Mechanism of Vasoconstriction Induced by Chronic Inhibition of Nitric Oxide in Rats

Norman Bank, Hagop S. Aynedjian, Ghazali A. Khan

Abstract Either acute or chronic inhibition of nitric oxide synthesis by L-arginine analogues results in increases in mean arterial pressure and reductions in renal blood flow. The role of endogenous vasoconstrictors in mediating these effects is not entirely clear. In the present study, nitric oxide was inhibited in male Sprague-Dawley rats by oral administration of nitro-L-arginine for 3 weeks. At the end of this time, mean arterial pressure was 30 to 40 mm Hg higher than in normal controls, renal blood flow and glomerular filtration rate were 25% to 30% lower, and renal vascular resistance was markedly increased. Intravenous infusion of receptor antagonists for angiotensin II, thromboxane, epinephrine, and endothelin-I had no significant effect on the hypertension. Inhibition of prostaglandin synthesis and furosemide-induced diuresis in the presence of nitro-L-arginine also had no effect on blood pressure. Renal vascular resistance was also unaffected by these interventions, except that saralasin did reduce renal resistance in both control and nitric oxide–inhibited groups. However, the absolute level of renal vascular resistance remained higher in the latter group. Calcium channel blockade partially corrected blood pressure and renal resistance, but the levels remained significantly higher than the control animals. The findings are consistent with the view that the increase in vascular smooth muscle tone caused by inhibition of nitric oxide synthesis cannot be accounted for by overexpression of common endogenous vasoconstrictors. Rather, the generalized increase in vascular smooth muscle tone appears to be due to a direct effect of reduced nitric oxide availability, which may lead to an increase in intracellular calcium concentration or sensitivity. (Hypertension. 1994;24:322-328.)

Key Words • nitric oxide • muscle, smooth, vascular • renal circulation • vasoconstriction

A number of recent studies have found that acute or chronic inhibition of nitric oxide (NO) synthesis in normal rats leads to hypertension and a reduction in renal blood flow (RBF).1-10 A generalized increase in peripheral vascular resistance occurs, involving many organs, whereas cardiac output is reduced.5 The mechanisms that have been proposed to account for the increase in peripheral vascular resistance are an imbalance between endogenous vasoconstrictors and the diminished vasodilating effect of NO11-17 and renal sodium and water retention.1,18 Alternatively, reduced NO production might, by its effect on vascular smooth muscle cyclic GMP and intracellular calcium, increase smooth muscle tone independent of an increase in the responsiveness to endogenous vasoconstrictors. In addition, there is evidence that NO inhibition has different effects on renal vascular resistance (RVR) versus peripheral vascular resistance.6,8,9 We carried out the present study to compare the effects of receptor blockade of several endogenous vasoconstrictors on mean arterial pressure (MAP) and renal hemodynamics in control versus experimental rats given nitro-L-arginine (NLA) for 3 weeks to inhibit NO synthesis. The effects of receptor antagonists for angiotensin II (Ang II), thromboxane A2/prostaglandin H2 (TXA2/PGH2), epinephrine, and endothelin-I had no significant effect on the hypertension. Inhibition of prostaglandin synthesis and furosemide-induced diuresis in the presence of angiotensin blockade also had no effect on blood pressure. Renal vascular resistance was also unaffected in addition, diuretic administration, prostaglandin inhibition, and calcium channel blockade were examined. The observations suggest that hypertension and reduced renal perfusion induced by chronic inhibition of NO synthesis are not mediated by unopposed action of the endogenous vasoconstrictors studied.

