <20 μm in diameter (28), AlkPhos preferentially stains arteriolar ends of capillaries (2), and DPEP IV preferentially stains venular ends of capillaries (37). Labeled microvessel density was determined using fluorescent (for GS-1) or light microscopy (for AlkPhos and DPEP IV) as described previously (22).

Leukocyte-endothelial cell interactions. In a separate cohort of LZR and OZR at 7 and 10 wk of age, an in situ extensor digitorum longus muscle was prepared as described previously (50). After preparation of the muscle for imaging, 10 random high magnification fields of view (Plan-Neo 20×/0.5 and 40×/0.75; Zeiss) were recorded.

Fig. 2. Data describing the dilator reactivity of ex vivo first order skeletal muscle (gracilis) resistance arterioles from LZR and OZR at 7 wk of age. Data are presented as means ± SE; n = vessels from 8–10 animals in for OZR and n = vessels from 6–8 animals for LZR. *P < 0.05 in the upper bound (A–C) or magnitude of the response (D) vs. LZR; †P < 0.05 in the upper bound (A–C) or magnitude of the response (D) vs. OZR. L-NAME, Nω-nitro-l-arginine methyl ester.
using a high-speed cooled digital imaging system (Axiocam HSM; Zeiss) for later analyses. Animals received a bolus injection of rhodamine 6G (0.3 mg/kg; Sigma) to permit visualization of leukocytes. Rhodamine 6G-labeled vascular cells were visualized by epillumination at 510–560 nm, using a 590-nm emission filter. The number of rolling and adherent leukocytes in venules was expressed as cells per squared millimeter of endothelial surface, calculated from the diameter and length of the venular segment. Leukocytes were considered adherent if they remained stationary for a 30-s observation period. Leukocytes were considered rolling if their velocity was less than 250 μm/s (54).

**Contribution of TNF-α and TxA2.** In a separate cohort of rats, OZR were treated with daily injections of either pentoxifylline (TNF-α production inhibitor; 30 mg·kg⁻¹·day⁻¹) or SQ-29548 (PGH₂/TxA₂ receptor antagonist; 2 mg·kg⁻¹·day⁻¹) from 7 wk to 13 wk of age to determine the impact of cessation of treatment on the progression of microvascular rarefaction. Animals were euthanized at 7, 10, or 13 wk of age, and microvessel density was determined as described above, with biochemical determinations of plasma TNF-α, TxA₂, and nitrotyrosine. In a final group of animals, OZR that had received chronic injections of SQ-29548 had treatments terminated at 13 wk of age and were left untreated out to 20 wk of age. These OZR were used at 17 and 20 wk of age to determine the impact of cessation of treatment on the progression of microvascular rarefaction.

**Data and statistical analyses.** The mechanical responses of isolated arterioles following pharmacological challenge were fit with the three-parameter logistic equation:

\[ y = \min + \{\max - \min\}/\left(1 + 10^{(\log ED_{50} - x)}/H11001\right) \]

where

\[ y \]

represents the change in arteriolar diameter; \( \min \) and \( \max \) represent the lower and upper bounds, respectively, of the change in arteriolar diameter with increasing agonist concentration; \( x \) is the logarithm of the agonist concentration; and \( \log ED_{50} \) represents the logarithm of the agonist concentration at which the response \( (y) \) is halfway between the lower and upper bounds.

All data are presented as means ± SE. Statistically significant differences in measured and calculated parameters in the present study were determined using ANOVA. In all cases, Student-Newman-Keuls post hoc test was used when appropriate, and \( P < 0.05 \) was taken to reflect statistical significance. For the analyses of inflammatory and other putative biomarkers between groups, we used discriminant techniques to eliminate the univariate nature of ANOVA and issues of independent variable co-linearity, which minimizes the utility of regression techniques. Discriminant analyses represent a classification technique for evaluating the contribution of multiple variables to the establishment of differences between experimental groups. These analyses result in a rank ordering of ceteris paribus correlation coefficients in terms of their significance for the establishment of differences between groups.

