Relaxin Ameliorates Hypertension and Increases Nitric Oxide Metabolite Excretion in Angiotensin II But Not Nω-Nitro-L-Arginine Methyl Ester Hypertensive Rats

Jennifer M. Sasser, Miklos Molnar, Chris Baylis

Abstract—Previous findings suggest a potential therapeutic action of relaxin, the putative vasodilatory signal of normal pregnancy, in some forms of cardiovascular disease. However, the mechanisms underlying the beneficial effects of relaxin have not been fully elucidated. The purpose of this study was to determine whether the vasodilatory effects of relaxin are dependent on activation of NO synthase. We examined the effect of relaxin in male Sprague-Dawley rats given angiotensin II (Ang II; 200 ng/kg per minute SC by minipump), the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 1.5 mg/100 g IV followed by 150 mg/L in drinking water), or vehicle for 3 weeks. After 7 days of Ang II or L-NAME, mean arterial pressure was elevated compared with baseline. Relaxin was administered (4 μg/h, SC by minipump) for the next 2 weeks of Ang II, L-NAME, or vehicle treatment. Two-week relaxin treatment alone slightly reduced mean arterial pressure in normotensive rats. Three weeks of either Ang II or L-NAME treatment alone produced hypertension, albuminuria, mild glomerular sclerosis, reduced nitric oxide metabolite excretion, and increased oxidative stress (excretion of hydrogen peroxide and thiobarbituric acid reactive substances and renal cortex nitrotyrosine abundance). Relaxin reduced mean arterial pressure, albumin excretion, and oxidative stress markers and preserved glomerular structure and nitro oxide metabolite excretion in Ang II–treated rats; however, relaxin did not attenuate these changes in the rats treated with L-NAME. None of the treatments affected protein abundance of neuronal or endothelial NO synthase in the kidney cortex. These data suggest that the vasodilatory effects of relaxin are dependent on a functional NO synthase system and increased NO bioavailability possibly because of a reduction in oxidative stress. (Hypertension. 2011;58:197-204.)

Key Words: NO synthase ● blood pressure ● albuminuria ● oxidative stress

Relaxin may be useful in the treatment of cardiovascular disease in both males and females because of its vasodilatory, antifibrotic, and angiogenic properties (for review, see References 1–3). Relaxin is a potent, endothelium-dependent vasodilator of human resistance arteries obtained from gluteal biopsies, and in rodents relaxin promotes dilation of microvessels in the kidney and heart, as well as in reproductive organs. In addition, acute administration of relaxin opposes the actions of angiotensin II (Ang II) in vitro and in vivo.5–7 Although some animal studies have shown that relaxin can reduce blood pressure when infused into spontaneously hypertensive rats (SHRs)8 and rats with 5/6 renal ablation/infarction,9 others have shown no effect of relaxin infusion on blood pressure in the SHR after 1 to 7 days or 2 weeks of relaxin treatment in aged (17-month) SHRs despite reduced vascular resistance and improved arterial compliance.5,10 In addition, Lekgabe et al11 demonstrated that relaxin reduces cardiac and renal fibrosis in the SHRs and suggested that relaxin has therapeutic potential in hypertension. Therefore, the first goal of this study was to determine whether chronic relaxin treatment can reduce blood pressure during chronic Ang II–induced hypertension.

There is evidence that some of the cardiovascular benefits of relaxin treatment are mediated by increased production of NO12 and that the vasodilatory effects of relaxin are dependent on NO.6,13 Baccari and Bani14 have recently reviewed the actions of relaxin to stimulate the NO pathway in both reproductive and nonreproductive organs. NO, produced by NO synthase (NOS), is an important regulator of blood pressure and regional blood flow, vascular smooth muscle proliferation, platelet aggregation, and leukocyte adhesion.15 NO deficiency is associated with endothelial dysfunction and the development of hypertension and associated target organ damage.16,17 Therefore, agents that increase NO bioavailability are of potential therapeutic use in the treatment of hypertension. Various studies have implicated all 3 of the NOS enzymes in the NO-dependent actions of relaxin according to the cell type studied.14 Within the kidney and heart,
relaxin is a potent vasodilator and improves renal and coronary blood flow in an NOS-dependent manner,6,13,18 and in vitro data suggest that relaxin stimulates NO production in endothelial and smooth muscle cells.19–22 We hypothesize that the antihypertensive effects of relaxin are dependent on activation of NOS and increased production of NO. To determine the importance of the endogenous NO system in the response to relaxin, we assessed the effects of relaxin treatment on blood pressure during chronic NOS inhibition.

