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ALTERED DISTRIBUTION OF ADRENERGIC CONSTRICTOR RESPONSES CONTRIBUTES TO SKELETAL MUSCLE PERFUSION ABNORMALITIES IN METABOLIC SYNDROME

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Abstract

Purpose—Although studies suggest elevated adrenergic activity paralleling metabolic syndrome in obese Zucker rats (OZR), the moderate hypertension and modest impact on organ perfusion questions the multi-scale validity of these data.

Methods—To understand how adrenergic function contributes to vascular reactivity in OZR, we utilized a multi-scale approach to investigate pressure responses, skeletal muscle blood flow and vascular reactivity following adrenergic challenge.

Results—For OZR, adrenergic challenge resulted in increased pressor responses vs. lean Zucker rats (LZR); mediated via α_1 receptors, with minimal contribution by either ROS or NO bioavailability. *In situ* gastrocnemius muscle of OZR exhibited blunted functional hyperemia, partially restored with α_1 inhibition, although improved muscle performance and VO_2 required combined treatment with TEMPOL. Within OZR *in situ* cremaster muscle, proximal arterioles exhibited a more heterogeneous constriction to adrenergic challenge, biased toward hyperresponsiveness, vs. LZR. This increasingly heterogeneous pattern was mirrored in *ex vivo* arterioles, mediated via α_1 receptors, with roles for ROS and NO bioavailability evident in hyperresponsive vessels only.

Conclusions—These results support the central role of the α_1 adrenoreceptor for augmented pressor responses and elevations in vascular resistance, but identify an increased heterogeneity of constrictor reactivity in OZR that is presently of unclear purpose.

INTRODUCTION

As has been well established, the development of peripheral vascular disease (PVD) risk factors of sufficient severity can lead to profound alterations in the ability of resistance vessels to regulate their degree of tone, and thus the levels of perfusion to and within the tissues and organs they serve (17). Multiple previous studies across laboratories have implicated alterations to vascular nitric oxide (NO) bioavailability and effectiveness (3), altered arachidonic acid metabolism and the ensuing impacts on vascular tone (7, 15), the impacts of reactive oxygen species (ROS; 13, 19), myogenic activation (16, 23, 28) and alterations mediated via adrenergic signaling and vascular reactivity (1, 6, 21). However, it is unclear that conclusions from previous studies on the impact of altered adrenergic signaling/responses represent an accurate reflection of the true alterations to the integrated system of microvascular perfusion and control within the setting of elevated PVD risk.

The metabolic syndrome is a multi-pathology state represented by the combined presentation of multiple risk factors for PVD, including obesity, impaired glycemic control, atherogenic dyslipidemia, and moderate hypertension; with the additional systemic outcomes of a pro-oxidant, pro-inflammatory and pro-thrombotic state (4, 18). Arguably the most important outcome of this condition is that it results in the impairment of perfusion (both bulk perfusion and the spatial-temporal matching of perfusion with metabolic demand) in the tissues and organs of the afflicted subject (8, 12). Previous studies have provided compelling evidence that adrenergic traffic (5), adrenergic signaling (21) and adrenergic vascular responses (9) may all be elevated within the metabolic syndrome (the setting for elevated PVD risk). Further, it can clearly be demonstrated that treatment of the moderate hypertension within metabolic syndrome in the obese Zucker rat (OZR) model with prazosin (50 µg/kg) not only abolishes the elevated blood pressure that develops within this state, it can equalize blood pressure characteristics to those determined in the control strain, the lean Zucker rat (LZR, 27). However, what is unclear at this point is how a general elevation in vascular adrenergic output with the potential for multiple contributing elements, producing significant elevations in vascular resistance, functions within the *in vivo* setting to produce the relatively mild/moderate elevations in arterial pressure that have been determined (9, 29). Clearly, we have not arrived at an accurate understanding of adrenergic control over muscle perfusion in the OZR model.

The purpose of the present study was to employ a multi-scale approach, integrating *in vivo*, *in situ*, and *ex vivo* conditions to garner a more accurate understanding of the changes in adrenergic control that are implicit for the development of skeletal muscle microvasculopathy within the metabolic syndrome. *In vivo* approaches will incorporate whole animal pressor responses with hindlimb blood flow measurements in anesthetized animals, while *in situ* approaches will employ perfusion/muscle performance relationships for gastrocnemius muscle and direct microscopic evaluation of vascular reactivity in cremaster muscle, and *ex vivo* approaches will employ studies of isolated vascular responses under specific challenged states. Taken together, these data will allow for a more accurate understanding of adrenergic function in the control of muscle blood flow in OZR with the full manifestation of the metabolic syndrome.

MATERIALS AND METHODS

Animals

Male LZR (n=27) and OZR (n=45) were delivered at 6–7 weeks of age, and after one week of acclimation to the local environment, were aged to ~17 weeks for final experiment usage. All animals were used between 16 and 18 weeks of age. Animals were fed standard chow and tap water *ad libitum* for all experiments unless otherwise noted. Animals were housed in an accredited animal care facility, and all protocols received prior IACUC approval from the West Virginia University. At ~17 weeks of age, each rat was anesthetized with injections of sodium pentobarbital ($50 \text{ mg} \cdot \text{kg}^{-1}$ i.p.), and all rats 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 intravenous infusion of additional substances as necessary (e.g., anesthetic, heparin, etc.). In addition, an aliquot of mixed venous blood was drawn from the jugular vein cannula for a full profiling of metabolic and endocrine biomarkers (see below).