**RESULTS**

Data describing the baseline characteristics of LZR and OZR at the different age ranges in the present study are summarized...
in Table 1. Although both strains of animals demonstrated a steady growth in their size with maturation, OZR were consistently much heavier than LZR at the same age and gradually demonstrated an increase in the presentation of the metabolic syndrome with age. By ∼13 wk of age, OZR were exhibiting the full manifestation of the metabolic syndrome, with profound obesity, impaired glycemic control, and dyslipidemia and the establishment of a moderate hypertension.

Figure 1 summarizes the progressive rarefaction of the skeletal muscle microcirculation in LZR and OZR across the age ranges in the present study (Fig. 1A). Although microvessel density was similar between LZR and OZR at ∼7 wk of age, OZR demonstrated an initial phase of microvascular rarefaction, such that microvessel density was significantly decreased by 10 wk of age. Microvessel density was stable in OZR from that point until ∼13 wk of age wherein a second phase of rarefaction occurred that resulted in another significant reduction to microvessel density. Microvessel density was only modestly reduced from that point to 20 wk of age in OZR. Figure 1B presents the magnitude of microvessel loss over the four age ranges of the present study. These results further clarify that there are two periods of time in which the rate of rarefaction in skeletal muscle of OZR is substantially elevated, between 7 and 10 and 13 and 17 wk of age, with more quiescent periods with regard to microvessel density changes between 10 and 13 and 17 and 20 wk of age.

Data describing arteriolar reactivity to dilator stimuli from LZR and OZR at 7 wk of age are summarized in Fig. 2. In response to increasing concentrations of ACh (Fig. 2A), the dilator response in arterioles from OZR was not different from that in LZR. Pretreatment of vessels with the antioxidant TEMPOL had no impact on dilator responses to ACh in either strain, although pretreatment with the NO synthase inhibitor L-NAME virtually abolished all mechanical reactivity to ACh in OZR. For increasing concentrations of arachidonic acid (Fig. 2B), arterioles from OZR exhibited a similar dilator response compared with LZR, and this was not impacted by TEMPOL treatment. However, application of L-NAME reduced arachidonic acid-induced dilation in arterioles from OZR. Dilator responses to sodium nitroprusside (Fig. 2C) did not differ between arterioles of LZR and OZR at 7 wk of age and were unaffected by treatment with either TEMPOL or L-NAME.

Hypoxic dilation of resistance arterioles from OZR was not different from that in LZR and was unaffected following
pretreatment with TEMPOL. Pretreatment with L-NAME resulted in a consistent reduction to hypoxic dilation in OZR. In all cases, responses of LZR following treatment with TEMPOL were not significantly impacted, whereas L-NAME treatment significantly attenuated responses to ACh, arachidonic acid, and hypoxia (data not shown).

Figure 3 describes dilator responses of arterioles from LZR and OZR at 10 wk of age in response to imposed stimuli. ACh caused a concentration-dependent dilation of arterioles from OZR that was very similar to that in LZR and was not significantly impacted by pretreatment with TEMPOL (Fig. 3A). However, pretreatment with L-NAME abolished reactivity to ACh in arterioles from OZR. Dilator responses to arachidonic acid (Fig. 3B) were reduced in vessels from OZR versus LZR, and pretreatment with TEMPOL restored much of this lost reactivity. Pretreatment with L-NAME reduced dilator responses of arterioles from OZR with increasing concentrations of arachidonic acid. Dilator responses to increasing concentrations of sodium nitroprusside were not different between LZR and OZR. Hypoxic dilation of resistance arterioles from OZR was reduced compared with that for vessels from LZR, but was nearly restored following pretreatment with TEMPOL (Fig. 3D). Incubation of vessels with L-NAME resulted in a significant reduction to hypoxic dilation in vessels from OZR. In all cases, responses of vessel from LZR following treatment with TEMPOL were not impacted, whereas L-NAME treatment significantly attenuated responses to ACh, arachidonic acid, and hypoxia (data not shown).