Methods

Animal Models
All of the experiments were performed using male Sprague-Dawley rats (400 to 500 g; Harlan Laboratories, Indianapolis, IN) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the University of Florida Institutional Animal Care and Use Committee. Animals were housed under conditions of constant temperature and humidity and exposed to a 12:12-hour light-dark cycle. All of the rats were given free access to regular rat chow and water. All of the surgeries were conducted with rats under general isoflurane anesthesia (Abbott, North Chicago, IL) using full sterile technique. A vascular catheter (Tygon) was placed in the left femoral artery, threaded under the skin by trocar, exteriorized at the back of the animal, and then rats were randomly assigned to 1 of 3 groups: (MAP) measurement were obtained from the indwelling arterial catheter, and then rats were placed in metabolic cages to facilitate 24-hour urine collection, after which the rats were given free access to regular rat chow and water. All of the surgeries were conducted with rats under general isoflurane anesthesia, rats were returned to individual cages in which they moved freely. All of the catheterized rats were trained to accept handling and the activity in the laboratory.

After 1 week, all of the rats were placed on a low-nitrate diet (ICN AIN 76C, MP Biomedicals, Aurora, OH) and 24 hours later were threaded under the skin by trocar, exteriorized at the back of the animal, and then rats were randomly assigned to 1 of 3 groups: sham, Ang II-infused, and Nω-nitro-l-arginine methyl ester (l-NNAME; an NOS inhibitor) treated as shown in Table 1. After the first week of treatment, recombinant human relaxin (relaxin) was then given to half of the rats in each of the 3 cohorts: Ang II and l-NNAME were from Sigma (St Louis, MO), and relaxin was a gift from Drs. Douglas Steinberg and Andrew Krieger (Corthera, Inc. San Mateo, CA). In both Ang II–treated groups, ALZET Model-2001 minipumps (Durect Corporation, Cupertino, CA) were used to deliver Ang II at 200 ng/kg per minute for 1 week. For the l-NNAME–treated groups, a bolus dose was given IV (1.5 mg/100 g of body weight bolus via tail vein) followed by l-NNAME ad libitum in the drinking water (150 mg/L) for the duration of the experiment. After 7 days, minipumps were replaced with ALZET 2ML2 minipumps containing Ang II (200 ng/kg per minute) or relaxin (4 μg/h) alone or in combination for 2 weeks. Sham minipumps containing vehicle only were used in rats not receiving Ang II or relaxin. Rats were placed on a low NOx diet 24 hours before being placed in metabolic cages, and then MAP and blood samples were taken at the end of each week. At the end of the study, both mean and systolic blood pressures were measured. Rats were anesthetized with isoflurane, and aortic blood sample was collected via the abdominal aorta. The kidneys were perfused blood free with cold PBS, and the right kidney was separated into cortex and medulla and snap frozen in liquid nitrogen. Then, the left kidney was then perfused with 2% paraformaldehyde, lysine, and meta-periodate for 8 to 10 minutes and removed.