Experimental Series #1: In Vivo Whole Pressor Responses

Following the initial surgical preparation (above), the femoral artery was isolated midway between the femoral triangle and the knee and a perivascular blood flow probe was placed around the vessel (Transonic 0.7V) which was held in place via a micromanipulator. After a period of equilibration, each animal was challenged with an intravenous infusion of the α_1/α_2 -adrenoreceptor agonist norepinephrine ($10 \mu\text{g}/\text{kg}$), with pressor responses and hindlimb blood flow continuously recorded. Subsequently, rats received a bolus intravenous infusion of the α_1 adrenoreceptor antagonist prazosin ($1 \text{ mg}/\text{kg}$), the α_2 adrenoreceptor antagonist yohimbine ($5 \text{ mg}/\text{kg}$), or the α_1/α_2 adrenoreceptor antagonist phentolamine ($10 \text{ mg}/\text{kg}$), each followed by 30 minutes of equilibration, in order to remove different components of adrenergic tone from the system (n=5 for each antagonist; total n=15 for each strain). After a second norepinephrine challenge with the respective antagonist present, animals were treated with bolus intravenous infusions of TEMPOL ($50 \text{ mg}/\text{kg}$) and a final subsequent treatment with L-NAME ($100 \text{ mg}/\text{kg}$) to assess the roles of reactive oxygen species and nitric oxide bioavailability in contributing to responses following adrenergic challenge. After treatment with TEMPOL and again after the treatment with L-NAME, a new challenge with norepinephrine was performed as described above. Alterations in mean arterial pressure and femoral artery perfusion were monitored following agonist infusion in order to determine peak responses and the restoration of baseline (pre-treatment) conditions. All infused intravenous doses of drugs were corrected for differences in circulating blood volume between LZR and OZR at this age (10, 24).

Experimental Series #2: In Situ Skeletal Muscle Perfusion

In a separate cohort of LZR and OZR (n=5 for LZR; n=15 for OZR), the left gastrocnemius muscle was isolated *in situ* as fully described previously (9). Subsequently, a perivascular flow probe (Transonic 0.5V or 0.7V) was placed around the femoral artery, immediately proximal to its entry into the muscle group, in order to measure blood flow to the gastrocnemius muscle. At the conclusion of these procedures, an angiocatheter (24 gauge) was inserted into the femoral vein to allow for sampling of venous blood from the

contracting muscle to determine blood gas levels (done using a Corning Rapid Lab Blood Gas Analyzer). The preparation was covered in PSS-soaked gauze and plastic film to minimize evaporative water loss and was placed under a heat lamp to maintain temperature at 37°C. At this time, heparin (500 IU/kg) was infused via the jugular vein to prevent blood coagulation.

Upon completion of the surgical preparation, the gastrocnemius muscle was stimulated to perform (via the sciatic nerve) bouts of isometric twitch contractions (4 Hz, 0.4 ms duration, 5V) lasting for 3 minutes followed by 15 minutes of self-perfused recovery time, with arterial pressure and femoral artery blood flow continuously monitored. Following the initial contraction regimen under control conditions, rats were given an intravenous injection of either prazosin (1 mg/kg; n=5), phentolamine (5 mg/kg; n=5) or yohimbine (5 mg/kg; n=5) and the contraction regimen was repeated. Each animal was treated with only one adrenoreceptor antagonist. As above, following treatment of the animal with either adrenoreceptor antagonist, animals were treated with TEMPOL and L-NAME as described above and the contraction regimen was repeated with all data and blood collection also repeated as described above.

Experiment Series #3: In Situ Skeletal Muscle Arterioles

In a dedicated cohort of rats (n=7 for LZR; n=15 for OZR), the left cremaster muscle was prepared for television microscopy (9). After completion of the preparation, the cremaster muscle was superfused with PSS, equilibrated with a gas mixture containing 5% CO₂ and 95% N₂, and maintained at 35°C as it flowed over the muscle. The ionic composition of the PSS was as follows (mM): NaCl 119.0, KCl 4.7, CaCl₂ 1.6, NaH₂PO₄ 1.18, MgSO₄ 1.17, and NaHCO₃ 24.0. Arteriolar diameter was determined with an on-screen video micrometer. After an initial post-surgical equilibration period of 30 minutes, proximal (~75 µm diameter) and distal arterioles (~30 µm diameter) were selected for investigation in a clearly visible region of the muscle. 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 10⁻⁵ M adenosine. All arterioles that were studied were located in a region of the muscle that was away from any incision.

Following an equilibration period, the responses of selected arterioles within the cremaster muscle of LZR and OZR were assessed in response to increasing concentrations of norepinephrine (10⁻¹⁰ – 10⁻⁵ M) or phentolamine (10⁻¹⁰ – 10⁻⁵ M), as described above, to establish baseline reactivity to increasing adrenergic receptor activation and inhibition, respectively. As above, following washout, the cremaster muscle was treated with TEMPOL by adding it to the superfusate (10⁻⁴ M), and the challenge with increasing concentrations of norepinephrine and phentolamine was repeated. Finally, the TEMPOL-treated cremaster muscle was also treated with L-NAME (10⁻⁴ M; in the superfusate) and challenge with the adrenergic agonist and antagonist was repeated.

