Angiotensin II Stimulates Synthesis of Endothelial Nitric Oxide Synthase

Bettye S Hennington, Hummin Zhang, M. Todd Miller, Joey P Granger, Jane F. Reckelhoff

Abstract—Previous studies have suggested that NO may play an important role in protecting the renal vessels from angiotensin II (ANGII)-mediated vasoconstriction. One possible mechanism for this interaction is that ANGII could stimulate NO production in the kidney by increasing endothelial NO synthase (NOS III). The present studies were performed in rats to determine whether acute or chronic elevations in ANGII are associated with enhanced renal NOS III mRNA or protein synthesis. In both acute and chronic studies captopril (20 μg/kg/min) was given IV to inhibit endogenous ANGII production. Acute suprarenal infusion of ANGII (5 ng/kg/min) for 110 minutes had no effect on arterial pressure but decreased GFR and renal plasma flow by 20% and 30%, respectively, and increased renal vascular resistance by 70%. Acute ANGII increased renal NOS III mRNA by 70% (as determined by ribonuclease protection assay), but had no effect on renal NOS III protein concentration (as detected by Western blot analyses). In contrast, chronic infusion of ANGII (5 ng/kg/min) for 10 days, increased arterial pressure by 30% and tended to reduce GFR and renal plasma flow. Chronic ANGII had no effect on renal NOS III mRNA levels, but increased NOS III protein by 90%. These data suggest that ANGII can stimulate NOS III synthesis and suggest that this may be one of the mechanisms whereby ANGII may enhance NO production (Hypertension. 1998;31[part 2]:283-288.)

Key Words: GFR ■ renal hemodynamics ■ mRNA ■ ribonuclease protection assay ■ Western blot

In recent years there has been considerable interest in the interaction between NO and angiotensin II (ANGII). While ANGII is known to play an important role in controlling renal hemodynamics via its actions on postglomerular vessels, recent studies have implied that ANGII may have an effect on preglomerular vessels that is modulated by the effects of nitric oxide (NO) or prostaglandins. To and colleagues, using isolated rabbit arterioles, demonstrated that production of NO is more important in modulation of afferent arteriolar tone rather than having an effect on the efferent arteriole. They further found that NO modulated ANGII-induced vasocostriction of the afferent arteriole but not the efferent arteriole. We have also recently reported that the acute effect of ANGII on preglomerular vessels in dogs is importantly modulated by nitric oxide. These data suggested that NO may play an important protective role in modulating the vasocostrictor effect of ANGII on preglomerular vessels.

The mechanisms by which NO could protect the renal vasculature from vasoconstrictor ANGII have not been fully elucidated. One possible mechanism is that ANGII could enhance NO production and NO would offset a direct vasocostrictor effect of ANGII. Whether ANGII is capable of stimulating renal NO production is unclear. Deng and colleagues found that acute infusion of pressor doses of ANGII increased urinary nitrate/nitrite excretion, metabolites of NO and indicators of NO production. However, chronic ANGII (5–6 days) had no effect on nitrate/nitrite excretion. Although this study suggests a role for ANGII in stimulating NO since urinary nitrate/nitrite excretion estimates whole body NO production, it is unclear whether ANGII stimulates renal NO production in these studies.

There are at least two mechanisms by which ANGII could increase renal NO production. First, ANGII could increase nitric oxide synthase enzyme activity by causing an increase in intracellular calcium concentration. Studies have been performed which document that ANGII is capable of increasing intracellular calcium in a concentration-dependent manner in endothelial cells in culture. Secondly, ANGII could increase NO by increasing endothelial NO synthase (NOS III) synthesis, either on a transcriptional level, typically by increasing and/or stabilizing the mRNA levels for NOS III, or on a translational level, and could thereby increase the amount of NOS III protein. Both transcriptional and translational increases in NOS III would increase NO production, if it is assumed that an increase in NOS III protein would be translated into an increase in NOS III activity in the cell and if the levels of substrate arginine and cofactors are not rate-limiting. A direct effect of ANGII on NOS III synthesis has not been previously investigated, however.

The present studies were performed to determine if acute intrarenal nonpressor doses of ANGII could stimulate NOS III synthesis. In addition, pressor doses of ANGII were given chronically for 10 days to determine the long-term effect of ANGII on transcription and translation of NOS III.

