Interaction Between Microvascular $\alpha_1$- and $\alpha_2$-Adrenoceptors and Endothelium-Derived Relaxing Factor

Mitsumasa Ohyanagi, Kazuhiko Nishigaki, and James E. Faber

Intravital microscopy was used to study the effect of endothelium-derived relaxing factor (EDRF) on microvascular adrenoceptor sensitivity in rat cremaster skeletal muscle. N(G)-Monomethyl l-arginine (L-NMMA, 1–300 $\mu$M), an inhibitor of EDRF formation, produced concentration-dependent constriction of arterioles and venules. When an intermediate amount of $\alpha_1$-versus $\alpha_2$-adrenoceptor tone was first produced with bath-added norepinephrine (NE) in the presence of rauwolscine or prazosin, L-NMMA caused constriction with greater potency and efficacy during $\alpha_2$ than during $\alpha_1$ tone. During localized $\alpha_1$ or $\alpha_2$ constriction along an arteriole by perivascular micropipette suffusion of NE in the presence of rauwolscine or prazosin, again, bath-added L-NMMA produced constriction with greater potency during $\alpha_2$ than during $\alpha_1$ constriction. Like L-NMMA, disruption of EDRF release by microembolization caused baseline arteriole constriction and selectively increased $\alpha_2$ sensitivity 75-fold. Although these findings support the hypothesis that endothelial cells possess $\alpha_2$-adrenoceptors that promote EDRF release, a greater susceptibility of $\alpha_2$ than $\alpha_1$ constriction to EDRF inhibition could also account for the results. In support of this latter possibility, $\alpha_2$ constriction was approximately 50-fold more susceptible than $\alpha_1$ constriction to inhibition by the EDRF-like nitrodilator nitroprusside. The similarity in magnitude of this difference in sensitivity with the difference obtained in the embolization experiments does not support the hypothesis that microvascular endothelial cells in skeletal muscle possess EDRF-promoting $\alpha_2$-adrenoceptors. However, these data do suggest that endogenous EDRF release modulates basal arteriole and venule tone and that $\alpha_2$-adrenoceptor constriction is more sensitive than $\alpha_1$ constriction to inhibition by EDRF. (Circulation Research 1992;71:188–200)

**Key Words** • endothelium-derived relaxing factor • $\alpha$-adrenergic receptors • arterioles • venules • N(G)-monomethyl l-arginine • endothelial cells • microcirculation

Since the discovery$^1$ that acetylcholine (ACh) can stimulate endothelial cells to release a potent vasodilator substance—endothelium-derived relaxing factor (EDRF) — that is nitric oxide or a nitric oxide adduct,$^2,^3$ much has been learned about its synthesis, metabolism, release, and mode of relaxant action on vascular smooth muscle.$^4,^5$ On the other hand, the significance of EDRF in vascular regulation is not as well understood. The dilator properties of a number of vasoactive substances, e.g., ACh, bradykinin, and histamine, are mediated partly or entirely by EDRF.$^5$ Also, flow-mediated dilation appears to involve a shear-stress–induced increase in EDRF release.$^6,^7$ In certain$^8,^9$ but not all$^{10–12}$ vessels, myogenic constriction may be mediated in part by endothelial cell vasoactive factors including EDRF. Inhibition of EDRF synthesis by substituted L-arginine compounds that serve as substrate inhibitors of EDRF synthetase$^{13}$ produces increases in blood pressure and vascular resistance in several tissues.$^{14,15}$ This suggests that tonically released EDRF is a determinant of basal resistance and flow.

There have been relatively few studies of EDRF interactions with vessels of the microcirculation.$^{16–22}$ Most of what is known about the vascular actions of EDRF has come from in vitro studies of smooth muscle from large arteries and veins.$^5$ These studies have revealed that considerable heterogeneity in the contribution of EDRF mechanisms can exist among tissues and species.$^{23,24}$ In the few studies in which comparisons have been made, responses to ACh and other endothelium-dependent dilators are often$^{25–28}$ but not always$^{29}$ much lower in veins than in arteries. These large vessels may differ in their responses from small arteries and arterioles (resistance vessels) and muscular venules, which are the main determinants of resistance and venous return for vascular regulation.

Under normal conditions, basal vascular resistance in certain tissues, such as skeletal muscle, is dominated by $\alpha$-adrenergic constriction and/or intrinsic smooth muscle tone of metabolic, myogenic, and/or autocoid origin. Thus, clarifying a physiological role for EDRF requires knowledge of how it interacts with adrenergic constriction. There is controversy concerning interaction between EDRF and $\alpha$-adrenergic vascular regulation. In vitro studies have found indirect evidence both supporting$^{30–32}$ and in conflict$^{33,34}$ with the concept that endo-

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endothelial cells can possess \( \alpha \)-adrenergic receptors that mediate release of EDRF. Species and tissue differences may prevent generalization of this concept. However, autoradiography has been unable to detect the presence of these receptors on endothelial cells of large vessels from several species.\(^{35}\) Moreover, previous functional studies have often been complicated by the presence of \( \alpha \)-receptors on vascular smooth muscle that can mediate contraction.

Based on functional studies, \( \alpha \)-adrenergic receptor subtypes appear to be distributed heterogeneously across the vasculature. Catecholaminergic constriction is mediated by \( \alpha_1 \)-receptors for most large arteries and by both \( \alpha_1 \)- and \( \alpha_2 \)-receptors for resistance vessels, muscular venules, and veins.\(^{36-38}\) We have found that constriction mediated by these two adrenoceptor populations exhibits very different sensitivities to modulation by humoral,\(^{39,40}\) local metabolic,\(^{41}\) and myogenic\(^{38}\) regulation. This has led us to hypothesize that a differential distribution of \( \alpha \)-adrenergic receptor types and susceptibility to modulation among functionally distinct vascular segments may be important in determining the site of interaction of neural with humoral and local control mechanisms. It is not known whether EDRF interacts differently with \( \alpha_1 \) and \( \alpha_2 \) constriction of arterioles and venules, which could be important in clarifying which functional segments of the vasculature are under EDRF regulation. The present study examined this question in the rat cremaster skeletal muscle microcirculation, using the EDRF substrate inhibitor \( N^\text{G} \)-monomethyl l-arginine (L-NMMA) and disruption of endothelial cell EDRF release by microembolization.