Methods

Male Sprague-Dawley rats (275 to 325 g) were housed in individual metabolic cages and fed preweighed rations of rat pellets (25 g/d) (Purina Mills). For inhibition of NO synthesis, 5 mg NLA (Aldrich Chemical Co) was added daily to 15 mL drinking water that had been sweetened with saccharin (0.5 g/100 mL). The rats consumed the entire 15 mL within a few hours, after which they were given an unlimited amount of drinking water for the remainder of the day. This regimen was continued for 3 weeks. On the day before the acute experiment, an overnight 16-hour urine collection was obtained for measurement of NO2- plus NO3-. Thymol was added to the collection vials as an antiseptic, and specimens were stored at −20°C until analyzed. The brucine method was used to measure urinary NO2- plus NO3- as previously described.19 Briefly, urinary NO3- is oxidized to NO2- by titration with KMNO4. The sample is then mixed with the brucine reagent (strychnine in H2SO4) and heated to boiling for 10 minutes. A sulfur-yellow color results, which is read on a spectrophotometer at 410 nm. The method is sensitive to 0.01 ppm and is suitable for biological fluids.20 Normal control rats were fed the rat pellet diet (25 g/d) and allowed tap water ad libitum.

On the day of study, the rats were anesthetized with 10 mg/100 g body wt IP thiamylal. A carotid artery was cannulated for continuous monitoring of MAP using instruments previously described,19 and a jugular vein was catheterized for infusion of Ringer's solution and the various biological agents described below. The left kidney was exposed via a midline abdominal incision, the kidney was dissected free of perirenal fat tissue, and an ultrasonic flow probe was placed...
around the left renal artery. The left ureter was catheterized with PE-10 tubing for timed urine collections. Ringer's lactate solution was infused continuously throughout the experiment at 1 mL/h per 100 g body weight. [14C]Inulin (New England Nuclear) was infused in a bolus dose of 2.5 μCi IV followed by 2 μCi/h IV. Glomerular filtration rate (GFR) was determined by measurement of [14C]inulin in urine (U) and plasma (P) (obtained at the midpoint of timed urine collections) using the equation GFR=U/PXV, where V is the urine flow rate in milliliters per minute.

Seven groups of animals were studied. In each animal, MAP, RBF, and GFR measurements were made during a baseline period lasting 45 to 60 minutes. After this period, one of the substances listed below was infused intravenously, and repeat measurements of MAP, RBF, and GFR were made.

**Group 1: Saralasin**

In six rats pretreated with NLA for 3 weeks and in six control rats, MAP, RBF, and GFR were measured during an initial period. After this, saralasin ([Sar 1 Ala8]Ang II, Sigma Chemical Co) was infused in a bolus dose of 10 μg/kg IV in 0.5 mL. Ringer's solution followed by a continuous infusion of 10 μg/kg per minute. This dose was previously found to block the renal and blood pressure effects of low doses of exogenous Ang II. Saralasin was chosen because it is a nonselective blocker of angiotensin receptors and presumably blocks both AT1 and AT2 receptor subtypes in the kidney. Measurements of MAP, RBF, and GFR were then continued for 45 minutes.

**Group 2: Saralasin Plus Furosemide**

In four NLA-treated animals, baseline measurements were obtained, and then saralasin infusion was begun as described above. Forty-five minutes later, furosemide (Lasix) was administered in a bolus dose of 1 mg IV. Measurements of MAP, RBF, and GFR were then continued for an additional 45 minutes.

**Group 3: TXA2/PGL2 Receptor Antagonist**

In six NLA-treated rats and six normal control rats, the TXA2/PGL2 receptor antagonist SKF 96148 (kindly supplied by SmithKline & French Laboratories) was infused as a bolus dose of 1.0 mg/kg IV followed by 17 μg/kg per minute after baseline measurements had been completed. This dose was previously shown to reverse the renal hemodynamic effects of excess endogenous TXA2 production. Repeat measurements of MAP, RBF, and GFR were made over the next 60 minutes.

**Group 4: Indomethacin**

In six rats pretreated with NLA for 3 weeks and in six control rats, baseline measurements were made first followed by bolus infusion of indomethacin at 5 mg/kg IV. This dose has previously been found to reduce urinary PGF2α excretion by more than 90%. MAP, RBF, and GFR measurements were repeated over the next 60 minutes to allow time for the effects of reduced prostaglandin synthesis to become apparent.