Data describing the dilator responses of arterioles from LZR and OZR at 13 wk of age in response to imposed challenges are summarized in Fig. 4. In response to increasing concentrations of ACh, dilator responses from arterioles of OZR were reduced from that in LZR and were restored by pretreatment with TEMPOL (Fig. 4A). As above, pretreatment with L-NAME abolished arteriolar reactivity to ACh in vessels from OZR. Dilator responses to arachidonic acid were reduced in OZR at 13 wk of age, and pretreatment with TEMPOL was able to improve this response (Fig. 4B). Pretreatment of vessels with L-NAME resulted in a significant reduction to arachidonic acid-induced dilation in OZR. Dilator responses of resistance arterioles from LZR and OZR in response to sodium nitroprusside were not impacted at this age (Fig. 4C). Hypoxic dilation (Fig. 4D) in arterioles from OZR was reduced compared with that in LZR, and pretreatment with TEMPOL resulted in a significant improvement to dilator responses to reduced PO2. As above, pretreatment with L-NAME significantly reduced...
dilator responses to low oxygen tension in OZR at this age range. In all cases, responses of vessel from LZR following treatment with TEMPOL were not impacted, whereas l-NAME treatment significantly attenuated responses to ACh, arachidonic acid, and hypoxia (data not shown).

Figures 5 and 6 present the data describing arteriolar dilator reactivity from LZR and OZR at 17 and 20 wk of age, respectively. Although the magnitude of the individual impairments and the specific effects of acute pharmacological intervention were somewhat different between the two ages, they were directionally consistent. In OZR, dilator responses to increasing concentrations of ACh were impaired versus responses in LZR (Figs. 5A and 6A). In both cases, treatment with TEMPOL resulted in significant improvement to the response, whereas treatment with l-NAME abolished all reactivity to ACh. Similarly, responses of arterioles from OZR to arachidonic acid were significantly blunted compared with that in LZR (Figs. 5B and 6B). Pretreatment with TEMPOL improved reactivity, although this appeared to have a diminished effectiveness at the 20 wk age, and treatment with l-NAME was virtually without effect. Responses to nitroprusside were largely intact, although the maximum dilator response was increasingly blunted in vessels from OZR with increasing age, and this was not altered by treatment with TEMPOL or l-NAME, suggesting that this may reflect a structural effect (Figs. 5C and 6C). Dilator responses to hypoxia were reduced in OZR at both age groups. Although TEMPOL improved the response at 17 and 20 wk of age, pretreatment with l-NAME did not have a significant impact.

Figure 7 summarizes data describing the agonist-induced NO bioavailability in arteries of LZR and OZR at increasing age. In arteries of rats at 7 wk (Fig. 7A) and 10 wk (Fig. 7B) of age, vascular NO bioavailability was not different between LZR and OZR and was unaffected as a result of pretreatment with the antioxidant TEMPOL (Fig. 7A). However, by 13 wk (Fig. 7C), 17 wk (Fig. 7D), and 20 wk (Fig. 7E) of age, the vascular NO bioavailability from OZR was significantly reduced compared with that in LZR and demonstrated a worsening with increasing age. Treatment with TEMPOL was effective at restoring NO bioavailability in OZR with increasing age, although the magnitude of this effect appeared to be diminished with increasing age. At all ages, pretreatment of vessels with l-NAME nearly abolished any methacholine-induced NO bioavailability in LZR or OZR.
Figure 8 presents the correlation of vascular NO bioavailability with skeletal muscle microvessel density in LZR and OZR at two age ranges, 7–10 wk (Fig. 8A) and 17–20 wk (Fig. 8B). In younger OZR, vascular NO bioavailability is an extremely poor predictor of the early loss in microvessel density, suggesting that NO-independent processes may be responsible for the early pulse of rarefaction in these animals. However, in older OZR, the severity of the reduction in NO bioavailability is a very strong predictor of microvessel density (Fig. 8B).

