Acute intrarenal infusion of ANG II does not stimulate immediate early gene expression in the kidney

AMANDA J. EDGLEY, NANCY R. NICHOLS, AND WARWICK P. ANDERSON
Department of Physiology, Monash University, Clayton, Victoria 3800, Australia

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Edgley, Amanda J., Nancy R. Nichols, and Warwick P. Anderson. Acute intrarenal infusion of ANG II does not stimulate immediate early gene expression in the kidney. Am J Physiol Regulatory Integrative Comp Physiol 282: R1133–R1139, 2002; 10.1152/ajpregu.00187.2001.—ANG II is known to stimulate both hypertrophy and proliferation of vascular smooth muscle cells in culture (5, 11, 12, 32) and has also been reported to stimulate expression of early growth response genes such as egr-1 and c-fos in a variety of cultured cells, including cells of renal origin. To investigate whether ANG II can stimulate early growth response gene expression in vivo, we studied the effects of acute renal artery infusion of low-dose ANG II (2.5 ng·kg⁻¹·min⁻¹) or vehicle on the renal expression of c-fos and egr-1 genes in rats. ANG II infusion for 30 or 240 min decreased renal vascular conductance by ~13 and 8%, respectively, compared with the vehicle group. Expression of the early growth response genes c-fos and egr-1 was analyzed using Northern blot hybridization. No significant upregulation of c-fos or egr-1 mRNA levels was detected in rats that received ANG II for either 30 or 240 min, compared with the vehicle groups. We conclude that ANG II, at doses that cause significant physiological effects, does not increase the renal expression of c-fos or egr-1 genes over periods of up to 4 h in vivo.

ANG II is known to stimulate both hypertrophy and proliferation of vascular smooth muscle cells in culture (5, 11, 12, 32) and has also been reported to stimulate expression of early growth response genes such as early growth response gene-1 (egr-1; zif-268) and c-fos (18, 22, 33, 35). Likewise, ANG II has trophic actions on many cells of renal origin in culture, including renal arteriolar smooth muscle cells (8, 40–43).

Once transcribed to proteins, c-Fos and EGR-1 may play a role in growth promotion through regulating the expression of other genes, including known smooth muscle growth factors such as platelet-derived growth factor and transforming growth factor-β1. For example, c-Fos can form a heterodimer with members of the jun family of transcription factors, which together can bind to activator protein sites in the DNA and regulate transcription of genes (15, 21).

We have shown recently that chronic intrarenal infusion of ANG II causes renal vascular changes that are consistent with growth and remodeling of the renal vasculature (37). In these experiments conducted over several days, the rats also developed hypertension (37). However, in contrast to the extensive evidence gathered from cultured cells, there is little direct evidence concerning the trophic actions of ANG II on vascular smooth muscle cells in vivo, and even less is known about such effects in the kidney.

The present experiments were constructed to determine whether acute infusion of ANG II at physiologically relevant levels into the kidney increased renal expression of early growth response genes c-fos and egr-1 as indicators of the initiation of cell growth. The dose of ANG II chosen caused a measurable reduction in renal blood flow without systemic pressor effects when infused directly in the renal artery.

MATERIALS AND METHODS

Systemic and renal hemodynamic responses to an acute infusion of ANG II (2.5 ng·kg⁻¹·min⁻¹; Auspep) or vehicle (10 IU/ml heparinized saline) directly in the left renal artery were investigated in 24 rats, with subsequent measurement of c-fos and egr-1 mRNA levels using Northern blot analysis. All experiments were conducted in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Ten-week-old male outbred Sprague-Dawley rats (240–360 g) were obtained from Monash University Animals Service, maintained on a standard rat chow (GR2; Barastoc Stockfeeds, Pakenham, Victoria, Australia), and allowed free access to water. Animals were randomly allocated into one of four groups (n = 6 in each group) consisting of an intrarenal infusion of vehicle and ANG II for 30 or 240 min.

The dose of ANG II used in the study was determined in a pilot study where increasing doses of ANG II (1, 2, 5, and 10 ng·kg⁻¹·min⁻¹) were infused directly in the renal artery of anesthetized rats for 5 min, resulting in mean renal blood flow reductions of ~13, 25, 40, and 51%, respectively.