**Group 5: Propranolol**

In four rats pretreated with NLA for 3 weeks, MAP, RBF, and GFR were measured during a baseline period lasting 30 to 45 minutes. After this, propranolol was administered in a dose of 4 μg IV over a 5-minute period. This dose was extrapolated from human intravenous propranolol dosage and is the equivalent of 1 mg IV in an adult. All measurements were continued for the next 45 minutes. Since intravenous propranolol has a very rapid pharmacological effect, 45 minutes of observation was considered sufficient to detect hemodynamic changes.

**Group 6: Verapamil**

In six rats pretreated for 3 weeks with NLA and six control rats, MAP, RBF, and GFR were measured during a baseline period. Verapamil was then infused in a bolus dose of 5 μg followed by 150 μg/h. This dose was previously found to block the effect of calcium infusion on renal hemodynamics. All measurements were repeated over the next 60 minutes.

**Group 7: Endothelin Antagonist BQ-123**

In three NLA-treated rats, after an initial period of baseline measurements, the endothelin-A (ET-A)-selective receptor antagonist BQ-123 (kindly supplied by Banyu Pharmaceutical Co) was infused in a dose of 1.2 mg/kg per hour IV. This dose was recommended by Banyu Pharmaceutical Co. MAP, RBF, and GFR were measured during 60 minutes of infusion of BQ-123.

In three normal rats not pretreated with NLA, endothelin-1 (ET-1) (Boehringer Mannheim Pharmaceutical Co) was infused at a dose of 4 μg/kg per minute IV for 40 minutes. MAP rose 25 to 30 mm Hg over this period of time. At the end of the 40 minutes, the ET-1 infusion was stopped, and the ET-A receptor antagonist BQ-123 was infused at the same dose as described above. Measurements of MAP were made continuously in these experiments. Because of the limited supply of BQ-123, a larger number of animals could not be studied.

In all experiments, RVR was calculated from the expression RVR=(MAP−3/RBF). A value of 3 mm Hg was assumed in this equation for renal venous pressure.

Statistical analysis was carried out by paired Student's t test comparing the initial period to values during the second period when the various agents were infused. Comparisons between NLA-treated rats and normal controls were made by unpaired Student's t test. A value of P<.05 was considered significant.

All animal experimentation was conducted in accord with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and the procedures followed were in accord with the Montefiore Medical Center Animal Research Committee guidelines.

**Results**

Oral administration of NLA for 3 weeks resulted in a significant reduction in urinary excretion of the stable metabolic products of NO, i.e., NO2− and NO3−. The mean 24-hour excretion was 22.5±2 μmol in control rats and 16±3 μmol in NLA-treated rats (P<.05). Since all animals were fed a predigested daily ration of diet pellets, which they consumed completely, the protein and mineral intake was virtually the same in all animals. Therefore, the decrease in NO2− plus NO3− excretion in the urine can be attributed to the competitive inhibitory effect of NLA rather than to a decrease in ingested precursor l-arginine or nitrates.

Fig 1 shows MAP data for the various animal groups. In the rats fed NLA, MAP ranged from 158 to 168 mm Hg during the baseline period, shown as hatched bars, and in the control rats it ranged from 112 to 122 mm Hg (P<.001). The effects of the various infusions are shown as the solid bars in Fig 1. It is clear that none of the receptor antagonists had a significant effect on MAP in either the normal or NLA-treated rats. In the NLA-treated rats, bolus infusion of furosemide in the presence of saralasin also did not reduce MAP significantly. Urine flow rate increased in these experiments from a baseline of 5.3±0.4 to 35.5±4 μL/min after furosemide administration. Administration of the calcium channel blocker verapamil caused a marked fall in MAP in both the control and NLA-treated groups.
However, blood pressure remained significantly higher in the NLA-treated rats than in the normal rats (136 versus 107 mm Hg, \(P<0.01\)). Fig 2 shows a blood pressure tracing from a representative experiment in which verapamil was given. As can be seen, a large fall in MAP occurred (from 165 to 138 mm Hg) within 10 minutes.

Fig 3 shows GFR for the control and NLA-treated groups. In the baseline period (hatched bars), GFR was significantly lower in the NLA-treated animals, ranging from 0.68 to 0.83 mL/min versus 1.03 to 1.19 mL/min in the normal controls (\(P<.001\)). None of the experimental interventions affected GFR in the normal rats. In the NLA-treated rats, saralasin and propranolol infusions led to small but statistically significant increases in GFR, whereas none of the other agents had an effect.

