Mechanisms of flow and ACh-induced dilation in rat soleus arterioles are altered by hindlimb unweighting

WILLIAM G. SCHRAGE,1,2 CHRISTOPHER R. WOODMAN,2,3 AND M. HAROLD LAUGHLIN1,2,3
Departments of 1Physiology and 2Veterinary Biomedical Sciences, and 3Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211

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Schrage, William G., Christopher R. Woodman, and M. Harold Laughlin. Mechanisms of flow and ACh-induced dilation in rat soleus arterioles are altered by hindlimb unweighting. J Appl Physiol 92: 901–911, 2002; 10.1152/japplphysiol.00642.2001.—The purpose of this study was to test the hypothesis that endothelium-dependent dilation (flow-induced dilation and ACh-induced dilation) in rat soleus muscle arterioles is impaired by hindlimb unweighting (HLU). Male Sprague-Dawley rats (~300 g) were exposed to HLU or weight-bearing control (Con) conditions for 14 days. Soleus first-order (1A) and second-order (2A) arterioles were isolated, cannulated, and exposed to step increases in luminal flow at constant pressure. Flow-induced dilation was not impaired by HLU in 1A or 2A arterioles. The cyclooxygenase inhibitor indomethacin (Indo; 50 μM) did not alter flow-induced dilation in 1As or 2As. Inhibition of nitric oxide synthase (NOS) with Nω-nitro-L-arginine (L-NNA; 300 μM) reduced flow-induced dilation by 65–70% in Con and HLU 1As. In contrast, L-NNA abolished flow-induced dilation in 2As from Con rats but had no effect in HLU 2As. Combined treatment with L-NNA + Indo reduced tone in 1As and 2As from Con rats, but flow-induced dilation in the presence of L-NNA + Indo was not different from responses without inhibitors in either Con or HLU 1As or 2As. HLU also did not impair ACh-induced dilation (10−9–10−4 M) in soleus 2As. L-NNA reduced ACh-induced dilation by ~40% in Con 2As but abolished dilation in HLU 2As. Indo did not alter ACh-induced dilation in Con or HLU 2As, whereas combined treatment with L-NNA + Indo abolished ACh-induced dilation in 2As from both groups. We conclude that flow-induced dilation (1As and 2As) was preserved after 2 wk HLU, but HLU decreased the contribution of NOS in mediating flow-induced dilation and increased the contribution of a NOS-and cyclooxygenase-independent mechanism (possibly endothelium-derived hyperpolarizing factor). In soleus 2As, ACh-induced dilation was preserved after 2-wk HLU but the contribution of NOS in mediating ACh-induced dilation was increased.

skeletal muscle; microcirculation; physical inactivity; simulated microgravity; reduced blood flow

AFTER SPACEFLIGHT, ASTRONAUTS exhibit impaired exercise capacity characterized by lower cardiac output and lower maximal oxygen uptake during maximal intensity exercise (15). The mechanisms accounting for the reduction in exercise capacity after spaceflight may involve altered vascular control leading to an impaired ability to increase skeletal muscle blood flow during exercise.

Hindlimb unweighting (HLU) is a useful model to investigate the effects of a non-weight-bearing environment on vascular control in skeletal muscles and whether altered vascular control mechanisms contribute to impaired exercise capacity on return to upright posture in gravity (3, 17, 20, 31). Rats exposed to 14 days of HLU exhibit reductions in maximal oxygen uptake similar in magnitude to the reductions reported in astronauts (3, 20, 30). The decline in exercise capacity in rats is associated with a decrement in maximal cardiac output and an impaired ability to increase blood flow to active skeletal muscle during exercise (20, 30). In addition, during HLU, rats experience reduced soleus blood flow that mimics the reduced calf blood flow observed in astronauts during spaceflight (17, 26). A chronic reduction in blood flow may induce vascular remodeling (2, 8, 14, 23) and changes in arterial vaso-motor responsiveness (2, 8, 23). If HLU induces vascular remodeling and altered arterial vasomotor responsiveness, such changes would be expected to contribute to reduced exercise capacity after HLU (3, 17, 20, 31) or spaceflight (15).

The fact that blood flow is reduced in calf muscle in humans during spaceflight (26) and rats during HLU (17) highlights the utility of the HLU rodent model for assessing skeletal muscle vascular adaptations produced by decreased weight-bearing activity. Adaptations reported to be induced by HLU in skeletal muscle vascular beds include reduced artery diameter (1, 2, 8, 23), impaired endothelium-dependent dilation (2, 8, 23), and reductions in endothelial nitric oxide synthase (eNOS) and superoxide dismutase-1 expression (8, 23, 29). Importantly, the magnitude and type of vascular adaptation observed in the arterial tree appear to vary with skeletal muscle fiber type (2, 29), the arterial branch order within a muscle (8, 23, 29), and the duration of HLU (2). In addition, the importance of vascular control mechanisms varies throughout the arteriolar network of muscle tissue (4, 9, 13). To allow evaluation of spatial differences in vascular control

Address for reprint requests and other correspondence: M. H. Laughlin, E102 Veterinary Biomedical Sciences, Univ. of Missouri, Columbia, MO 65211 (E-mail: LaughlinM@missouri.edu).

