Effects of aging, TNF-\(\alpha\), and exercise training on angiotensin II-induced vasoconstriction of rat skeletal muscle arterioles

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AEROBIC EXERCISE CAPACITY has been shown to decline with advancing age. This decline in exercise capacity is partly due to an impaired ability to increase blood flow to working muscles during exercise (35, 46). One local mechanism that could mediate the impaired exercise hyperemia in skeletal muscle is a dysfunctional endothelium-dependent nitric oxide (NO)-mediated vasodilator function (12, 16, 17, 26, 41, 63). Long-term aerobic exercise training has been reported to ameliorate this old age-associated dysfunction through the endothelium-dependent vasodilation and the NO synthase (NOS) signaling pathway in humans and animals (17, 50, 51, 58). Although the mechanisms through which aging and exercise training alter endothelium-dependent vasodilation have been previously investigated, the effects of aging and exercise training on the modulation of vasconstrictor responses, such as angiotensin II (ANG II), in the peripheral resistance vasculature have not been as clearly delineated.

ANG II is the main biologically active peptide of the renin-angiotensin system, which exerts both hemodynamic and renal effects. For example, ANG II plays an important physiological role in the regulation of blood pressure, plasma volume, and sympathetic nerve activity in the cardiovascular system (5). In regard to the regulation of blood pressure, ANG II is a potent substance capable of constricting arterioles mainly through smooth muscle cell ANG II type 1 receptors (AT1R). However, the endothelium also appears to play an important role in ANG II-induced vasoconstriction because AT1R and ANG II type 2 receptors (AT2R) are expressed in endothelial cell, and activation of these receptors evokes vasodilation (7, 9, 31, 37, 48, 60). Therefore, ANG II-induced vasconstriction is determined by the interaction of vasoconstrictor and vasodilator influences mediated through these vascular cells, and understanding the role of the smooth muscle and endothelial cells is important to elucidate the mechanisms of adaptation in ANG II-induced vasoconstriction of resistance arterioles with aging and exercise training.

The etiology of old age-associated vascular dysfunction has been postulated to be related to oxidative stress and vascular inflammation (10, 11, 43). Elevated levels of the proinflammatory cytokine tumor necrosis factor (TNF-\(\alpha\)) in plasma have been reported in humans and rodents with aging (4, 10, 19), while greater paracrine secretion of TNF-\(\alpha\) by vascular cells with aging has been linked to increased levels of superoxide (\(\mathrm{O}_2^-\)) generated through stimulation of nicotinamide adenine dinucleotide phosphate oxidase by TNF-\(\alpha\) and the local renin-angiotensin system (10, 11). TNF-\(\alpha\) mRNA expression in carotid arteries from rats remains relatively stable at 3, 9, 12, and 18 mo of age, whereas expression is elevated at 24 and 29 mo of age (10). These data suggest that aging and vascular dysfunction are not necessarily a linear process across the lifespan, but may occur primarily in the more advanced stages of life. Impairment of endothelial cell vasodilator function through a proinflammatory cytokine, such as TNF-\(\alpha\), could also exaggerate vasconstrictor responses to ANG II in the skeletal muscle microcirculation.
Limited information is available regarding changes in ANG II vasoreactivity in the microcirculation. Moreover, no direct studies have been conducted regarding the effects of aging and heightened levels of physical activity on ANG II-induced vasoconstriction of skeletal muscle arterioles. Therefore, the purpose of this study is to determine whether and through what mechanism(s) aging affects ANG II-mediated vasoconstriction in rat skeletal muscle arterioles, and whether a proinflammatory state might contribute to putative alterations in vasoconstrictor responses to ANG II with aging. In addition, studies were conducted to determine whether habitual exercise might provide similar results to an anti-inflammatory treatment. Based on previous results showing that aging enhances α-adrenoceptor-mediated vasoconstriction via a diminished endothelium-dependent NOS mechanism (21), we hypothesized that aging would likewise enhance ANG II-induced vasoconstriction through an impaired endothelium-dependent NOS signaling pathway, and that chronic inhibition of TNF-α and exercise training would ameliorate the increased ANG II-mediated vasoconstriction.

MATERIALS AND METHODS

Animals

Young (3–4 mo) and old (22 mo) male Fischer 344 rats were obtained from the National Institute on Aging (NIA/Harlan) and housed in a temperature-controlled (23 ± 2°C) room with a 12:12 light-dark cycle. Water and rat chow were provided ad libitum. All animal procedures were approved by the Texas A&M University, West Virginia University, and the University of Florida Laboratory Animal Care Committees and complied with the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23, revised 1996).