Experiment Series #4: Ex Vivo Isolated Skeletal Muscle Resistance Arterioles

In anesthetized rats, prior to the preparation of the cremaster muscle (above), the intramuscular continuation of the right gracilis artery was identified, it's *in vivo* diameter

determined using an eyepiece micrometer, and the vessel was surgically removed. In LZR, arteriolar diameter estimated using this method was $105 \pm 4 \mu\text{m}$, while in OZR, the value was reduced to $98 \pm 4 \mu\text{m}$. Arterioles were placed in a heated chamber (37°C) that allowed the vessel lumen and exterior to be perfused and superfused, respectively, with physiological salt solution (PSS; equilibrated with 21% O_2 , 5% CO_2 ; 74% N_2) from separate reservoirs. Vessels were cannulated at both ends and were secured to inflow and outflow pipettes connected to a reservoir perfusion system allowing intraluminal pressure and luminal gas concentration to be controlled. Vessel diameter was measured using television microscopy and an on-screen video micrometer. Arterioles were extended to their *in situ* length and were equilibrated at $\sim 80\%$ of the animal's mean arterial pressure ($\sim 80 \text{ mmHg}$ for LZR, $\sim 100 \text{ mmHg}$ for OZR). Active tone for vessels in the present study, calculated as $(D/D_{\text{max}}) \cdot 100$, where D is the diameter increase from rest in response to Ca^{2+} -free PSS, and D_{max} is the maximum diameter measured at the equilibration pressure in Ca^{2+} -free PSS, averaged $33 \pm 3\%$ in LZR and $35 \pm 3\%$ in OZR. To get a more accurate presentation of the impact of altered adrenergic function on microvascular perfusion, we elected to reduce the inclusion criteria for isolated arterioles in this study. Traditionally, three criteria are employed to assess the viability of an individual vessel: 1) active tone $>25\%$, 2) robust constrictor response to challenge with phenylephrine, 3) viable endothelial layer (dilator response to pharmacological challenge such as methacholine). However, as one of the inclusion criteria represent the key outcome variable of the present study, this has the potential to cause experimental bias in terms of which vessels get included into experiments/analyses and which vessels are omitted for failing to achieve all three criteria. For the purposes of the present study, we omitted criterion #2 and simply considered vessels with sufficient active tone and viable endothelial function as having met the inclusion requirements.

Prior to subsequent evaluation of arteriolar reactivity, the *in vivo* diameter of vessels was restored through addition of low levels of norepinephrine to the vessel chamber. This process required $\sim 3 \times 10^{-10} \text{ M}$ norepinephrine in vessels from OZR. While vessels from LZR usually regained their *in vivo* diameter without treatment with norepinephrine ($n=12$), some vessels required a maximum norepinephrine concentration of $\sim 1 \times 10^{-10} \text{ M}$ ($n=3$). Following an equilibration period, arteriolar constriction was assessed in response to increasing concentrations of phenylephrine ($10^{-10} \text{ M} - 10^{-5} \text{ M}$) or clonidine ($10^{-10} \text{ M} - 10^{-5} \text{ M}$) to establish baseline reactivity to α_1 - and α_2 -adrenoreceptor agonists, respectively. Subsequently, reactivity of these isolated arterioles from LZR and OZR was assessed following treatment of the vessel with TEMPOL (10^{-4} M), and following incubation with L-NAME (10^{-4} M) to the TEMPOL-treated vessel.

Data and Statistical Analyses

In all cases, $p < 0.05$ was taken to reflect statistical significance. All data are presented as mean $\pm$ SE.

In Vivo Pressor Response Experiments

All arterial pressure, hindlimb blood flow, and calculated hindlimb vascular resistance (pressure/flow) data are presented as the change in the parameter following treatment or challenge (mmHg, ml/g/min, mmHg/(ml/g/min), respectively).

Vascular Reactivity Experiments

Arteriolar constrictor responses following challenge with adrenergic agonists or antagonists were fit with a three-parameter logistic equation:

$$y = \min + \left[\frac{\max - \min}{1 + 10^{\log ED_{50} - x}} \right]$$

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 (x) at which the response (y) is halfway between the lower and upper bounds. Statistically significant differences in lower bound employed ANOVA followed by Student-Newman-Keuls-test post-hoc as appropriate. Statistically significant differences in the distribution of adrenergic responses between LZR and OZR utilized Student’s t -test for mean and variance.

For the purposes of categorizing arteriolar responses to adrenergic challenge into “high”, “normal”, and “low” responders, the reactivity of all vessels (both OZR and LZR) was compared to mean responses in LZR. Given the degree of heterogeneity in the responses in OZR, a value of $\pm 20\%$ was used as the threshold for inclusion into the “high” or “low” responder categories.

In Situ Gastrocnemius Muscle Experiments

Muscle blood flow data were normalized to gastrocnemius muscle mass, which did not differ between LZR (2.29 ± 0.08 g) and OZR (2.25 ± 0.09 g). Muscle oxygen uptake (VO_2) was calculated using the Fick equation:

$$VO_2 = Q \times (CaO_2 - CvO_2)$$

where Q represents blood flow, CaO_2 represents arterial oxygen content and CvO_2 represents venous oxygen content. Muscle fatigue curves were fit using a semi-logarithmic relationship and the slope (β) coefficient was determined using curve fitting techniques. Differences in muscle blood flow, O_2 extraction, VO_2 and the slope coefficient describing muscle fatigue curves of contraction were determined using ANOVA, with Student-Newman-Keuls-test post-hoc as appropriate.

RESULTS

As summarized in Table 1, ~17 week old OZR demonstrated the full complement of the metabolic syndrome. This included obesity, impaired glycemic control, dyslipidemia and moderate hypertension. In addition, OZR also demonstrated a high level of chronic oxidant stress and inflammation, with elevated levels of nitrotyrosine and TNF- α , respectively.

Table 2 presents the initial conditions for the vascular/systemic phenotypes across the conditions of the present study. In these data, blockade of the α_1 adrenergic system was effective in blunting the development of hypertension in OZR, as well as many of the vascular diameter and perfusion responses system. Further, the impact altering the vascular NO bioavailability in OZR in terms of contributing to integrated vascular function was relatively modest compared to the impact of alterations to adrenergic function.