**Materials and Methods**

**Drugs**

Normetanephrine and desipramine (Sigma Chemical Co., St. Louis, Mo.), rauwolscine (RWL, Boehringer-Ingelheim, FKG), and prazosin (PRZ, kindly donated by Pfizer Pharmaceutical, Groton, Conn.) were frozen in 1 mM ascorbate saline for <6 weeks and diluted with water on the day of the experiment. Propranolol, ACh, and nitroprusside (Sigma) were dissolved in saline and kept frozen ≤6 weeks. L-NMMA (dissolved in water, Calbiochem Corp., La Jolla, Calif.), norepinephrine (NE, dissolved in ascorbate saline, Sigma), and prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\), dissolved in sodium carbonate [wt/wt] in water, Sigma) were prepared daily. All agents were added in 27–40 \( \mu \)l aliquots to a 40-ml cremaster bath except for local micropipette suffusion of NE (dissolved in Krebs’ solution).

**Surgical Procedures**

Detailed methods of surgical procedures are given elsewhere.\(^{40,42}\) Fifty-three male, 6–7-week-old Sprague-Dawley rats (mean ± SEM body weight, 168±5 g) were anesthetized with urethane and \( \alpha \)-chloralose (425 and 100 mg/kg i.m., respectively) and tracheotomized; rectal temperature was maintained at 37°C. Mean arterial pressure and heart rate (left carotid cannula) at the beginning of the experiments were 98±3 mm Hg and 486±8 beats per minute, respectively, and at the end were 95±3 mm Hg and 480±9 beats per minute, respectively.

The right cremaster muscle was denervated via nerve transection\(^{40,41}\) to prevent release of endogenous NE and possible complications of interpretation presented by interaction of ligands with presynaptic \( \alpha \)-adrenoceptors. The muscle was prepared with intact cremasteric and deferential artery circulations\(^{42}\) and was extended over an optical port in a 40-ml tissue bath filled with a modified Krebs’ solution. A gas mixture of nitrogen and carbon dioxide was bubbled through both the tissue bath and the Krebs’ stock reservoir to provide mixing and to maintain tissue bath and stock solution \( P_{\text{O}_2} \) (15–30 mm Hg), \( P_{\text{CO}_2} \) (35–45 mm Hg), and pH (7.4±0.05). Tissue bath parameters were digitized (1 Hz), and 1-minute averages were graphically displayed and stored for later analysis of preparation stability. The preparation was transilluminated with a 20-W filtered (420–600-nm bandpass) halogen light source, and vessel inner diameter was measured with a videomicroscopic digital image analysis system accurate to ±1 \( \mu \)m.\(^{43}\)

Beginning with the major cremasteric arteriole and paired venule designated as the first-order arteriole (1A arteriole) and first-order venule (1V venule), consecutive branches were numbered accordingly. Arterioles branching at approximately right angles from the first 2A branch that supplied the anterolateral portion of the cremaster muscle and that had diameters ≤50% of the 2A arteriole were designated as third-order arterioles (3A arterioles). In each experiment, either a 1A/1V or 2A/2V “large” arteriole and venule pair or a 3A “small” arteriole was chosen for study. After completion of the surgery, the cremaster was allowed to equilibrate for approximately 30 minutes. The preparation was examined during this period and judged to be acceptable if 1) mean arterial pressure was ≥80 mm Hg, 2) terminal arterioles in the area of study exhibited vasomotion, and 3) no venous stasis, leukocyte adhesion, or petechial hemorrhages were present. Failure of these criteria to be maintained over the duration of an experiment resulted in the exclusion of 13 of 66 preparations. Surgical procedures required approximately 80 minutes, and the experimental protocols were generally 2–3 hours in duration. The cremaster bath solution contained at all times propranolol (1 \( \mu \)M) for blockade of \( \beta \)-adrenergic receptors and desipramine (10 nM) and normetanephrine (10 \( \mu \)M) for blockade of neuronal and nonneuronal catecholamine uptake mechanisms, respectively.\(^{37,40,42}\) Drug concentrations expressed here and elsewhere represent final bath concentrations.

**Experimental Protocol**

*Experiment I: Effect of L-NMMA on \( \alpha \)-adrenergic tone.* The effect of L-NMMA on large arterioles and venules was evaluated after preconstriction with a low concentration of NE (10 nM) in the presence of the \( \alpha_1 \)-antagonist PRZ (0.1 \( \mu \)M) or the \( \alpha_2 \)-antagonist RWL (1 \( \mu \)M) to select for activation of \( \alpha_1 \) or \( \alpha_2 \)-adrenoceptors, respectively (Figure 1, top panel). A given antagonist, which was added at least 15 minutes before the first 5-minute control period (C\(_t\)), remained in the bath throughout the protocol for this and all subsequent experiments. The potency and selectivity of these antagonist concentrations, which were also used in all subsequent protocols, have been determined previously for cremasteric arterioles and venules.\(^{37,41}\) After \( \alpha_1 \) or \( \alpha_2 \)
tone was induced, an L-NMMA concentration-
response curve was obtained by stepwise cumulative
addition of L-NMMA to the cremaster bath in approx-
imately half-log increments at 5-minute intervals that
were sufficient to achieve steady constrictor responses.
After completion of the curve and a 30-minute wash
interval to reestablish control diameter (C2), mainte-
nance of adrenoceptor sensitivity was verified by a
second application of 10 nM NE for 10 minutes (in most
experiments, not shown in Figure 1). Maximal NE
constriction (30 μM) was then obtained for normaliza-
tion of L-NMMA responses. After a wash interval,
maximal diameter was obtained with complete dilation
by nitroprusside (30 μM). The data obtained from each
experiment in this and subsequent experiments (see
below) were judged to be acceptable if 1) no significant
difference existed between arteriole diameter during C1
and C2, 2) no significant difference was evident in the
NE response obtained initially and at the end of the experi-
ment, 3) maximal responses to NE were similar to
data obtained in previous studies from this laboratory,
and 4) arterioles had intrinsic tone during control as
determined by nitroprusside dilation.