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along the soleus arteriolar tree and to evaluate whether mechanisms of HLU-induced adaptation in vascular control occur uniformly in the soleus arteriolar network, we studied two different arteriolar branch orders (first- and second-order arterioles, 1A and 2As, respectively) from the soleus muscle of control and HLU rats.

Rationale supporting this experimental design includes observations of impaired flow-induced dilation in soleus feed arteries after 14 days HLU (8) and impaired ACh-induced dilation with reduced eNOS expression in soleus feed arteries and soleus 1A arterioles from HLU rats (8, 23). The degree of endothelial impairment was greater in soleus 1A arterioles than feed arteries (8, 23). Furthermore, Woodman et al. (29) reported that eNOS protein content is decreased in soleus feed arteries and 1A arterioles but not 2A arterioles. These results suggest that HLU-induced adaptations do not occur uniformly in the soleus arteriolar network (8, 23, 29). The purpose of the present study was to test the hypothesis that flow-induced dilation is impaired in soleus 1A arterioles and that both flow- and ACh-induced dilation are impaired in soleus 2A arterioles of rats exposed to 14 days of HLU. To test this hypothesis, we examined flow-induced dilation in 1As and both flow- and ACh-induced dilation in 2As isolated from control and HLU rats. In addition, we used pharmacological inhibitors to assess whether the contributions of nitric oxide synthase (NOS) and/or cyclooxygenase (COX) pathways mediating flow-induced dilation or ACh-induced dilation are changed by 14 days HLU.

**METHODS**

**Animals and HLU procedures.** Male Sprague-Dawley rats (n = 91) (Harlan Sprague Dawley, Indianapolis, IN) (275–300 g) were housed two per cage in a room with constant temperature (24°C) and 12-h light-dark cycle. Food and water were provided ad libitum. The Institutional Animal Care and Use Committee of the University of Missouri approved all experimental protocols. Rats were randomly assigned to HLU or control (Con) groups. Rats in the HLU group were familiarized to HLU procedures for 1–2 h/day for 2–3 days before the HLU treatment as described previously (8, 18). After this familiarization period, rats were anesthetized with ketamine and xylazine (intraperitoneal, 35 mg/kg and 2.25 mg/kg, respectively). Four strips of mole foam were secured with adhesive tape in parallel with the caudal vein to maintain tail circulation. A stiff mesh cast was attached to the base and tip of the tail cast and hooked to chains suspended from a wire spanning the cage. HLU rats were housed individually in Plexiglas cages and suspended in a manner that prevented the hindlimbs from touching any surface while allowing free, 360° movement about the cage on cooling (4°C) over a 1-h equilibration period, during which time the pressure was increased three to four times with warm PSS (37°C). Arterioles were exposed to 80 mM KCl to test for viability. If arterioles did not constrict to KCl by 20% of initial diameter, and blood was flushed out of the arteriole with PSS + albumin. The free end of the arteriole was then tied to a size-matched micropipette. Under an inverted microscope (Nikon Diaphot 200; ×40 magnification), the arteriole image was displayed. Internal arteriole diameter was measured continuously with a video micrometer (Microcirculation Research Institute, Texas A&M University) calibrated to <1 μm. Data were acquired and stored with the Macintosh/ MacLab data acquisition system. Pressure with no flow was applied to the vessel lumen by raising to the same level two reservoirs connected to the micropipettes. Because soleus feed artery pressure in vivo has been reported to be 90 cmH2O (28), we selected an intraluminal pressure of 80 cmH2O for 1As and 60 cmH2O for 2As.

Each arteriole was set at its in situ length, pressurized, and checked for leaks over 5 min. The arteriole was washed three to four times with warm PSS (37°C) over a 1-h equilibration period, during which time the pressure was increased from 30 to 80 cmH2O in the first 30 min (60 cmH2O for 2As). Arterioles were exposed to 80 mM KCl to test for viability. If arterioles did not constrict to KCl by 20% of initial diameter, the arteriole was considered nonfunctional and discarded. After KCl was replaced with PSS, all arterioles developed at least 20% spontaneous tone.

**Experiments to assess vasodilator responses to flow or ACh.** Arterioles used in flow experiments were cannulated with resistance matched pipettes as described by Kuo et al. (12). We sought first to determine whether HLU altered flow-
induced dilation in 1A and 2A arterioles. Control flow-diameter curves (i.e., no inhibitors of NOS or COX) were examined first. The flow-diameter relationship was assessed by producing a pressure gradient between the proximal and distal pipettes while maintaining constant pressure at the midpoint of each vessel (12). Vasodilator responses were assessed at pressure gradients of 1, 2, 4, 6, 8, 10, 15, and 20 cmH$_2$O, which corresponded to flow rates of 0.4, 1.2, 2.6, 3.9, 5.0, 6.0, 7.7, and 8.5 μl/min measured by an Omega ball flowmeter calibrated with a Razin micropump. Flow was maintained for 3–5 min at each pressure gradient, although a stable dilation was usually achieved in the first minute. In all rats, the first flow-diameter curve represented the control curve (no inhibitors of endothelium-dependent dilation). Next, flow-diameter relationships under pharmacological inhibition of the NOS and/or COX pathways were examined to determine the relative contribution of nitric oxide (NO) and prostanoids mediating flow-induced dilation. Specifically, arterioles were washed with PSS and allowed to return to baseline diameter.