Received September 17, 1997; first decision October 10, 1997, revision accepted October 24, 1997
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Enhanced Synthesis of NOS III by Angiotensin II

Methods

Experimental Animals

For these studies, 53 male Sprague Dawley rats (Harlan SD, Indianapolis, Ind), aged 3 to 5 months, were used. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Rats were maintained on rat chow (Teklad) and tap water in a 12 hours/12 hours light/dark cycle until the time of study.

Short-Term Effect of ANGII on Renal Hemodynamics and NOS III Synthesis

The short-term effect of AngII on renal hemodynamics and NOS III synthesis was examined in rats pretreated with converting enzyme inhibitors. Prior to renal function studies, rats were placed on 4% NaCl diet for 24 to 48 hours, and during the experiment, converting enzyme inhibitors were given to all rats to block endogenous production of AngII. A diagram of the acute AngII Protocol is shown in Fig 1A. Rats were divided into two groups. Rats in group 1 (n=17) were treated with captopril (20 μg/kg/min) and suprapenal infusions of isotonic saline in the first and second periods of the experiment. Rats in group 2 (n=15) received intravenous captopril and suprapenal infusions of saline in the first period and captopril and suprapenal infusions of ANGII (8 ng/kg/min) in the second period of the experiment. Renal hemodynamics were measured in 6 control rats and 8 ANGII-treated rats.

Specifically, rats were anesthetized by intraperitoneal injection of the thiobarbiturate, Inactin (100 to 110 mg/kg body weight, RBI) and placed on a temperature-regulated surgery table to maintain rectal temperature at 36 to 38°C. Femoral and jugular catheters were placed, and a tracheostomy was performed as described previously.6,10 A catheter (PE-10) was also inserted below the bifurcation of the aorta on the left and advanced above the renal arteries for suprapenal infusion of isotonic saline or ANGII. A 23g needle connected to PE-50 tubing was inserted into the left renal vein in the retrograde direction for blood sampling for determination of extraction across the kidney for calculation of renal plasma flow. During catheter placement, the rats received an intravenous injection of recombinant porcine thromboplatin (2.0 μg/g bovine immunoglobulin, 2.5 μg/g bovine serum albumin in Ringer's solution) at 12.5 mL/kg/h for 45 minutes and thereafter at 1.5 mL/kg/h throughout the experimental period to maintain an euveolemic preparation.6,11 H-inulin (15 to 20 μCi/ml saline, New England Nuclear), was infused at 1.5 mL/h.

As shown in Fig 1A, following a 50-minute equilibration period for the H-inulin and captopril (20 μg/kg/min), two 30-minute clearances were performed during the first (control) period. Following this control period, ANGII (8 ng/kg/min) was infused suprapenally for 50 minutes. After equilibration of the ANGII, two 30-minute clearances were performed (period 2). Following the experiments, kidneys were removed, weighed, and placed in liquid nitrogen for assessment of NOS III mRNA and protein.

Long-Term Effect of ANGII on Renal Hemodynamics and NOS III Synthesis

A diagram of the chronic ANGII protocol is shown in Fig 1B. Rats were divided into two groups. Rats in group 1 (time control) (n=9) received captopril (20 μg/kg/min) IV throughout the control (4 days) and the experimental periods (10 days). Rats in group 2 (n=12) received captopril during the control period (4 days) and captopril and ANGII (5 ng/kg/min) in the experimental period (10 days). Abdominal aortic and femoral venous catheters were placed into rats under pentobarbital (30 mg/kg) anesthesia. A midline abdominal incision was made, and the abdominal aorta was separated from the inferior vena cava. A small hole was made in the aorta with a 20g needle and a catheter of medical vinyl tubing (0.24/0.40, BOLAB) in the shape of an “s” was inserted into the aorta. The femoral vein was also cannulated with vinyl tubing (0.24/0.40, BOLAB). Both catheters were advanced subcutaneously along the back and exteriorized at the nape of the neck through a button sutured to the skin and connected to a spring. Rats were placed in individual metabolism cages, and the spring tether was connected to a two-channel hydraulic swivel above the cage. The femoral catheter was connected via the swivel to an infusion pump at a rate of 0.2 mL/h. The arterial catheter was connected via the swivel to a transducer (Cobe) connected to an A/D converter for 24-hour blood pressure recording. Rats were provided with normal sodium intake (2.2 mEq/d) via the combination of diet and saline infusion.