In nine rats the sensitivity to L-NMMA of large
arterioles and venules (n=6 pairs) and small arterioles
(n=3) in the absence of adrenergic constriction was
studied to determine the effect of blockade of EDRF
synthesis on basal tone. The protocol was similar to the
previous experiment, but no NE was administered ex-
cept for the maximal constrictor concentration that
was tested near the end of each experiment. Maximal
constriction of 3A arterioles was obtained with a lower NE
concentration (1 μM),37 whereas 30 μM was used for the
large vessel experiments. Time-averaged diameter of
3A arterioles was continuously measured43 because
these vessels exhibit vasomotion, i.e., periodic contrac-
tion and relaxation cycles of 5–20 per minute.

The specificity of L-NMMA for substrate inhibition
of nitric oxide synthase was evaluated in five rats during
the above protocol after obtaining the response to 300
μM L-NMMA. Before washout of L-NMMA, d-arginine
(300 μM) was added to the bath (10 minutes), and then
L-arginine (300 and 600 μM) was added sequen-
tially at 10-minute intervals. A single 2A and 2V pair
was studied in each of four additional animals to
determine the specificity of L-NMMA for inhibition of
EDRF synthesis. Previous studies in this laboratory
have determined that first- and second-order vessels
have similar α1- and α2-adrenoceptor sensitivity.37 Sec-
ond-order vessels were studied in this dilator protocol
because the 2A arteriole possesses greater intrinsic tone
than the 1A arteriole.37 After a 30-minute stabilization
and 5-minute control period, ACh (0.1 μM) was added
to the bath for 10 minutes to evoke dilation. Subsequent
control and drug testing periods consisted of 2- and
10-minute intervals, respectively, and the wash intervals
lasted 10 minutes between drugs. After a wash interval
and return to control diameter, nitroprusside (1 μM)
was tested. After a wash and control interval, 100 μM
L-NMMA was tested, followed by L-NMMA plus ACh.
This was followed by a wash interval, return to control,
and addition of L-NMMA for 10 minutes. Thereafter,
L-NMMA plus nitroprusside were added. After a wash
interval of 30 minutes and after control measurements
were made, 6 μM NE was added to the bath to produce
an amount of constriction similar to that produced by
L-NMMA. Ten minutes later, ACh was tested in the
presence of NE. After a wash interval and a final
control period, constriction to 6 μM NE was reexam-
ined, followed by the addition of nitroprusside in the
presence of NE.

Experiment 2: Effect of L-NMMA on focal α-adrener-
gic constriction. In the above experiments, application of
a vasoactive agent to the cremaster bath induces diam-
eter changes in vessels in addition to the vessel under
study. This may produce hemodynamic changes in the
network that could obfuscate the direct effect of the
agent on the vessel of interest. To minimize this poten-
tial complication, experiments were performed with
perivascular suffusion of NE by micropipette to produce
focal α1 or α2 constriction of the 1A arteriole, followed
by bath-added L-NMMA and determination of the
additional amount of L-NMMA constriction. 1A arte-
rioles were studied in a region where no 2A branches
were present and where 1A diameter along 500–700 μm
of length was similar. At least 15 minutes before the
start of the first 3-minute control period, either PRZ or
RWL was added to the bath and was without effect. The
tip of a glass micropipette (tip diameter, 3.8±0.2 μm)
filled with NE (3 nM in Krebs' solution) was positioned
within 15–20 μm of the arteriole wall with a hydraulic
micromanipulator. Continuous NE ejection was
achieved with a Picospritzer II (General Valve Co.,
Fairfield, N.J.) triggered by an external stimulator that
delivered 13-V rectangular pulses. A desired amount of
focal constriction was achieved by varying the Pi-
cospritzer pressure (2–5 psi) and the duration (7–9
msec) and frequency (0.1–0.8 Hz) of the square-wave
stimuli. Constriction reached a steady-state value within
2–3 minutes and was adjusted to decrease vessel diam-
eter by a different amount (∼10–50%) in each experi-
ment (Figure 5, top panel). In other studies from this
laboratory,44 focal α1 or α2 constriction persisted un-
changed for at least 40 minutes during continuous
ejection at the same rate; comparable ejection of the
Krebs' vehicle had no effect. The distances between
the point of maximal constriction adjacent to the pipette
tip and points upstream and downstream over which the
constriction extended were determined in each experi-
ment to define the limits of NE constriction. No con-
striction extended more than 300–400 μm above or
below the pipette tip. A site 50-μm upstream from the
limit of detectable NE constriction was defined as the
"upstream site" and was studied to allow determination
of the effect of bath-added L-NMMA, per se, in the
absence of concomitant NE constriction (Figure 5,
bottom panel). After NE suffusion alone for 10 minutes,
L-NMMA (0.1 mM) was added to the bath for 10
minutes, during which time NE suffusion was main-
tained. Thereafter, suffusion was stopped, and there
was a wash interval of 30 minutes. After the vessel had
returned to the initial control diameter, NE (30 μM)
was added to the bath to obtain maximal constriction.
After a wash interval, nitroprusside was added for 5
minutes to determine the amount of intrinsic tone
present in the 1A arteriole during control conditions.

