Endothelium-Dependent Relaxation and L-Arginine Metabolism in Genetic Hypertension

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This study characterizes the effects of L-arginine and N\(^{G}\)-monomethyl L-arginine on dilator responsiveness of vascular tissue from Wistar-Kyoto rats and stroke-prone spontaneously hypertensive rats. Rings of abdominal aorta were suspended in tissue baths for measurement of isometric force. After contraction induced by phenylephrine, cumulative addition of acetylcholine, L-arginine, or A23187 to the muscle bath caused a similar relaxation of aortic rings in both animal groups. To test the hypothesis that arginine metabolism is altered in hypertension, aortic rings were incubated with N\(^{G}\)-monomethyl L-arginine. N\(^{G}\)-monomethyl L-arginine (10–300 \(\mu\)M) did not affect contractile responses to phenylephrine (10\(^{-8}\) to 10\(^{-4}\) M) in either animal group (EC\(_{50}\), 10\(^{-7}\) M). Exposure of aortic rings to N\(^{G}\)-monomethyl L-arginine resulted in a greater inhibition of relaxation response to acetylcholine (10\(^{-10}\) to 10\(^{-5}\) M) in hypertensive animals. N\(^{G}\)-monomethyl L-arginine (300 \(\mu\)M) caused complete inhibition of relaxation to acetylcholine in the hypertension group. Incubation with L-arginine (10–100 \(\mu\)M) overcame the inhibition of acetylcholine-induced relaxation produced by N\(^{G}\)-monomethyl L-arginine in both groups. Exposure of aortic ring segments to N\(^{G}\)-monomethyl L-arginine attenuated relaxation responses to A23187 (10\(^{-10}\) to 3\(\times\)10\(^{-7}\) M) in both groups. L-Arginine-induced reversal of the inhibitory effect of N\(^{G}\)-monomethyl L-arginine on the relaxation responses to A23187 was similar between groups. We conclude that the inhibitory effect of N\(^{G}\)-monomethyl L-arginine on endothelium-derived relaxing factor production in response to acetylcholine is greater in stroke-prone spontaneously hypertensive rats as compared with Wistar-Kyoto rats. This finding reflects differences in L-arginine metabolism in genetic hypertension that may be an important factor in contributing to altered vascular reactivity. (Hypertension 1992;19:435–441)

KEY WORDS • endothelium • genetic hypertension • arginine • rat studies

Endothelium-derived relaxing factor (EDRF), first described by Furchgott and Zawadski, has been characterized as nitric oxide (NO) or an unstable nitroso-related compound. There is substantial experimental evidence suggesting that vascular endothelial cells use L-arginine as an endogenous substrate for the synthesis of NO. This enzymatic process has been demonstrated to be stereoselective because the D-enantiomer of L-arginine does not produce NO. Further, the L-arginine analogue N\(^{G}\)-monomethyl L-arginine (L-NMMA) competitively inhibits the synthesis of NO in a dose-dependent fashion. Several investigators have hypothesized that NO production and L-arginine use may be important in the regulation of blood pressure. Therefore, altered arginine metabolism or NO production may be a significant factor in the genesis of hypertension. We hypothesized that L-arginine metabolism is altered in rats with genetic hypertension. We therefore compared the effects of the L-arginine analogue L-NMMA on relaxation responses to acetylcholine (ACH) in abdominal aortic rings of normotensive Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP). The objective of the present study was to determine whether the L-arginine: NO pathway is impaired in arteries from SHRSP. The SHRSP was chosen as a model of experimental hypertension in the present work because it is a commonly studied rat model that is characterized by a genetic association between elevated blood pressure and altered vascular reactivity. The WKY strain was selected for comparison because it is used as a point of reference in most studies on SHR and SHRSP.

