**L-Arginine Evokes Both Endothelium-Dependent and -Independent Relaxations in L-Arginine–Depleted Aortas of the Rat**

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This study was designed to investigate the effects of L-arginine (the substrate for the formation of endothelium-derived nitric oxide) in vascular tissues. Rat aortic rings, with or without endothelium, were suspended in organ chambers for the measurement of isometric tension; they were contracted with phenylephrine (10^{-6} M). After a short incubation period (0.5 hour) in physiological salt solution, L-arginine induced minor changes in both types of rings. In contrast, when the incubation time was increased (2, 4, 6, and 8 hours), L-arginine evoked concentration- and time-dependent relaxations in aortic rings both with and without endothelium. The relaxations were larger in rings with endothelium. The presence of L-arginine (10^{-3} M) in the incubation medium inhibited subsequent relaxations evoked by the amino acid. The concentration–relaxation curves associated with acetylcholine in rings with endothelium and the curves associated with Sin-1, a spontaneous donor of nitric oxide, in rings with or without endothelium were slightly but significantly shifted to the right after a 6-hour incubation. Nitro-L-arginine (3\times10^{-5} M) and methylene blue (3\times10^{-7} M) attenuated the relaxations evoked by L-arginine in rings both with and without endothelium. Other basic amino acids (D-arginine, L-homoarginine, L-citrulline, L-lysine, and L-ornithine; all tested at 10^{-3} M) either had no effect or induced small relaxations and did not affect the response to L-arginine. These observations suggest that L-arginine specifically and stereoselectively relaxes aortic rings with and without endothelium, probably by restoring the endogenous pool of the amino acid, which is likely depleted by prolonged incubation. Since the relaxations in response to L-arginine are inhibited by nitro-L-arginine in rings both with and without endothelium, the present experiments demonstrate that both the endothelial cells and the vascular smooth muscle possess biochemical pathways converting L-arginine to nitric oxide. (*Circulation Research* 1991;68:209–216)

Nitric oxide is produced from the conversion of the semessential amino acid L-arginine into L-citrulline by nitric oxide synthetase in endothelial cells,1–4 cytotoxic activated macrophages,5,6 neutrophils,7–9 adrenal glands,10 EMT-6 mammary adenocarcinoma cells,11 and Kupffer cells12 and in the central nervous system.13–15 In the vascular system, the biosynthesis of nitric oxide by endothelial cells accounts for the relaxation of the vessels16 and also for the inhibition of platelet aggregation and activation evoked by endothelium-derived relaxing factor (for review see References 17–19). Nitric oxide is a potent and direct activator of soluble guanylate cyclase20,21 and increases the production of cyclic GMP, which in turn mediates the biological responses. Nitric oxide synthetase is located in the cytosol, and its activation necessitates the presence of NADPH and divalent cations as cofactors.3–5,10 The objective of this study was to investigate the effects of L-arginine, the substrate of the formation of nitric oxide, in vascular tissues.

**Materials and Methods**

Male Wistar rats (12–17 weeks old) were killed, and the thoracic aortas were removed and placed in a physiological salt solution containing (mM) NaCl 118.3, KCl 4.7, MgSO_{4} 1.2, KH_{2}PO_{4} 1.2, CaCl_{2} 2.5, NaHCO_{3} 25.0, CaEDTA 0.016, and glucose 11.1. The aortas were cleaned of loose connective tissue, and special care was taken not to touch the luminal
surface. Each aorta was cut in eight to 10 rings (2–3 mm wide). In some preparations, the endothelium was removed mechanically by inserting a pair of small forceps into the lumen and rolling the tissue back and forth several times on a paper towel wetted with physiological salt solution. The aortic rings were mounted horizontally between two stainless-steel stirrups in organ chambers filled with 25 ml physiological salt solution (37°C, pH 7.4) and bubbled with 95% O₂-5% CO₂. One of the stirrups was anchored to the organ chamber, and one was connected to a strain gauge (model UC2, Gould, Cleveland, Ohio) for the recording of isometric tension. The aortic rings were stretched progressively to optimal length for maximal isometric contraction (2.5 g; determined in preliminary experiments with 10⁻⁶ M phenylephrine, data not shown) before the addition of phenylephrine (10⁻⁶ M). Once the maximal contraction to phenylephrine was obtained, the aortic rings were rinsed three times with warm (37°C) physiological salt solution. After a resting period (30 minutes), during which the tension was readjusted to 2.5 g, the aortic rings were exposed again to phenylephrine (10⁻⁶ M). When the contraction stabilized, acetylcholine (10⁻⁶ M) was added to test the presence of functional endothelium. The organ chambers were rinsed three times with warm (37°C) physiological salt solution before the addition of indomethacin (10⁻⁵ M) to prevent the production of endogenous vasoactive prostanooids. This step was considered to be the beginning (t=0 hour) of the experimental protocol. After various incubation periods (0.5, 2, 4, 6, or 8 hours), the aortic rings were contracted with phenylephrine (10⁻⁶ M) before the addition of the test relaxing agent in a cumulative fashion. Methylene blue (3×10⁻⁷ M) or nitro-L-arginine (3×10⁻⁵ M) were added 30 minutes before the contraction of the tissue by phenylephrine, and the amino acids (d-arginine, L-lysine, L-ornithine, and L-citrulline) were added 5 minutes before the contraction by phenylephrine.