Experiment 3: Effect of disruption of EDRF release on
α-adrenergic constriction. A nonpharmacological
method of EDRF inhibition was also studied for com-
parison with L-NMMA. Endothelial release of EDRF
was disrupted by microembolization. In six rats a pulled-out PE-10 catheter was inserted into the right femoral artery. The catheter was advanced into the right common iliac artery, and the tip was placed at the bifurcation of the pudic–epigastric trunk to allow syringe pump infusion of CO₂ into the cremaster arterial supply. After a 3-minute control period with measurement of 1A diameter, CO₂ was infused (0.25 ml over 1 minute), and a subsequent 30-minute period was observed during which the bath was changed several times (Figure 6, top panel). The rate of CO₂ infusion was determined in preliminary experiments on the basis of cremaster wet weight (320±10 mg, n=3) and elimination of ACh dilation. During CO₂ infusion, emboli entered the 1A arteriole intermittently and were distributed throughout the branching arterioles, with cessation of flow in some arterioles in which emboli became lodged. After termination of CO₂ infusion, emboli were absorbed within 5–10 minutes with no detectable thrombogenic effects. Thirty minutes were observed with several bath changes to allow the vessels to regain tone, which was absent during and immediately after reabsorption of microemboli. Experiments were terminated in three animals because arterial pressure became unstable after CO₂ infusion; there was no effect on arterial pressure or heart rate in the remaining animals. After a second control period (C₂), several concentrations of ACh were added cumulatively to the bath at 5-minute intervals (Figure 6, top panel), followed by a wash, a third control period (C₃), and several concentrations of nitroprusside. After a wash interval and a fourth control period (C₄), L-NMMA (0.1 mM) was added for 10 minutes, followed by a wash period. At least 15 minutes before the start of the fifth control period (C₅), either PRZ or RWL was added to the bath. An α₁- or α₂-adrenergic concentration–response curve was then constructed by cumulative addition of NE to the bath at 5-minute intervals. After a 30-minute wash in the presence of the opposite adrenergic antagonist, a second NE curve was obtained. The order of PRZ or RWL present during the two sequential curves was randomized.

Four additional animals were studied as a control for experiment 3 to determine the effect on α₁- and α₂-adrenoceptor sensitivity (EC₅₀) of the increase in basal tone after disruption of EDRF release by embolization. The protocol was identical to experiment 3 (Figure 6, top panel) except that PGF₂α (0.3–5.0 µM) was added to the bath after C₁, instead of CO₂ infusion, in sufficient concentration to reduce diameter by a similar amount. PGF₂α remained in the bath throughout the rest of the protocol.

Experiment 4: Sensitivity of α-adrenoceptor constriction to nitroprusside. Differences in the sensitivity of α₁ and α₂ constriction to L-NMMA and microembolization could be dependent not only on EDRF release by stimulation of putative endothelial α₂-adrenoceptors but also (or instead) on a greater sensitivity of α₂ constriction to cGMP-dependent dilator mechanisms activated by EDRF. To test this hypothesis, focal constriction of 1A arterioles was produced by peryvascular micropipette suction of NE in the presence of bath-added PRZ or RWL (Figure 8, top panel) as in the previous experiment. A concentration–response curve for addition to the bath of the EDRF-like nitrodiolator nitroprusside, which, like EDRF, produces cGMP-dependent dilation, was then obtained. After a 30-minute wash to reestablish the control diameter, NE (30 µM) was added to the bath to produce maximal constriction.

**Data Analysis**

Vessel diameter values are the average of two measurements taken at 1-minute intervals over the last 2 minutes of each control period or test interval. For construction of concentration–response curves, drug responses are expressed as a percentage of the maximal response to NE: response = [(Dc–Dx)/(Dc–Dmr)]×100, where Dc is the control diameter, Dx is the diameter produced by x concentration of drug, and Dmr is the diameter obtained during maximal constriction with NE (30 µM for large arterioles and venules or 1 µM for small arterioles). In experiment 3, concentration–response curves and EC₅₀ values were derived from nonlinear, least-squares, sigmoid regression analysis. Drug responses in experiment 4 are expressed as a percentage of dilation: response = [(Dc–Dpc)/(Dc–Dmr)]×100, where Dpc is the diameter obtained during preconstriction by micropipette suction of NE. Data were analyzed with paired and unpaired two-tailed t tests where appropriate. Bonferroni procedures were used for multiple comparisons. Data from experiment 2 were subjected to analysis of covariance (general linear model Systat, Inc., Evanston, Ill.). Results are expressed as mean±SEM, with p<0.05 representing significance.

**Results**

**Effect of L-NMMA on α-Adrenergic Tone**

L-NMMA induced concentration-dependent constriction of both 1A and 1V vessels in the presence of NE added to the bath to impose a low level of α₁ (+RWL) or α₂ (+PRZ) tone (Figures 1 and 2). The potency of L-NMMA was significantly greater in the presence of α₂ than in the presence of α₁ tone. The amounts of basal NE constriction of 1A and 1V vessels (NE₁ in Table 1) were not significantly different during α₁ versus α₂ stimulation. In the absence of adrenergic tone, L-NMMA also produced constriction of microvessels (Figure 2, bottom panel). 1A and 1V vessels exhibited similar sensitivity to L-NMMA, whereas small 3A arterioles were more sensitive.

Control (baseline) data for experiment 1 are given in Table 1. 1A diameter for the RWL and PRZ groups during the first control period was not significantly different. The same lack of baseline differences was noted for the 1V groups. There was no significant difference in the maximal NE constriction of 1A arterioles in the presence of RWL versus PRZ; this was also the case for the 1V groups. These similarities indicate that the greater L-NMMA potency during α₂ tone is not due to differences in initial diameters or maximal NE responses. The stability of the preparations is evidenced by 1) the lack of significant differences in diameter between the C₁ and C₂, 2) the consistent amount of intrinsic tone present during control periods in arterioles, as revealed by significant nitroprusside dilation, and 3) the similarity of response to the low concentration of NE retested at the end of the experiment (NE₂ on Table 1) when compared with the response to the same concentration tested earlier (NE₁).
The specificity and efficacy of L-NMMA were evaluated in several ways. The constriction of 1A and 1V vessels produced by 300 μM L-NMMA (the highest concentration tested) was unaffected by sequential application of D-arginine but was antagonized in a concentration-dependent manner by L-arginine (Figure 3). In a separate study of 2A and 2V vessels, L-NMMA inhibited dilation by ACh, which produces dilation by release of EDRF, but had no effect on dilation by the EDRF-independent dilator nitroprusside (Figure 4). The abolishment of ACh dilation by L-NMMA could not be attributed to the L-NMMA constriction, per se, since ACh (and nitroprusside) produced dilation when vessels were constricted with NE to the same degree as that produced by L-NMMA (Figure 4). 2A and 2V vessels returned to control diameters (C1–C6) that did not differ significantly (2A diameter for C3–C6, 79±7 to 81±7 μm, respectively; 2V diameter for C3–C6, 156±19 to 157±19 μm, respectively) among the individual interventions in the experiment shown in Figure 4.