The vascular production of 6-keto-PGF$_1$α (Fig. 9A; the stable breakdown product of PGI$_2$) and 11-dehydro-TxB$_2$ (Fig. 9B; the stable breakdown product of TxA$_2$) in arteries from LZR and OZR in response to hypoxia over the age ranges of the present study is summarized in Fig. 9. As shown in Fig. 9A, hypoxia-stimulated production of PGI$_2$ was comparable between LZR and OZR at younger ages. However, as OZR continued to age, and with increasing severity of the metabolic syndrome, these responses began to diverge and the production of PGI$_2$ in arteries of OZR was significantly reduced compared with responses in LZR. In contrast, vascular production of TxA$_2$ was consistently elevated in OZR compared with LZR under resting conditions at all ages (Fig. 9B).
was exacerbated with increasing age, such that the vascular production of TxA2 in OZR was consistently much higher than in LZR, although it did appear to plateau between 13 and 17 wk of age.

With the use of differential immunohistochemistry, the changes in microvessel density in the skeletal muscle of LZR and OZR at increasing ages are summarized in Fig. 10. Although staining with GS-1 presented an aggregate change in microvessel density and clearly demonstrated the progressive rarefaction in OZR with increasing age, staining with AlkPhos (which preferentially stains terminal arterioles and the arteriolar ends of capillaries) (2, 34) demonstrated an extremely consistent response to that determined with GS-1 at 7 wk (Fig. 10A), 10 wk (Fig. 10B), 13 wk (Fig. 10C), 17 wk (Fig. 10D), and 20 wk (Fig. 10E) of age. However, staining with DPEP IV (which preferentially stains small venules and venular ends of capillaries) demonstrated reduced venular capillary/venular staining that preceded any reduction in either GS-1 or AlkPhos staining at 7 wk of age and demonstrated a more severe reduction at each age in OZR compared with GS-1 and AlkPhos.

Figure 11 presents data describing leukocyte adhesion and rolling in post-capillary skeletal muscle venules of LZR and OZR at 7–10 wk of age (data from both ages were combined to produce 1 panel for each). The number of adherent (Fig. 11A) and rolling (Fig. 11B) leukocytes in these vessel segments in the skeletal muscle of OZR was significantly elevated compared with that in LZR at both 7 and 10 wk of age.

Based on these results, we engaged in a discovery process to determine biomarkers that correlated well with the early pulse of microvascular rarefaction in OZR, with the results summarized in Table 2. Although plasma levels of the inflammatory biomarkers TNF-α, monocyte chemoattractant protein (MCP-1), sICAM, and sVCAM all demonstrated the appropriate temporal characteristics, TNF-α and both oxidant stress (plasma nitrotyrosine; Table 1) and vascular production of TxA2 (Fig. 9B) were the three strongest predictors of early reduction in microvessel density in skeletal muscle of OZR based on discriminant analysis (Table 3). MCP-1, sICAM, and sVCAM represented the grouping of the next three strongest predictors of the early phase of rarefaction with all other measured biomarkers assuming an inconsistent or not statistically significant role.

Data describing the effects of chronic treatment of OZR with either pentoxifylline (inhibitor of TNF-α production) or SQ-29548 (PGH2/TxA2 receptor blocker) from 7 to 13 wk of age on microvessel density in OZR and LZR are presented in Fig. 12.
12. In control OZR, microvascular rarefaction proceeded as described above. However, in response to chronic treatment with either pentoxifylline or SQ-29548, the rarefaction that normally develops in OZR up to 13 wk of age was severely attenuated, and there was almost no reduction in microvessel density compared with responses in LZR (Fig. 12A). As a result of the chronic treatment with pentoxifylline, levels of both vascular TxA2 production (Fig. 12C) and systemic oxidant stress (Fig. 12D) were reduced, although still elevated compared with levels in LZR. However, chronic inhibition of the effects of TxA2 did not alter circulating levels of TNF-α (Fig. 12B) or oxidant stress (Fig. 12D) compared with untreated OZR.