**Drugs**

The following drugs were used: L-arginine hydrochloride, D-arginine hydrochloride, L-homoarginine hydrochloride, L-lysine dihydrochloride, L-ornithine hydrochloride, L-citrulline, acetylcholine, methylene blue, phenylephrine, indomethacin (all from Sigma Chemical Co., St. Louis, Sin-1 (a gift from Laboratoires Hoechst, Paris), and nitro-L-arginine (Aldrich Chemical Co., Milwaukee, Wis.). Unless otherwise stated, drugs were prepared in distilled water and kept at 4°C. Indomethacin was prepared in an equimolar concentration of sodium carbonate (10⁻⁵ M). All concentrations are expressed in terms of the base.

**Statistical Analysis**

Results are expressed as mean±SEM. The number of rings is represented by n. Statistical evaluation of the data was performed by Student’s t test for paired observations. Values were considered to be statistically different at p<0.05. The negative logarithm of the effective molar concentration of agonist causing 50% relaxation (IC₅₀) of the contraction to phenylephrine was calculated for each concentration–response curve.

**Results**

**Phenylephrine**

Phenylephrine (10⁻⁶ M) evoked similar contractions in rat aortic rings with or without endothelium; the rings had been incubated for 0.5 hour (2.61±0.25 and 3.01±0.45 g, respectively, n=5 each), 4 hours (2.68±0.16 and 3.24±0.28 g, respectively, n=4 each), 6 hours (2.44±0.09 and 2.76±0.14 g, respectively, n=24 each), and 8 hours (2.15±0.38 and 2.88±0.40 g, respectively, n=4 each) in physiological salt solution. After a 2-hour incubation period, phenylephrine evoked a slightly larger contraction in rings without endothelium compared with those with endothelium (3.36±0.24 and 2.56±0.13, respectively, p<0.05, n=4 each).

**L-Arginine**

L-Arginine induced only minor relaxations of aortic rings with or without endothelium after incubation for 0.5 hour in physiological salt solution before contraction by phenylephrine (10⁻⁶ M) (Figure 1). When the incubation period was increased to 2 hours, L-arginine induced a concentration-dependent relaxation in rings with endothelium but not in those without endothelium (Figure 1). When the aortic rings were equilibrated for longer periods (4, 6, or 8 hours), L-arginine evoked relaxations in aortic rings both with and without endothelium (Figure 1); however, the relaxations were significantly greater in rings with endothelium (n=4) compared with those without endothelium (n=4) (IC₅₀ were 5.41±0.19 and 4.88±0.17, respectively, after an 8-hour incubation). L-Arginine (10⁻⁴ M) reduced phenylephrine-evoked contractions by 6% and 3% after 0.5 hour, 37% and 0% after 2 hours, 55% and 10% after 4 hours, 79% and 45% after 6 hours, and 87% and 81% after 8 hours in aortic rings with and without endothelium, respectively.

Incubation of aortic rings, with or without endothelium, for 6 hours in physiological salt solution containing L-arginine (10⁻⁵ M; followed by washout three times with fresh physiological salt solution and by a 5-minute equilibration period in the presence of 10⁻⁵ M indomethacin before the addition of phenylephrine) significantly reduced the relaxations evoked by L-arginine (Figure 2).