Exercise Training

Young and old rats were randomly assigned to one of four groups, young sedentary (YS), young exercise trained (YT), old sedentary (OS), and old exercise trained (OT). Rats in the training groups were habituated to walk on a motor-driven treadmill at 10 m/min (0° incline) for several minutes, and then speed was increased to 15 m/min, 5 min/day, for 3 days. After habituation, the rats performed treadmill exercise at 15 m/min on a 15° incline, 60 min/day, 5 days/wk, for 10–12 wk, as previously described (3, 14, 15, 52). A minimum of 48 h was allowed between the execution of experiments and the final bout of exercise to avoid any possible acute effects of exercise on vasomotor responses.

Microvessel Preparation

Rats were deeply anesthetized with pentobarbital sodium (60 mg/kg ip) and euthanized by decapitation at 5–6 mo of age in the young groups and 24–25 mo of age in the old groups. It is at this age that our laboratory has previously reported old age-associated alterations in intrinsic vasomotor function in the skeletal muscle resistance vasculature (12, 20, 21, 40, 41, 50, 51) and TNF-α mRNA expression is greater in carotid arteries (10). The gastrocnemius-plantaris-soleus muscle group from both hindlimbs was carefully dissected free and placed in cold (4°C) physiological saline solution (PSS) that contained 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.17 mM MgSO₄, 1.2 mM Na₂HPO₄, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 ml BSA at pH 7.4. With a dissecting microscope (Olympus SVH10), first-order (1A) arterioles from the soleus muscle, composed primarily of slow-twitch fibers (13), and the white portion of the gastrocnemius muscle, composed primarily of fast-twitch glycolytic fibers (13), were isolated and removed from the surrounding muscle tissue, as previously described (38, 40). The arterioles were transferred to a Lucite chamber that contained PSS equilibrated with room temperature. Each end of the arteriole was cannulated with a glass micropipette and secured with 10–0 ophthalmic nylon suture, and the microvessel chamber was transferred to the stage of an inverted microscope (Olympus IX70) equipped with a video camera (Panasonic BP310), video caliper (Microcirculation Research Institute), and data acquisition system (MacLab/Macintosh). The arterioles were initially pressurized to 70 cmH₂O with two independent hydrostatic pressure reservoirs; this intraluminal pressure is equivalent to that measured in vivo in skeletal muscle arterioles of similar size (34, 39). Leaks were detected by pressurizing the vessel and verifying that intraluminal diameter remained constant; arterioles that exhibited leaks were discarded. The arterioles were warmed to 37°C and allowed to develop spontaneous tone during a 60-min equilibration period. Vessels that did not develop at least 20% tone were excluded from the study.

Experimental Design

Protocol I. Concentration-response relations to the cumulative addition of ANG II (10⁻¹¹ to 10⁻⁴ M) were determined in arterioles from the soleus and gastrocnemius muscles from YS (n = 13), OS (n = 10), YT (n = 7), and OT (n = 8) groups (n = the number of animals studied per group). Intraluminal diameter was recorded for 3 min following each addition of ANG II.

Protocol II. Results from protocol I indicated that aging and exercise training altered arteriolar responses to the cumulative addition of ANG II. To determine whether these differences were the result of time-dependent or cumulative dose-dependent phenomenon, responses to a single dose of ANG II (10⁻⁸ M) were determined in arterioles from the soleus and gastrocnemius muscles from YS (n = 8), OS (n = 8), YT (n = 8), and OT (n = 8) groups. Diameter was recorded after ~3 min of exposure to ANG II.

Protocol III. To determine whether alterations induced by aging and exercise training from protocol I were mediated through the vascular endothelium, the endothelium was denuded from the gastrocnemius and soleus muscle arterioles from YS (n = 12), OS (n = 13), YT (n = 7), and OT (n = 5) rats by passing 10 ml of air through the lumen of the vessel. To ensure adequate removal of the endothelium, the arterioles were exposed to acetylcholine (3 × 10⁻⁵ M). Vessels that exhibited vasodilatation of >5% were excluded from further study. Following the acetylcholine test, the vessels were washed several times with PSS and allowed to develop spontaneous tone before the ANG II dose response. The diameters of denuded 1A arterioles from gastrocnemius and soleus muscles were measured in response to increasing concentrations of ANG II (10⁻¹¹ to 10⁻⁴ M).