Renal Histology
Paraffin-embedded kidneys were sectioned at a thickness of 5 μm onto Superfrost plus slides, and kidney sections were stained using a periodic acid–Schiff stain kit (Sigma) with hematoxylin as the secondary stain. Up to 100 glomeruli were scored and blinded, as follows: 0=healthy glomeruli, +1=<25% damage, +2=25% to 50% damage, +3=51% to 74% damage, and +4=>75% damage. A glomerulosclerosis index score was calculated using the following equation: (#of +1)+ 2(#of +2)+ 3(#of +3)+ 4(#of +4)/total glomeruli observed as described previously.23

Western Blotting
Protein abundances were detected using Western blotting, as described previously.24 Briefly, 200 μg of kidney cortex homogenate were loaded on 6.0% or 7.5% polyacrylamide gels and separated by electrophoresis. Membranes were incubated overnight with specific antibodies: mouse monoclonal NOS1 (1:50 dilution, Santa Cruz Biotechnology, SC-5302), rabbit polyclonal NOS1 (1:500 dilution, Thermo Scientific, PAI-033), mouse monoclonal NOS3 (1:250 dilution, BD Transduction, 610297), and mouse monoclonal nitro-tyrosine (1:500, Upstate, 05-233). The membranes were then incubated with corresponding secondary antibodies: goat antirabbit antibody (1:3000 dilution; Bio-Rad 170-6515) or goat antimouse antibody (1:2000 dilution; Bio-Rad 170-6516). Bands of interest were visualized using enhanced chemiluminescence reagent and quantified by densitometry (VersaDoc imaging system and Quantity One Analysis software, Bio-Rad) as integrated optical density after subtraction of background. Integrated optical density was factored for Penceau red staining (Sigma) to correct for any variations in total protein loading and for an internal standard, and protein abundance is represented as integrated optical density/Penceau red/standard.

Urine and Plasma Analysis
Urine albumin concentrations were measured using a commercially available kit (Cayman Chemical) according to the manufacturer’s specifications. Total NO production (from NOx=NO3–+NO2–) was measured in urine Griess reaction.25 Plasma and urine creatinine concentrations were measured by high-performance liquid chromatography as described by us previously.26 Urine concentrations of hydrogen peroxide and thiobarbituric acid reactive substances were measured using commercially available kits (Amplex Red, Molecular Probes and Oxitek, Zeptometrix, respectively) according to the manufacturer’s specifications.

Statistical Analysis
Results are presented as mean±SE. For multiple comparisons, ANOVA with Newman-Keuls post hoc analysis was used. MAP and urinary NOx excretion were compared using repeated-measures ANOVA with Bonferroni posttest to compare the effects of both time (within group comparisons) and treatment (between group comparisons) using Prism 4 software (Graph Pad Software, San Diego, CA). Histological (nonparametric) data were analyzed by Kruskal-Wallis test with Dunn posttest. P<0.05 was considered statistically significant.

Results
As shown in Figure 1, the control rats maintained a constant MAP over the 3-week study period. After 1 week, Ang II and l-NNAME treatments increased MAP to a similar extent (145±4 mm Hg in all of the rats receiving Ang II treatment

### Table 1. Treatment Protocol

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 2 to 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (n=9)</td>
<td>Sham minipump</td>
</tr>
<tr>
<td>2</td>
<td>Relaxin (n=11)</td>
<td>Relaxin minipump</td>
</tr>
<tr>
<td>3</td>
<td>Ang II (n=10)</td>
<td>Ang II minipump</td>
</tr>
<tr>
<td>4</td>
<td>Ang II + relaxin (n=10)</td>
<td>Ang II + relaxin minipump</td>
</tr>
<tr>
<td>5</td>
<td>l-NNAME (n=9)</td>
<td>l-NNAME (initial IV bolus, then via water)</td>
</tr>
<tr>
<td>6</td>
<td>l-NNAME + relaxin (n=9)</td>
<td>l-NNAME (water) + relaxin minipump</td>
</tr>
</tbody>
</table>

