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VASCULAR MYOCYTE-DERIVED NITRIC OXIDE IS AN AUTOCRINE THAT LIMITS VASOCONSTRICTION[1]

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Vascular myocytes and endothelial cells possess the enzymatic machinery to generate nitric oxide from L-arginine. This study tests the hypothesis that myocyte-derived nitric oxide has an autocrine function to inhibit contraction. Rat aortic rings were placed in muscle baths for isometric force measurement. Denuded and intact rings contracted to N^{ω}nitro-L-arginine; L-arginine reversed these contractions. Compared to intact rings, contractile sensitivity to phenylephrine was increased in denuded rings; N^{ω}nitro-L-arginine caused a further enhancement of phenylephrine sensitivity. Acetylcholine contracted denuded rings but not intact rings; these contractions were also potentiated by N^{ω}nitro-L-arginine. In intact rings contracted with phenylephrine, acetylcholine caused relaxation that was inhibited by N^{ω}nitro-L-arginine. Denuded rings did not relax to acetylcholine. In summary, contractile responses of rat aortae to interventions that alter nitric oxide production are the composite of enzymatic activity in both the endothelial cells and myocytes. Thus, myocyte-derived nitric oxide modulates vascular tone.

Control of vascular tone is complex and appears to represent a balance between locally active vasoconstricting and relaxing factors that are primarily derived from the endothelium. Multiple agonists, including acetylcholine, thrombin, bradykinin, serotonin, ADP, and the calcium ionophore A23187, have all been shown to cause release of vasodilator substances, the most important of which is nitric oxide (NO), from several different vascular beds and cultured endothelial cells.[1-4] Recently, it has become apparent that vascular smooth muscle cells not only respond to endothelium-derived NO but possess the enzymatic machinery to manufacture

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; L-NNA, N^{ω}nitro-L-arginine; EDRF, endothelium-derived relaxing factor.

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NO from L-arginine directly in the absence of endothelial input.[5] The relative importance of vascular myoctye-derived NO to overall regulation of vascular tone remains to be determined. The purpose of this paper is to explore the contribution of NO derived from vascular smooth muscle cells from the thoracic aorta of the rat to regulation of vascular tone in the basal state and in response to contractile agonists. The hypothesis tested was that NO derived from the vascular myocyte has an autocrine function to inhibit contractile activity.

METHODS

Aortic tissue preparation. Adult male Wistar-Kyoto rats (270-350 mg; University of Michigan, Ann Arbor, MI, and Harlan Industries, Chicago, IL) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and exsanguinated. Thoracic aortae were removed and placed into cold physiologic salt solution (PSS; 130 mmol/l NaCl, 4.7 mmol/l KCl, 1.18 mmol/l KH_2PO_4, 1.17 mmol/l $MgSO_4$, 1.6 mmol/l $CaCl_2$-$2H_2O$, 14.9 mmol/l $NaHCO_3$, 5.5 mmol/l dextrose, and 0.03 mmol/l $CaNa_2EDTA$). The vessels were cleaned of adventitia and cut into four to six 4mm cylindrical segments under a dissecting microscope. When necessary, the endothelium was removed from the arterial ring preparations by cannulating the lumen with microforceps and gently rolling the vessel between the forceps and palm. Previous histological work has demonstrated that this rubbing procedure removes at least 95% of the endothelium.[6] In all experiments (see below), the absence of endothelium was confirmed by no relaxation to acetylcholine (10^{-7} M) following contraction induced by a concentration of phenylephrine (10^{-8} M) that produced a near half-maximal response (EC_{50}).

Muscle bath experiments. The vessels were transferred to warm PSS (37°C) and were mounted in 50 ml jacketed organ baths for measurement of contractile activity. Vessels were bubbled with 95% O_2 and 5% CO_2 throughout the experiment. The lumen of the vessel was threaded with two wires fastened to two stainless steel support blocks. One block was mounted on a force transducer (Grass FT.03, Quincy, MA) and the other on a displacement device, so that passive force could be applied (3 g passive force). After preparation, the vessel segment was allowed to equilibrate for ninety minutes in PSS followed by an additional thirty minute incubation in PSS with 10^{-5} M indomethacin to inhibit cyclo-oxygenase activity. Contractile force was measured (in grams) after exposure of the vascular segments to increasing concentrations of an agonist.