A second flow-diameter curve was then generated in the presence of the arginine analog, N$	ext{^6}$-nitro-L-arginine (L-NNA; 300 μM) or the COX inhibitor indomethacin (Indo; 50 μM). In remaining rats, the second flow-diameter curve was performed in the combined presence of L-NNA + Indo. Last, we tested the responsiveness to the endothelium-independent dilator sodium nitroprusside (SNP; 10$^{-9}$–10$^{-4}$ M). The experimental protocol included three dose-response curves for each arteriole: 1) flow alone, 2) flow + inhibitor(s), and 3) SNP. In all experiments, maximal passive arteriolar diameter was determined by replacing the PSS solution with calcium-free PSS (2 mM EDTA) for 30 min. Finally, we determined whether ACh-induced dilation in soleus 2As was altered by HLU similar to 1As (23). To address this, the approach was identical to the previous study (23). In brief, the protocol included three ACh concentration-response curves (10$^{-9}$–10$^{-4}$ M in whole log increments) with or without L-NNA and/or Indo followed by a concentration-response curve to SNP (1) ACh alone, 2) ACh + L-NNA or Indo, 3) ACh + L-NNA + Indo, 4) SNP). Because three ACh curves were reproducible in pilot studies, the effects of inhibitors were studied in the same arterioles, with the treatment order for L-NNA or Indo reversed in half of the rats.

Reproducibility of responses to flow and ACh. Experiments to determine whether flow-induced vasodilator responses were repeatable were performed on 1As from six rats. The highest flow rate used (8.5 μl/min) caused similar flow-induced dilation on two consecutive curves (curve 1: 61 ± 14 to 109 ± 15 μm; curve 2: 67 ± 12 to 124 ± 14 μm). ANOVA indicated that flow-diameter curves were not different. Experiments to determine whether ACh-induced vasodilator responses were repeatable were performed on 2As from six rats. ACh (10$^{-10}$–10$^{-4}$ M) induced similar dilation for three consecutive dose-response curves (curve 1: 54 ± 8 to 98 ± 6 μm, curve 2: 58 ± 10 to 90 ± 7 μm, and curve 3: 52 ± 7 to 98 ± 4 μm). ANOVA indicated that the three ACh-diameter curves were not different.

Endothelial dependence of ACh-induced and flow-induced vasodilator responses. To determine whether the vasodilator responses to ACh and flow were endothelium dependent, we examined vasodilation responses in arterioles from three rats before and after the arterioles were denuded. Specifically, arterioles were exposed to ACh (100 μM) and the vasodilator response was measured. ACh was then washed from the vessel chamber, and the arteriole was allowed to acquire a stable diameter. Arterioles were subsequently exposed to three different flow rates at pressure differences of 6, 10, and 20 cmH$_2$O. The arterioles were then denuded by passing 3 ml of room air through the arterioles as described previously (5, 10, 16). Arterioles were allowed to attain a stable diameter, and vasodilator responses to ACh and flow were reexamined. Results of these experiments revealed that, before denudation, ACh increased diameter 61 ± 4 μm. In contrast, ACh changed diameter −1 ± 4 μm after denudation. Exposure to flow increased diameter 26 ± 8 μm in intact arterioles, but the highest flow rate changed diameter 0 ± 2 μm after denudation. Finally, arterioles were exposed to SNP (100 μM) to confirm that smooth muscle was still responsive to NO. SNP increased diameter 48 ± 4 μm, which is similar to responses in our 1A experiments. These experiments confirm many reports in the literature indicating that ACh-induced and flow-induced vasodilator responses in skeletal muscle arterioles are endothelium dependent (5, 10, 16).

Solutions and drugs. Reagents were obtained from Sigma Chemical (St. Louis, MO). ACh and SNP were dissolved in water and PSS, respectively. SNP was further diluted in PSS. l-NNA was dissolved in hydrochloric acid, which did not alter the pH of PSS. Indo was dissolved in ethanol (0.6% vol/vol final concentration). In control experiments, ethanol did not alter steady-state diameter.

Statistical analysis. Dose-response data were expressed and analyzed four ways: 1) absolute diameter, 2) relative diameter, 3) change from resting diameter, and 4) percentage of possible change in diameter. In general, relative diameter data (relative to maximal passive diameter, which was defined = 1.0) are presented here because maximal diameters were different between groups for 1As and 2As. Percent possible dilation (%) possible was used to identify the SNP (or ACh) dose producing half-maximal response (EC$_{50}$). EC$_{50}$ values were determined using a sigmoid curve-fitting model for log[ACh] values (Prism program Graphpad software). Student’s unpaired t-tests were used to compare EC$_{50}$ values between groups. Percent possible dilation was calculated as [(D$_{max}$ − D$_{b}$)/D$_{b}$] × 100, where D$_{max}$ is measured diameter for a given perfusate flow rate (or dose of ACh or SNP), D$_{b}$ is baseline diameter before flow (or ACh or SNP), and D$_{b}$ is maximal passive diameter.

All dose-response curves were analyzed by two-way ANOVA, with repeated measures on one factor (flow rate, ACh concentration, or SNP concentration). Post hoc analyses were performed using Duncan’s new multiple range test. Group comparisons for all other factors were analyzed by unpaired Student’s t-test. Level of significance for all analyses was $P < 0.05$.