Ang indicates angiotensin; l-NNAME, Nω-nitro-l-arginine methyl ester.
and 156±3 mm Hg in all of the rats receiving L-NAME treatment; 127±3 mm Hg in vehicle-treated rats, P<0.05 versus control for both). After the first week of hypertension, relaxin was then given randomly to half of the rats in each treatment cohort (vehicle, Ang II, and L-NAME). MAP remained elevated in the groups that received either Ang II or L-NAME alone throughout the study period. Seven days of relaxin treatment had no effect on MAP in any of these 3 groups. After 2 weeks, however, relaxin treatment slightly reduced MAP in normotensive rats (P<0.05 versus baseline and P<0.01 versus week 1); however, MAP in the relaxin-treated group was not statistically lower than control-treated rats. Relaxin reduced MAP in the Ang II+relaxin group so that MAP was similar to values observed in the control group (P>0.001 versus Ang II alone) but had no effect on MAP in the L-NAME+relaxin group. Similarly, systolic blood pressure (Table 2) was elevated after 3-week treatment with Ang II or L-NAME (P<0.01 versus control). Relaxin treatment normalized systolic pressure in the Ang II hypertensive group (P<0.001 versus Ang II alone) but had no effect on systolic pressure in the control or L-NAME-treated rats.

As shown in Table 2, there was no change in creatinine clearance with relaxin treatment with or without Ang II or L-NAME. Urinary albumin excretion, a marker of renal injury, was elevated in both Ang II–(P<0.05 versus control) and L-NAME–(P<0.01 versus control) treated groups. Relaxin treatment preserved glomerular structure during chronic Ang II treatment but had no effect during L-NAME treatment. Please see the online Data Supplement at http://hyper.ahajournals.org for representative images of the renal histology (Figure S1).

Urinary excretion of NO metabolites, NOx, was not changed by 1 week of treatment with Ang II (P=0.14 versus baseline) but was significantly reduced by 1 week of L-NAME treatment (P=0.04 versus baseline; Figure 3). After 2 weeks of hypertension, NOx excretion was decreased in both Ang II– and L-NAME–treated rats (P<0.01 versus baseline) and remained low at week 3 in these groups. Relaxin treatment alone had no effect on NOx excretion (P=0.35 versus baseline); however, rats that received relaxin along with Ang II showed restoration of NOx excretion to normal values (P<0.05 versus Ang II alone at weeks 2 and 3). The fall in NOx excretion in rats given L-NAME persisted despite relaxin administration. Western blot analysis revealed that neither hypertensive treatment nor relaxin had an effect on the protein abundance of NOS1 or NOS3 in the kidney cortex (Figure 4).

After 3 weeks of treatment with either Ang II or L-NAME, renal excretion of 2 markers of oxidative stress, hydrogen peroxide and thiobarbituric acid reactive substances (P<0.05 versus control for both markers), was increased compared with control (Figure 5). In addition, Western blot analysis showed that there is increased nitrotyrosine content in the renal cortex of both hypertensive groups (P<0.05 for Ang II versus control; P<0.01 for L-NAME versus control; Figure 5E through 5G). Two-week relaxin treatment in normotensive rats had no effect on these markers; however, relaxin treatment normalized the excretion of both hydrogen peroxide (P<0.05 versus control) and thiobarbituric acid reactive substances (P<0.01 versus control) and normalized nitrotyrosine immunoreactivity in rats treated with Ang II. No effect of relaxin treatment was observed in the L-NAME–treated group.

**Discussion**

The main novel findings of this study are that relaxin lowers blood pressure, reduces albuminuria and glomerular sclerosis, and increases NOx excretion during chronic Ang II infusion.
In contrast, relaxin was ineffective in preventing hypertension or renal injury during L-NAME administration, demonstrating that the antihypertensive and renoprotective effects of relaxin are dependent on a functional NOS system. Relaxin treatment had no effect on the protein abundance of the constitutive NOS enzymes, NOS1 and NOS3, in the kidney cortex, indicating that the protective effect of relaxin in Ang II hypertension is not simply attributed to an increase in NOS enzyme abundance. Relaxin treatment reduced oxidative stress in rats treated with Ang II, suggesting that the beneficial effects of relaxin are attributed to an antioxidant mechanism (see below).