**Effect of L-NMMA on Focal α-Adrenergic Constriction**

Continuous perivascular micropipette suffusion of NE (in the presence of bath-added RWL or PRZ) was used to produce focal α1 or α2 constriction along the 1A arteriole (Figure 5, top panel). This was done to eliminate hemodynamic changes at the site of observation produced by bath-added NE acting elsewhere in the microvascular network. For all experiments (n=12), and irrespective of whether α1- or α2-adrenoceptors were selected, the focal constriction extended 208±37 μm upstream and significantly less (141±23 μm, p<0.05) downstream from the pipette tip; there was no significant difference in the length of focal constriction obtained during α1 versus α2 constriction. The pipette tip was located adjacent to the vessel and at mid depth in the cremaster (=100 μm from either cremaster surface). A similar range of α1 constriction (8–53% of control diameter) and α2 constriction (14–42% of control diameter) was produced in the two groups adjacent to the pipette tip. After focal NE constriction, bath-added L-NMMA (10−4 M) produced additional constriction. The “additional L-NMMA constriction” produced at the site of focal NE constriction was normalized against the 1A constriction produced by L-NMMA alone at the “upstream site” located 50 μm beyond the upstream limit of NE constriction (Figure 5, bottom panel). Before NE or L-NMMA application, diameter at the upstream site and pipette site did not differ significantly. Also, L-NMMA produced constriction at the upstream site that did not differ significantly between the α1 and α2 groups. The amount of additional L-NMMA constriction at the pipette site, when regressed against the amount of initial α1 versus α2 constriction, indicated that L-NMMA was significantly more potent and efficacious during α2 activation (Figure 5, middle panel).

Control diameters did not differ significantly (C1, 112±5 μm; C2, 110±5 μm; n=12); the maximal constric-
L-arginine (LMA) norepinephrine of first-order arterioles, 1A, 1V, per arteriole; 3A, third-order arteriole.

**FIGURE 2.** Top panel: Line graph showing NG-monomethyl L-arginine (LMA) sensitivity of large venules during α1 versus α2 preconstriction. 1V, First-order venule; n, number of vessels (one per rat). Bottom panel: LMA sensitivity in the absence of norepinephrine preconstriction for different vessel types. 1A, First-order arteriole; 3A, third-order arteriole.

**TABLE 1.** Baseline Control Data for Large Arterioles, Large Venules, and Small Arterioles in Experiments Examining Interaction Between Adrenergic Tone and NG-Monomethyl l-arginine and Effect of NG-Monomethyl l-arginine Alone

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Control diameter</th>
<th>NP (μM)</th>
<th>NEmax (% of C2)</th>
<th>NE1 (% of C1)</th>
<th>NE2 (% of C2)</th>
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<td>C1 (μM)</td>
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<td>127±3</td>
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<td>17±1</td>
<td>38±4*</td>
<td>39±7</td>
<td>...</td>
</tr>
</tbody>
</table>

n, Number of vessels in each group; C1, control diameter before norepinephrine (NE) or NG-monomethyl l-arginine (L-NMMA); C2, control diameter after 30-minute change of bath; NP, nitroprusside (30 μM); NEmax, maximal percent decrease in diameter from control during constriction with highest concentration of NE (30 μM for first-order arteriole [1A] and first-order venule [1V], 1 μM for third-order arteriole [3A]); NE1 and NE2, diameter during low concentration (10 nM) of NE after C1 and C2, respectively; RWL, rauwolscine (1 μM); PRZ, prazosin (0.1 μM); experiment 1, interaction between adrenergic tone and L-NMMA; experiment 2, effect of L-NMMA alone. Values are mean±SEM.

In all experiments propranolol (1 μM), desipramine (10 nM), and normetanephrine (10 μM) were present, except for control arterioles when no drugs were present. Arterioles were maximally preconstricted by norepinephrine (NE); L-NMMA was added to the bath to 0.1 μM before NE was added. NE concentration at corresponded with the graphs of Figs. 2 bottom and 3. Values are mean±SEM.

**FIGURE 3.** Bar graph showing specificity of NG-monomethyl L-arginine (LMA) constriction of first-order arterioles and venules for substrate antagonism of endothelium-derived relaxing factor synthesis by L-arginine (L-Arg) but not D-arginine (D-Arg). n, Number of vessels (one per rat).

Interaction to bath-added NE (3×10⁻⁵ M) reduced diameter to 42±2% of C2, and nitroprusside produced significant dilation (110±4% of C1, p<0.05). These control data also did not differ significantly when compared between the α1 and α2 groups.