On the day of the experiment, each rat was anesthetized (Inactin; thioptabarbitral, 175 mg/kg ip; Research Biochemicals International), a tracheotomy was performed, and a jugular vein catheter (PE-50) was inserted for infusion of maintenance fluids (2% BSA; Sigma Chemical). Arterial pressure was recorded by catheterizing the femoral artery and connecting the catheter to a disposable pressure transducer (Cobe, Avarda, CO). Heart rate was measured by a cardiotachometer activated by the arterial pressure pulse. The left renal artery was catheterized using a tapered PE-10...
Northern Blot Analysis of c-fos mRNA Levels in the Kidney

In a series of Northern blots, c-fos and egr-1 mRNA levels were determined in left kidney tissue from animals that had received an intrarenal infusion of vehicle (10 IU/ml heparinized saline) or ANG II (2.5 ng·kg⁻¹·min⁻¹) for 30 or 240 min.

Positive control. c-fos mRNA levels were determined in left kidney samples from animals that received a supraphysiological dose of ANG II (100 ng·kg⁻¹·min⁻¹), infused intravenously for 1 h to establish if detection of the c-fos mRNA levels was possible in the kidney (Fig. 1).

Total RNA was extracted from left and right kidneys using a standard Tri-reagent (Sigma Chemical) protocol that is a modification of the method of Chomczynski and Sacchi (6). Samples of total RNA were separated according to size using agarose gel electrophoresis, and levels of c-fos mRNA in left kidneys from animals infused with ANG II commenced with variables measured every 5 min for the 30-min infusion period and every 10 min for the 240-min infusion period. Renal vascular conductance was calculated by dividing renal blood flow by mean arterial pressure.

At the end of the infusion, left kidneys were harvested from the animals, snap-frozen on dry ice, and stored at −70°C before Northern blot analysis of immediate early gene expression.

Northern Blot Analyses of Immediate Early Gene Expression in the Kidney

Duplicate membranes were hybridized with an [α-³²P]UTP (NEN Life Sciences and Amersham)-labeled c-fos RNA probe, synthesized by in vitro transcription of a linearized antisense c-fos cDNA template using RNA polymerase T7. Membranes were prewashed in 1% wt/vol SDS and 0.1× vol/vol standard saline citrate buffer (SSC: 20× stock; 3 M NaCl and 0.3 M sodium acetate, pH 7.0) for 30 min at 77°C. The prewash was discarded, and 15 ml of prehybridization solution containing 5× SSC vol/vol, 0.5% wt/vol "blooto" (instant skim milk powder; Carnation), 1% wt/vol SDS, 10% wt/vol dextran sulfate, 25 µg/ml poly A, poly C, and 100 µg/ml salmon sperm DNA was added (Sigma Chemical). The poly A,poly C and salmon sperm DNA were boiled and kept on ice for 5 min before addition to the hybridization solution. Blots were prehybridized for at least 3 h at 77°C. Prehybridization solution was discarded after 3 h, and 10 ml of fresh solution were added. Radioactive RNA probe was added to achieve 10⁶ counts·min⁻¹·(cpm)·ml⁻¹ of hybridization solution. Blots were hybridized for 18 h at 77°C before being washed one time at 77°C with 5× SSC vol/vol, 0.5% wt/vol blooto, and 1% wt/vol SDS for 1 h followed by two washes (30 min each) at 77°C in 2× SSC vol/vol and 0.1% wt/vol SDS and two times in 0.5× SSC vol/vol.

Hybridized membranes were sealed in plastic bags and exposed to a blanked PhosphorImage screen (Molecular Dynamics) for 2 wk at room temperature. The exposed phosphor screen was then scanned using a STORM PhosphorImager (Molecular Dynamics), and the bands were visualized as pixels using ImageQuaNT software (Molecular Dynamics).