Methods

Animal and Tissue Preparation

Adult male and female WKY rats and SHRSP (aged 12 weeks) were obtained from a colony maintained in the Department of Anatomy and Cell Biology, University of Michigan, Ann Arbor, Mich. Sex distribution was similar between groups. Endothelium-dependent and endothelium-independent relaxation of rings of WKY rats and SHRSP were studied. Systolic blood pressures were measured by the tail-cuff method. WKY rats (\(n=44\), blood pressure less than 120 mm Hg) and SHRSP (\(n=40\), blood pressure greater than 160 mm Hg) were killed with an overdose of sodium pento-
barbital (50 mg/kg i.p.). The abdominal aorta was carefully dissected, trimmed of adherent fat and connective tissue, and cut into 4-mm rings. The endothelium was removed from some rings by gently rubbing the intimal surface over a stainless steel wire. The presence or absence of endothelium was confirmed by either a relaxation response or no relaxation response to ACh (0.1 μM). Rings were mounted on stainless steel hooks in 50-ml organ chambers and connected to isometric force transducers (FT .03, Grass Instrument Co., Quincy, Mass.). Resting tension was adjusted to 3.0 g to allow for maximal active force generation. The bathing medium was maintained at 37°C and aerated with a mixture of 95% O₂–5% CO₂. The composition of the solution was as follows: NaCl 130, KCl 4.7, MgSO₄ • 7 H₂O 1.7, KH₂PO₄ 1.18, NaHCO₃ 14.9, dextrose 5.5, CaCl₂ • 2 H₂O 1.6, and CaNa₂EDTA 0.03. Before the initiation of experiments, the strips were allowed to equilibrate for 90–120 minutes. All experiments were done in the presence of 1.4 x 10⁻⁵ M indomethacin (Sigma Chemical Co., St. Louis, Mo.). Indomethacin was dissolved in ethanol; the final concentration of ethanol did not exceed 0.1%. Control experiments demonstrated this concentration of ethanol to have no effect on abdominal aortic ring tone.

Experimental Protocols

Tissues were then submaximally contracted with 0.1 μM phenylephrine (Sigma). Relaxation responses to cumulative additions of the following drugs were studied: ACh (Michol, Cooper Vision Pharmaceuticals, San German, Puerto Rico), A23187 (solvent, DMSO, Calbiochem, La Jolla, Calif.), and l-arginine (Sigma). In one series of experiments, tissues were incubated with 30 μM L-NMMA (Calbiochem), and cumulative concentration–response curves were generated to ACh and A23187 after the addition of increasing concentrations of L-arginine (10⁻¹⁰ to 10⁻μM). A final series of experiments was performed in which tissues were incubated with increasing concentrations of L-NMMA (10⁻³ to 10⁻⁶ M), and cumulative concentration–response curves were generated to ACh (10⁻¹⁰ to 10⁻⁶ M).

Statistics

Data are reported as mean±SEM. ED₅₀ values (concentration of drug that produced 50% maximal response) were determined by graphic analysis. Student’s unpaired t test was applied to compare differences between the means. The Bonferroni correction was applied when multiple comparisons were made. A value of p<0.05 was considered to be statistically significant.

Results

Endothelium-Dependent Vascular Relaxation

Phenylephrine (10⁻⁶ to 10⁻⁴ M) caused a concentration-dependent contraction in abdominal aortic rings from both WKY rats (n=6) and SHRSP (n=6). ED₅₀ was approximately 10⁻⁷ M in aortic rings from both SHRSP and WKY rats. Maximal force generation to phenylephrine occurred at 10⁻⁴ M in both animal groups, and the magnitude of contraction in aortic rings from SHRSP did not differ from WKY values (2.0±0.05 versus 1.9±0.5 g, p=NS). The presence of endothelium did not alter the contractions to phenylephrine in WKY or SHRSP aortic rings. In aortic rings contracted with 10⁻⁴ M phenylephrine, exposure to increasing concentrations of ACh (10⁻⁸ to 10⁻⁶ M) resulted in a concentration-dependent relaxation in both animal groups. This relaxation response was endothelium-dependent and did not differ significantly between WKY and SHRSP aortic rings (see Figure 1; ED₅₀ 10⁻⁶ M).

Effect of Addition of L-Arginine After Incubation With L-NMMA

ACh (10⁻¹⁰ to 10⁻⁶ M) and A23187 (10⁻¹⁰ to 3 x 10⁻⁷ M) caused similar endothelium-dependent, concentration-dependent relaxations in both WKY and SHRSP aortic rings. To test the hypothesis that arginine metabolism is altered in SHRSP aortic rings, were incubated with 30 μM L-NMMA, a competitive inhibitor of arginine metabolism, for 15 minutes before the initiation of experiments. The tone of quiescent aortic rings was not altered by incubation with L-NMMA. Further, the addition of L-NMMA (10⁻³ to 300 μM) did not result in a shift of the dose–response curve to phenylephrine (10⁻⁴ to 10⁻⁴ M) in WKY or SHRSP aortic rings (data not shown). Exposure of aortic ring segments to 30 μM L-NMMA resulted in an attenuation of the relaxation responses to ACh (10⁻¹⁰ to 10⁻⁴ M) in both WKY and SHRSP as compared with control values (Figure 2). Further, SHRSP demonstrated a greater attenuation of the relaxation responses to ACh as compared with WKY after incubation with 30 μM L-NMMA (Figure 2). Concomitant addition of l-arginine (10⁻¹⁰ to 10⁻³ M) overcame, in a concentration-dependent manner, the inhibition of ACh-induced relaxation produced by L-NMMA (30 μM) in both WKY and SHRSP aortic rings. However, in WKY the addition of 10 μM l-arginine resulted in a significantly greater reversal of L-NMMA–induced inhibition of ACh relaxation response toward control values as compared with SHRSP (Figure 2). Additionally, experiments were performed using the receptor-independent agonist A23187. Exposure of aortic ring segments to 30 μM L-NMMA resulted in a significant attenuation of the relaxation responses to cumulative additions of A23187 (10⁻⁸ to 3 x 10⁻⁶ M) in both WKY and SHRSP as compared with controls. L-Arginine reversed the inhibitory effect of L-NMMA on the relaxation responses to the cumulative addition of A23187 was identical for WKY and SHRSP (Figure 3).