**Effect of Disruption of EDRF Release on α-Adrenergic Constriction**

Disruption of EDRF release by CO₂ microembolization produced sustained vasoconstriction of 1A arterioles (58±4% of C1 diameter, top panels of Figures 6 and 7). PGF2α was applied to the bath in a separate group to induce a similar amount of constriction (62±3% of C1, Figure 7, bottom panel). Control diameters of the two groups (C1) were similar (123±4 versus 127±1 μm). α1-Adrenoceptor sensitivity was significantly greater...
than \(\alpha_1\)-adrenoceptor sensitivity in EDRF-disrupted animals; \(\alpha_2\)-adrenoceptor sensitivity was also significantly greater (and \(\alpha_1\)-adrenoceptor sensitivity significantly less) than adrenoceptor sensitivity during a similar amount of baseline PGF\(_{2\alpha}\) constriction (Figure 6, bottom panel; Table 2). Significant differences were also evident when these groups were compared with 1A \(\alpha_1\)-adrenoceptor sensitivity that was obtained in a comparable previously published study\(^{42}\) without embolization or PGF\(_{2\alpha}\) pretreatment (Table 2; see “Discussion”). Control diameters (C\(_2\)-C\(_6\)) did not differ after embolization (C\(_2\), 70±5 \(\mu m\); C\(_3\), 71±4 \(\mu m\); C\(_4\), 70±5 \(\mu m\); C\(_5\) [+RWL], 71±5 \(\mu m\); C\(_6\) [+PRZ], 72±5 \(\mu m\)) nor after PGF\(_{2\alpha}\) (C\(_2\), 77±4 \(\mu m\); C\(_3\), 78±4 \(\mu m\); C\(_4\), 78±3 \(\mu m\); C\(_5\), 78±3 \(\mu m\); C\(_6\), 78±4 \(\mu m\)). Maximal response to NE (10 \(\mu M\)) for both embolization and PGF\(_{2\alpha}\) groups was different between \(\alpha_1\) (e.g., 34±3 \(\mu m\) for embolization, 27\% of C\(_1\)) and \(\alpha_2\) (53±4 \(\mu m\) for embolization, 43\% of C\(_1\)), in agreement with previous studies\(^{37}\); however, maximal NE responses for \(\alpha_1\) stimulation in the embolization and PGF\(_{2\alpha}\) groups were not significantly different, nor were those for \(\alpha_2\) stimulation in the two groups.

Efficacy and specificity of microembolization to disrupt EDRF release was evidenced by elimination of ACh dilation after microembolization but not after an equivalent amount of PGF\(_{2\alpha}\) constriction (Figure 6, top panel; Figure 7). Also, nitroprusside dilation was evident in both groups but was less effective after microembolization (Figure 7). Finally, L-NMMA constriction was abolished after microembolization (Figure 7).

**Sensitivity of \(\alpha_1\)-Adrenoceptor Constriction to Nitroprusside**

Sensitivity of \(\alpha_1\) and \(\alpha_2\) constriction to cGMP-dependent dilation was assessed with the EDRF-like nitroilator nitroprusside. This was done to distinguish whether the enhanced \(\alpha_1\) constriction during inhibition of EDRF with L-NMMA or embolization, observed in the previous experiments, might be related to a greater sensitivity of \(\alpha_1\) constriction to EDRF’s cGMP-dependent mechanism of action. Perivascular micropipette suflusion of NE in the presence of either RWL or PRZ was used to produce focal \(\alpha_1\) or \(\alpha_2\) constriction of the 1A arterioles (Figure 8, top panel). Focal constriction extended 272±57 \(\mu m\) upstream and 218±58 \(\mu m\) downstream from the pipette tip. The percentage of imposed NE constriction measured adjacent to the pipette tip...
did not differ for $\alpha_1$ (39±7%) and $\alpha_2$ (43±9%) activation (constriction was normalized to C1 diameter and the maximal response to bath-added NE [30 μM] obtained at the end of the experiment; Figure 8, top panel). $\alpha_2$ constriction was significantly more sensitive than $\alpha_1$ constriction to bath-added nitroprusside (Figure 8, bottom panel).

Control diameters did not differ significantly (C1, 113±4 μm; C2, 116±5 μm; n=12), the maximal constriction to bath-added NE (3×10⁻⁵ M) reduced diameter to 45±2% of C2, and the highest concentration of nitroprusside produced significant dilation relative to C1. These control data also did not differ significantly when compared between the $\alpha_1$ and $\alpha_2$ groups.

**Discussion**

Interactions between EDRF and adrenoceptor constriction were analyzed in this study using several strategies. In the first design, a low level of $\alpha_1$ or $\alpha_2$ constriction (=10–20% of maximum) was induced in large arterioles and venules with bath-added NE in the presence of either $\alpha_1$- or $\alpha_2$-selective antagonists. The endogenous ligand was used rather than selective adrenergic agonists because synthetic agonists often exhibit different efficacies and, especially for $\alpha_2$-agonists, different coupling efficiencies and partial agonist properties compared with those exhibited by NE. Use of low-to-intermediate levels of NE constriction ensured that the antagonists would select for $\alpha_1$ and $\alpha_2$-receptor stimulation. During $\alpha_2$ tone, bath-added L-NMMA examined over a 500-fold concentration range produced concentration-dependent additional constriction of both arterioles and venules that was greater during $\alpha_2$ than during $\alpha_1$ tone; the sensitivity to L-NMMA was similar for arterioles and venules. This suggests that EDRF, either released at basal levels (see below) or further induced by NE itself, inhibits $\alpha_2$ constriction more than $\alpha_1$ constriction in both arterioles and venules.

After elimination of basal adrenergic tone by acute denervation, L-NMMA alone produced dose-depen-
dent constriction of arterioles and venules. This suggests that EDRF is released tonically in this tissue to significantly influence vascular resistance and capacitance. In this regard, L-NMMA had similar potency for large arterioles and venules and exhibited approximately 10-fold greater potency for small 3A arterioles. Comparison of L-NMMA constriction in the absence and presence of adrenergic tone indicates that L-NMMA produced less constriction in the presence of \( \alpha_1 \) tone than in its absence. This could be interpreted to suggest that \( \alpha_1 \) stimulation impairs EDRF release or action. However, in light of no evidence in the literature for such an effect, it appears more likely that partial \( \alpha_1 \)-or \( \alpha_2 \)-adrenergic constriction, per se, reduced the sensitivity to additional constriction by L-NMMA. This latter interpretation is consistent with other experiments in this study that support the conclusion that EDRF is more efficacious for inhibition of \( \alpha_2 \) than \( \alpha_1 \) tone.