**Acetylcholine and Sin-1**

Acetylcholine evoked concentration- and endothelium-dependent relaxation in rings incubated in physiological salt solution for either 0.5 or 6 hours before the addition of phenylephrine (10⁻⁶ M) (Figure 3). After 6 hours, the concentration–response curve was shifted slightly but significantly to the right (IC₅₀ were 7.26±0.05 and 6.76±0.10, p<0.01, after 0.5 and 6 hours, respectively, n=5 each), and the maximal
reduction of the phenylephrine-evoked contraction evoked by acetylcholine \((10^{-5} \text{M})\) was reduced significantly (from 94\% to 82\%, \(p<0.02\), after 0.5 and 6 hours, respectively) (Figure 3).

Sin-1, a spontaneous donor of nitric oxide,\(^{22}\) elicited similar concentration-dependent relaxations in aortas, with or without endothelium, contracted by phenylephrine \((10^{-6} \text{M})\) after either 0.5 or 6 hours of incubation (Figure 4). The concentration–response curves in both types of rings were shifted slightly to the right after 6 hours \((\text{IC}_{50} \approx 6.31 \pm 0.26 \text{ and } 5.91 \pm 0.20 \text{ in rings with endothelium} \ [n=5], \ p=\text{NS}, \text{ and } 6.52 \pm 0.08 \text{ and } 6.14 \pm 0.09 \text{ in rings without endothelium} \ [n=5], \ p<0.01, \text{ after 0.5 and 6 hours, respectively}).

**Inhibitors of Endothelium-Derived Nitric Oxide**

After a 6-hour incubation in physiological salt solution, treatment of aortic rings with nitro-L-arginine (an inhibitor of the conversion of L-arginine to L-citrulline and nitric oxide; \(3 \times 10^{-5} \text{M} \text{ for } 30 \text{ minutes}\)) enhanced significantly the contraction evoked by phenylephrine \((10^{-6} \text{M})\) in preparations both with and without endothelium (from 2.08\%±0.23 to 3.43\%±0.23, \(p<0.02\), in rings with endothelium \([n=9]\)

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**FIGURE 1.** Graphs showing effects of L-arginine on rings of rat aortas, with or without endothelium, incubated in physiological salt solution \((37^\circ\text{C}, 95\% \text{ O}_2-5\% \text{ CO}_2)\) for 0.5, 2, 4, 6, and 8 hours before being contracted by phenylephrine \((10^{-6} \text{M})\). All experiments were performed in the presence of indomethacin \((10^{-5} \text{M})\). Results are presented as mean±SEM from four or five experiments.

**FIGURE 2.** Graphs showing effects of incubation of rings, with endothelium (panel A) or without endothelium (panel B), in physiological salt solution \((37^\circ\text{C}, 95\% \text{ O}_2-5\% \text{ CO}_2)\) for 6 hours in the presence of L-arginine \((10^{-4} \text{M})\) on the subsequent relaxations evoked by L-arginine during contractions to phenylephrine \((10^{-6} \text{M})\). After the 6-hour incubation period, L-arginine \((10^{-3} \text{M})\) was washed out by changing the bath three times with fresh physiological salt solution followed by a 5-minute equilibration of the rings before the addition of phenylephrine. All experiments were performed in the presence of indomethacin \((10^{-5} \text{M})\). Results are presented as mean±SEM from five experiments.
and from 2.14±0.18 to 3.37±0.22 g, p<0.005, in rings without endothelium [n=9] in the absence and presence of nitro-L-arginine, respectively.

Nitro-L-arginine (3×10^{-5} M) shifted the concentration-response curve of L-arginine to the right in rings both with and without endothelium (Figure 5). In the presence of nitro-L-arginine, L-arginine evoked relaxations only at concentrations greater than the inhibitor of nitric oxide synthesis (3×10^{-5} to 1×10^{-3} M); these relaxations were comparable in rings with and without endothelium. Treatment of aortas with endothelium with nitro-L-arginine (3×10^{-5} M) abolished the relaxation evoked by acetylcholine (Figure 6).