As shown in Fig 1B, after a 7 day recovery from surgery, the rats were given continuous infusions of captopril (15 μg/kg/min) via the femoral venous catheter. On day 3 of captopril infusion in 2 rats, bolus IV injections of angiotensin I (ANGI, 0.3 μg/kg) were given to verify adequate blockade of converting enzyme. Prior to captopril treatment, the ANGII bolus caused increases in systemic arterial pressure from 111 mm Hg to 150 mm Hg. On day 3 of captopril in the same animals, Ang I bolus caused no increase in systemic blood pressure (96 to 96 mm Hg).

On day 4 of the captopril infusion, GFR and estimated renal plasma flow (RPF) were measured by infusion of [14C]-triolethalamate (Isotex...
Inactm (150 mg/kg), and the kidneys were removed, weighed, and analyzed for NOS III were performed as previously described by us,3 using a mouse monoclonal anti-NOS III primary antibody (1:1000, Transduction Laboratories) and a horse radish-peroxidase-conjugated, chromogenically for 10 days. On day 9, GFR and RPF measurements were performed by phosphomager system (Biorad) and was resuspended in DEPC-treated water, stored at -20°C, and used lsopropropyl precipitation, and three 70% ethanol washes The pellet was homogenized 20% (weight/volume) in 20 mmol/L Hepes, pH 7.5, containing 100 μM pepstatin, 100 μg/ml aprotinin, 10 mmol/L EDTA, 100 μg/mL leupeptin, and 1 mmol/L phenanthroline, and 1 mmol/L E-64 (Sigma) Western blot analyses for NOS III were performed as previously described by us,3 using a mouse monoclonal anti-NOS III primary antibody (1:1000, Transduction Laboratories) and a horse radish-peroxidase-conjugated, goat anti-mouse IgG (1:1000) (Amersham) secondary antibody. The bound antibody was detected using the ECL kit (Amersham) and quantified by densitometry (Biorad).

Ribonuclease Protection Assays (RPA)

Liquid nitrogen frozen whole kidney tissue was ground with a mortar and pestle, and total RNA was isolated by homogenization in RNAstat (Teltest), followed by two chloroform extractions, one isopropanol precipitation, and three 70% ethanol washes. The pellet was resuspended in DEPC-treated water, stored at -20°C, and used for isolation of RNA. A full-length probe was radioactively labeled with α-35P-UTP and used for hybridization to the RNA sample. The hybridized RNA/probe was separated by electrophoresis on 5% denaturing polyacrylamide gels. Quantiﬁcation was performed by phosphomager system (Biorad).

Western Blot Analyses

Liquid nitrogen frozen kidneys were homogenized 20% (weight/volume) in 20 mmol/L Hepes, pH 7.5, containing 100 μM pepstatin, 100 μg/mL aprotinin, 10 mmol/L EDTA, 100 μg/mL leupeptin, 1 mmol/L phenanthroline, and 1 mmol/L E-64 (Sigma) Western blot analyses for NOS III were performed as previously described by us,3 using a mouse monoclonal anti-NOS III primary antibody (1:1000, Transduction Laboratories) and a horse radish-peroxidase-conjugated, goat anti-mouse IgG (1:1000) (Amersham) secondary antibody. The bound antibody was detected using the ECL kit (Amersham) and quantified by densitometry (Biorad).

Statistical Analyses

The renal functional data were analyzed by analyses of variance (ANOVA), using Statview 5.0 software for the Macintosh. Significance was defined as P<0.05. All data values are expressed as mean±SEM. For RPAs and Western blots, differences between groups (controls and AngII-treated) were assessed by Student's t test. P<0.05, defined as significant.

Results

Acute Response to AngII

As shown in Table 1, there were no changes in mean arterial pressure, GFR, renal plasma flow (RPF), or renal vascular resistance within group 1 (control) rats. Supranephric infusion of AngII (group 2 rats) had no effect on mean arterial pressure, but caused GFR to decrease by 20% RPF also decreased by 30% with AngII, and renal vascular resistance increased by 70% Renal NOS III mRNA was increased by 74% with acute supranephric infusion of AngII (Fig 2A). In contrast, there was no change in renal NOS III protein concentration in response to AngII (Fig 2B).