Conrad et al demonstrated a reduction in systemic vascular resistance in normal nonpregnant female rats but without a change in blood pressure after 10 days of relaxin treatment. In the present study, relaxin treatment in normotensive rats had no effect on any of the measured parameters after 7 days, but 14 days of relaxin reduced MAP compared with baseline levels. The current study is the first to determine the effect of chronic relaxin treatment during Ang II–induced hypertension. Here, we found that relaxin reduced blood pressure in rats with Ang II–induced hypertension after 2 weeks of treatment. Our findings are consistent with in vitro and in vivo evidence that relaxin blunts the constrictor response to Ang II.5–7 Debrah et al found that acute relaxin treatment in Ang II–hypertensive rats reduced arterial load and increased cardiac output within 2 hours of treatment but did not affect blood pressure. Likewise, we did not observe an antihypertensive effect of relaxin during the first week of treatment, but relaxin normalized blood pressure after 2 weeks.

There is some controversy in the literature regarding the effects of long-term relaxin treatment on blood pressure regulation in animal models of hypertension. Evidence for the vasodilatory and antihypertensive roles of relaxin has been reported in both male and female SHRs and in the 5/6 renal
ablation/infarction model in male rats,\textsuperscript{8–9,11} whereas others found no effect of relaxin on blood pressure in male SHRs despite increased arterial compliance.\textsuperscript{5,10} We cannot speculate on the reasons for the differences in the blood pressure–lowering effects of relaxin in the SHRs because we have not used this model; however, even when blood pressure did not fall with relaxin there was evidence of some cardiovascular benefit in every study.

Figure 4. Relaxin treatment does not affect renal cortex abundance of NO synthase (NOS) 1 or NOS3. Effects of angiotensin (Ang) II and relaxin (A through C) and effects of \(N^\omega\)-nitro-L-arginine methyl ester (L-NAME) and relaxin (D through F) on abundance of the constitutive NOS enzymes in the renal cortex. Values are mean±SEM, \(n=7\) to 10. G, Representative images of Western blots for NOS1\(\alpha\), NOS1\(\beta\), and NOS3 in kidney cortex homogenates from control (C), relaxin (R), Ang II (A), Ang II+relaxin (A+R), L-NAME (L), and L-NAME+relaxin (L+R)–treated rats. + indicates positive control. Black block arrow on left side of image indicates 150 kDa as measured using the BioRad Kaleidoscope Precision Plus Protein Standards. Thin black arrow indicates specific protein band.

The decrease in blood pressure in Ang II–treated rats with relaxin was associated with a restoration of NO\textsubscript{x} excretion, a marker of NO bioavailability, to normal values. In contrast, relaxin had no effect on either blood pressure or NO\textsubscript{x} excretion during chronic NOS inhibition with L-NAME. This is the first study that illustrates that the blood pressure–lowering effects of relaxin require a functional NOS system. Our findings are consistent with previous work by Danielson.
that showed that acute NOS inhibition abolished the renal vasodilatory response to relaxin, and the effects of relaxin on coronary blood flow are also dependent on stimulation of NO. The molecular mechanisms by which relaxin activates the NO system are currently unknown. Based on the current studies, we are unable to determine which NOS isoform(s) is involved in the response to relaxin, because L-NAME inhibits all NOS. In addition, the specific isoform of NOS activated by relaxin varies according to cell type. For example, relaxin activates NOS II in cultured coronary endothelial cells and aortic vascular smooth muscle cells and upregulates NOS III in uterus. In the kidney, the hemodynamic effects of relaxin occur via NO, possibly via activation of endothelin B receptors, but the source of this NO has not been defined. In the current study, we saw no effect of relaxin on level of the constitutive NOS enzymes (NOS1 and NOS3) in the renal cortex. Of course, in addition to protein abundance, the activity of the NOS enzymes is regulated by many factors, including substrate and cofactor availability, posttranslational modifications, such as phosphorylation, and the presence of oxidative stress.