Northern Blot Analysis of egr-1 mRNA Levels in the Kidney

A 2.1-kb fragment (OC68 insert) of egr-1, including three zinc-finger domains, was used as a DNA probe (38). The egr-1 cDNA was labeled with the radioactive nucleotide [α-³²P]dCTP (Easytide; NEN Life Sciences and Amersham) using a random priming labeling kit (Oligolabeling Kit, Pharmacia, Amirad, Australia).

Northern blot membranes with left kidney RNA from control and ANG II-infused kidneys in the 30- and 240-min group were prehybridized at 42°C for at least 3 h with gentle agitation. The hybridization solution contained 50% vol/vol formamide (Sigma Chemical), 7% wt/vol SDS, 0.1 mg/ml salmon sperm DNA (Sigma Chemical), 0.75 M NaCl, 0.05 M sodium dihydrogen orthophosphate (NaH₂PO₄·H₂O), and 5 mM EDTA, pH 7.4. After at least 3 h, the radioactive probe was added to fresh hybridization solution (2× 10⁶ cpм/ml hybridization solution), and the membranes were incubated at 42°C for 18 h, after which they were washed three times (20 min each) in 2× SSC vol/vol, 1% wt/vol SDS at room temperature.
Northern Blot Analysis of 18S rRNA Levels

Blots that had been previously hybridized with egr-1 and c-fos underwent a second hybridization with a [α-32P]cDNA probe directed against 18S rRNA, using the same protocol as for the egr-1 cDNA probe. Hybridized membranes were exposed to a blanked PhosphorImage screen overnight. The expression of the ribosomal gene 18S rRNA was used to correct for gel loading differences.

Data Analysis

Each lane in the Northern blot contained RNA from an individual animal. With the use of the ImageQuaNT software package, total integrated density (the integrated density of all the pixels in a designated area) was determined for each transcript, and a corresponding background area of the lane was subtracted from the density of the transcript. The total integrated density of the transcript was then divided by the total integrated, background-adjusted density of the 18S rRNA transcript. The mRNA levels are expressed as a ratio of 18S rRNA to standardize for minor loading differences in total RNA between lanes and as such are presented as arbitrary units.

All data were analyzed using the SYSTAT 5.0 statistical software package (SPSS). Resting hemodynamic variables before infusion of vehicle or ANG II were compared using an unpaired t-test. The hemodynamic effects of acute infusion of ANG II compared with its vehicle were tested using repeated-measures ANOVA (19).

The expression of c-fos mRNA and egr-1 mRNA was adjusted for background and expressed as a ratio of 18S rRNA expression. Statistical comparisons of the relative levels of c-fos mRNA and egr-1 mRNA in kidneys from animals treated with ANG II or vehicle were analyzed using the rank sign test for nonparametric data (36). For all data, P < 0.05 was considered statistically significant.

RESULTS

Acute Infusion of ANG II

Resting values before commencement of the 30- or 240-min infusion of vehicle (10 IU/ml heparinized saline) or ANG II (2.5 ng·kg⁻¹·min⁻¹) are given in Table 1. There was no significant difference in resting mean arterial pressure, heart rate, renal blood flow, renal vascular resistance, or conductance between animals before commencement of the 30- or 240-min infusion of vehicle (10 IU/ml heparinized saline) or ANG II (2.5 ng·kg⁻¹·min⁻¹). Figure 2 shows the changes in mean arterial pressure, renal blood flow, and renal vascular conductance (from preinfusion control values) in animals receiving either vehicle or ANG II infusion for 30 or 240 min. ANG II infusion for both 30 and 240 min significantly decreased renal vascular conductance relative to vehicle controls (P = 0.004 and 0.016, respectively; Fig. 2C). Renal blood flow was decreased by ~8% after 30 min of ANG II infusion and by ~25% after 240 min of ANG II infusion compared with preinfusion control measurements (P = 0.28 and 0.026, respectively, compared with vehicle; Fig. 2B). Mean arterial pressure did not change significantly during intrarenal infusion of ANG II for 30 or 240 min compared with the vehicle group (Fig. 2A).