Protocol IV. Since results from protocol III indicated that the effects of aging and exercise training were endothelium dependent, another series of studies was performed in gastrocnemius and soleus muscle arterioles from YS (n = 11), OS (n = 11), YT (n = 8), and OT (n = 5) animals to determine whether the alteration of the endothelium by aging and exercise training was mediated through the NOS signaling pathway. After the arterioles were allowed to develop spontaneous tone, they were incubated for 20 min with N⁵-nitro-L-arginine methyl ester (L-NAME; 10⁻⁵ M) (41, 51), and the ANG II dose response (10⁻¹⁰ to 10⁻⁴ M) was performed.
young and old control rats. At the end of the prolonged TNF-α inhibition period, gastrocnemius muscle arterioles were isolated, and concentration response relations to the cumulative addition of ANG II (10^{-11} to 10^{-5} M) were determined. Results from protocols I–IV indicated that the effects of aging were similar in soleus and gastrocnemius muscle arterioles, so only gastrocnemius muscle arterioles were studied in this protocol.

Protocols I–V. At the end of each ANG II dose response, maximal intraluminal diameter of arterioles was determined after two 15-min incubations in Ca^{2+}-free PSS at 70 cmH_{2}O. A bolus dose of sodium nitroprusside (10^{-4} M) was added during the second 15-min incubation in Ca^{2+}-free PSS to ensure complete smooth muscle relaxation.

Muscle Citrate Synthase Activity and Heart Mass

Sections of the soleus and white gastrocnemius muscles from each animal were stored at −80°C for determination of citrate synthase activity (53), a measure of muscle oxidative capacity, to determine the efficacy of the training regimen (14, 15, 51). Likewise, the heart was removed to determine whether exercise training elevated the heart-to-body mass ratio, an indicator of an exercise-trained state.

Data Analysis

Actual intraluminal diameter was measured in response to ANG II and was expressed as a percent constrictor response according to the formula:

\[
\text{Vasoconstriction} \, (\%) = \left[\left(\frac{D_b - D_s}{D_b}\right) \times 100\right]
\]

where \(D_b\) is the initial baseline diameter recorded immediately before the addition of the ANG II, and \(D_s\) is the steady-state diameter measured after each dose of ANG II. Spontaneous tone was expressed as a percentage of the maximal diameter (\(D_m\)) as follows:

\[
\% \text{ Spontaneous tone} = \left(\frac{D_m - D_b}{D_m}\right) \times 100
\]

Vascular sensitivity, the concentration of ANG II exhibiting 50% maximal contraction (EC_{50}), was determined by logarithmic curve-fitting equations. Dose-response curves were analyzed by two-way ANOVA with repeated measures on one factor (ANG II dose). Pairwise comparisons between specific levels were made through postanalysis (least significant difference). A one-way ANOVA was performed to determine the significance of differences among groups in vessel characteristics, body and tissue masses, and citrate synthase activity. All values are presented as means ± SE. Significant differences are indicated by \(P < 0.05\).

RESULTS

Animal Characteristics

At the time of the experiments, the young adult rats were ~6 mo old (range, 4–7 mo), and the senescent animals were ~25 mo old (range, 24–25 mo). Body mass was greater in OS than in YS rats, and exercise training reduced body mass in old rats, but not in the young rats (Table 1). Although soleus muscle mass was greater with age, both soleus and gastrocnemius muscle-to-body mass ratios decreased with aging. Soleus muscle-to-body mass ratios were increased with exercise training in young and old rats (Table 1). Heart mass was greater in old rats, and heart-to-body mass and left ventricle-to-body mass ratios were greater in the exercise-trained groups (Table 1). Citrate synthase activity was higher in soleus muscle from both YT and OT rats and in the white portion of gastrocnemius muscle in OT rats (Table 1), demonstrating the efficacy of the exercise-training regimen.

Arteriolar Characteristics

Maximal intraluminal diameters of soleus muscle arterioles were not different among groups. Maximal intraluminal diameters of gastrocnemius muscle arterioles were greater with age, and exercise training increased maximal diameter of gastrocnemius muscle arterioles in old rats (Table 2). In animals treated with PEG sTNF-RI, there was a trend \((P < 0.1)\) for a larger maximal diameter in gastrocnemius arterioles from the old vs. young animals (Table 3). Initial spontaneous tone developed in arterioles was not different among groups, including those from rats treated with PEG sTNF-RI, from either soleus or gastrocnemius muscles (Tables 2 and 3).