Drugs. L-Phenylephrine, indomethacin, acetylcholine, N^{ω}Nitro-L-arginine (L-NNA) were purchased from Sigma Chemical (St. Louis, MO). Norepinephrine was purchased from Winthrop Pharmaceuticals (New York, NY).

Statistics. Concentration-response curves were generated by calculating the geometric means and standard error of the means (SEM) for all observations. Where appropriate, EC_{50}'s were calculated using a SYSTAT computer program. Paired and unpaired Student's t-tests were calculated using Microsoft Excel. When multiple Student t-tests were used, the Bonferroni correction was employed. For all tests, a p value less than 0.05 was considered statistically significant.

RESULTS

Cumulative addition of L-NNA (10^{-8} to $3x10^{-4}$ M) to the muscle bath caused contractions in aortic segments (Figure 1). The magnitude of contraction induced by the inhibitor of nitric oxide synthase (NOS) tended to be larger in aortic segments without endothelium (- E) compared to intact segments (+ E). However, for any given concentration of L-NNA this difference was not statistically significant. Maximal force development to phenylephrine or norepinephrine in denuded segments (1.83±0.18 g) was not significantly different from that in intact aortic rings (2.10±0.22 g).

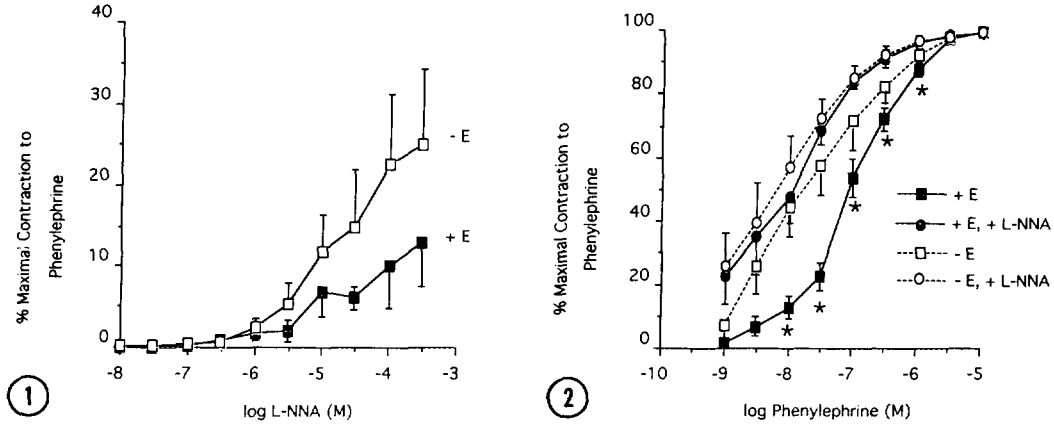

FIGURE 1. Contractile responses to N^{ω}nitro-L-arginine (L-NNA). Aortic segments without endothelium (- E; n=7-10 rats) contracted to L-NNA to a greater extent than those with intact endothelium (+ E; n=4-7 rats). Values are the mean ± SEM. There was no statistical significance between the two curves.

FIGURE 2. Contractile responses to phenylephrine. Compared to endothelium intact segments (+ E), contractile responsiveness to phenylephrine was increased in denuded aortic rings (- E) as evidenced by the leftward shift in the concentration-response curve. Incubation with L-NNA potentiated contractile responses to phenylephrine in both intact and denuded segments. Values are the mean ± SEM for 7-9 rats. Asterisks indicate a statistically significant difference between endothelium intact and all other segments ($p<0.05$).

In aortic segments without endothelium, contractile sensitivity to phenylephrine was increased compared to intact aortic segments as evidenced by the shift to the left in the concentration-response curve (Figure 2) and the lower concentration of the agonist required to produce a half-maximal response (EC_{50}(- E)=log[-7.9±0.2] or $1.3x10^{-8}$M, and EC_{50}(+ E)= log[-7.0±0.1] or $1.0x10^{-7}$M, p=0.005). Incubation with L-NNA ($3x10^{-4}$M) potentiated contractile responses to phenylephrine in both intact (EC_{50}=log[-8.3±0.2] or $5x10^{-9}$M) and denuded (EC_{50}=log[-8.4±0.2] or $4x10^{-9}$M) aortic segments.