RESULTS

Efficacy of HLU procedures. HLU rats used in this study had lower body weights than Con rats (Table 1). In addition, soleus muscle weight was reduced ∼35% whether expressed in absolute units (mg) or relative to body weight (Table 1), indicating that the HLU protocol was effective in achieving the typical responses to 2 wk HLU.

Arteriole characteristics. Passive diameters of HLU 1As were 13% smaller than those of Con 1As (Table 1). 1As from both groups exhibited similar spontaneous tone (Table 1) before generation of the first flow-induced dilation curve. Thus 1As from HLU rats exhibited smaller baseline diameter in the presence of spontaneous tone (Table 1).

Passive diameters of 2As from HLU rats were 9.5% smaller than those of 2As from Con rats (Table 1). 2As exhibited similar spontaneous tone and similar base-
line diameter (before exposure to flow or ACh) (Table 1) compared with Con rats.

Flow-induced dilation. Soleus 1A and 2As from Con and HLU rats exhibited a flow rate-dependent dilation in response to intraluminal flow (Fig. 1). ANOVA of relative diameter data indicated that flow-induced dilation of HLU arterioles was not different compared with Con arterioles. Dilation in response to intraluminal flow was an endothelium-dependent process because denuding the arterioles (with air) abolished flow-induced dilation without altering responses to constriction to 80 mM KCl or dilation to SNP in 1As from three Con rats (data not shown).

ACh-induced dilation in 2A arterioles. Soleus 2As from Con and HLU rats exhibited a dose-dependent dilation to ACh (Fig. 1). ANOVA of relative diameter data indicated that the dilator response to ACh for HLU rats was not different from that in Con rats. The sensitivity to ACh, reflected in the EC50, was similar between groups (Con 1.4 ± 2.1 × 10⁻⁷ M vs. HLU 5.1 ± 2.7 × 10⁻⁸ M).

Basal release of endothelium-derived dilators. Table 2 summarizes the effects of pharmacological inhibitors on baseline diameter (spontaneous tone). L-NNA treatment alone did not alter baseline diameter in 1As from Con rats but significantly decreased baseline diameter (increased tone) of 1As from HLU rats (Table 2). Indo treatment did not alter 1A baseline diameter in either group (Table 2).

In 2As from Con rats (Table 2), L-NNA treatment alone did not alter baseline diameter in either group. Indo treatment did not alter baseline diameter of 2As from Con rats, whereas Indo modestly diluted 2As from HLU rats. Indo did not dilate 2As used for the ACh experiments.

Double blockade with l-NNA + Indo induced a marked increase in baseline diameter (reduced spontaneous tone) of 1As from Con rats but did not alter baseline diameter in 1As from HLU rats (Table 2). In 2As from both groups, l-NNA + Indo increased baseline diameter (Table 2).

In 2A arterioles studied in the ACh experiments, l-NNA treatment tended to decrease baseline diameter (P = 0.06), and l-NNA + Indo treatment did not significantly alter baseline diameter. Indo did not alter baseline diameter of 2As from Con rats. Indo treatment, l-NNA treatment, or combined l-NNA + Indo did not alter baseline diameter in HLU 2As.

Relative roles of NOS and COX mediating flow-induced dilation. Treatment with 300 μM l-NNA blocked ~70% of the flow-induced dilation in 1A arterioles from Con rats (Fig. 2A) and similarly inhibited ~65% of

Fig. 1. Comparison of flow-induced dilation (A and B) and ACh-induced dilation (C) of soleus arterioles from control (Con) and hindlimb unweighted (HLU) rats. A: values are means ± SE for 25 Con and 23 HLU rats. First-order (1A) arterioles from both groups exhibit similar myogenic tone under zero-flow conditions and dilate to flow in a similar manner (ANOVA interaction effect: P = 0.99). B: values are means ± SE for 17 Con and 17 HLU rats. Second-order (2A) arterioles from both groups exhibit similar myogenic tone under zero flow conditions, and dilate to flow in a similar manner (ANOVA interaction effect: P = 0.22). Values are means ± SE for 12 Con and 13 HLU rats.

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flow-induced dilation in 1A arterioles from HLU rats (Fig. 2B). Indo did not alter flow-induced dilation in 1As of either group (data not shown). Surprisingly, combined treatment with L-NNA + Indo resulted in reduced spontaneous tone in Con 1As, which nearly abolished the capacity for flow-induced dilation (Fig. 2C). In HLU 1As, L-NNA + Indo did not alter flow-induced dilation or baseline diameter (Fig. 2D). To compare these effects, data were expressed as % possible dilation to correct for differences in baseline diameter. Results of this analysis indicated (Fig. 2, E and F) that there was no significant difference between flow-induced dilation in untreated arterioles (no inhibitors) compared with arterioles exposed to L-NNA + Indo.