Figures 1 and 2 summarize data describing the pressor responses (Panels A), hindlimb blood flow (Panels B) and perfusion resistance (Panels C) in OZR versus LZR under control conditions and following treatment with phentolamine (Figure 1) and prazosin or yohimbine (Figure 2). Any differences between LZR and OZR in terms of blood pressure and the pressor response to intravenous infusion of norepinephrine itself were abolished by treatment with either prazosin (the α_1 blocker) or phentolamine (the α_1/α_2 blocker), with minimal impact of yohimbine (the α_2 blocker). Treatment of OZR with TEMPOL (to blunt ROS and increase NO bioavailability) or TEMPOL/L-NAME (to remove NO bioavailability from a low ROS condition) had minimal impact on systemic responses to adrenergic challenges.

Data describing the fatigue curves (Panel A), active hyperemia (Panel B), oxygen extraction (Panel C) and oxygen uptake (VO_2 ; Panel D) for *in situ* gastrocnemius muscle of LZR and OZR contracting at 4Hz (isometric twitch) under control conditions and following treatment with phentolamine are presented in Figure 3. Following three minutes of imposed elevations in metabolic demand, OZR demonstrated an increased development of muscle fatigue, coincident with a blunted hyperemic response. While O_2 extraction was very similar between the two strains, the combination of extraction and reduced blood flow resulted in a significant reduction in VO_2 . Treatment of OZR with the combined α_1/α_2 adrenoceptor antagonist phentolamine improved hyperemic responses when given alone, with additional improvements to muscle performance and VO_2 when followed up with antioxidant (TEMPOL) treatment. Treatment of OZR with prazosin (Figure 4) mirrored the effect of phentolamine, with an improved hyperemic responses when given alone and improved muscle performance and VO_2 when given with the antioxidant. Any beneficial impacts of TEMPOL in OZR were abolished by combined treatment with TEMPOL and L-NAME. Treatment of OZR with the α_2 -adrenoceptor antagonist yohimbine had no consistent or significant effect from control values on muscle performance, hyperemic responses or blood gas exchange measurements (Figure 5).

Table 3 presents the baseline characteristics of *in situ* cremaster muscle proximal and distal arterioles from LZR and OZR for the experiments described in Figures 6–9. Figures 6 and 7 summarize data describing the responses of *in situ* cremaster muscle arterioles located proximally within the microvascular network to bi-directional manipulation of adrenergic stimulation. In response to increasing adrenergic stimulation (increased norepinephrine) proximal arterioles from both LZR and OZR exhibited robust constrictor responses (Figure 6). However, while arterioles from LZR exhibited a relatively consistent response, with 38/50 vessels constricting by at least 60% of their resting diameter with 10^{-6} M norepinephrine, there was a significant shift in the distribution of reactivity with the development of the metabolic syndrome in OZR as only 19/50 vessels demonstrated a

comparable response, while 21/50 vessels exhibited a stronger degree of reactivity, with a significantly reduced ED₅₀ value, essentially closing off fully at lower concentrations of stimulation, and 10/50 vessels exhibited a significant reduction to constrictor responses, with an elevated ED₅₀. In both LZR and OZR, treatment with either TEMPOL or L-NAME had minimal consistent impacts on adrenergic responses in either strain.

With increasing phentolamine treatment (Figure 7), used to simulate progressive removal of adrenergic tone to proximal arterioles of LZR and OZR, “normal” responders in both strains exhibited comparable dilation. However, arterioles in both strains that were identified as being “high” responders exhibited a very limited dilator response with increasing phentolamine concentration, while arterioles that were “low” responders to norepinephrine challenge demonstrated the greatest dilation in response to increasing concentration of phentolamine. In both strains, neither TEMPOL nor L-NAME treatment had a consistent and significant impact on the phentolamine-induced dilation in “normal” and “high” responders. However, in “low” responders of either strain, treatment of arterioles with L-NAME blunted dilator responses to phentolamine.

Using the data presented above, Figure 8 presents the relationship between *in situ* proximal arteriolar constrictor responses to 10⁻⁸ M norepinephrine and the number of occurrences (out of a total n=50 for each strain). These data clearly illustrate the changing distribution in constrictor responses to adrenergic challenge, where LZR exhibit a tighter distribution of responses with lower variability than OZR, where proximal arteriolar responses to adrenergic challenge are more distributed, with a greater occurrence of constrictor responses at the “tails” of the distribution. Statistical analysis of the mean and variance between LZR and OZR demonstrated that the distributions of adrenergic responses were significantly different between the two strains.

Figure 9 presents the responses of distal *in situ* cremaster muscle arterioles of LZR and OZR to increasing concentrations of norepinephrine (Panels A and B) and phentolamine (Panels C and D), respectively. Under neither control conditions, nor pre-treatment conditions (TEMPOL or L-NAME) were responses of *in situ* distal arterioles significantly different between strains, nor was any evidence for an altered distribution of reactivity present (Panel E).

Figure 10 presents the reactivity of *ex vivo* gracilis muscle first order resistance arterioles of LZR and OZR to increasing concentrations of the α₁ adrenoreceptor agonist phenylephrine (for data describing the baseline characteristics of *ex vivo* arterioles, please see Table 3). When the inclusion criteria of “sufficient adrenergic reactivity” was eliminated (please see above), a similar widening of constrictor responses to adrenergic challenge was determined in OZR as compared to LZR (Panel A). In “high” responders, the increased constrictor reactivity was significantly attenuated following treatment of the vessel with TEMPOL, while treatment with L-NAME was without effect (Panel B). This effect was less evident in “normal” (Panel C) and “low” (Panel D) responding arterioles from OZR, such that treatment with either TEMPOL or L-NAME resulted in minimal impact to phenylephrine-induced reactivity. Constrictor responses to increasing concentrations of the α₂

adrenoreceptor agonist clonidine did not exhibit a difference between LZR and OZR (Panel E), and this was not significantly impacted by treatment with TEMPOL or L-NAME.