Figure 3 characterizes contractile properties of acetylcholine in intact and denuded aortic rings. Aortic segments without endothelium contracted in response to the cumulative addition of acetylcholine whereas intact segments did not. In the presence of L-NNA, contractile responses to acetylcholine were potentiated in intact and denuded aortic rings. Denuded aortic rings treated with L-NNA were significantly more sensitive to acetylcholine than intact rings treated with L-NNA as seen by the leftward shift of the dose-response curve. Furthermore, L-NNA treatment caused a similar potentiation of contractile responses to acetylcholine in intact and denuded aortic rings. For example, at 3 mM acetylcholine L-NNA increased contractile responses approximately 18% in aortic rings with endothelium and 17% in denuded aortic rings (Figure 3, inset). The enhanced contractile responses to acetylcholine in the presence of L-NNA were completely reversed with 10^{-4}M L-arginine.

Figure 4 shows the relaxation response to acetylcholine in intact and denuded aortic rings contracted with an EC_{50} of phenylephrine. Intact rings relaxed completely in a dose-dependent fashion (EC_{50}(+ E)=log[-7.8±0.6] or $1.6x10^{-8}$M), however removal of the endothelium and/or addition of L-NNA prevented relaxation to acetylcholine. At doses of acetylcholine greater than 10^{-5}M, L-NNA treatment caused a contractile response in aortic rings with endothelium.

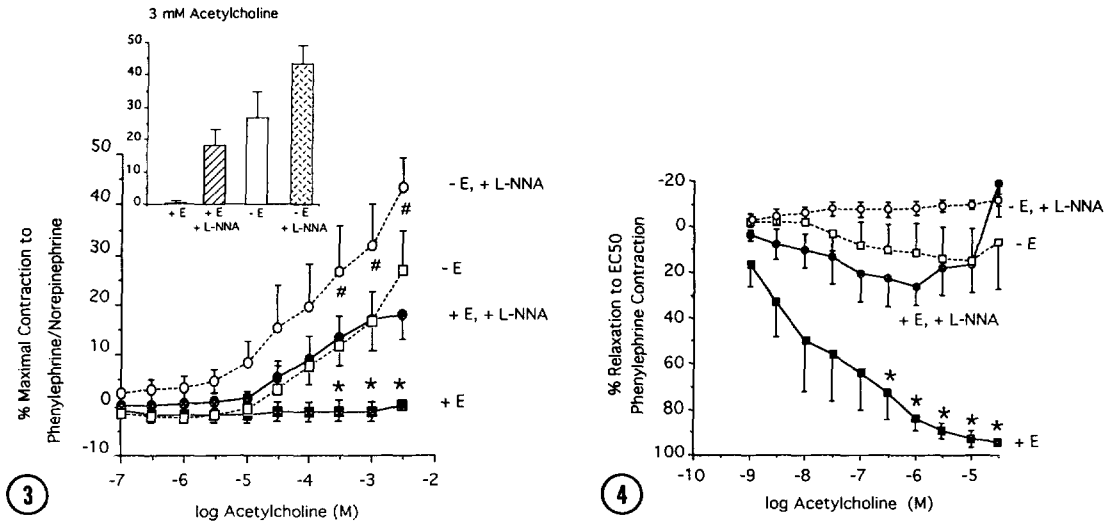

FIGURE 3. Contractile responses to acetylcholine. Aortic segments that were denuded of endothelium (- E) contracted in response to the cumulative addition of acetylcholine to the muscle bath whereas those with intact endothelium (+ E) did not. Incubation with L-NNA potentiated contractile response to acetylcholine in both intact and denuded segments and denuded aortic segments treated with L-NNA were significantly more responsive to acetylcholine contraction than intact rings treated with L-NNA (#; $p<0.05$). Values are the mean $\pm$ SEM for 6 rats. Asterisks indicate a statistically significant difference between endothelium intact and all other segments ($p<0.05$).

FIGURE 4. Relaxation responses to acetylcholine. Following contraction to phenylephrine (EC_{50}) aortic rings with intact endothelium (+ E) relaxed to a greater extent in response to acetylcholine than those without endothelium (- E). Incubation with L-NNA inhibited relaxation responses to acetylcholine in both intact and denuded aortic rings. Values are the mean $\pm$ SEM for 4 rats. Asterisks indicate a statistically significant difference between endothelium intact and all other segments ($p<0.05$).