Treatment with L-NNA abolished flow-induced dilatation in 2A arterioles from Con rats (Fig. 3A) but did not alter flow-induced dilation in HLU 2As (Fig. 3B). Indo

Table 2. Effects of inhibitors on baseline diameter

<table>
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<th>Arteriole</th>
<th>Group</th>
<th>Baseline</th>
<th>Baseline + L-NNA</th>
<th>Baseline</th>
<th>Baseline + Indo</th>
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<td>1A</td>
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<td>70 ± 7 (10)</td>
<td>78 ± 12</td>
<td>82 ± 12 (7)</td>
<td>71 ± 6</td>
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<td>55 ± 2 (8)</td>
<td>64 ± 4</td>
<td>64 ± 5 (9)</td>
<td>59 ± 5</td>
<td>65 ± 7 (8)</td>
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<tr>
<td>2A</td>
<td>Con</td>
<td>64 ± 6</td>
<td>59 ± 5 (9)</td>
<td>55 ± 3</td>
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<td>48 ± 8</td>
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<tr>
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<td>52 ± 4 (6)</td>
<td>47 ± 5</td>
<td>59 ± 4 (7)</td>
<td>45 ± 8</td>
<td>57 ± 8 (4)</td>
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<tr>
<td>2A</td>
<td>Con</td>
<td>63 ± 5</td>
<td>51 ± 6</td>
<td>57 ± 9 (10)</td>
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<td>52 ± 9 (7)</td>
<td>64 ± 4</td>
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</table>

Values are means ± SE (in μm). By themselves, Nω-nitro-arginine (L-NNA) or indomethacin (Indo) exerted little or no influence on baseline diameter (spontaneous tone) in 1A or 2A arterioles from either group. The exceptions are that L-NNA increased tone in HLU 1As and Indo reduced tone in HLU 2As. Combined L-NNA + Indo reduced tone in Con 1As and in both Con and HLU 2As. Inhibitors had no significant effects on baseline diameter of 2As used in the ACh experiments. Number of arteries studied indicated by parentheses.

Significant difference between baseline diameter without inhibitors compared baseline diameter in the presence of L-NNA, Indo, or L-NNA + Indo (P < 0.05).
did not alter flow-induced dilation in 2As from either group (data not shown), similar to results from 1As. Double blockade with L-NNA/Indo increased baseline diameter in 2As from Con and HLU rats. In 2As from Con and HLU rats, flow-induced dilation was not significantly different between uninhibited and L-NNA + Indo conditions in either Con (n = 5) or HLU (n = 4) rats. E and F: changes in baseline diameters caused by double blockade were corrected by calculating % possible dilation. This analysis revealed that flow-induced dilation is not significantly different between untreated and L-NNA + Indo conditions in either group.

Relative role of NOS and COX mediating ACh-induced dilation in 2A arterioles. Treatment with 300 µM L-NNA reduced ACh-induced dilation by ~40% in Con 2As (Fig. 4A). L-NNA abolished ACh-induced dilation in 2As from HLU rats (Fig. 4B). Combined treatment with L-NNA + Indo abolished ACh-induced dilation in Con 2As and HLU 2As (Fig. 4A and B). When the order of inhibitors was reversed, 50 µM Indo did not significantly alter ACh-induced dilation in Con or HLU 2As, and there was no difference between groups (data not shown). Also, in arterioles in which Indo was the first inhibitor, subsequent L-NNA + Indo treatment abolished dilation to ACh in both Con and HLU 2As (data not shown), identical to the L-NNA + Indo results seen in Fig. 4, A and B.

Calculated shear stress. Because shear stress is believed to signal flow-induced dilation, we determined whether HLU altered the shear stress required to cause dilation for each arteriole. Shear stress was calculated at each level of flow as \( \tau = 4 \eta Q/r^3 \), where \( \eta \) is viscosity of perfusate (0.008 dyn·s·cm\(^{-2}\)), \( Q \) is flow rate (in ml/s), and \( r \) is vessel radius (in cm). Results from control flow-diameter curves in 2As from Con and HLU rats are shown in Fig. 5. Calculated shear stress was not different between 2As from Con and HLU rats. Treatment with L-NNA increased shear stress equally in both groups, whereas Indo had no effect on the relationships between shear stress and flow in 2As from either group (data not shown). Treatment with L-NNA + Indo did not significantly alter the shear stress relationship in either group (data not shown). Furthermore, 1As from Con and HLU rats exhibit similar shear stress when untreated as well as under conditions of inhibition using L-NNA or Indo.

Endothelium-independent dilation. Dilation of 1As to SNP did not differ between Con and HLU rats (ANOVA \( P = 0.53 \), data not shown). Maximal dilation
Values are means ± SE for 17 Con and 17 HLU rats.

DISCUSSION

The purpose of these experiments was to test the hypothesis that HLU leads to impaired endothelium-dependent dilation characterized by blunted flow-induced dilation in soleus muscle 1As and 2As and impaired ACh-induced dilation in 2As. The new findings of this study are as follows: 1) flow-induced dilation in soleus 1A and 2A arterioles was not impaired by 14 days HLU. 2) Flow-induced dilation in Con and HLU 1As was mediated primarily by NOS. 3) Flow-induced dilation in Con 2As was primarily mediated by NOS, but 14 days HLU shifted flow-induced dilator responses from primary reliance on NO produced by NOS to reliance on a dilator mechanism that was NOS independent and COX independent. 4) In soleus 1A and 2A arterioles of HLU rats, flow-induced dilation can be mediated by an L-NNA- and Indo-insensitive mechanism, perhaps endothelium-derived hyperpolarizing factor (EDHF). 5) ACh-induced dilation is similar between 2As from Con and HLU rats, but, after HLU, soleus 2A arterioles exhibit complete reliance on NO produced by NOS for ACh-induced dilation. Taken together, these observations indicate that vascular remodeling in response to 14 days HLU, combined with shifts in the relative importance of endothelium-derived dilators, may influence vasodilation and thus exercise hyperemia on return to weight-bearing activity.