Effect of L-NMMA on Endothelium-Dependent Relaxation

In a final series of experiments, aortic ring segments were incubated with increasing concentrations of
L-NMMA (10−300 μM) and exposed to the cumulative addition of ACh (10−10 to 10−6 M). Incubation with L-NMMA did not affect tone in quiescent or contracted aortic rings. Exposure of aortic ring segments to increasing concentrations of L-NMMA (10−300 μM) resulted in a concentration-dependent attenuation of the relaxation response to ACh (10−10 to 10−6 M) in both WKY and SHRSP as compared with WKY (Figure 4). Incubation with L-NMMA (300 μM) resulted in complete inhibition of the relaxation to ACh in the SHRSP as compared with the WKY aortic rings. Incubation with 300 μM L-NMMA resulted in complete inhibition of relaxation to ACh (10−6 M) in the SHRSP, whereas in WKY aortic rings the relaxation was inhibited by 75%.

**Discussion**

The present study is the first to show differential impairment of the L-arginine: NO pathway between normotensive and hypertensive animals in response to stimulation with the endothelium-dependent dilator ACh. Our findings demonstrate that aortic rings from WKY and SHRSP respond in a similar fashion to phenylephrine, L-arginine, and to the endothelium-
FIGURE 2. Inset: Bar graphs show relaxation response of aortic rings to increasing concentrations of acetylcholine (ACH) in Wistar-Kyoto (WKY) rats (left panel) and stroke-prone spontaneously hypertensive rats (SHRSP) (right panel) after incubation with 30 nM Nω-monomethyl L-arginine (L-NMMA) and after subsequent addition of increasing concentrations of L-arginine (L-Arg). Controls (solid bar) (no inhibitor and no added L-arginine); 100 nM L-arginine (solid hatched bar) added to tissue bath after incubation with 30 nM L-NMMA; 10 nM L-arginine (checkered bar) added to tissue bath after incubation with 30 pM L-NMMA; and inhibitor alone (open hatched bar) (0 mM L-arginine). *Statistically significant (p<0.05) as compared with control. Values reported in the inset are replotted to demonstrate more clearly the differences between WKY and SHRSP. Lower figure. Panel A: Bar graph shows relaxation responses of aortic rings of SHRSP (solid bar) versus WKY (hatched bar) to increasing concentrations of ACH in the absence of L-NMMA or L-arginine. Panel B: Bar graph shows relaxation responses of aortic rings of SHRSP (solid bar) versus WKY (hatched bar) to increasing concentrations of ACH after incubation with 30 nM L-NMMA and subsequent addition of 100 pM L-arginine. Panel C: Bar graph shows relaxation responses of aortic rings of SHRSP (solid bar) versus WKY (hatched bar) to increasing concentrations of ACH after incubation with 30 nM L-NMMA and subsequent addition of 100 pM L-arginine. Panel D: Bar graph shows relaxation responses of aortic rings of SHRSP (solid bar) versus WKY (hatched bar) to increasing concentrations of ACH after incubation with 30 nM L-NMMA alone. *Statistically significant (p<0.05) between animal groups. Data are expressed as mean±SEM.
of these mechanisms. 2) L-Arginine may not be the only possible that the SHRSP may be deficient in any or all the constant intracellular supply of L-arginine. It is that intracellular recycling of L-citrullene accounts for cells can generate arginine from an intracellular source. Confirming this observation, Hecker et al suggested 12 that when incubation with 30 μM N\textsuperscript{\textcircled{c}}-monomethyl L-arginine (L-NMMA) and after subsequent addition of increasing concentrations of L-arginine: controls (○) (no inhibitor and no added L-arginine); 100 μM L-arginine added to tissue bath after incubation with 30 μM L-NMMA (●); 10 μM L-arginine added to tissue bath after incubation with 30 μM L-NMMA (●); and inhibitor alone (0 μM L-arginine) (■). *Statistically significant (p<0.05) as compared with control Data are expressed as mean±SEM. dependent dilators ACh and A23187. However, after incubation with L-NMMA, a specific inhibitor of NO formation from L-arginine, SHRSP demonstrate greater depression of relaxation responses to ACh as compared with WKY aortic rings. This difference between SHRSP and WKY aortic rings is not evident when A23187 is used to induce relaxation even though L-NMMA inhibits relaxation to this agent. After the addition of L-arginine to the media to “reverse” the effects of L-NMMA, SHRSP rings demonstrate a persistent impaired relaxation to ACh as compared with those from WKY. Our data show that ACh- and A23187-mediated relaxation is endothelium-dependent and inhibited by L-NMMA. This observation suggests that NO is synthesized from L-arginine in the endothelial cells of the WKY and SHRSP abdominal aorta. This finding confirms published data by numerous investigators implicating L-arginine conversion to NO, which results in endothelium-dependent relaxation.