ANG II Vasoconstrictor Studies

Effects of aging. Aging enhanced ANG II-mediated vasoconstriction responses in arterioles from both the soleus (Figure 1A) and gastrocnemius (Fig. 1B) muscles. In soleus muscle arterioles, vasoconstrictor responses were greater at \(10^{-10}\) to \(10^{-5}\) M ANG II, whereas, in gastrocnemius muscle, arteriole constriction was greater at \(10^{-5}\) to \(10^{-6}\) M ANG II with aging. However, aging did not alter vascular sensitivity (EC_{50}) to

<table>
<thead>
<tr>
<th>Table 1. Animal and tissue characteristics</th>
<th>Sedentary</th>
<th>Exercise Trained</th>
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<tbody>
<tr>
<td></td>
<td>YS</td>
<td>OS</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>342 ± 6</td>
<td>431 ± 4*</td>
</tr>
<tr>
<td>Soleus muscle mass, mg</td>
<td>150 ± 4</td>
<td>170 ± 4*</td>
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<tr>
<td>Gastrocnemius muscle mass, mg</td>
<td>1,756 ± 33</td>
<td>1,804 ± 37</td>
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<tr>
<td>Soleus muscle mass/body mass</td>
<td>441 ± 10</td>
<td>395 ± 10*</td>
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<td>Ratio, mg/kg</td>
<td>5,160 ± 98</td>
<td>4,182 ± 85*</td>
</tr>
<tr>
<td>Gastrocnemius muscle mass/body mass</td>
<td>2,157 ± 60</td>
<td>3,053 ± 97*</td>
</tr>
<tr>
<td>Heart mass, mg</td>
<td>909 ± 33</td>
<td>1,168 ± 37*</td>
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<tr>
<td>Heart/mass/body mass ratio, mg/kg</td>
<td>2,657 ± 124</td>
<td>2,709 ± 94</td>
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<tr>
<td>LV/body mass ratio, mg/kg</td>
<td>1,822 ± 71</td>
<td>1,887 ± 35</td>
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<td>Soleus muscle citrate synthase activity, (\mu)mol·min^{-1}·g wet wt^{-1}</td>
<td>20.0 ± 0.6</td>
<td>17.5 ± 0.9*</td>
</tr>
<tr>
<td>White portion of gastrocnemius muscle citrate synthase activity, (\mu)mol·min^{-1}·g wet wt^{-1}</td>
<td>12.0 ± 0.3</td>
<td>12.1 ± 0.6</td>
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Values are means ± SE. LV, left ventricle; YS, young sedentary; OS, old sedentary; YT, young trained; OT, old trained. Significant difference between *YS and OS, †YS and YT, and ‡OS and OT: \(P < 0.05\).
Table 2. Vessel characteristics from young and old sedentary and trained rats

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<th>Sedentary</th>
<th>Exercise Trained</th>
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<tr>
<td></td>
<td>YS</td>
<td>OS</td>
</tr>
<tr>
<td>Soleus muscle maximal arteriolar lumen diameter, µm</td>
<td>120 ± 3</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>Gastrocnemius muscle maximal arteriolar lumen diameter, µm</td>
<td>151 ± 4</td>
<td>172 ± 5*</td>
</tr>
<tr>
<td>Soleus muscle arteriolar spontaneous tone, %</td>
<td>51 ± 3</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Gastrocnemius muscle arteriolar spontaneous tone, %</td>
<td>45 ± 3</td>
<td>40 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Significant difference between *YS and OS, and ‡OS and OT: P < 0.05.

ANG II in either muscle type (soleus: young $2.3 \times 10^{-9} \pm 0.6 \times 10^{-9}$ M, old $3.0 \times 10^{-9} \pm 1.5 \times 10^{-9}$ M; gastrocnemius: young $1.5 \times 10^{-9} \pm 0.3 \times 10^{-9}$ M, old $1.6 \times 10^{-9} \pm 0.5 \times 10^{-9}$ M). Likewise, ANG II-induced vasoconstriction to a single dose of ANG II ($10^{-8}$ M) resulted in a higher vasoconstrictor response in aged arterioles from soleus (Fig. 2A) and gastrocnemius (Fig. 2B) muscles. These results indicate that differences in ANG II-induced vasoconstriction between young and old skeletal muscle arterioles are not time dependent or cumulative dose-dependent phenomenon.

**ENDOTHELIUM REMOVAL.** The biphasic response of ANG II-mediated vasoconstriction, i.e., the lower vasoconstrictor response at the highest concentrations of ANG II, was abolished with the removal of the endothelium in both young and old rats (Fig. 3). Furthermore, the removal of the endothelium eliminated the age-associated difference in ANG II-mediated vasoconstriction of soleus (Fig. 3A) and gastrocnemius (Fig. 3B) muscle arterioles.