A problem with this first experiment was that NE was applied topically to the entire cremaster microvasculature. This could permit the direct effects of NE to be obscured by changes in pressure and flow at the site of measurement arising from actions of NE elsewhere in the network. To avoid this potential complication, in a second experiment, local \( \alpha_1 \) or \( \alpha_2 \) constriction was produced by perivascular suffusion of NE with a micropipette positioned adjacent to the large arteriole while either the \( \alpha_1 \)- or \( \alpha_2 \)-antagonist was present in the bath. In other experiments, we have verified that \( \alpha_1 \)- and \( \alpha_2 \)-receptor populations are selected by this protocol. The local \( \alpha_1 \) and \( \alpha_2 \) constriction in this experiment
and in the nitroprusside experiments occurred over similar lengths of the vessel, extending slightly farther upstream from the pipette tip (see "Results"). Thus, no propagated constriction was detected beyond 272 μm from the pipette tip in either protocol. In the presence of nitroprusside, the arteriole was acutely denervated. The criteria for a1 and a2 constrictions spanning approximately 25–75% of maximal response, 100 μM L-NMMA caused the arteriole to constrict approximately 50% more during a2 than during a1 preconstriction. Since, by a number of criteria established in control experiments, 300 μM L-NMMA was judged to be selective in its effect to inhibit EDRF production (Figures 3 and 4), these data strengthen the conclusion that a2 constriction is more sensitive than a1 constriction to interference by EDRF; when EDRF production was prevented pharmacologically, a2 constriction was enhanced to a greater degree than a1 constriction.

Despite the apparent specificity of L-NMMA as demonstrated here and elsewhere (see References 13–15, but compare with Reference 48), we conducted an additional series of experiments on large arterioles using interference of EDRF synthesis by microembolization. This technique has been used both in vitro with air45,46 and in vivo with CO247 to transiently prevent the endothelium from releasing EDRF for at least several hours. This was verified in the present study. Microembolization caused a large sustained increase in baseline tone, similar in magnitude to that produced by the highest concentration of L-NMMA tested in the previous experiments. This supports the conclusion from the L-NMMA experiments concerning tonic EDRF release.

**Table 2. Effect of Disruption of Endothelium-Derived Relaxing Factor Release by Embolization on α-Adrenoceptor Sensitivity (–Log EC50) of Arterioles**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>(NE+RWL) α1</th>
<th>(NE+PRZ) α2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>6.83±0.09*</td>
<td>7.04±0.04*</td>
</tr>
<tr>
<td>Embolization</td>
<td>6</td>
<td>5.94±0.10†</td>
<td>7.35±0.07†</td>
</tr>
<tr>
<td>PGF2α</td>
<td>4</td>
<td>6.56±0.06*</td>
<td>6.77±0.05‡</td>
</tr>
</tbody>
</table>

n. Number of first-order arterioles (one per rat); NE, norepinephrine; RWL, rauwolscine (1 μM); PRZ, prazosin (0.1 μM); control, values taken from a previous study43; PGF2α, prostaglandin F2α. Values are mean±SEM.

In all experiments, propranolol (1 μM), desipramine (10 nM), and normetanephrine (10 μM) were present, the deferential collateral circulation was intact, and the cremaster muscle was acutely denervated.

*p<0.01 vs. corresponding value for embolization by unpaired two-tailed t test.

†p<0.01 vs. corresponding value for α1 by unpaired two-tailed t test.

‡p<0.01 vs. corresponding control value by unpaired two-tailed t test.

**Figure 7. Bar graphs showing specificity of carbon dioxide embolization for inhibition of endothelium-derived relaxing factor release (top panel) (see Figure 6, top panel, for protocol) versus effect of similar amount of baseline constriction with prostaglandin F2α (PGF2α, bottom panel). n, Number of vessels (one per rat); Cont, control diameter; ACh, acetylcholine; NP, nitroprusside; LMA, NG-monomethyl L-arginine.**

**Figure 8. Top panel: Time course showing response of first-order arteriole to local micropipette suffusion of norepinephrine (NE) in the presence of bath-added rauwolscine or prazosin to produce α1 or α2 constriction, followed by nitroprusside (NP) addition and maximal NE constriction (30 μM). C1 and C2, first and second control periods, respectively; ΔB, change of bath. Bottom panel: Line graph showing sensitivity of α1 versus α2 constriction to inhibition by NP. n, Number of vessels (one per rat).**
in the control of arteriolar resistance. Other studies have shown substantial increases in arterial pressure, regional resistance, and in vitro tone after inhibition of EDRF synthesis by pharmacological or nonpharmacological methods.

Like L-NMMA, microembolization eliminated dilation to ACh but not to the endothelium-independent dilator nitroprusside; L-NMMA constriction was now abolished, which supports the specificity of this inhibitor. Because microembolization caused baseline constriction (57±4% of control), the specificity of these controls and α1 and α2 sensitivity were compared with arterioles in sham-embolized animals during arteriole constriction with PGF2α (61±5% of control). Here, ACh-like nitroprusside produced potent dilation, and L-NMMA produced additional constriction. A minor point is that nitroprusside was more effective at inhibiting constriction produced by PGF2α than that produced by embolization (Figure 7). This may indicate that microembolization partially impairs guanylate cyclase induction. Alternatively, this difference may simply reflect a greater sensitivity of tone produced by PGF2α than tone produced by removal of basal EDRF action to inhibition by nitroprusside.

After impairment of EDRF release by embolization, sensitivity was approximately 75-fold greater to α2 than to α1 stimulation (Figure 6). By comparison, during a similar amount of baseline constriction with PGF2α, NE demonstrated similar potency for stimulation of either the α1- or α2-receptor. In contrast, NE α1 potency during PGF2α tone was not significantly different from values obtained in a previous comparable study without induced tone, although α2 potency was slightly reduced by PGF2α tone (Table 2). Regardless of comparison with either PGF2α responses or those obtained in the absence of imposed tone, removal of EDRF by embolization augmented α2 and attenuated α1 sensitivity (Table 2). It is difficult to predict the effect of changes in baseline tone, together with potential amplification of adrenergic constriction by PGF2α, on the positions of these curves. However, within the limitations of the experimental design, these results support the conclusion drawn from the two previous designs using L-NMMA that EDRF interacts to a greater extent with α2 than with α1 constriction.