After a 6-hour incubation period, treatment of aortic rings with methylene blue (an inhibitor of soluble guanylate cyclase activation; 3×10^{-7} M for 30 minutes) did not affect the contraction evoked by phenylephrine (10^{-6} M) in preparations with endothelium (n=4) but enhanced significantly that in preparations without endothelium (n=4) (from 2.16±0.20 to 3.25±0.10 g, p<0.05, in the absence and presence of methylene blue, respectively). Methylene blue abolished the relaxations evoked by L-arginine in aortic rings both with and without endothelium (Figure 7).

**Other Basic Amino Acids**

D-Arginine, L-citrulline, and L-lysine caused small concentration-dependent relaxations in rings with but not in those without endothelium; the rings had been incubated for 6 hours in physiological salt solution before contraction with phenylephrine (10^{-6} M) (Figure 8). However, these differences were not significant (by Student's paired t test). L-Homoarginine also evoked small concentration-dependent relaxations that were similar in both types of preparations; L-ornithine, up to a concentration of 10^{-3} M, did not affect the contraction evoked by phenylephrine (Figure 8). D-Arginine, L-citrulline, L-lysine, or L-ornithine (all tested at 10^{-3} M for 5 minutes) affected only minimally the relaxations evoked by L-arginine in rings with or without endothelium after a 6-hour incubation period in physiological salt solution (Figure 9).
The semiessential amino acid L-arginine is converted to L-citrulline and nitric oxide by nitric oxide synthetase. The nitric oxide synthetase uses NADPH and divalent cations as cofactors in the rat cerebellum, the enzyme requires calmodulin. The enzyme is present in the cytosol of endothelial cells, cytotoxic activated macrophages, neutrophils, Kupffer cells, and adrenal glands and in the central nervous system, indicating that the synthesis of nitric oxide from L-arginine is a widespread pathway for the regulation of cell function and communication.

The addition of L-arginine to rat aortic rings, with or without endothelium, that had been incubated in physiological salt solution for a short period (0.5 hour) before being contracted by phenylephrine evoked only minor changes in tension. Small relaxations, or absence of relaxations, to L-arginine have also been observed in rat and rabbit aortas and in bovine pulmonary artery and vein. In contrast, this study shows that L-arginine evoked concentration- and time-dependent relaxations in rings, with or without endothelium, that had been incubated under tension for several hours in physiological salt solution at 37°C and aerated with 95% O₂-5% CO₂. After an 8-hour incubation period, L-arginine caused nearly complete relaxation in rings both with and without endothelium contracted by phenylephrine. These relaxations were not due to an alteration of the contractile responses of the blood vessel, because phenylephrine evoked similar contractions in rings, with or without endothelium, that had been incubated for several hours. The relaxations evoked by Sin-1 were shifted slightly to the right after a 6-hour incubation period. The fact that the relaxations evoked by L-arginine were concentration-dependent and incubation time-dependent seems to be explained best by a selective and progressive enhancement of a biological pathway activated by L-arginine and leading to the relaxation of the vessel. Incubation of rat aortic rings in physiological solution for several hours is presumably associated with a gradual reduction of the tissue content of a number of small molecular weight components, one of which may be L-arginine. This process may be brought about as a consequence of both cellular efflux and metabolic utilization. In a similar study performed on isolated rings of bovine pulmonary artery incubated for a much longer period in physiological salt solution (24 hours) than those in the present study (up to 8 hours), L-arginine evoked concentration-dependent relaxations in rings with endothelium without affecting those without. These relaxations were associated with a threefold reduction of the tissue concentration of L-arginine. A reduction of the concentration of L-arginine in the vessel is likely to be associated with a hypersensitivity of the biological systems using L-arginine as a substrate, such as the L-arginine–nitric oxide pathway. This interpretation is supported by the observation that incubation of the rat aorta in physiological salt solution in the presence of a high concentration of L-arginine (10⁻⁵ M) abolishes the relaxation evoked by the amino acid, presumably by preventing the depletion of its endogenous content.