**NOS INHIBITION.** ANG II elicited a linear dose-dependent vasoconstriction with L-NAME treatment rather than the biphasic response (Fig. 4). The age-related difference in ANG II-mediated vasoconstriction of soleus (Fig. 4A) and gastrocnemius (Fig. 4B) muscle arterioles were abolished with NOS inhibition.

**Effect of exercise training.** Exercise training diminished ANG II-mediated vasoconstrictor responses in arterioles from the soleus (Fig. 1A) and gastrocnemius (Fig. 1B) muscles of old rats. In soleus and gastrocnemius muscle arterioles, this training-induced difference was evident at the four highest doses of ANG II tested ($10^{-7}$ to $10^{-4}$ M); training did not alter responses of arterioles from young rats to a single dose of ANG II ($10^{-8}$ M).

**ENDOTHELIUM REMOVAL.** Following removal of vascular endothelium, the exercise training-mediated alterations in ANG II-induced vasoconstriction of soleus (Fig. 5A) and gastrocnemius (Fig. 5B) muscle arterioles from old rats were abolished.

**NOS INHIBITION.** In the presence of L-NAME, the training-related decrease in ANG II-mediated vasoconstriction of old rat soleus (Fig. 6A) and gastrocnemius (Fig. 6B) muscle arterioles was eliminated.

**Comparison between removal of endothelium and L-NAME.** ANG II-mediated vasoconstrictor responses with the endothelium removed and those treated with L-NAME were not different between arterioles from the soleus or gastrocnemius muscles in young and old rats (Fig. 7).

**Effect of chronic TNF-α inhibition.** Chronic TNF-α inhibition diminished the vasoconstrictor response of arterioles from the old treated rats relative to that in the old untreated animals and eliminated the age-associated difference (young vs. old) in ANG II-mediated vasoconstriction (Fig. 8).

**DISCUSSION**

The purpose of this study was to determine whether 1) aging alters ANG II-induced vasoconstriction of skeletal muscle arterioles; 2) the proinflammatory cytokine TNF-α contributes to alterations in ANG II-mediated vasoconstriction with aging; 3) exercise training modulates ANG II-induced vasoconstriction in young and old animals; and 4) mechanism(s) of putative aging and exercise training induced alterations in ANG II-induced vasoconstriction. The results provide several unique findings. First, aging enhances ANG II-induced vasoconstrictor responses in arterioles from both the highly oxidative soleus muscle and low-oxidative superficial portion of gastrocnemius muscle. Second, the old age-associated enhancement of ANG II-induced vasoconstriction occurs through an endothelium-dependent NOS signaling mechanism. The endothelial dysfunction underlying the enhanced vasoconstrictor response to ANG II appears to be related to a proinflammatory state, as

Table 3. Animal and vessel characteristics from young and old sedentary and trained rats, with and without TNF-α inhibition

<table>
<thead>
<tr>
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<th>No TNF-α Inhibition</th>
<th>TNF-α Inhibition</th>
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<tbody>
<tr>
<td></td>
<td>YS</td>
<td>OS</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>364 ± 20</td>
<td>443 ± 19*</td>
</tr>
<tr>
<td>Gastrocnemius muscle maximal arteriolar lumen diameter, µm</td>
<td>157 ± 15</td>
<td>186 ± 11*</td>
</tr>
<tr>
<td>Gastrocnemius muscle arteriolar spontaneous tone, %</td>
<td>39 ± 6</td>
<td>42 ± 8</td>
</tr>
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</table>

Values are means ± SE. *Significant difference between young and old (P < 0.05). ‡Significant difference between young and old with TNF-α inhibition (P < 0.05). †Difference between young and old with TNF-α inhibition (P = 0.089).
Soleus Muscle