DISCUSSION

The underlying logic of the present study stemmed from a lack of clarity between the results of previous studies suggesting an increase in sympathetic activity (5), adrenergic constrictor reactivity (9, 11) and an increase in some elements of adrenergic intracellular signaling (21) in metabolic syndrome, despite the consistent observation that the development of both hypertension and any elevations in vascular resistance of adrenergic origin were relatively modest (4, 12, 16, 29). This suggests that a potential disconnect may exist between the interpretations from data collected at higher resolutions (e.g., *in situ* vascular networks, *ex vivo* resistance vessels), and responses determined under *in vivo* conditions. The present study was designed to address this discrepancy by identifying the source for this lack of clarity.

At the lowest level of spatial resolution, the *in vivo* preparation, OZR clearly exhibited an increased pressor response following adrenergic challenge as compared to responses determined in LZR, while use of adrenoreceptor antagonists demonstrated that this increased response was largely mediated via the α_1 receptors. While consistent with previous evidence (9, 11), results from the present study also provide insight into the role of vascular oxidant stress/nitric oxide bioavailability balance on the pressor response to adrenoreceptor stimulation. Despite the clearly established presence of elevated vascular oxidant stress in OZR, the current results suggest that the reduction in vascular ROS levels following TEMPOL treatment was without significant impact on the magnitude of the pressor response in OZR, nor did the subsequent L-NAME-based abolition of rescued nitric oxide bioavailability consistently impact the magnitude of the pressor responses between groups. Given the well-established relationships in question and the effectiveness of the treatment interventions in impacting ROS and NO levels, the most reasonable interpretation of these data is that, in comparison to the α_1 -mediated responses, a manipulation of vascular nitric oxide bioavailability was of insufficient significance to impact pressor responses at this level of resolution.

When taking these observations to the next higher level of resolution, the *in situ* skeletal muscle, a roughly comparable condition was evident to that for the *in vivo* setting. Specifically, the accelerated rate of muscle fatigue and blunted active hyperemia with 4Hz twitch contraction in OZR vs. LZR were improved following α_1 receptor blockade (but only with concurrent antioxidant treatment); with no significant effect associated with α_2 receptor antagonism under any condition. This enhanced function was associated with the greatest improvements to VO_2 which also suggests that the combination of adrenoreceptor antagonism and antioxidant therapy results in an overall improvement to the perfusion-based elements of mass transport and exchange beyond that for α_1 adrenoreceptor antagonism alone. Further, the importance of “rescued” vascular nitric oxide bioavailability on contributing to skeletal muscle blood flow, especially during periods of elevated metabolic demand, was demonstrated following the application of L-NAME under TEMPOL-treated conditions.

These results also provide support for previous observations indicating that general blunting of adrenergic constriction can improve hyperemic responses in skeletal muscle in metabolic syndrome (8, 9, 11, 21), but are of limited effectiveness in terms of improving muscle performance unless treatments are combined with antioxidant agents to directly improve endothelial function (8). As antioxidant treatment alone was without impact on either bulk flow responses or muscle performance in the absence of α_1 adrenoreceptor inhibition, these observations suggest that a conceptual division may be appropriate for the impact of the metabolic syndrome muscle performance outcomes. It may be that the modest reduction in skeletal muscle blood flow in OZR primarily reflects adrenergic constraint sufficient to hinder metabolic sympatholysis, while endothelial dysfunction (although ubiquitously present) is more important for the higher resolution matching of perfusion distribution to metabolic need within tissue.

Previous studies have suggested that there is a general increase in sympathetic traffic, intracellular signaling, and/or vascular constrictor responses to adrenergic challenge (5, 9, 21). However, as stated above, *in vivo* data do not strongly support this contention. Results from the *in situ* cremaster muscle of OZR as compared to responses in LZR may provide insight into this discrepancy, as constrictor responses of proximal resistance arterioles to increasing adrenergic stimulation exhibited a much more consistent response in LZR than in OZR, with a narrower distribution. In contrast, the responses to adrenergic challenge in OZR exhibited a larger spread in their magnitude with a greater proportion of vessels demonstrating both increased and decreased responsiveness (although more commonly toward increased reactivity). Similarly, when treated with increasing concentrations of the α_1/α_2 adrenoreceptor antagonist phentolamine, arterioles that were classified as “high responders” exhibited the lowest degree of dilation, while the reverse was true for vessels that were “low responders”. Obviously, this somewhat skewed, but broader distribution of adrenergic constrictor responses, may contribute to the *in vivo* outcomes that have been identified in OZR, and may explain why they are more modest than would otherwise be predicted.

Based on the current data, some of this heterogeneity in reactivity may reflect variability in the initial conditions. Arterioles with increased tone under resting conditions will be unable to exhibit the full range of constriction in response to any stimulus as compared to levels from those of a greater initial diameter/reduced level of tone. Conversely, those with less tone (greater diameter) at rest have less ability to respond to the removal of adrenergic constriction as compared to those with a greater degree of tone. Given this, the greater heterogeneity of reactivity in the microcirculation of OZR vs. LZR may reflect the concept of the “optimal diameter” or “optimal wall tension” as originally described by Gore (14). In that work, the author determined that the greatest degree of reactivity was realized when vessels had the ability to constrict or dilate through a set range of optimal responsiveness and that moving the initial condition away from this range in either direction was associated with a severe decline in stimulus-induced reactivity. It may be that the more heterogeneous reactivity of proximal resistance arterioles from OZR in response to adrenergic challenge could partially reflect a loss of coordination through the sympathetic nerves that results in an increasingly heterogeneous initial “resting” condition which impacts adrenergic control over resistance at both the individual vessel and network levels. Recent work by Rachev and

colleagues (22) has shed further light on this concept and provided compelling evidence of an optimal state of vascular mechanics that may result in the most efficient adaptability to changing conditions.