The effects of chronically treating OZR with SQ-29548 from 7 wk to 13 wk of age, followed by termination of treatment and the animal being allowed to age out to 20 wk are summarized in Fig. 13. As above, chronic treatment of OZR with the antagonist of the PGH2/TxA2 receptor nearly abrogated the early pulse of rarefaction in OZR to 13 wk of age. Subsequent withdrawal of treatment resulted in gradual rarefaction of the microvascular network, although the rate of this reduction in microvessel density in OZR was sufficiently slow that by 20

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**Fig. 10.** The change in skeletal muscle microvessel density in LZR and OZR at 7 (A), 10 (B), 13 (C), 17 (D), and 20 (E) wk of age. Results are presented following tissue staining with Griffonia simplicifolia 1 lectin (GS-1 lectin), alkaline phosphatase (AlkPhos), and dipeptidylpeptidase IV (DPEP IV). Data are presented as means ± SE; n = 8–10 animals in each age group for OZR and n = 6–8 animals in each age group for LZR. *P < 0.05 vs. LZR at that age.
wk of age, the microvessel density in OZR was still not different from that determined in untreated age-matched LZR.

DISCUSSION

One of the established microvascular consequences of the progressive development of the metabolic syndrome in both rodent models and human subjects is an evolving rarefaction of the microvessel networks. This progressive reduction in microvessel density has multiple implications with regard to the integrated processes of mass transport and exchange within tissue/organisms and represents a significant contributor to the clinical manifestation of peripheral vascular disease in afflicted subjects and the inherent reduction in patient quality of life and resistance to treatment and healing, as well as accelerated mortality. However, although there is a general understanding of the extent of microvessel rarefaction in the OZR model of the metabolic syndrome, and preliminary evidence that the magnitude of the reduction to microvessel density is associated with the reduction in vascular NO bioavailability, there is a very limited understanding of the initiating factors underlying microvascular rarefaction and how the condition develops. This study was designed to address this current gap in our understanding.

The primary observation from the current study is that the evolution of microvascular rarefaction in the skeletal muscle of OZR occurs via two general phases, and early phase of rarefaction between 7 and 10 wk of age, followed by a slower rate of decay between 13 and 17 wk of age, culminating with a final later phase that occurs between 13 and 17 wk of age (Fig. 1). There is an additional lower rate of microvessel loss after 17 wk of age, but our unpublished work suggests that net microvessel loss develops asymptotic behavior after 17 wk of age. What was most interesting about this observation is that our previous results suggested that the change in skeletal muscle microvessel density would parallel the changes to vascular NO bioavailability (21, 23). However, as demonstrated in Figs. 2–6 and especially in Fig. 7, this assumption is not supported by the present data. Although vascular NO bioavailability does decay with progression of the metabolic syndrome in OZR, this is delayed by several weeks compared with the decay in skeletal muscle microvessel density, such that the initial reduction in vascular density cannot be predicted by a change in vascular NO levels (Fig. 8A). Interestingly, the ultimate extent of rarefaction is well correlated by the loss in NO bioavailability, as suggested by the results from previous studies (8, 12, 17, 26, 40), where physiological and pharmacological interventions to maintain NO bioavailability also blunt the severity of the reductions to microvessel density. Taken together, the results of the present study and the prior ones suggest that although the ultimate loss in vascular NO bioavailability is a strong predictor and potentially a causal contributor to the extent of microvascular rarefaction in OZR, it cannot and does not explain the initial phase of rarefaction that occurs between 7 and 10 weeks of age where NO bioavailability remains intact.