Our results with L-NMMA and embolization are consistent with one or both of two possibilities. First, α2 contraction may be more sensitive than α1 contraction to inhibition by guanylate cyclase stimulation. Second, endothelial cells may possess α2-receptors that promote EDRF release, although the generality of this hypothesis remains controversial. To test the first possibility, we evaluated the sensitivity of local pipette constriction of arterioles with an intermediate level of α1 or α2 stimulation to inhibition by nitroprusside. Nitroprusside, like EDRF, interacts positively with the regulatory subunit of guanylate cyclase. α2 constriction was approximately 50-fold more sensitive than α1 constriction to reversal by nitroprusside. Thus, our previous results with L-NMMA and embolization could reflect, at least in part, a greater susceptibility of α2 constriction to reversal by EDRF-dependent increases in cGMP. Had nitroprusside had a similar inhibitory potency against α1 and α2 constrictions, our results would have been consistent with the existence of EDRF-promoting α2-receptors on microvascular endothelial cells. However, this was not the case; moreover, the 50-fold greater susceptibility of α2 than α1 tone to nitroprusside inhibition, together with the similar magnitude (~75-fold) of difference in α2 and α1 sensitivity after embolization, does not support a major contribution of endothelial α2-receptors to EDRF release in this tissue.

The greater sensitivity of α2 constriction to nitroprusside inhibition is consistent with the observation that nitroglycerin also inhibits α2 constriction more than α1 constriction and that nitroglycerin and nitroprusside act similarly to stimulate soluble guanylate cyclase. It is interesting that atrial natriuretic peptide, which stimulates the particulate fraction of guanylate cyclase, reverses α1 but not α2 constriction. Such a difference in susceptibility of α1 and α2 constriction to reversal by guanylate cyclase stimulation may extend from differences in enzyme localization and modes of intracellular signal transduction between α-adrenoceptor types.

The mechanism by which EDRF and nitroprusside, activators of soluble guanylate cyclase, preferentially inhibit α1 contraction can only be speculated on. Changes in levels of second messenger molecules activated by α2 stimulation to signal contraction may be subject to greater attenuation by EDRF stimulation than those activated by α1 stimulation. One possible mechanism for such an interaction is the degradation of cAMP and cGMP by phosphodiesterases. Stimulation of guanylate cyclase by tonic release of EDRF may “tie up” phosphodiesterase metabolism of cGMP and thus elevate cAMP. This could counteract the effect of α2-adrenoceptor stimulation, which inhibits adenylyl cyclase. In this case, inhibition of EDRF would decrease cGMP and permit diesterases to lower cellular cAMP and augment α2-mediated constriction. Since α2-receptors are coupled to phosphoinositide metabolism, contraction mediated by these receptors may be less affected by changes in cyclic nucleotides. However, a potential problem with this hypothesis is evidence, though limited, suggesting that α2-mediated inhibition of adenylyl cyclase has little influence on the contractile response in vascular smooth muscle (reviewed in Reference 36). Direct measurement of second messenger signaling of adrenoceptor contraction and EDRF stimulation will be required to clarify the cellular basis for the differential effect of EDRF on α1- and α2-adrenergic stimulation.

This study did not determine whether microvascular endothelial cells within this tissue possess α2-adrenoceptors that promote EDRF release. Additional experiments and methodology are required to determine if such receptors are present. The presence of α2-receptors on endothelial cells has been inferred indirectly from functional studies of mostly large arteries and veins. In precontracted pig coronary artery and various canine vessels α2-agonists in the presence of PRZ cause, depending on the vessel type, either relaxation that is abolished by endothelial removal or enhanced constriction after endothelial cell removal or treatment with EDRF inhibitors. However, other studies do not support the generalization of this concept. Removal of the endothelium in rat aorta augmented responses to a wide variety of agonists with varying selectively for α1- and α2-receptors. Also, there is wide variability among species and vessels in the ability to produce EDRF.
to evoke responses consistent with the presence of EDRF-promoting endothelial \alpha_2-receptors. Thus, it is not possible to assume the presence of such receptors on microvascular endothelial cells. Moreover, in vessels from a variety of species, including rats, no \alpha_2-adrenergic receptors were detected by autoradiography on endothelial cells, although both \beta-adrenergic and substance P receptors were localized to endothelial cells and \alpha_2-adrenergic receptors on vascular smooth muscle. The authors of this previous study concluded that either the endothelial \alpha_2-receptor population was extremely sparse or that stimulation of smooth muscle \alpha_2-receptors might cause endothelial cells to release EDRF. In addition, vascular smooth muscle itself is capable of synthesizing EDRF, although apparently in much smaller quantities than the endothelium.

Several previous studies support the presence of EDRF interactions at the arteriolar level, although venular data are extant. In rat cremaster muscle, L-NMMA inhibited dilation of small arterioles by ACh, and an EDRF-like factor dilated small arterioles in the hamster cheek pouch and rodent pial microcirculation. In particular, L-NMMA was observed to increase basal tone and augment \alpha_2 more than \alpha_1 adrenoceptor sensitivity in rat spinotrapezius muscle, findings that are in agreement with the present study. In rabbit skeletal muscle, L-NMMA produced constriction of arterioles but had no effect on dilation during muscle contraction or reductions in perfusion pressure. This suggests that EDRF may not play an important role in local metabolic or myogenic regulation in this tissue.

In conclusion, our studies suggest that 1) basal EDRF release exerts a substantial restraining influence on intrinsic tone in both resistance arterioles and capacitance venules, and 2) \alpha_2 constriction is more susceptible than \alpha_1 constriction to interference by EDRF and other nitrovasodilators (i.e., nitroprusside). The relative densities of \alpha_1- and \alpha_2-adrenoceptors on vascular smooth muscle vary considerably among different tissues and vascular segments; the constrictions evoked by these populations show remarkable differences in their sensitivity to modulation by humoral and local vascular controls. The present findings suggest, in addition, that the relative reliance of different vascular segments on \alpha_1 versus \alpha_2-receptors for generation of adrenergic tone is a determinant of the sensitivity of these segments to local modulation by EDRF-dependent mechanisms.

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