The concentration–relaxation curves evoked by L-arginine in rings with or without endothelium were shifted significantly to the right by nitro-L-arginine, an inhibitor of the conversion of L-arginine to nitric oxide and of the endothelium-dependent relaxation of vessels. Similarly, relaxations due to L-arginine...
were abolished by methylene blue, an inhibitor of soluble guanylate cyclase activation\textsuperscript{28} and also of the conversion of L-arginine to L-citrulline.\textsuperscript{3} These observations suggest that the addition of L-arginine to rings incubated for several hours enhances the activity of the nitric oxide synthetases that catalyze the formation of nitric oxide both in endothelial cells and in vascular smooth muscle. The presence of the L-arginine—nitric oxide pathway in the smooth muscle is suggested also by the increased contraction evoked by phenylephrine in rings without endothelium in the presence of either nitro-L-arginine or methylene blue. The relaxations evoked by L-arginine are more resistant to inhibition by nitro-L-arginine in rings without endothelium than in those with endothelium. This may suggest that nitric oxide synthetases in endothelial cells and in the smooth muscle have different properties. The biochemical characterization of the nitric oxide synthetases in the cytosolic fraction of homogenates from endothelial cells, macrophages, and central nervous tissues also suggests the presence of a family of enzymes.\textsuperscript{3–5} Alternatively, high concentrations of the amino acid may have nonselective inhibitory effects on vascular smooth muscle; this interpretation is made unlikely by the relative lack of effect of other basic amino acids on preparations without endothelium.

Basic cationic amino acids such as D-arginine, L-homoarginine, L-citrulline, L-lysine, and L-ornithine evoked only minor changes in tension after prolonged incubation in physiological salt solution. These observations indicate that L-arginine induced relaxation of rings both with and without endothelium in a specific and stereoselective fashion. L-Arginine, but not its enantiomer or other basic amino acids, reverses the inhibition by nitro-L-arginine or N\textsuperscript{6}-monomethyl-L-arginine of the relaxation evoked by acetylcholine in the rabbit aorta.\textsuperscript{2,27} Neither D-arginine nor L-citrulline affect the conversion of L-arginine to L-citrulline in cultured porcine aortic endothelial cells.\textsuperscript{3} Taken in conjunction with the present findings, these observations are consistent with the view that L-arginine is a specific and stereoselective substrate for nitric oxide synthetase in both endothelial cells and vascular smooth muscle.

The study of the transport of amino acids in cultured endothelial cells of the human umbilical vein indicates the presence of a saturable transport system for large neutral amino acids resembling system L, and of an uptake of the cationic amino acids, L-arginine and L-ornithine.\textsuperscript{29} In eukaryotic cells, the uptake of cationic amino acids occurs by a common saturable and stereoselective transport system, designated system y\textsuperscript{+}.\textsuperscript{30} The y\textsuperscript{+} transporter is Na\textsuperscript{+}- and pH-independent, stereoselective, inhibited by other exogenous cationic amino acids, and stimulated by cationic amino acids inside the cells ("trans-stimulation").\textsuperscript{31–34} In the present experiments, high concentrations (10\textsuperscript{-3} M) of either D-arginine, L-citrulline, L-lysine, or L-ornithine affected only mini-
Normally the relaxations evoked by L-arginine in rings with or without endothelium. This surprising lack of effect by other basic amino acid suggests that the uptake of L-arginine in both endothelial cells and vascular smooth muscle may be highly selective. However, the present observations do not rule out the possibility that L-arginine may selectively activate membrane-associated receptors. These receptors may enhance the activity of the cytosolic nitric oxide synthetase, which induces the production of nitric oxide in endothelial cells and smooth muscle and results in relaxation.

The concentration-relaxation curves evoked by acetylcholine in rings with endothelium were shifted slightly to the right after a 6-hour incubation of the tissues in physiological salt solution in comparison with those obtained after 0.5 hour. Nitro-L-arginine abolished the endothelium-dependent relaxation evoked by acetylcholine. Thus, as a function of time, the tissues became slightly less sensitive to acetylcholine and markedly more sensitive to L-arginine. These results indicate that acetylcholine may have access to mobilization of a pool of L-arginine relatively resistant to depletion.

The present study demonstrates that the semielemental amino acid L-arginine evokes marked relaxations in rat aortic rings with endothelium as well as in preparations in which the endothelium was removed deliberately. The relaxations evoked by L-arginine were specific and stereoselective and were a function of concentration and incubation time, suggesting that they may result from progressive depletion of the tissue content of L-arginine. The relaxations evoked by L-arginine were impaired by inhibitors of the synthesis and/or biological responses of endothelium-derived nitric oxide, suggesting that endothelial cells, as well as the smooth muscle, possess biochemical pathways converting L-arginine to nitric oxide.

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