Fig 4 shows RBF. Although there was some overlap between the two groups during the baseline period (hatched bars), in most cases RBF was lower than 5 mL/min in the NLA-treated animals and above 5 mL/min in the normal rats. Infusion of saralasin resulted in marked increases in RBF in both the control and NLA-treated rats, whereas none of the other agents had any effect in either group.

Fig 5 shows the calculated values for RVR in the control and NLA-treated rats. Baseline RVR was markedly elevated in the NLA-treated rats compared with controls (\(P<.001\)). Saralasin infusion caused large decreases in RVR in both animal groups, but the absolute level of RVR remained significantly higher in the NLA-treated group (29.2 versus 18.3 mm Hg/mL per minute, \(P<.01\)). Furosemide infusion after saralasin administration had no further effect on RVR in the NLA-treated animals despite a sevenfold to eightfold increase in urine flow. Calcium channel blockade with verapamil also decreased RVR markedly in both rat groups. Again, the absolute level remained significantly higher in the NLA-treated group (27.6 versus 19.6 mm Hg/mL per minute, \(P<.01\)). None of the other experimental interventions reduced RVR.

The Table shows the results of experiments with infusion of the ET\(_A\) receptor antagonist BQ-123 in NLA-treated rats. The dose used, 1.2 mg/kg per hour, is close to that administered by Kivlihgh et al\(^2\) (1 mg/kg bolus followed by 0.1 mg/kg per hour). Pollock and Opgenorth\(^2\) studied much higher doses of BQ-123,
infusing 0.1 to 0.2 mg/kg per minute (6 to 12 mg/kg per hour). In our experiments, MAP fell gradually over a 60-minute period, but the fall was not statistically significant. The lack of statistical significance is probably due to the small number of animals we were able to study. GFR and RBF both declined below the already low values in the baseline period, and RVR tended to rise further. To establish the efficacy of the ETα receptor antagonist, we infused ET-1 into three normal rats for 40 minutes, followed by infusion of BQ-123. In each of these animals, MAP rose by 18 to 20 mm Hg during ET-1 infusion and fell almost immediately to baseline when BQ-123 was infused. Fig 6 shows a representative blood pressure tracing. These observations demonstrate that BQ-123 is effective in the dose used in rapidly lowering blood pressure elevations caused by exogenous ET-1 infusion.

Discussion

The present experiments confirm several previous studies that found that acute or chronic inhibition of NO synthesis leads to marked elevations of systemic blood pressure1,2,6-9 and reductions in RBF and GFR.2-13-30 The renal hemodynamic changes are associated with increased afferent and efferent arteriolar resistance and a decrease in the glomerular ultrafiltration coefficient (Kf).2,10,30 The magnitude of these effects depends on the specific inhibitor of NO synthesis used as well as its dose and the duration of the inhibition.31 In the present study, administration of 5 mg/d NLA to male rats for 3 weeks led to a 30– to 40-mm Hg rise in MAP and a 25% to 30% fall in RBF and GFR.

The mechanism underlying these changes in peripheral and renal vascular tone has not been fully explained. Since NO is synthesized continuously by endothelial cells and acts locally to modulate vascular smooth muscle tone,17 a reduction in its supply might in itself result in a higher setting of smooth muscle tone. Alternatively, a decrease in NO supply could allow other vasoactive substances produced by the endothelium or nerve endings or those circulating in the blood to elevate vascular smooth muscle tone. It has been proposed that the balance among vasoconstrictors versus vasodilators is important in setting vascular tone.4-13-17

### Effects of Infusion of Endothelin-A Receptor Antagonist BQ-123

<table>
<thead>
<tr>
<th></th>
<th>Control Period</th>
<th>Experimental Period</th>
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<tr>
<td>MAP, mm Hg</td>
<td>166±1.2</td>
<td>149±3.8</td>
<td>NS</td>
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<td>RBF, mL/min</td>
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<td>&lt;.01</td>
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<td>RVR, (mm Hg/mL)/min</td>
<td>30.8±0.9</td>
<td>34.5±2.1</td>
<td>NS</td>
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</table>