Interestingly, the increased variability in resistance arteriolar adrenergic reactivity in OZR following development of the metabolic syndrome in proximal arterioles, was not evident in distal arterioles. Using intravital microscopy, our group has shown that adrenergic vasoconstriction, in the rat gluteus maximus microcirculation, displays spatial-dependency. In this work, we reported that the greatest α_{1R} and α_{2R} effects were noted in lower order (proximal) arterioles (i.e., 1A and 2A); whereas, sympathetically-mediated peptidergic and purinergic control dominated in higher order distal and terminal arterioles (2). Such spatial heterogeneity in sympathetic control provides a plausible explanation for the lack of adrenergic influences measured in distal microcirculation of OZR. Thus, future work addressing the contributions of NPY and ATP to microvascular regulation/dysregulation in the metabolic syndrome will likely reveal new mechanisms of sympathetic dysregulation in the distal microcirculation.

An important component of the present study was the removal of “robust adrenergic reactivity” as an inclusion criterion for the study of *ex vivo* resistance arterioles. When this was incorporated, a comparable pattern of divergence in adrenergic constrictor reactivity is present in the larger resistance arterioles of OZR vs. LZR to that for the proximal arterioles within the cremaster muscle. The increased variability in the adrenergic constrictor reactivity of *ex vivo* arterioles of OZR appears reflect intrinsic vascular, and perhaps endothelial cell, function itself, as treatment of “high responders” with TEMPOL blunted the adrenergic responses, which was subsequently abolished following additional treatment with L-NAME. These effects were not observed in “normal” or “low” responders where the ability of TEMPOL and/or L-NAME to impact adrenergic constriction was severely attenuated. As a result of a relaxed inclusion criteria, the magnitude of the net increase in adrenergic constriction in large resistance arterioles of OZR was elevated as compared to LZR, but to a far less extent than previously estimated.

The results of the present study are important for several reasons. Foremost, these data provide evidence for the role of altered adrenoreceptor reactivity in the metabolic syndrome on integrated vascular function from a multi-scale perspective. Clearly, the increased pressor response to adrenergic challenge largely reflects activity mediated through the α_1 receptor and does not appear to be substantially impacted by treatment against elevated oxidant stress or a potential loss in vascular nitric oxide bioavailability. While responses from the hindlimb preparation suggested that hyperemic responses of OZR skeletal muscle can be improved by α_1 adrenoreceptor inhibition, additional antioxidant treatment of OZR was required to improve muscle performance, suggesting divergent roles for adrenergic constraint on bulk flow and endothelial function for higher resolution perfusion:demand matching. Finally, results at the highest levels of spatial resolution, the *in situ* cremaster muscle and *ex vivo* microvessel revealed an increased diversity of vascular reactivity in OZR following adrenergic challenge. The immediate question from these results is why an increased diversity of adrenergic constrictor reactivity develops in OZR with progression of metabolic syndrome.

There does not appear to be evidence that a compensatory change in constrictor reactivity for the shifts in adrenergic responses develops in OZR, as there is no evidence that changes in myogenic activation between strains or within an individual animal that would match with increased adrenergic reactivity heterogeneity exists (i.e., vessels with low adrenergic reactivity having elevated myogenic responses, etc.). Additionally, there is no prior evidence to which the authors are aware that constrictor responses to other agents (e.g., endothelin, angiotensin II, serotonin) are significantly altered in OZR (27). However, there is evidence from some investigators that pressor reflexes may be blunted in OZR, although the mechanism underlying this and its generalizability for the model remain unclear (25). As such, one is left with speculation. Do vessels with “low” adrenergic constriction help the networks maintain an appropriate active hyperemic response, or at least largely maintain it? In an animal model that has been established as suffering from a profound loss of the microvascular network flexibility necessary to respond to imposed stressors (8), does the increased heterogeneity of adrenergic responses help to maintain system flexibility?

Perhaps even more importantly for clinical/population health outcomes, alterations in adrenergic tone and function have been previously implicated as contributing to the regulation of microvessel density, and chronic α_1 adrenoreceptor inhibition with prazosin is an established systemic model of angiogenesis (30). Of greatest relevance to the present study, may be recent work from the Hass group, where chronic prazosin treatment blunted the development of microvessel rarefaction in chronic corticosterone-treated rats (20). Given the presence of high cortisol/corticosterone levels in OZR, it may be that tissue regions containing resistance arterioles with elevated adrenergic reactivity may be associated with earlier or more severe levels of microvascular rarefaction (12). At this point, answers to the above questions are unknown. However, the challenge that the metabolic syndrome and PVD risk pose for public health outcomes (26), combined with the clear potential for improving microvascular network function and perfusion with appropriate intervention, make this a compelling avenue for future investigation.

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List of Abbreviations

LZR	lean Zucker rat
OZR	obese Zucker rat
PRZ	prazosin
PHT	phentolamine
YOH	yohimbine
ROS	reactive oxygen species
NO	nitric oxide

PVD	peripheral vascular disease
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl
L-NAME	L-N ^G -Nitroarginine methyl ester
PSS	physiological salt solution
VO₂	oxygen uptake
Q	muscle blood flow
C_aO₂	arterial oxygen content
C_vO₂	venous oxygen content

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Figure 1.

In vivo pressor responses (Panel A), *in situ* hindlimb blood flow (Panel B) and calculated vascular resistance across the hindlimb (Panel C) for LZR and OZR following intravenous infusion of 10 mg/kg norepinephrine. Data are presented as the change in the respective parameter from unstimulated, under control conditions and following pre-treatment with phentolamine (PHT), phentolamine+TEMPOL (PHT-TEM) or phentolamine+L-NAME (PHT-LNM). * $p < 0.05$ vs. LZR in that condition; † $p < 0.05$ vs. CON within that strain. Data are presented as mean $\pm$ SE; $n = 5$ for LZR; $n = 15$ for OZR; please see text for details.