This study represents one of a limited number that not only details the progression of specific characteristics of the meta-

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**Table 2. Plasma markers of oxidant stress and inflammation in the present study**

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<th>Age, weeks</th>
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<th>OZR</th>
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<td>7</td>
<td>8 ± 2</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>10 ± 2</td>
<td>22 ± 3*</td>
</tr>
<tr>
<td>13</td>
<td>14 ± 3</td>
<td>41 ± 5*</td>
</tr>
<tr>
<td>17</td>
<td>16 ± 4</td>
<td>54 ± 6*</td>
</tr>
<tr>
<td>20</td>
<td>20 ± 3</td>
<td>56 ± 7*</td>
</tr>
<tr>
<td>sVCAM, pg/ml</td>
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<tr>
<td>7</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
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<tr>
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<td>10 ± 1</td>
<td>14 ± 2</td>
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<tr>
<td>13</td>
<td>11 ± 1</td>
<td>21 ± 3*</td>
</tr>
<tr>
<td>17</td>
<td>12 ± 2</td>
<td>27 ± 4*</td>
</tr>
<tr>
<td>20</td>
<td>13 ± 3</td>
<td>29 ± 5*</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
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<tr>
<td>7</td>
<td>1.6 ± 0.4</td>
<td>4.0 ± 1.0*</td>
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<tr>
<td>10</td>
<td>2.4 ± 0.4</td>
<td>9.9 ± 0.9*</td>
</tr>
<tr>
<td>13</td>
<td>2.8 ± 0.3</td>
<td>11.4 ± 1.8*</td>
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<tr>
<td>17</td>
<td>3.2 ± 0.4</td>
<td>13.4 ± 1.6*</td>
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<tr>
<td>20</td>
<td>3.4 ± 0.5</td>
<td>13.2 ± 1.7*</td>
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<tr>
<td>Monocyte chemoattractant protein-1, pg/ml</td>
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<td>29 ± 5</td>
<td>35 ± 6</td>
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<tr>
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<td>32 ± 6</td>
<td>65 ± 8*</td>
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<tr>
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<td>91 ± 11*</td>
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<tr>
<td>17</td>
<td>38 ± 5</td>
<td>102 ± 12*</td>
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<tr>
<td>20</td>
<td>40 ± 6</td>
<td>114 ± 11*</td>
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<tr>
<td>sICAM, pg/ml</td>
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<tr>
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<td>40 ± 6</td>
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<tr>
<td>10</td>
<td>29 ± 7</td>
<td>63 ± 5*</td>
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<tr>
<td>13</td>
<td>34 ± 6</td>
<td>64 ± 6*</td>
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<tr>
<td>17</td>
<td>38 ± 5</td>
<td>72 ± 7*</td>
</tr>
<tr>
<td>20</td>
<td>42 ± 5</td>
<td>80 ± 7*</td>
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*P < 0.05 vs. LZR.
bolic syndrome in OZR but also ties this risk factor progression to specific indexes of vascular/microvascular dysfunction. With its severe leptin resistance and impaired satiety reflex, OZR are significantly heavier than LZR by 7 wk of age, and this difference continues to grow out to 20 wk of age. This is paralleled by a steadily increasing severity of insulin resistance out to about 17 wk of age, although after this time, our OZR began exhibiting type II diabetes mellitus. The development of hypertension also was delayed somewhat, becoming clearly evident by 17 wk of age. The development of the more traditional characteristics of the metabolic syndrome can also be tracked with the progression of chronic inflammation, since levels of TNF-α were elevated very quickly with the presence of obesity and impaired glycemic control, followed by MCP-1 and the adhesion molecules (Table 2). This allows for a very interesting observation - that microvascular network structure (i.e., microvessel density) may be altered in the OZR before there is a significant deterioration to arteriolar endothelial function or vascular reactivity. This is a particularly intriguing possibility, since it is frequently assumed that the changes to endothelial function are the initiating factor for the genesis of microvasculopathy. Clearly, this may not be fully accurate under conditions of the metabolic syndrome, and the contributions of venular dysfunction (rather than arteriolar) and other

Table 3. Structure matrix of results from discriminant analyses for markers of inflammation between animal groups in the present study