MAP indicates mean arterial pressure; RBF, renal blood flow; GFR, glomerular filtration rate; FF, filtration fraction; and RVR, renal vascular resistance. n=3. Data for the experimental period were obtained 60 minutes after BQ-123 infusion was started.
The purpose of the present experiments was to examine the effect of blockade of receptors for several endogenous vasoconstrictors on blood pressure and renal hemodynamics in rats with long-term (3 weeks) NO inhibition. We used receptor antagonists against Ang II, TXA_2/PGH_2, epinephrine, and endothelin. We also examined the effect of furosemide diuresis in angiotensin-blocked animals and studied prostaglandin synthesis inhibition.

As shown in Fig. 1, blockade of receptors for Ang II, TXA_2/PGH_2, and _β_-adrenergics had no significant effect on systemic blood pressure in the hypertensive NLA-treated rats. If inhibition of NO synthesis resulted in overexpression of any of these endogenous vasoconstrictors, it would be expected that receptor blockade would result in a sharp fall in blood pressure. In previous studies of Ang II blockade, a specific receptor antagonist for AT_1 receptors was used, i.e., DuP 753.5,6,8,9 Most physiological responses to Ang II are thought to be mediated by AT_1 receptors, including vascular contraction.22 AT_1 receptors are highly expressed in glomerular, vasa recta, and outer medullary vascular bundles as well as in tubules.22 A second subtype of Ang II receptor, AT_2, has binding sites in large preglomerular vessels.22 Chatziantoniou and Arendshorst23 have demonstrated that both AT_1 and AT_2 receptors are physiologically significant in normal rats, in that specific inhibitors for each subtype were additive in blocking the renal effect of Ang II infusion in normal rats.23 In our study, the nonspecific Ang II receptor antagonist saralasin was used, which presumably blocked both AT_1 and AT_2 receptors in the kidney and elsewhere. The failure of saralasin to reduce blood pressure in the rats with chronic NO inhibition therefore provides further evidence that Ang II did not contribute to the rise in peripheral vascular resistance. Samsell et al22 found that either DuP 753 or prazosin given alone did not lower blood pressure or RVR in awake rats with long-term NO inhibition, but when given in combination, the two drugs significantly lowered both blood pressure and RVR. The mechanism of the combined effect in the absence of an effect of each drug alone is not clear.

In the case of propranolol, the drug blocks _β_-receptors in both the heart and peripheral blood vessels, and because the cardiac effect predominates, the most reasonable conclusion is that the hypertension caused by NO inhibition is not mediated by epinephrine-augmented cardiac output. Manning et al26 infused phenylephrine into dogs given N^ω^-nitro-L-arginine methyl ester for 11 days and found no increased sensitivity in terms of the blood pressure fall or increase in heart rate. This also argues against increased expression of epinephrine in animals with inhibition of NO synthesis.ucci et al29 examined acute _α_-adrenergic receptor blockade with prazosin, ganglionic blockade with chlorisondamine, and a vasopressin antagonist followed by infusion of an inhibitor of NO synthesis. These drugs also failed to influence the rise in blood pressure when NO was acutely inhibited. Taken together, these studies and ours indicate that increases in blood pressure induced by inhibition of NO synthesis are not mediated by overexpression of _α_ or _β_-adrenergic vasoconstrictors, vasopressin, Ang II, or TXA_2/PGH_2. Our experiments with furosemide given in conjunction with saralasin suggest that volume overload also did not contribute to the hypertension, because a sharp fall in blood pressure would be expected under these experimental conditions.