\textsuperscript{9,10} In our present study, the L-NMMA-induced inhibition of ACh-induced relaxation was significantly less for WKY as compared with SHRSP. These data suggest that SHRSP have altered metabolism or mobilization, or both, of L-arginine and therefore altered production of NO as compared with WKY. Possible considerations for this difference in L-arginine metabolism include: 1) WKY rats may have a larger store of intracellular L-arginine. Mitchell et al\textsuperscript{11} have convincingly demonstrated that L-arginine levels are maintained despite constant EDRF stimulation and release. Mitchell et al\textsuperscript{11} noted that L-arginine concentrations were conserved, whereas the levels of other amino acids fell. This finding argues that endothelial cells can generate arginine from an intracellular source. Confirming this observation, Hecker et al\textsuperscript{12} suggested that intracellular recycling of L-citrullene accounts for the constant intracellular supply of L-arginine. It is possible that the SHRSP may be deficient in any or all of these mechanisms. 2) L-Arginine may not be the only substrate for conversion to NO in the WKY rat. Our data support the possibility that the WKY rat may have an alternative pathway for NO production that is not present in the SHRSP. Since very high concentrations of L-NMMA failed to completely inhibit ACh-induced relaxation in WKY aortic rings as contrasted to those of SHRSP in which relaxation to ACh was completely inhibited. One could hypothesize that release of other endothelium-derived relaxing factors, as has been suggested by numerous investigators, may be operative. From our data, we cannot exclude the possibility that L-NMMA might have greater access to NO synthase in aortic rings from SHRSP as compared with those from WKY rats. The similar responses to A23187, which stimulates EDRF release via a receptor-independent mechanism, would strongly suggest that L-NMMA has equal access to NO synthase in our model. Differences in responses to ACh-mediated relaxations, however, may be secondary to differences in receptor-mediated signal transduction or stimulation of different NO syntheses between animal groups. A23187 induced similar endothelium-dependent relaxations in both WKY and SHRSP aortic rings. Relaxation responses to A23187 were similarly inhibited by L-NMMA in both animal groups. Further the L-NMMA inhibition was reversed to the same degree with the subsequent addition of L-arginine. In contrast to the findings with ACh, the similarities in responsiveness to A23187 suggest that the calcium ionophore may directly stimulate conversion of L-arginine to NO contrasted to ACh, which must stimulate muscarinic receptors and then, in some unelucidated method, effect the conversion of L-arginine to NO. In contrast to other reports, in the present study the basal release of EDRF did not appear to be a significant factor affecting tone in the quiescent or contracted abdominal aortic rings of WKY or SHRSP.\textsuperscript{13,14} Dohi et al\textsuperscript{14} reported a significant leftward shift in the dose-
response curve to norepinephrine and phenylephrine after incubation with L-NMMA. This shift was further augmented by removal of the endothelium. In our preparations, removal of the endothelium, exposure to L-NMMA, or both, did not result in a shift of the dose–response curve to phenylephrine. Explanations
for these differences are probably related to experimental design and the type of vessel used. Dohi et al. noted these effects in pressurized WKY and SHR mesenteric arteries where shear stress may account for higher release of basal EDRF as compared with vascular stretch, which was used in our protocol. Further basal release of EDRF may vary within vascular beds as well as between rat strains.

In conclusion, our current study demonstrates a differential sensitivity between WKY and SHRSP in response to the inhibitory effects of L-NMMA to ACh-induced relaxation in aortic rings. This finding strongly supports the hypothesis that there are differences in the L-arginine: NO pathway that account for this differential sensitivity to L-NMMA. Differences in L-arginine metabolism between WKY and SHRSP may be an important factor contributing to altered vascular reactivity in hypertension.

References
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