<table>
<thead>
<tr>
<th>Age</th>
<th>7 Weeks</th>
<th>10 Weeks</th>
<th>13 Weeks</th>
<th>17 Weeks</th>
<th>17 Weeks</th>
<th>17 Weeks</th>
</tr>
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<tr>
<td>Functions</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Percent variance</td>
<td>86.4</td>
<td>11.8</td>
<td>80.8</td>
<td>10.9</td>
<td>64.2</td>
<td>8.4</td>
</tr>
<tr>
<td>Canonical correlation</td>
<td>0.841</td>
<td>0.720</td>
<td>0.812</td>
<td>0.684</td>
<td>0.622</td>
<td>0.564</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.584*</td>
<td>0.633*</td>
<td>0.588*</td>
<td>0.663*</td>
<td>0.424</td>
<td>0.468</td>
</tr>
<tr>
<td>Thromboxane A2</td>
<td>0.622*</td>
<td>0.708*</td>
<td>0.637*</td>
<td>0.589*</td>
<td>0.324</td>
<td>0.408</td>
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<tr>
<td>Reactive oxygen species</td>
<td>0.588*</td>
<td>0.628*</td>
<td>0.484*</td>
<td>0.824*</td>
<td>0.624*</td>
<td>0.588*</td>
</tr>
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</table>

*Represents largest absolute correlation between each variable and any discriminant function.
indexes of endothelial function under these conditions of elevated CVD risk may warrant future investigation.

With the use of differential immunohistochemistry, the present results suggest that microvessel rarefaction does not reflect a general global degradation of microvessels or microvessel segments. Rather, based on the results presented in Fig. 10, it is apparent that microvessel degradation is initiated at the venular ends of capillaries or at the level of the smallest venules and proceeds in a retrograde fashion toward the arteriolar ends of capillaries/terminal arterioles, as the fall in DPEP IV staining preceded the fall in either GS-1 lectin or alkaline phosphatase staining at all ages. This observation suggests that changes in venular endothelial function may be the initiating factor for the development of rarefaction in the skeletal muscle of OZR. This hypothesis received further support since indexes of leukocyte adhesion (Fig. 11A) and rolling (Fig. 11B) for in situ skeletal muscle of OZR were significantly increased in animals between 7 and 10 wk of age, during the initial phase of rarefaction.

Tightly intertwined with the general processes of leukocyte adhesion and rolling are elevations in circulating markers of inflammation, oxidant stress, and their associated sequelae. From this, we engaged in a biomarker discovery process to determine which (if any) markers of inflammation were associated with the development of the metabolic syndrome in OZR before 10 wk of age and, through the use of multivariate discriminant analyses, were strongly predictive of the change in microvessel density over that time period. As shown in Table 2, the inflammatory biomarkers MCP-1, TNF-α, sICAM, and sVCAM and the oxidant stress marker nitrotyrosine were all associated with the development of the metabolic syndrome at that age range. However, the markers TNF-α, nitrotyrosine, and TxA2 production were the most powerfully predictive of the early phase of rarefaction (Table 3), with MCP-1, sICAM, and sVCAM being somewhat less powerfully predictive.

To investigate the potential contributions of TxA2 and TNF-α on the early pulse of microvessel rarefaction in skeletal muscle of OZR, animals were given daily treatments to block the action of TxA2 (SQ-29548) or the production of TNF-α (pentoxifylline) from 7 wk of age out to 13 wk. As shown in Fig. 12A, treatment with either SQ-29548 or pentoxifylline was effective at blunting the initial phase of microvascular rarefaction in OZR compared with the reductions in microvessel density determined in the untreated control animals. Interestingly, treatment with pentoxifylline reduced plasma levels of TNF-α (Fig. 12B), nitrotyrosine (Fig. 12D), and vascular production of TxA2 (Fig. 12C) in OZR. However, treatment of OZR with SQ-29548 had virtually no effect on these three biomarkers, but had a very similar beneficial outcome for microvessel density. Although not definitive, these results suggest that increased oxidant stress and TNF-α that are present in the vasculature of OZR may reside upstream of the elevations in TxA2 and that alterations in arachidonic acid metabolism are an outcome of the changes to oxidant stress and inflammation rather than a contributing factor to them.