Of particular interest are the experiments with BQ-123 (Table). These data suggest that unopposed ET-1 also does not account for reduced RBF during NO inhibition and probably not the hypertension. Endothelin release has been found to increase in the isolated aorta after NO production is inhibited, under conditions of exposure to thrombin.14 Since ET-1 increases blood pressure and RVR when infused into experimental animals,14,24 it is a logical candidata to mediate vasoconstriction after NO inhibition. However, in the present study, infusion of the selective ET_A receptor antagonist BQ-123, which blocks endothelin receptors on vascular smooth muscle cells,20,26 failed to increase RBF and GFR in the NLA-treated rats (Table). ET-1, ET-2, and ET-3 binding sites have been localized in rat kidney,26,27 and rat afferent and efferent arterioles have been shown to be sensitive to endothelin.36,40 Thus, BQ-123 should have increased RBF and GFR if these endothelins activated ET_A receptors in the kidney. However, several issues remain to be resolved. First, Cristol et al41 reported that although BQ-123 largely prevents a blood pressure rise when ET-1, ET-3, or sarafotoxin is infused into rats, the drug failed to prevent renal vasoconstriction. Similarly, Pollock and Opgenorth28 found that BQ-123 given in much larger doses prevented ET-1-induced hypertension but failed to prevent a rise in RVR. Thus, BQ-123 given systemically does not reverse renal vasoconstriction caused by endothelin, even though ET_A receptors are present in the kidney.28 It has been suggested that ET-1 acts on ET_A receptors in the kidney rather than ET_A receptors.41 A second problem with BQ-123 is that it apparently has a partial agonist effect in that it transiently lowers blood pressure, renal plasma flow, and GFR in normal rats.27 These hemodynamic changes are similar to those we observed in NO-inhibited rats (Table), although the changes in our NO-inhibited animals lasted longer. The more prolonged effect may have been caused by a decreased availability of NO to counter an agonist effect of BQ-123. Although it is difficult to draw conclusions, the agonist effect of BQ-123 in the NO-inhibited rats (Ta-
able) argues that ET\(_A\) receptors were not fully occupied by endogenous endothelin in our animals. It should also be noted that although BQ-123 rapidly reversed the hypertension caused by exogenous ET-1 infusion (Fig 6), it lowered MAP very gradually and incompletely in the NO-inhibited animals.

With regard to RVR, none of the receptor blocking agents except saralasin caused a decrease. As shown in Figs 4 and 5, saralasin led to a striking rise in RBF and fall in RVR in both control and NLA-treated rats. Thus, it is clear that Ang II contributed to renal vascular constriction in both animal groups. It should be noted, however, that RVR remained significantly higher in the NO-inhibited rats than in the normal rats after saralasin. This suggests that Ang II was not solely responsible for renal vasoconstriction in the NO-inhibited group. This view is supported by previous studies which showed that when renin was suppressed by a high salt diet, Ang II played no role in renal vasoconstriction in acutely NO-inhibited animals.\(^5\) Also, Ang II blockade in awake rats, in which the renin-angiotensin system is not activated, fails to lower RVR caused by NO inhibition.\(^5,23\)

Thus, NO appears to play an independent role in regulating renal vascular tone. The saralasin effect observed in both the control and NO-inhibited rats in our study was probably caused by the experimental conditions of general anesthesia and surgery, which activated the renin-angiotensin system.

It is clear that voltage-regulated calcium channel blockade with verapamil was able to reduce both MAP and RVR markedly in the normal controls as well as in the NO-inhibited group. As in the case of saralasin, however, both MAP and RVR remained higher in the NO-inhibited rats than in the controls, suggesting that intrinsic vascular tone had been set at a higher level by chronic NO inhibition.

In summary, hypertension and reduced RBF caused by chronic NO inhibition do not appear to be due to unopposed angiotensin, vasoconstrctor prostaglandins, epinephrine, or sodium retention. Blockade of ET\(_A\) receptors with BQ-123 did not restore renal hemodynamics or blood pressure, but the data are difficult to interpret because of complex actions of the drug. Other investigators\(^29\) have excluded norepinephrine and vasoressin. The weight of findings indicates that overexpression of endogenous vasoconstrictors is not responsible for increased vascular tone in NO-inhibited animals. Rather, the reduced availability of NO per se appears to cause a resetting of intrinsic vascular smooth muscle tone, presumably mediated by an increase in intracellular calcium concentration or sensitivity.

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**References**


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