The present study demonstrates that the mechanisms for the old age-associated enhancement of ANG II-induced vasoconstriction are dependent upon the endothelium (Fig. 3). Previous work has shown that aging increases norepinephrine-mediated vasoconstrictor responses through an endothelium-dependent mechanism (21), whereas an aging-induced enhancement of endothelin-1-mediated vasoconstriction occurs through smooth muscle cell endothelin type A receptors (20). Results from the present study are also consistent with the finding in humans that exogenous ANG II infusion induces greater vasoconstriction and reductions in resting leg blood flow in elderly individuals relative to young subjects (64). Several factors can induce endothelium-dependent vasodilation, including NO, prostacyclin, and endothelium-derived hyperpolarizing factor. The present results indicate that the NOS signaling pathway plays the major role in determining the age-associated enhancement of ANG II-induced vasoconstriction through the endothelium. Results from the present study are consistent with previous findings that endothelial vasodilator function declines with old age (12, 16, 41, 50, 63). In soleus muscle arterioles, for example, flow-induced vasodilation and tetrahydrobiopterin content are diminished by aging (12, 50), with no change in arteriolar anti-oxidant SOD-1, catalase, or glutathione peroxidase-1 protein levels (unpublished observations). This limited availability of tetrahydrobiopterin appears to promote an endothelial NOS (eNOS) uncoupling that results in greater eNOS-derived O$_2^-$ generation and lower NO bioavailability (50). The degradation of NO through its interaction with O$_2^-$ indicated by the lack of an enhanced vasoconstriction to ANG II in old animals chronically treated with the TNF type 1 receptor inhibitor. Finally, exercise training ameliorates the old age-associated increase in ANG II-mediated vasoconstriction in both soleus and gastrocnemius muscles, which also occurs through an endothelium-dependent NOS signaling pathway.

**ANG II-Induced Vasoconstriction**

Unlike other vasoconstrictor responses in the vasculature, ANG II-induced vasoconstriction is a biphasic response, where the most potent vasoconstriction occurs at the lower concentrations of ANG II (66). The present results in skeletal muscle arterioles (Fig. 1) are similar to these previous findings. Numerous studies have reported that activation of AT$_1$R results in vasoconstriction through increased smooth muscle intracellular Ca$^{2+}$ availability, whereas ANG II binding to AT$_1$R and AT$_2$R on the endothelium causes vasodilation through increased NO synthesis (7, 9, 31, 37, 48, 60). These studies indicate that the net ANG II-induced vasoconstriction is the result of an interaction between the vasoconstrictor influence via smooth muscle cell AT$_1$R and the vasodilator influence through endothelial cell AT$_1$R and AT$_2$R. Therefore, the endothelium plays an important role in modulating the balance between ANG II-induced contraction and relaxation (7, 29, 30, 67). Consequently, the focus of the present study was on the location of receptors activated (i.e., smooth muscle vs. endothelium) rather than the type of receptors activated (AT$_1$R vs. AT$_2$R) as it relates to the mechanism of the biphasic response and alterations induced by aging and exercise training.

**Fig. 1.** Effects of aging and exercise training on vasoconstrictor responses to the cumulative addition of angiotensin II of soleus (A) and gastrocnemius (B) muscle arterioles from young sedentary (YS), old sedentary (OS), young exercise-trained (YT), and old exercise-trained (OT) rats. Values are means ± SE; n = number of animals studied per group. *Difference between groups (P < 0.05).

**Fig. 2.** Effects of aging and exercise training on vasoconstrictor responses of soleus (A) and gastrocnemius (B) muscle arterioles to the single dose of angiotensin II (10$^{-8}$ M). Values are means ± SE; n = number of animals studied per group. *Mean arteriolar response from OS animals different from that of all other groups (P < 0.05).
Although determination of whether TNF-α/H9251 is elevated (1, 25) through the NOS signaling mechanism (27, 36) is a proinflammatory cytokine that impairs endothelium-dependent vasodilation during exercise in older individuals (2, 18, 35, 46, 47). Although diminished limb perfusion during exercise has been attributed to an impairment of endothelium-dependent vasodilator function in humans (16, 17, 58) and rats (12, 41, 50, 51, 63), vasoconstrictor mechanisms that involve activation of endothelium-dependent vasodilator pathways may also be involved in a greater net vasoconstriction to ANG II with aging. The findings of the present study also concur with previous studies demonstrating that ANG II-induced vasoconstriction is modulated by the release of endothelium-derived NO in the arterial vasculature (7, 37, 47, 62). Moreover, hindlimb vascular conductance during ANG II infusion is significantly lower in the presence of a NOS antagonist in conscious rats, indicating NO plays an important role in maintaining muscle perfusion and oxygen delivery by opposing ANG II-mediated smooth muscle contraction (56).