Vascular remodeling. Present results and current literature indicate that vascular remodeling is a consistent finding in the arteriolar network of soleus muscles of HLU rats, but this adaptation is not uniformly distributed throughout the arteriolar network (1, 8, 23). Data in Table 1 indicate that HLU reduced maximal passive diameter by 13% in 1As and 9.5% in 2As. These results compare well with HLU-induced remodeling of 1As (2, 23) and 2As (1) previously reported. Comparing across the soleus arteriolar network reveals that vascular remodeling occurred in a nonuniform manner because feed arteries remodeled to 22% smaller diameter (8), 1As to 13–15% smaller diameter (2, 23), and 2As to 9.5% smaller diameter. These data suggest the hypothesis that soleus arterioles distal to 2As may undergo progressively less remodeling than these larger arterioles. The finding that feed arteries through 2As remodeled to smaller diameters suggests that soleus vascular resistance would be expected to be higher after 14 days HLU. An increase in vascular resistance produced by vascular remodeling after 14
days of HLU may contribute to reduced blood flow at rest (17) and during exercise (31).

It is interesting that vascular remodeling to a smaller diameter does not appear to occur in the arterial network of the gastrocnemius muscle after HLU. Delp et al. (2) reported that 1A arterioles from the white portion of gastrocnemius muscle from HLU rats exhibit similar passive diameter as Con rats. The finding that the soleus muscle exhibits a decrease in blood flow during HLU, whereas the white gastrocnemius muscle exhibits no change in blood flow during HLU (17), is consistent with the hypothesis that a major signal for arteriolar remodeling is reduced muscle blood flow and/or shear stress (14).

Reduced shear stress during HLU could lead to lower NO production; thus lower NO levels during HLU may be one mechanism leading to remodeling. Importantly, eNOS expression is decreased in arterioles from soleus but not white gastrocnemius after HLU (29). Additional support for a role of NO in remodeling comes from experimental evidence indicating that eNOS knockout mice do not exhibit reductions in vascular diameter in response to chronic reductions in blood flow (22). These observations combined with the present data support the idea that vascular remodeling in the soleus arterial network is related to lower shear stress and production of NO during HLU.

Flow-induced dilation. Contrary to our hypothesis, flow-induced dilation of soleus 1A and 2A arterioles is not impaired after 14 days of HLU (Fig. 1). The lack of effect of HLU on flow-induced dilation in 1A and 2A arterioles is surprising given that soleus feed arteries exhibit impaired flow-induced and ACh-induced dilation after HLU (8). In addition, 1A arterioles have been reported to exhibit impaired ACh-induced dilation after HLU (2, 23). These results indicate that HLU effects on soleus muscle arteries are specific to the endothelial agonist (flow vs. ACh), as well as to the artery within a vascular network. Because previous work revealed that HLU altered the relative roles of NOS and COX in mediating ACh-induced dilation of soleus 1A arterioles (23), we assessed whether the mediators of flow-induced dilation are altered after HLU.

Baseline effects of L-NNA or Indo treatment. Before examining effects of L-NNA or Indo on flow-induced and ACh-induced dilation, it was important to determine whether these inhibitors exerted influence on spontaneous tone. Although in one subset of 1A arterioles L-NNA produced constriction, and in one subset of 2As Indo produced dilation, data in Table 2 indicate that, in general, L-NNA or Indo exerted little influence on spontaneous tone. Integration of these results indicates that NO and cyclooxygenase products are relatively unimportant in establishing basal or spontaneous tone in these arterioles of rat soleus muscle.

Our results suggest that the effect of combined treatment with L-NNA + Indo (“double blockade”) on baseline diameter was different depending on whether or not the arterioles were exposed to flow in vitro. By experimental design, the only difference between arterioles used in ACh and flow experiments was that the former were exposed to an ACh dose-response curve before treatment with double blockade and the latter were exposed to flow in vitro before treatment with double blockade. Our results indicate that exposure to the present flow protocol altered basal release of NO and COX products. This is demonstrated by the finding that double blockade exerted no significant effects on baseline diameter of 2As used in ACh experiments or 1As studied in ACh experiments (23). In contrast, double blockade after the flow protocol resulted in an altered baseline response to double blockade: the arterioles dilated in response to double blockade (Table 2). It is possible that some other NOS-independent and COX-independent pathway (perhaps EDHF) contributes to spontaneous tone after exposure to flow but not after exposure to an ACh dose-response curve. We are unaware of a mechanism by which prior exposure to flow could activate production of EDHF or other vasoactive compounds. It is interesting that the differential effects of double blockade on ACh vs. flow experiments suggest that intraluminal flow and ACh do not activate production of endothelium-derived factors equally.