Figure 2.

In vivo pressor responses (Panel A), *in situ* hindlimb blood flow (Panel B) and calculated vascular resistance across the hindlimb (Panel C) for LZR and OZR following intravenous infusion of 10 mg/kg norepinephrine. Data are presented as the change in the respective parameter from unstimulated, under control conditions and following pre-treatment with prazosin (PRZ), prazosin+TEMPOL (PRZ-TEM), prazosin+L-NAME (PRZ-LNM), yohimbine (YOH), yohimbine+TEMPOL (YOH-TEM) or yohimbine+L-NAME (YOH-LNM).. * $p < 0.05$ vs. LZR in that condition; † $p < 0.05$ vs. CON within that strain. Data are presented as mean $\pm$ SE; $n = 5$ for LZR; $n = 15$ for OZR; please see text for details.

Figure 3.

Data describing the performance (Panel A) and active hyperemic responses (Panel B) of *in situ* gastrocnemius muscle of LZR and OZR contracting at 4Hz (isometric twitch). Also presented are O₂ extraction (Panel C) and oxygen uptake (VO₂, Panel D) at three minutes of the contraction regimen. Data are presented under untreated control conditions and following pre-treatment with phentolamine (PHT), phentolamine+TEMPOL (PHT-TEM) or phentolamine+L-NAME (PHT-LNM). * p<0.05 vs. LZR; † p<0.05 vs. OZR; ‡ p<0.05 vs. OZR + PHT. Data are presented as mean±SE; n=5 for LZR; n=15 for OZR; please see text for details.

Figure 4.

Data describing the performance (Panel A) and active hyperemic responses (Panel B) of *in situ* gastrocnemius muscle of LZR and OZR contracting at 4Hz (isometric twitch). Also presented are O₂ extraction (Panel C) and oxygen uptake (VO₂, Panel D) at three minutes of the contraction regimen. Data are presented under untreated control conditions and following pre-treatment with prazosin (PRZ), prazosin+TEMPOL (PRZ-TEM) or prazosin +L-NAME (PRZ-LNM). * p<0.05 vs. LZR in that condition; † p<0.05 vs. CON (100%) within that strain; ‡ p<0.05 vs. PRZ within that strain. Data are presented as mean±SE; n=5 for LZR; n=15 for OZR; please see text for details.

Figure 5.

Data describing the performance (Panel A) and active hyperemic responses (Panel B) of *in situ* gastrocnemius muscle of LZR and OZR contracting at 4Hz (isometric twitch). Also presented are O₂ extraction (Panel C) and oxygen uptake (VO₂, Panel D) at three minutes of the contraction regimen. Data are presented under untreated control conditions and following pre-treatment with yohimbine (YOH), yohimbine+TEMPOL (YOH-TEM) or yohimbine+L-NAME (YOH-LNM). * p<0.05 vs. LZR in that condition. Data are presented as mean±SE; n=5 for LZR; n=15 for OZR; please see text for details.

Figure 6.

Constrictor responses from *in situ* cremaster muscle proximal arterioles from LZR (Panel A) and OZR (Panel D) in response to increasing concentrations of norepinephrine. In Panels A and D, different levels of reactivity are colored such that “high” responders are red, “low” responders are green and “normal” responders are blue. These colors are “greyed” in subsequent panels to facilitate comparisons. Panels B and E present the impact of TEMPOL on arteriolar constrictor responses in LZR and OZR, respectively. Panels C and F present the impact of L-NAME on arteriolar constrictor responses in LZR and OZR, respectively. * $p < 0.05$ vs. “normal” in that strain; † $p < 0.05$ vs. responses in untreated arterioles within that strain and reactivity category. Data are presented as mean $\pm$ SE; $n=7$ for LZR; $n=15$ for OZR; please see text for details.

Figure 7.

Dilator responses from *in situ* cremaster muscle proximal arterioles from LZR (Panel A) and OZR (Panel D) in response to increasing concentrations of phentolamine. In Panels A and D, different levels of reactivity are colored such that “high” responders are red, “low” responders are green and “normal” responders are blue (classification of responders was performed for Figure 8). These colors are “greyed” in subsequent panels to facilitate comparisons. Panels B and E present the impact of TEMPOL on arteriolar dilator responses in LZR and OZR, respectively. Panels C and F present the impact of L-NAME on arteriolar dilator responses in LZR and OZR, respectively. * $p < 0.05$ vs. “normal” in that strain; † $p < 0.05$ vs. responses in untreated arterioles within that strain and reactivity category. Data are presented as mean $\pm$ SE; $n = 7$ for LZR; $n = 15$ for OZR; please see text for details.

Figure 8.

Distribution of constrictor responses of *in situ* cremaster muscle proximal arterioles to 10^{-8} M norepinephrine. Data are presented as the frequency of occurrence for a level of constrictor response (out of 50 occurrences) for arterioles from LZR and OZR; demonstrating the widening and flattening of the distribution in OZR as compared to that for LZR. The distribution for LZR passes the normality test ($p=0.834$), while that for OZR does not ($p=0.025$). As such, while the distributions for LZR and OZR are considered to be significantly different ($p=0.026$), they cannot both be classified as normal distributions.

Figure 9.

Mechanical responses from *in situ* cremaster muscle distal arterioles from LZR (Panel A) and OZR (Panel B) in response to increasing concentrations of norepinephrine (Panels A and B, respectively) or phentolamine (Panels C and D, respectively). Data are presented under untreated control conditions and following pre-treatment of the cremaster muscle with TEMPOL or L-NAME. Also presented is the distribution of constrictor responses of *in situ* cremaster muscle proximal arterioles to 10^{-8} M norepinephrine. Data are presented as the frequency of occurrence for a level of constrictor response (out of 50 occurrences) for arterioles from LZR and OZR; demonstrating the lack of a difference between the distribution for LZR and OZR ($p=0.909$). Data are presented as mean $\pm$ SE; $n=7$ for LZR; $n=15$ for OZR; please see text for details. Please see text for details.