One potential mechanism contributing to the old age-associated endothelial dysfunction in the skeletal muscle microcirculation is an enhanced vascular proinflammatory state resulting from elevated levels of TNF-α (8, 10, 28, 49). TNF-α is a proinflammatory cytokine that impairs endothelium-dependent vasodilation (1, 25) through the NOS signaling mechanism (27, 44, 65). Although determination of whether TNF-α is elevated in the skeletal muscle vasculature with aging has not been established, elevations of plasma TNF-α have been shown to occur in old rats, as well as increased TNF-α mRNA expression in the carotid arteries from aged rats (10). Anti-TNF-α treatment has also been shown to improve endothelial function in patients with chronic heart failure (24), systemic vasculitis (6), and type 2 diabetes (25, 44). In the present study, chronic (10 wk) inhibition of TNF-α eliminated old age-associated differences in ANG II-mediated vasoconstriction (Fig. 8).

These data suggest that the impairment of the NOS signaling pathway linked to AT1R and AT2R on arteriolar endothelial cells with old age may be the result of a proinflammatory state. Although results from the present study demonstrate enhanced ANG II-induced vasoconstriction through an endothelium-dependent mechanism in skeletal muscle resistance arteries with aging, Pinaud et al. (45) have shown enhanced ANG II-evoked constriction in mesenteric resistance arteries that occurs through greater AT2R-mediated contraction of smooth muscle cells. This enhanced smooth muscle constriction was attenuated by the antioxidant Tempol, indicating the old age-associated enhancement of AT2R-dependent contractions is mediated through reactive oxygen species in the mesentery. Similar results were also found in old rats chronically treated with hydralazine, which has antioxidant properties. The authors further suggested that the beneficial vascular effects of hydralazine treatment appeared comparable to that of exercise training (45).

In humans, old age is often associated with elevated blood pressure and systemic vascular resistance (33). Likewise, regional elevations in vascular resistance lower leg blood flow during exercise in older individuals (2, 18, 35, 46, 47). Although diminished limb perfusion during exercise has been attributed to an impairment of endothelium-dependent vasodilator function in humans (16, 17, 58) and rats (12, 41, 50, 51, 63), vasoconstrictor mechanisms that involve activation of endothelium-dependent vasodilator pathways may also be involved.

Fig. 3. Effects of endothelium removal on vasoconstrictor responses to the cumulative addition of angiotensin II in YS, endothelium-removed YS (YS-E), old sedentary (OS), and endothelium-removed OS (OS-E) arterioles from the soleus (A) and gastrocnemius (B) muscles. Vasoconstrictor responses of YS and OS with intact endothelium are the same as those in Fig. 1, A and B. Values are means ± SE; n = number of animals studied per group. *Difference between groups (P < 0.05).

Fig. 4. Effects of nitric oxide synthase (NOS) inhibition with Nω-nitro-L-arginine methyl ester (L-NAME) on vasoconstrictor responses to the cumulative addition of angiotensin II in YS, YS with L-NAME (YS+L-NAME), OS, and OS with L-NAME (OS+L-NAME) arterioles from the soleus (A) and gastrocnemius (B) muscles. Vasoconstrictor responses of YS and OS without NOS inhibition are the same as those in Fig. 1. A and B. Values are means ± SE; n = number of animals studied per group. *Difference between groups (P < 0.05).
Exercise training increases SOD-1 protein levels in soleus arterioles from old rats. In addition, we have found that diated vasodilation via the NOS pathway in rat skeletal muscle reported that exercise training enhances the endothelium-mediated vasodilation through the NOS signaling pathway was first tested in the rat abdominal aorta (14, 15). Subsequent findings of a training-induced enhancement of endothelium-dependent vasodilation and the upregulation of eNOS mRNA and protein expression in the skeletal muscle microcirculation (51, 55) has reinforced the concept that elevated intraluminal shear stress is an important mediator for endothelial adaptations. Exposure of the endothelium to exercise-induced increases in ANG II concentration is another possible mechanism to increase eNOS expression. Plasma concentrations of ANG II increase during dynamic exercise (59), with a reported doubling during exercise at 80% of maximal heart rate reserve (61), and such elevations in ANG II working through endothelial AT receptors have been reported to increase eNOS mRNA and protein expression (32, 42).

Effects of Exercise Training

Results from the present study demonstrate that the mechanism of the exercise training-induced reduction in ANG II-mediated vasoconstriction in skeletal muscle arterioles from old rats is through an endothelium-dependent NOS signaling pathway, since both endothelium removal and NOS inhibition abolished the training effect (Figs. 5 and 6). Although no direct studies were performed to investigate the possible prosacyclin and endothelium-derived hyperpolarizing factor vasodilator mechanisms on ANG II-induced vasoconstrictor responses, the notion that training primarily affects the eNOS signaling pathway is supported by the finding of Spier et al. (51), who reported that exercise training enhances the endothelium-mediated vasodilation via the NOS pathway in rat skeletal muscle arterioles from old rats. In addition, we have found that exercise training increases SOD-1 protein levels in soleus muscles from both young and old rats and glutathione peroxidase-1 protein content only in arterioles from old rats (unpublished observations), indicating that the enhancement of endothelial function by exercise training may result, in part, from a training-induced upregulation of anti-oxidant proteins that regulate levels of reactive oxygen species in the skeletal muscle resistance vasculature.