Northern Blot Analyses of Immediate Early Gene Expression in the Kidney

Hybridization of Northern blots with the c-fos riboprobe elicited a transcript ~2.2 kb in length, which corresponded to the published size of the full-length c-fos gene (34). The size of the transcript was determined using a methylene blue-stained RNA ladder that was run with the hybridized samples. Similarly, hybridization with the egr-1 cDNA probe elicited a 2.1-kb transcript that corresponded to the published size of the egr-1 gene (33).

Rank sign analysis of the Northern blot data demonstrates that c-fos mRNA levels were not significantly different in kidney tissue from animals that received an intrarenal infusion of ANG II for 30 or 240 min compared with the respective vehicle group (Fig. 3; 30-min ANG II infusion 52 ± 13 vs. vehicle 34 ± 8 arbitrary units and 240-min ANG II infusion 51 ± 4 vs. vehicle 51 ± 10 arbitrary units). c-fos mRNA levels were variable within the same treatment group and overlapped between treatment groups. In a positive control experiment, c-fos mRNA expression was de-

Table 1. Renal and systemic cardiovascular variables in anesthetized rats during the preinfusion control period, before commencement of intrarenal infusion of ANG II or vehicle

<table>
<thead>
<tr>
<th></th>
<th>30-min Infusion</th>
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<th>240-min Infusion</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>ANG II</td>
<td>t-Test</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>89 ± 3</td>
<td>82 ± 2</td>
<td>NS</td>
<td>87 ± 6</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>407 ± 13</td>
<td>387 ± 19</td>
<td>NS</td>
<td>381 ± 14</td>
</tr>
<tr>
<td>Renal blood flow, ml/min</td>
<td>4.97 ± 0.25</td>
<td>5.17 ± 0.5</td>
<td>NS</td>
<td>5.28 ± 0.65</td>
</tr>
<tr>
<td>Renal vascular resistance, ml/mmHg</td>
<td>18.2 ± 1.4</td>
<td>16.8 ± 1.9</td>
<td>NS</td>
<td>18.0 ± 2.9</td>
</tr>
<tr>
<td>Renal vascular conductance, ml·min⁻¹·mmHg⁻¹</td>
<td>0.057 ± 0.004</td>
<td>0.063 ± 0.008</td>
<td>NS</td>
<td>0.062 ± 0.009</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>300 ± 20</td>
<td>322 ± 17</td>
<td>NS</td>
<td>332 ± 6</td>
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</tbody>
</table>

Values are means ± SE; n = 6 rats in each group. Measurements in anesthetized rats before commencement of infusion of either vehicle (heparinized saline 10 IU/ml) or ANG II (2.5 ng·kg⁻¹·min⁻¹) in the renal artery. Measurements were recorded for 15 min and analyzed at 3-min intervals. An unpaired t-test was used to test for significant differences in control measurements between treatment groups. NS, not significant.
Repeated-measures ANOVA was used to test for significant differences between the averaged preinfusion period and levels at the indicated time after commencement of infusion of ANG II or vehicle. The results of this experiment performed in vivo contrast evidence gathered from cultured cells of many types. Upregulation of *egr-1* mRNA and protein after incubation with ANG II has been demonstrated in a number of cell culture systems, including vascular smooth muscle cells (8, 30, 33). Likewise, expression of *c-fos* mRNA has been shown to be upregulated by ANG II in cultured rat cardiomyocytes and smooth muscle cells (16, 22, 25). Activation of the AT$_1$ receptor by ANG II initiates multiple signal transduction pathways, including G protein-coupled phospholipases and some tyrosine kinase pathways that are also coupled to growth factor receptors (13). Ultimately, this complex series of second messenger pathways can lead to the expression of early genes such as *c-fos* and *egr-1* (4). Once transcribed to protein, c-Fos and EGR-1 can act as transcription factors or DNA-binding proteins that affect mean arterial pressure, indicating that ANG II did not spill over to affect systemic hemodynamics. Despite the physiologically significant renal vasoconstriction, the ANG II infusion did not produce a significant increase in expression of immediate early genes *c-fos* or *egr-1* in the kidney compared with vehicle controls. The results of this experiment performed in vivo contrast evidence gathered from cultured cells of many types. Upregulation of *egr-1* mRNA and protein after incubation with ANG II has been demonstrated in a number of cell culture systems, including vascular smooth muscle cells (8, 30, 33). Likewise, expression of *c-fos* mRNA has been shown to be upregulated by ANG II in cultured rat cardiomyocytes and smooth muscle cells (16, 22, 25). Activation of the AT$_1$ receptor by ANG II initiates multiple signal transduction pathways, including G protein-coupled phospholipases and some tyrosine kinase pathways that are also coupled to growth factor receptors (13). Ultimately, this complex series of second messenger pathways can lead to the expression of early genes such as *c-fos* and *egr-1* (4). Once transcribed to protein, c-Fos and EGR-1 can act as transcription factors or DNA-binding proteins that