Figure 10.

Constrictor responses from *ex vivo* gracilis muscle resistance arterioles from LZR and OZR in response to increasing concentrations of phenylephrine (α_1 agonist; Panels A–D) or clonidine (α_2 agonist; Panel E). Data for phenylephrine-induced constriction under control conditions are presented in Panel A, which also demonstrate the different classes of reactivity. These data are “greyed” in subsequent panels such that the impact of pre-treatment with TEMPOL, L-NAME or both are presented for vessels in the “high” (Panel B), “normal” (Panel C) and “low” responder (Panel D) categories. * $p < 0.05$ vs. “normal” in that strain; † $p < 0.05$ vs. responses in untreated arterioles within that strain and reactivity category. Data are presented as mean $\pm$ SE; $n = 5$ for LZR; $n = 15$ for OZR; please see text for details.

Table 1

Baseline characteristics of 17 week-old LZR and OZR used in the present study

	LZR	OZR
Mass (g)	358±11	679±12 *
MAP (mmHg)	98±4	129±6 *
[Glucose] _{plasma} (mg/dl)	105±9	164±11 *
[Insulin] _{plasma} (ng/ml)	1.4±0.2	7.6±1.0 *
[Cholesterol] _{plasma} (mg/dl)	84±7	132±11 *
[Triglycerides] _{plasma} (mg/dl)	91±10	361±20 *
[Nitrotyrosine] _{plasma} (ng/ml)	11±3	44±6 *

*
p<0.05 versus LZR.

Baseline hemodynamic characteristics of 17 week-old LZR and OZR used in the present study under control conditions and in response to the different interventional treatments.

Table 2

		MAP	Q _{FEM}	R _{FEM}	(a-v)O ₂		MAP	Q _{FEM}	R _{FEM}	(a-v)O ₂
LZR	Control	98±4	0.77±0.06	127±8	0.037±0.005	OZR	131±4 [*]	0.60±0.06 [*]	218±15 [*]	0.037±0.005
	+PRZ	82±5 [‡]	0.80±0.10	100±9 [‡]	0.038±0.004		91±5 [‡]	0.70±0.07	137±11 ^{**‡}	0.037±0.005
	+PRZ/TEM	82±6 [‡]	0.82±0.07	98±8 [‡]	0.038±0.004		90±5 [‡]	0.68±0.07 [*]	132±10 ^{**‡}	0.038±0.004
	+PRZ/LNM	88±5	0.78±0.06	113±7	0.041±0.005		95±5 [‡]	0.62±0.05 [*]	153±12 ^{**‡}	0.037±0.006
	+YOH	94±5	0.79±0.08	121±10	0.039±0.005		124±4 [*]	0.64±0.06 [*]	194±13 [*]	0.038±0.005
	+YOH/TEM	92±4	0.78±0.07	118±9	0.039±0.005		124±5 [*]	0.65±0.07 [*]	185±12 ^{**‡}	0.039±0.006
	+YOH/LNM	98±5	0.75±0.06	131±9	0.039±0.006		126±4 [*]	0.61±0.05 [*]	207±14 [*]	0.038±0.005
	+PHT	75±4 [‡]	0.82±0.08	92±9 [‡]	0.040±0.006		92±5 ^{**‡}	0.65±0.06 [*]	145±9 ^{**‡}	0.038±0.005
	+PHT/TEM	76±4 [‡]	0.82±0.06	93±6 [‡]	0.039±0.005		90±5 [‡]	0.66±0.07 [*]	136±10 ^{**‡}	0.040±0.006
	+PHT/LNM	85±5 [‡]	0.75±0.07	113±6	0.038±0.006		95±6 [‡]	0.62±0.05 [*]	157±9 ^{**‡}	0.037±0.005

AMP: mean arterial pressure (mmHg); Q_{FEM}: femoral artery blood flow (ml/g/min); R_{FEM}: resistance (mmHg/[ml/g/min]); (a-v)O₂: oxygen extraction (ml/ml blood).

^{*} p<0.05 versus LZR value.

[‡] p<0.05 vs. strain control.

Table 3

Baseline vascular characteristics of 17 week-old LZR and OZR used in the present study under control conditions and in response to the different interventions. Data are presented for *in situ* cremaster muscle proximal (CPA) and distal arterioles (CDA) as well as *ex vivo* gracilis muscle resistance arterioles (GA).

		ID _{CPA}	MaxD _{CPA}	AT _{CPA}	ID _{CDA}	MaxD _{CDA}	AT _{CDA}	ID _{GA}	MaxD _{GA}	AT _{GA}
LZR	Control	76±4	118±4	36±5	34±4	58±5	41±5	121±3	186±5	35±4
	+TEMPOL	76±5	----	35±5	34±5	----	41±4	----	----	----
	+L-NAME	72±4	----	39±4	32±4	----	45±4	----	----	----
OZR	Control	73±4	110±5	34±4	31±4	53±5	42±5	116±4	175±6	34±5
	+TEMPOL	76±4	----	31±4	34±4	----	36±4	118±4	----	33±5
	+L-NAME	72±3	----	35±5	31±5	----	42±4	114±5	----	36±4

ID: internal diameter (resting; μm); MaxD: maximum diameter (μm) under 10^{-3}M adenosine + 10^{-3}M sodium nitroprusside (*in situ*) or Ca^{2+} -free conditions (*ex vivo*); AT: active tone (%) calculated as: Despite multiple trends, no significant differences in the results below were determined.