DISCUSSION

In 1980, Furchgott and Zawadzki[7] demonstrated that vascular relaxation induced by the muscarinic receptor agonist acetylcholine was dependent on the presence of a functionally intact endothelium and they postulated the release by endothelial cells of a labile factor termed endothelium-derived relaxing factor (EDRF). Subsequently, it was shown that NO, which accounts for the biological activity of EDRF, is synthesized by the vascular endothelium from L-arginine.[8] Since identification of EDRF as NO, the role that endothelium-derived NO plays in regulation of vascular tone has been the subject of much investigation. Until recently, however, the vascular myocyte was perceived as a passive target cell for endothelium-derived NO. Schini and Vanhoutte[5] showed that relaxations in response to L-arginine are inhibited by L-NNA in aortic rings both with and without endothelium. These experiments demonstrated that both the endothelial cell and vascular smooth muscle cell possess biochemical pathways for converting L-arginine to NO. Subsequently, at least two different NOS have been cloned and expressed in cultured cell lines; neuronal nitric oxide synthase (n-NOS) and macrophage nitric oxide synthase (mac-NOS).[9] The two enzymes have significant sequence homology (50%) with similar recognition sites for FAD, FMN, and NADPH and a consensus calmodulin binding site. Mac-

NOS mRNA is inducible by cytokines but is absent in quiescent macrophages. In contrast, n-NOS is constitutively active and is dependent on Ca^{+2}-calmodulin binding. Busse and Mulsch[10] showed direct evidence that native and cultured vascular smooth muscle cells from rabbit aortae possess a cytokine-inducible NOS similar or identical to mac-NOS that they propose is a major factor responsible for the loss of vascular responsiveness observed in septic shock and during antitumor therapy with cytokines. In support of their argument, Beasley[11] and Wood *et al.*[12] provided indirect evidence for the existence of both a constitutive and an inducible NOS pathway in endothelium-denuded arterial rings.

Our results demonstrate the important contribution of the vascular smooth muscle cell to total NO production from the vascular wall of the rat aorta in response to contractile agonists. Intact aortic rings contracted in response to L-NNA and the magnitude of these contractions were not statistically different from those observed in aortic rings denuded of endothelium despite a trend towards greater contractions in aortic segments without endothelium. Incubation with L-arginine reversed the contractile effect of L-NNA. Sensitivity to phenylephrine was increased in aortic rings without endothelium and incubation of intact and denuded rings with L-NNA further increased sensitivity. However, the magnitude of the shift in the concentration-response curves did not differ statistically from that observed in denuded rings. Relaxation responses to acetylcholine were inhibited in aortic rings without endothelium and incubation with L-NNA blocked responsiveness to the muscarinic agonist. Taken together, these observations confirm the work of others[4,5,7,8] and demonstrate that the endothelium is an important source of NO under most circumstances. However, the observation that contractile responsiveness to acetylcholine is greatest in denuded aortic segments in the presence of L-NNA is compelling evidence to support the hypothesis that myocyte-derived NO may act as an autocrine to limit contractile activity. One potential criticism of these data is that the increased contractile responsiveness to agonists in denuded aortic segments is due to contamination by endothelial cells. We recognize that it is impossible to entirely remove all endothelial cells, specifically tiny branch vessels and vasa vasorum. However, given the lack of significant relaxation responses to acetylcholine and the histological evidence for greater than 95% de-endothelialization, we attribute our responses to the vascular smooth muscle. A second concern is that Rees *et al.*[13] showed that there was a time-dependent induction of NOS in rings of rat thoracic aorta due to contamination of their buffer with endotoxin that was detectable in homogenates after eight hours. Although we did not measure endotoxin levels or add antibiotics to our buffer (due to potential interference with ion channels and contractility), we did not detect a significantly decreased responsiveness to phenylephrine over the course of our experiments. Thus, inducible NOS probably contributes little to regulation of contractile activity in the short term.

We believe that this is the first study that has addressed the role of the myocyte as a vascular wall component contributing to total NO production and the regulation of contractile activity in the short term. We also observed that myocyte-derived NO does not appear to play a major role in the basal state or in response to acetylcholine vasorelaxation. However, the relaxation response is not entirely dependent on endothelium-derived NO either. The component of the endothelium-dependent vasorelaxant response not inhibited by L-NNA may be due to endothelium-derived hyperpolarizing factor[14] or to some other factor not yet described.

Regardless, these data demonstrate that synthesis and release of vascular myocyte-derived NO is more important than previously thought and may play a role in the determination of vascular tone. NO from vascular smooth muscle cells may also contribute to other functions traditionally attributed to the endothelium including inhibition of platelet function, renin release, and vascular myocyte growth and replication.

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