It is interesting that relaxin is able to fully restore NOx excretion during Ang II hypertension despite the known action of Ang II to increase oxidative stress via activation of NADPH oxidase. Increased superoxide production directly reduces NO bioavailability by binding with NO to produce peroxynitrite and by reducing the availability of tetrahydrobiopterin, an essential NOS cofactor, which reduces NO production and causes the NOS enzymes to switch to superoxide generation. Indeed, we observed that both

Figure 5. Relaxin reduces oxidative stress during angiotensin (Ang) II- and l-nitro-L-arginine methyl ester (l-NAME)-induced hypertension. Effects of Ang II and relaxin (A, C, and E) and effects of l-NAME and relaxin (B, D, and F) on excretion of hydrogen peroxide (A and B) and thiobarbituric acid reactive substances (TBARS) (C and D) and renal cortex nitrotyrosine abundance (E and F). Values are mean±SEM, n=8 to 9. *P<0.05 vs control. #P<0.05 vs Ang II. G. Representative images of Western blots for nitrotyrosine in kidney cortex homogenates from control (C), relaxin (R), Ang II (A), Ang II+relaxin (A+R), l-NAME (L), and l-NAME+relaxin (L+R)-treated rats. Black block arrows on right side of image illustrate molecular weights as measured using the BioRad Kaleidoscope Precision Plus Protein Standards.
chronic Ang II and L-NAME models exhibited significant oxidative stress and that relaxin reduced urinary excretion of hydrogen peroxide and thiobarbituric acid reactive substances and renal cortex nitrotyrosine content in the rats treated with Ang II. This is consistent with work by Masini et al.,34 which demonstrated that relaxin decreases lipid peroxidation in myocardial tissue after ischemia reperfusion injury and lipid peroxidation and 8-hydroxy-2’-deoxyguanosine levels in ileal tissue from animals subjected to splanchnic artery occlusion.35 However, we did not observe any decrease in oxidative stress markers during L-NAME hypertension, suggesting that the antioxidant effects of relaxin require stimulation of endogenous NO.

Our findings indicate that relaxin can also prevent Ang II–induced renal injury but is not protective during chronic NOS inhibition. The protective effect observed during Ang II–treatment may be predominantly attributed to the blood pressure–lowering effect of relaxin in this model. However, others have demonstrated renoprotective effects of relaxin in experimental models of renal disease with normal blood pressure,9,36 and it is likely that, with established injury, relaxin can directly remodel the extracellular matrix via reduced collagen synthesis, increased activity of matrix metalloproteinases, and antagonism of profibrotic factors.11 In cultured kidney cells, relaxin has been shown to promote fibronectin degradation via the ubiquitin-proteasomal pathway12 and to increase the activity of matrix metalloproteinases.38 Most likely, a combination of hemodynamic and direct actions of relaxin account for the remarkable ability of relaxin to preserve renal function and prevent/reverse renal injury in the male relaxin knockout mouse39 and in the aging rat.40

**Perspectives**

More than half of the people who are treated for hypertension do not have adequate blood pressure control. New, effective treatments for hypertension are desperately needed, and our data indicate that relaxin is a potential therapeutic agent for the treatment of hypertension and the resulting end organ damage. However, this study suggests that an assessment of the endogenous NO system is critical before the use of relaxin to treat cardiovascular disease, because in states where the endogenous NO system cannot be stimulated, relaxin may not be an effective treatment. This work also suggests a potential role for relaxin as an antioxidant and highlights the need for future studies to investigate the impact of relaxin on prooxidant and antioxidant pathways.

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**Disclosures**

None.

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Relaxin ameliorates hypertension and increases NO metabolite excretion in Angiotensin II but not L-NAME hypertensive rats

Jennifer M Sasser¹, Miklos Molnar¹,³, and Chris Baylis¹,²

Running Title: Antihypertensive effects of relaxin depend on NO

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Supplemental Figure 1. Representative images of renal histology in Periodic Acid-Schiff stained sections from Control (A), Relaxin (B), ANGII (C), ANGII + Relaxin (D), L-NAME (E), and L-NAME + Relaxin (F) treated rats. * indicates focal segmental glomerular sclerosis, # indicates protein cast, + indicates global glomerular sclerosis. No remarkable histological changes were observed in slides from Control (A), Relaxin (B), or ANGII + Relaxin (D) treated rats, and sections from each group showed many undamaged glomeruli. Original magnification = 10X.