To provide further insight into the effects of blunting the early phase of rarefaction on the final extent of the microvessel degradation in OZR, a separate cohort of rats was treated with daily injections of SQ-29548 out to 13 wk of age, after which time treatment was discontinued and the animal was allowed to age further without subsequent intervention. As shown in Fig. 13, treatment with the PGH2/TxA2 receptor antagonist provided the expecting blunting in the development of skeletal muscle microvascular rarefaction in OZR to 13 wk of age, after which the reduction in microvessel density began. However, whether this is a re-initiation of the TxA2-dependent early pulse of rarefaction or the NO-dependent later pulse of rarefaction (since this is the temporal window where NO bioavailability was becoming significantly compromised) is unknown at this time and represents a potential area for future investigation. Although an initial observation, this has the potential for excellent translational importance, since it suggests that if the early pulse of rarefaction can be prevented in subjects where the severity of the metabolic syndrome is still moderate, the rate at which microvessels degrade with further progression/duration of the metabolic syndrome can be blunted, with potential reductions to the severity of the negative outcomes associated with chronic metabolic syndrome. This may be an especially compelling observation that warrants further validation and targeted investment into the specific vascular, metabolic, and proteomic environments that may have been altered to impact the severity of microvascular rarefaction development.

No experimental study is without its limitations and the present one is no exception. In this study, we examined microvascular structure/function and made comparisons between three distinct muscles, gracilis muscle (resistance arterioles), gastrocnemius muscle (microvessel density), and EDL (leukocyte adhesion/rolling). Although this was a function of the infrastructure and expertise present in the laboratories of the research team, this does allow for the introduction of potential bias owing to disparities between the microvascular and skeletal muscle cell environment between the three muscles. It is unclear at this time if either this bias or its severity was actually present, and this does need to be kept in mind. Additionally, the agent used for inhibition of TNF-α (pentoxifylline) does have other nonspecific effects beyond simply blocking the production of this inflammatory biomarker, including its actions as a mild phosphodiesterase inhibitor and as a weak antagonist of adenosine type 2 receptors (52). Finally, although the results from the present study
provide compelling evidence for the pursuit of higher resolution mechanistic contributors to rarefaction, the loss of microvessel density in OZR clearly demonstrates an asymptotic relationship as the animal approaches 20 wk of age. Whether this represents a decline in the stimuli for rarefaction (unlikely) or an increase in the intensity of the stimuli for angiogenesis as a compensation is unknown, and the mechanistic integration of these stimuli represents areas for further investigation.

Summary. Although it has been well established that one of the microvasculopathies that accompanies the chronic presentation of the metabolic syndrome is a rarefaction of the skeletal muscle microvascular networks, how this develops from a temporal perspective and the mechanistic contributors have not been well elucidated. The results from the present study suggest that the reduction to microvessel density develops along a distinct time line, with an early phase of rarefaction that is dependent on the generation and actions of reactive oxygen species, specific markers of inflammation and dysfunction to the venular endothelium and the actions of TxA2. Subsequently, the networks enter into a moderately quiescent period, and then into a second phase of rarefaction that is associated with the evolving reductions to vascular NO bioavailability. Furthermore, the importance of the venular dysfunction is highlighted by the apparent retrograde behavior of microvessel degradation, implicating the venular endothelium as the initiating site of rarefaction. Although specific signaling pathways and cellular environmental influences underlying this loss in microvessel density in the metabolic syndrome remain to be elucidated, the temporally distributed nature of the mechanisms and the potential for an early intervention that can prevent a larger long-term negative outcome have clear implications for the possible reduction in the negative health outcomes associated with chronic presentation of the metabolic syndrome.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions


References