Mediators of flow-induced dilation in control rats. On the basis of previous reports in the literature that examined rat gracilis muscle arterioles (11, 24), we anticipated that flow-induced dilation in soleus arterioles from control rats would be mediated by nearly equal contributions from the NOS and COX pathways. Specifically, we expected treatment with L-NNA or Indo would each inhibit ~50% of flow-induced dilation in soleus arterioles as occurs in gracilis muscle arterioles. Similar rationale predicted that combined treatment with L-NNA + Indo would abolish dilation to flow (11, 24). In the present study, flow-induced dilation of Con soleus 1A and 2A arterioles was mediated primarily via release of NO from the NOS pathway as indicated by the finding that L-NNA decreased flow-induced dilation by 70% in 1As and 100% in 2As. Indo did not produce significant changes in flow-induced responses in Con 1A or 2A soleus arterioles.

Combined treatment with L-NNA + Indo had surprising effects in both 1A and 2A arterioles of Con soleus muscle. In 1A arterioles from Con rats, treatment with both inhibitors reduced spontaneous tone by ~50%, such that flow-induced dilator responses could not be directly compared between treated and untreated conditions. When differences in baseline diameter were accounted for by calculating % possible dilation, responses to flow were not significantly different in untreated compared with L-NNA + Indo treated condition (Fig. 2, E and F). Likewise, in 2As, flow-induced responses were not significantly different in untreated compared with L-NNA + Indo treated arterioles. These data suggest that flow-induced dilation of soleus 1A and 2A arterioles can be mediated by a pathway (or pathways) other than NOS or COX, perhaps EDHF (19). Although our experimental design did not test this hypothesis, these results are consistent with the idea that NO and/or prostaglandins exert
feedback inhibition on EDHF in skeletal muscle arterioles (33).

**HLU alters the contributions of mediators of flow-induced dilation.** Although the overall response to flow was not different between Con and HLU rats (Fig. 1), our results suggest that HLU altered the relative importance of, or interactions among, endothelium-derived mediators responsible for flow-induced dilation in these arterioles. In 1A arterioles from HLU rats, L-NNA treatment produced a significant increase in basal tone (Table 2) and blocked 70% of the flow-induced response. This effect on the response to flow is similar to that seen with L-NNA treatment in Con 1As. Interestingly, flow-induced vasodilator responses of soleus 1A arterioles from HLU rats treated with L-NNA + Indo were similar to untreated HLU 1As, and basal tone was not significantly altered by double blockade of 1As from HLU rats (Table 2).

In soleus 2As, HLU did not alter flow-induced dilation or the apparent sensitivity of arterioles to flow and/or shear stress (Fig. 1). L-NNA treatment did not produce a significant change in baseline diameter in HLU 2As (Table 2) and did not have a significant effect on the flow-induced response. Thus, different from 1A arterioles of Con and HLU rats, and different from 2A arterioles of Con rats, NO release appears to be of minor importance in flow-induced dilation of 2A arterioles after HLU. Indo treatment of HLU 2As had no effect on the response to flow. Finally, flow-induced vasodilator responses of soleus 2As from HLU rats treated with L-NNA + Indo were not significantly different from untreated HLU 2As.

The results of double blockade with L-NNA + Indo in the HLU 1A and 2A arterioles demonstrate that flow-induced vasodilator responses in the presence of double blockade are not signifi cantly different from the response to flow in arterioles without inhibitors (Fig. 2D and 3D). These results suggest that, after HLU, flow-induced dilation in both of these arteriolar branch orders can be mediated by a pathway other than NOS or COX, perhaps EDHF (19). Results from the 2A arterioles presented in Fig. 3 suggest that this alternative pathway becomes the dominant mechanism responsible for flow-induced dilation of soleus 2A arterioles after HLU because treatment with l-NNa, Indo, or L-NNA + Indo does not significantly change the response to flow. This adaptation in mechanisms producing flow-induced dilation is similar to reports that eNOS knockout mice mediate flow-induced dilation via EDHF (7, 33). It is possible that EDHF pathways are “silent” unless the arterioles are treated with combined L-NNA + Indo treatment. There is evidence that NO normally binds to the heme group of cytochrome P450 enzymes, believed to produce EDHF (19). This effect, combined with “shunting” of arachidonic acid toward production of EDHF after Indo, could set up the apparent enhanced production of EDHF after double blockade due to removal of NO suppression on P-450 enzymes and shunting of arachidonic acid metabolites toward the EDHF pathway.

**ACh-induced dilation of 2A arterioles.** ACh-induced dilation of Con 2As was reduced 40% by treatment with l-NNa (Fig. 4A) and Indo treatment did not affect ACh-induced dilation. The finding that l-NNa treatment only blocked 40% of ACh-induced dilation and that combined treatment with l-NNa + Indo abolished ACh-induced dilation suggests that prostacyclin (PGI2) release from the COX pathway contributes to ACh-induced dilation of Con 2A arterioles (Fig. 4). However, Indo treatment alone had no significant effect. Because it has been reported that NO can inhibit PGI2 production (6, 21), it is possible that inhibition of NO increases PGI2 release so that Indo only inhibits ACh-induced dilation during NOS blockade. In other words, blockade of NO production may remove a suppressive effect on PGI2 production. Whatever the mechanisms

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**Fig. 6. Role of NOS activity in flow-induced dilation of 1A (A) and 2A (C) arterioles and in ACh-induced dilation of 1A (B) and 2A (D) arterioles from Con and HLU rats.**

A: l-NNa nearly abolished flow-induced dilation in 1As from Con and HLU rats, indicating that NOS is an important mediator of flow-induced dilation in 1As and that HLU does not alter this. B: ACh-induced dilation is decreased only 40% by l-NNa in 1As from Con rats but is abolished by l-NNa in 1As from HLU rats. Data from Schrage et al. (23). C: l-NNa abolished flow-induced dilation in Con 2As but had no significant effect on flow-induced dilation of HLU 2As. D: l-NNa abolished ACh-induced dilation in HLU 2As but only decreased ACh-induced dilation by 40% in Con 2As.
for the interaction between the COX and NOS pathways observed in Con 2A arterioles, this interaction is no longer apparent in 2As from HLU rats.