Response to Chronic AngII

As shown in Table 2, there was no effect of time on mean arterial pressure, GFR, or RPF (group 1). In response to chronic AngII infusion, mean arterial pressure increased by approximately 30% (group 2). There was a tendency for GFR and RPF to decrease, but not significantly so. Chronic infusion of pressor doses of AngII for 10 days had no effect on renal NOS III mRNA levels (Fig 3A), but did increase NOS III protein in the kidney by 90% (Fig 3B).

Discussion

The present studies demonstrate that acute AngII-induced reductions in renal hemodynamics are associated with increased renal NOS III mRNA production. In addition, we report that long-term elevations in AngII result in increased NOS III protein synthesis, supporting the hypothesis that AngII can chronically increase NO production in the kidney.

Acute AngII infusions at nonpressor doses caused a reduction in GFR and renal plasma flow. In addition, AngII increased the renal mRNA levels of NOS III. However, there was no effect on renal NOS III protein concentrations. The lack of an acute effect of AngII to increase renal NOS III protein is not surprising since the AngII infusion time was only 110 minutes and was probably not long enough to allow translation of NOS III protein to be upregulated or, if upregulated, to be detected using the technique of Western

### Table 1. Hemodynamic Response to Acute Angiotensin II Infusion in Male Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Period 1</th>
<th>Period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAP (mm Hg)</td>
<td>GFR (ml/min)</td>
</tr>
<tr>
<td>Group 1 (n=6)</td>
<td>99±4</td>
<td>3.24±0.40</td>
</tr>
<tr>
<td>Group 2 (n=8)</td>
<td>110±4</td>
<td>4.11±0.26</td>
</tr>
<tr>
<td>Group 1 (n=8)</td>
<td>106±5</td>
<td>2.92±0.30</td>
</tr>
<tr>
<td>Group 2</td>
<td>114±3</td>
<td>3.11±0.40</td>
</tr>
</tbody>
</table>

*Period 1: Rats received intravenous infusion of captopril (20 μg/kg/min) and supranephric infusion of isotonic saline. Period 2: Group 1 rats received intravenous infusion of captopril and supranephric saline. Group 2 rats received intravenous captopril and supranephric infusion of angiotensin II (8 ng/kg/min). MAP, mean arterial pressure, GFR, glomerular filtration rate, FF, filtration fraction, RVR, renal vascular resistance. Data are expressed as mean±SEM. Data were analyzed by ANOVA. P<0.05, compared to rats receiving captopril alone in Period 1.
Enhanced Synthesis of NOS III by Angiotensin II

Figure 2. Effect of acute ANGII on renal mRNA and protein for endothelial NO synthase (NOS III). ANGII (8 ng/kg/min) was given for 110 minutes to anesthetized rats pretreated with captopril. A. Top: Representative ribonuclease protection assays (RPAs) taken from phosphorimaging scans: Lanes 1–5, ANGII-treated rats; lanes 6–11, control rats; lanes 12 and 13, controls containing yeast tRNA, labeled RNA probe in the presence (lane 12) and absence (lane 13) of RNase. Bottom: Densitometric analyses of RPAs (total n=17 control and 15 ANGII-treated rats) of the acute effect of ANGII on renal NOS III mRNA. B. Top: Representative Western blots of renal NOS III in response to acute ANGII. Lanes 1–3, control; lanes 4–6, ANGII-treated. Bottom: Averages of densitometric scans (n=7 control, 8 ANGII-treated rats) of Westerns. *, P<.05, in ANGII-treated group compared to control group.

TABLE 2. Hemodynamics in Rats Given Chronic Angiotensin II

<table>
<thead>
<tr>
<th>Groups</th>
<th>GFR (ml/min)</th>
<th>RPF (ml/min)</th>
<th>MAP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captopril alone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (n=9)</td>
<td>1.92±0.07</td>
<td>11.00±1.79</td>
<td>90±4</td>
</tr>
<tr>
<td>Group 2 (n=12)</td>
<td>2.11±0.17</td>
<td>12.54±0.99</td>
<td>88±4</td>
</tr>
<tr>
<td>Captopril and vehicle or ANGII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>2.02±0.20</td>
<td>11.58±1.24</td>
<td>92±2</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.49±0.15</td>
<td>11.13±1.12</td>
<td>119±4</td>
</tr>
</tbody>
</table>

P value NS NS 0.001

Rats in Group 1 were infused chronically with captopril (20 μg/kg/min) for 14 days. Rats in Group 2 were infused chronically with captopril for 14 days and Ang II (5 ng/kg/min) was given for the last 10 days. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were measured at days 4 and 14 of the infusions. Data are expressed as mean±SEM and differences between groups were analyzed by ANOVA.