**DISCUSSION**

Infusion of a low, physiological dose of ANG II directly in the kidney for 30 or 240 min produced a sustained renal vasoconstriction. This dose did not
regulate the expression of other genes by binding to activator sequences on the DNA that are often located in the promoter regions of genes (4). One way in which c-Fos can modulate gene transcription is by forming a heterodimer with members of the jun family of transcription factors (15, 21). Together, the fos-jun heterodimer has a high affinity for activator protein-1 binding sites on the DNA (15, 21). The EGR-1 protein has a structural “zinc finger” component that is highly conserved and mediates binding to the DNA to regulate gene transcription (7). It is possible that induction of transcription factors like c-fos and egr-1 could regulate expression of growth factor genes such as platelet-derived growth factor and transforming growth factor-β1, which could in turn regulate ANG II-induced growth in the kidney in vivo.

Unlike the extensive information gathered in vitro, there are few previous studies investigating the effect of ANG II on renal c-fos and egr-1 mRNA expression in vivo. Rosenberg and Hostetter (29) reported that ANG II infused at 50 ng·kg⁻¹·min⁻¹ intravenously into anesthetized rats for 1 h produced a significant increase in renal expression of c-fos and egr-1 compared with the left, uninfused control kidneys in the same animals (29). However, this dose of ANG II is ~20 times higher than the dose used in our present study (29). In the pilot study, we found that renal blood flow was reduced by up to 50% at even 10 ng·kg⁻¹·min⁻¹ ANG II infusion in the renal artery. Rosenberg and Hostetter (29) reported decreases in renal plasma flow and glomerular filtration rate (26 and 22%, respectively), measured by para-aminohippuric acid (PAH), and [³²H]inulin clearance. Measurement of renal blood flow by clearance of PAH provides an average renal plasma flow reading for the entire experimental period and thus does not provide information about transient fluctuations in renal blood flow. Previous studies have also reported marked initial reductions in renal blood flow with intrarenal doses of ANG II as low as 0.5 ng·kg⁻¹·min⁻¹ in dogs (10) and 0.3 pmol/min in rats (9). Therefore, we suggest that the differences in gene expression seen between the study by Rosenberg and Hostetter and the current study presented here may be the result of a much larger stimulus produced by the higher dose of ANG II. Possibly, a transient severe renal ischemia could have been induced by the high dose of ANG II, and this may have stimulated expression of early genes in the kidney via mechanisms not directly attributable to a trophic effect of ANG II. Indeed, in the current experiment, supraphysiological doses of ANG II, infused intravenously, raised arterial pressure and resulted in large and readily detectable levels of c-fos mRNA in the kidney.

Omiya and colleagues (26) have reported a rapid, increased c-fos expression in the renal cortex and medulla after an intravenous bolus of ANG II (1–10 M) in anesthetized rats. The authors noted that there was an increase in blood pressure just after the bolus of ANG II but did not provide any data on the size or duration of the increase (26). As above, it is likely that this dose caused marked reductions in renal blood flow and this, together with the increased arterial pressure, may explain why c-fos expression was increased in study but not in ours, in which the dose of ANG II did not produce marked reductions in renal blood flow or any increase in arterial pressure.