After HLU, responses of soleus 2A arterioles to ACh were not impaired, but 2As exhibited increased reliance on release of NO to mediate ACh-induced dilation. This interpretation is supported by the observation that treatment with L-NNA abolished ACh-induced dilation in 2As from HLU rats whereas Con 2As exhibited significant dilation after L-NNA treatment (Fig. 4). Also, Indo treatment (with or without L-NNA treatment) had no effect on ACh-induced dilation of HLU 2As. The increased contribution of NO as a mediator of ACh-induced dilation in 2A arterioles after HLU is similar to that previously reported for soleus 1A arterioles (23).

Comparison of mediators of flow-induced vs. ACh-induced dilation. It is interesting that the relative contributions of mediators producing flow-induced dilation are not the same as those producing ACh-induced dilation in either 1A or 2A arterioles of soleus muscle. For example, blockade of the NOS pathway in Con 1A arterioles nearly abolished flow-induced dilation but only produced a small decrease in ACh-induced dilation (Fig. 6, A and B). Perhaps of greater interest, the effects of HLU on the relative contributions of mediators producing flow-induced dilation are not the same as the effects of HLU on the relative contributions of mediators producing ACh-induced dilation. Specifically, in soleus 1A arterioles, HLU did not appear to alter the relative importance of NO in flow-induced dilation (Fig. 6A) whereas HLU resulted in a dramatic increase in the relative importance of NO for ACh-induced dilation (23) (Fig. 6B). The differential effects of HLU on flow-induced and ACh-induced dilation in soleus 2A arterioles are also striking. HLU decreased the relative importance of NO in flow-induced dilation of 2As (Fig. 6C) but increased the relative importance of NO in ACh-induced dilation of 2As (Fig. 6D). In fact, flow-induced dilation was not significantly altered by L-NNA treatment in HLU 2A arterioles, whereas L-NNA abolished ACh-induced dilation in 2A arterioles from HLU rats. We do not know of an established mechanism by which HLU could induce opposing effects on the role of the NOS pathway in these two types of endothelium-dependent dilation in soleus 2A arterioles. These results, combined with available literature, indicate that the complex interactions and cross talk among endothelial mediators and biochemical pathways for producing dilation differ among various skeletal muscle arterioles (7, 23, 25, 33). Furthermore, the compensatory mechanisms produced by chronic absence of any of these mediators depends on the mediator removed, gender, and skeletal muscle examined (7, 23, 33).

It is important to emphasize that the structural and functional adaptations observed in soleus 1A and 2A arterioles after 2 wk HLU may not reflect adaptations present at other times after HLU. Previous studies of the HLU-induced response of skeletal muscle microcirculation indicate that the response to HLU is a dynamic temporal response (2). For example, soleus 1As exhibit impaired ACh-induced dilation at 2 wk HLU (2, 23), which is normalized at 4 wk HLU (2). Therefore it is important to consider the duration of HLU when comparing results from different studies because adaptations may be different when examined after various durations of HLU.

In summary, the results of this study reveal that 14 days HLU does not result in impaired flow-induced dilation in soleus 1A or 2A arterioles or impair ACh-induced dilation of soleus 2A arterioles. Jasperse et al. (8) reported that HLU produced decreased flow-induced and ACh-induced dilation of soleus feed arteries and that these changes were associated with decreased expression of eNOS in feed arteries. Recently, Woodman et al. (29) reported that 14 days of HLU produced decreased eNOS protein content in soleus feed arteries and 1A arterioles but not 2A arterioles. No change in eNOS protein content in 2A arterioles is consistent with present findings that flow-induced and ACh-induced dilation of 2A arterioles are not decreased after 14 days HLU and is consistent with the concept that structural, molecular, and functional adaptations to HLU are dynamic and temporally and spatially specific. However, the increased relative importance of NO in ACh-induced responses and decreased role of NO in flow-induced responses cannot be explained simply by these changes in eNOS expression. Present results, demonstrating that flow-induced dilation was not significantly different before and after treatment with L-NNA + Indo in either 1A or 2A soleus arterioles from HLU rats, suggest that 14 days of HLU increases the contribution of EDHF (or other NOS- and COX-independent pathways) in mediating flow-induced dilation in these arterioles. Although it is reasonable that these HLU-induced changes in endothelial function contribute to altered soleus muscle blood flow and limited exercise capacity after HLU, our results do not allow us to directly relate these changes to altered control of blood flow in soleus muscle. Integration of available results reveals two exciting concepts. First, the relative importance of endothelium-dependent vascular control mechanisms in soleus muscle arterioles, and the adaptations induced by 14 days of HLU, are not the same in all branch orders in the arteriolar tree. Second, the changes produced by HLU in one endothelium-dependent dilator system may not be apparent in another endothelial dilator system.

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