These data suggest that it is unlikely that the increase in NOS III synthesis plays an important role in the acute ANGII-NO interaction. However, the fact that NOS III mRNA is increased suggests an important initiating step in the interaction between NO and ANGII which goes beyond the transient acute interactions.

In support of the latter hypothesis, chronic ANGII infusion for 10 days increased mean arterial pressure and tended to reduce GFR and renal plasma flow. Chronic ANGII infusion had no effect on renal NOS III mRNA levels. However, renal NOS III protein levels were increased significantly with ANGII. This pattern of increased protein in the absence of increases in mRNA is consistent with an increase in basal production of NOS III enzyme, perhaps due to stabilization of the message, thus not requiring an increase in mRNA to increase translation of the protein. Barring any change in L-arginine substrate or NO synthase cofactors, the increase in NOS III protein would cause increased production of NO in the kidney. These data suggest then that ANGII can stimulate renal NO production by increasing NOS III protein content.

Consistent with our findings is a preliminary report by Zou and colleagues, who found the ANGII directly increases NO

blot. These data suggest that it is unlikely that the increase in NOS III synthesis plays an important role in the acute ANGII-NO interaction. However, the fact that NOS III mRNA is increased suggests an important initiating step in the interaction between NO and ANGII which goes beyond the transient acute interactions.
in the medulla of the kidney as measured by the oxyhemoglobin microdialysis technique. 

Although the present studies describe the effect of ANGII to increase NOS III synthesis, these studies do not explain the mechanism(s) by which NOS III synthesis could be increased by ANGII. Since there are shear stress elements in the 5' untranslated region of NOS III gene, it is possible that ANGII could cause an increase in NOS III synthesis due to an increase in shear stress. However, since Ito and colleagues have demonstrated an increased NO response to acute ANGII in isolated afferent arterioles, a preparation independent of flow, the mechanism for increased NOS III synthesis with ANGII may also be independent of shear stress.

It is also possible that ANGII could increase NO synthesis by directly affecting the second messenger systems in the endothelial cells and thereby increase synthesis of NOS III. Siragy and Carey have recently reported that the renal response of increased cGMP, an indicator of NO production, to acute ANGII was mediated by angiotensin AT2 receptors. Other investigators have shown that ANGII increases intracellular calcium concentrations in cultured endothelial cells in a dose-dependent manner. Since NOS III activity is calcium-dependent, this may also be a mechanism for the acute increase in NO production mediated by NOS III. However, future studies will be necessary to determine if the NOS III synthetic response to chronic ANGII is also mediated via the AT2 receptors, increases in intracellular calcium, or other second messenger systems.

In the present study, our results suggest that chronic infusion of ANGII increases NOS III activity. However, we have not directly measured the levels of NOS III activity. The major reason for this is that it is difficult to assess the activity of the NOS III isoform separately from the activity of the other NO synthase isoforms. There are no inhibitors specific for NOS III activity that do not also inhibit the activity of the other isoforms of NO synthase. Whether ANGII can indeed increase NOS III activity in the renal vasculature by increasing the NOS III protein concentration needs to be further investigated.

In summary, acute suprarenal infusion of nonpressor doses of ANGII increased renal mRNA for NOS III, but had no effect on renal NOS III protein concentration. In contrast, chronic ANGII infusion, at low pressor doses, had no effect on NOS III mRNA levels in the kidney, but significantly increased renal NOS III protein concentration. These data suggest that ANGII is capable of controlling local NO production in the renal vasculature, thus protecting against ANGII-induced vasoconstriction.
Acknowledgments
This work was supported by the American Heart Association, Mississippi Affiliate and by HL51971 from NIH

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Hypertension. 1998;31:283-288
doi: 10.1161/01.HYP.31.1.283

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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