In the present study, renal blood flow was measured continuously, and we induced a moderate and sustained renovascular constriction that was physiologically relevant (2, 3) yet did not stimulate increased expression of either c-fos or egr-1 in the kidney. Although basal levels of both egr-1 and c-fos mRNA levels were very low, duplicate Northern blot analyses performed on each experiment detected similar levels of both c-fos and egr-1 mRNA, indicating that the levels detected were reproducible and consistent. Therefore, the lack of measurable upregulation in c-fos or egr-1 gene expression in this study indicates that low-dose intrarenal ANG II infusion may not upregulate expression of these genes in the kidney in vivo.

Two time points were chosen to investigate the time course of induction of early gene expression in the kidney. In cultured rat and human vascular smooth muscle cells, cardiomyocytes, and rat mesangial cells, there is abundant evidence to show that egr-1 and c-fos gene expression is increased within 15 min when ANG II is added to the culture (14, 16, 20, 22, 25, 27, 30, 33).
The expression of both of these genes reaches a maximum at 30–60 min and then declines to basal levels by ~2–4 h (8, 14, 16, 20, 22, 23, 25, 27, 28, 30, 33, 35, 39). Although we cannot rule out the possibility that a peak response in early gene expression may have occurred beyond the 30-min time point, yet before the 240-min time point, we chose to look for expression at 30 min based on the findings of ANG II-induced early gene expression in cultured cells. We then wished to see if any early change was maintained with an ongoing stimulus (indicating perhaps the strength of the growth-promoting stimulus of ANG II). It might be that, in vivo, ANG II-induced early gene expression may be delayed in the whole organ compared with cell culture systems; however, this seems unlikely given the findings of a previous study in which ANG II induced c-fos expression in the renal cortex and medulla within 10 min of a bolus dose given systemically to anesthetized rats (26).

Changes in expression of both c-fos and egr-1 genes in vivo have been described previously in a number of organs and disease states (17, 44). In the present experiment, it may be that the infusion of ANG II has affected gene expression in some cell types but not others, and thus the increase in expression was diluted when total kidney gene expression was investigated. For example, in rats with induced experimental glomerulonephritis, there is no significant change in egr-1 mRNA expression between control animals and animals 6 days after disease induction when RNA was isolated from total kidneys (31). However, if RNA is extracted from isolated glomeruli, there is a maximal 14.9-fold increase in egr-1 mRNA expression in animals 6 days after the disease was induced, indicating that egr-1 expression is isolated to particular tissues within the kidney (31). In culture, too, differences in early gene expression and cell growth in response to ANG II have been noted in cultured rat mesangial cells vs. cultured glomerular endothelial cells (30, 43). Thus in vivo detection of ANG II-induced changes in gene expression may be enhanced by dissecting out the kidney tissue where these changes are thought to be important, such as the renal vasculature or the renal glomeruli. Alternatively, in situ hybridization could be used to localize expression of these genes to structures within the kidney and then compare differences in expression between animals. Finally, it should be noted that, in vivo, there are many factors that regulate the expression of genes and cell growth that are absent in culture conditions and thus cannot be compared with the situation in vivo.

In summary, the present experiment indicates that intrarenal infusion of ANG II in rats, at a dose that caused physiologically significant renal vasoconstriction, did not upregulate the renal expression of egr-1 and c-fos genes, early indicators of cell growth. However, we have previously published evidence that demonstrates that long-term, low-dose intrarenal and systemic infusion of ANG II causes renal structural changes that are consistent with vascular growth and hypertrophy (10, 37). Thus the molecular and cellular mechanisms activated within the kidney in vivo, in states of elevated ANG II, and the relationship these mechanisms have to the initiation or maintenance of hypertension still remain to be determined.

**Perspectives**

There is extensive evidence in cultured cells that ANG II has potent trophic actions, but extrapolation of such information to whole organs and animals should be made with caution. Analysis of the role that ANG II plays in the regulation of renal growth in vivo is complicated by an array of factors that influence cellular homeostasis and function. We have tried to use physiologically relevant doses of ANG II in vivo to assess whether ANG II sets in motion gene expression that may lead to trophic effects. The significance of such an action for ANG II on renal vessels is yet to be determined in terms of the development and maintenance of hypertension, although extensive evidence indicates elevated intrarenal ANG II levels in a number of forms of hypertension (1, 24).

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ANG II and Renal Expression of Early Genes in Vivo