Exercise is a potent stimulus to elevate O₂ delivery and nutrients to active skeletal muscles. The hypothesis that the repetitive muscle hyperemia and corresponding elevations in intravascular shear stress associated with chronic bouts of exercise provide a stimulus to improve endothelium-dependent vasodilation through the NOS signaling pathway was first tested in the rat abdominal aorta (14, 15). Subsequent findings of a training-induced enhancement of endothelium-dependent vasodilation and the upregulation of eNOS mRNA and protein expression in the skeletal muscle microcirculation (51, 55) has reinforced the concept that elevated intraluminal shear stress is an important mediator for endothelial adaptations. Exposure of the endothelium to exercise-induced increases in ANG II concentration is another possible mechanism to increase eNOS expression. Plasma concentrations of ANG II increase during dynamic exercise (59), with a reported doubling during exercise at 80% of maximal heart rate reserve (61), and such elevations in ANG II working through endothelial AT receptors have been reported to increase eNOS mRNA and protein expression (32, 42).
Previous work has shown that endurance training can increase leg blood flow in aged humans (36), and that a mechanism for exercise training-induced increases in blood flow in the elderly is through endothelium-dependent vasodilation (17, 58). The present results suggest that endurance exercise training may also ameliorate an age-associated enhancement of ANG II-induced vasoconstriction through an endothelium-dependent pathway, and that this may be one contributing mechanism to enhance blood flow in trained elderly individuals.

The same absolute exercise intensity (15 m/min on a 15° incline) was used in the present study for training young and old animals. Consequently, differences in aerobic capacity between YS and OS rats could result in a different training stimulus between the two groups (22). In the soleus muscle, any difference in exercise stimulus appears to be of negligible significance with this low-intensity exercise, given that the percent increase of soleus muscle citrate synthase activity, an indication of training efficacy, is similarly elevated in young (30%) and old (33%) rats (Table 1). However, in the white portion of the gastrocnemius muscle, citrate synthase activity was only elevated in the OT rats, indicating this portion of the muscle was chronically recruited during the training regimen. Despite differences in the training stimulus between these two muscles, the arteriolar adaptation to training was similar between the two muscle types, indicating that any differences in relative exercise intensity between young and old animals had little impact on the results.

Fig. 7. Effects of endothelium removal and l-NAME on vasoconstrictor response to the cumulative addition of angiotensin II in gastrocnemius muscle arterioles from YS, OS, YS with the TNF-α inhibitor (YS-TNFα), and OS with TNF-α inhibitor (OS-TNFα). Values are means ± SE; n = number of animals studied per group.

Fig. 8. Effects of chronic TNF type 1 receptor inhibition on vasoconstrictor response to the cumulative addition of angiotensin II in gastrocnemius muscle arterioles from YS, OS, YS with the TNF-α inhibitor (YS-TNFα), and OS with TNF-α inhibitor (OS-TNFα). Values are means ± SE; n = number of animals studied per group. *Difference between groups (P < 0.05).

**Conclusion**

ANG II-induced vasoconstriction is determined by the net effects of a potent smooth muscle cell vasoconstrictor response and a less potent endothelium-dependent vasodilator influence via a NOS signaling pathway. With aging, ANG II-mediated vasoconstriction in skeletal muscle arterioles is enhanced due to an old age-associated dysfunction of the endothelium-dependent NOS vasodilator mechanism. Experiments that chronically inhibited TNF-α suggest that the impairment of the eNOS signaling mechanism is associated with a proinflammatory state of the skeletal muscle microvasculature during aging, resulting in enhanced vasoconstrictor responses to ANG II. Exercise training can ameliorate the old age-associated elevation in ANG II-induced vasoconstriction through an enhancement in the endothelium-dependent NOS signaling pathway. Since aging and training can modify the ANG II-induced vasoconstrictor responsiveness of skeletal muscle arterioles, results from the present study suggest that vascular responsiveness to ANG II may play a role in the old age-associated reductions in skeletal muscle blood flow and elevations in systemic vascular resistance, as well as with the training-induced restoration of muscle perfusion during exercise.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

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