Angiotensin II inhibits interleukin-1β–induced nitric oxide production in cultured rat mesangial cells

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Background. Macrophage-type nitric oxide synthase (NOS-II) is expressed in glomerular mesangial cells in response to inflammatory cytokines. Nitric oxide (NO) has antithrombotic and cytostatic activities in glomerular diseases. Recent studies have suggested that several vasoactive substances and growth factors modulate NO production in a tissue-specific manner. The aim of this study was to examine whether angiotensin II and transforming growth factor-β (TGF-β) modulate cytokine-stimulated NO production and NOS-II gene expression in rat glomerular mesangial cells.

Methods. Cultured rat mesangial cells were incubated with interleukin-1β (IL-1β) for 24 hours. The effects of angiotensin II and TGF-β on stimulated nitrite accumulation and NOS-II mRNA levels were determined.

Results. Angiotensin II and TGF-β significantly decreased IL-1β–stimulated nitrite accumulation. The angiotensin type 1 receptor antagonist CV11974 prevented angiotensin II-mediated inhibition of NO production. TGF-β-neutralizing antibody reversed the effect of TGF-β without affecting angiotensin II-mediated inhibition of NO production. TGF-β markedly decreased steady-state levels of NOS-II mRNA and the half-life of the message, whereas angiotensin II did not alter these parameters.

Conclusions. These results suggest that in mesangial cells, angiotensin II and TGF-β participate in the inhibitory regulation of cytokine-induced NO production. TGF-β inhibits NO production by decreasing NOS-II mRNA levels, whereas angiotensin II may regulate NO production at the levels after NOS-II gene expression. An autocrine action of TGF-β induced by angiotensin II is unlikely to contribute to angiotensin II-mediated inhibition of NO production.

The biosynthesis of nitric oxide (NO) is catalyzed by three isoforms of nitric oxide synthase (NOS). The neuronal-type NOS (NOS-I) and endothelial-type NOS (NOS-III) are constitutively expressed, and their activities are mainly regulated by the Ca²⁺/calmodulin system [1]. In the kidney, NO derived from NOS-I and NOS-III is involved in the regulation of glomerular hemodynamics, water–electrolyte balance, and renin production [2–7]. The third isoform of NOS, the macrophage-type NOS (NOS-II), is normally undetectable in renal tissue. NOS-II is induced by the stimulation of inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α, and interferon-γ (INF-γ) in mesangial cells. Once induced, NOS-II is active for a prolonged period and produces large amounts of NO. In experimental glomerulonephritis, NO has been demonstrated to prevent glomerular thrombosis and ischemia [8–10]. Furthermore, NO attenuates mesangial cell proliferation and extracellular matrix protein synthesis [11]. Recent studies have demonstrated that angiotensin II inhibits NOS-II–derived NO production in vascular smooth muscle cells and astroglial cells [12, 13]. Currently, however, little is known about how this peptide modulates NO formation in mesangial cells. Angiotensin II plays an important role in the progression of glomerular diseases. It stimulates extracellular matrix protein synthesis and mesangial proliferation, thereby opposing the effects of NO in the glomerulus. The clinical relevance of the renal angiotensin system has been implicated by the beneficial effects of angiotensin-converting enzyme inhibitors on various types of glomerular diseases, such as glomerulonephritis, nephrosclerosis, and diabetic nephropathy [14–16]. Angiotensin II exhibits these actions at least partly through an induction of transforming growth factor-β (TGF-β) in mesangial cells [17–19]. It is therefore speculated that an inhibition of NO production might be one of the mechanisms by which angiotensin II accelerates glomerular diseases. The aim of this study was to investigate how angiotensin II modulates IL-1β–induced NO production in mesangial cells and, if so, whether the effect of angiotensin II is mediated through an autocrine action of TGF-β on mesangial cells.

Key words: NOS-II, TGF-β, inflammation, cytokines, gene expression, glomerular thrombosis, progression of renal disease.

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**METHODS**

**Materials**

RPMI 1640 medium, fetal bovine serum, trypsin, and ITS-Supplement® were purchased from Gibco BRL (Gaithersburg, MD, USA). Recombinant human IL-1β, IL-6, and INF-γ derived from E. coli were from Fujisawa-Pharmingen (Tokyo, Japan). Recombinant human TGF-β1 derived from CHO cells and chicken anti-TGF-β IgY antibody were purchased from R&D Systems (Minneapolis, MN, USA). Other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA).

The ED₅₀, as determined by the dose-dependent stimulation of thymidine uptake by mouse C3H/HeJ thymocytes, and the specific activity of IL-1β were 0.1 ng/ml and 10⁻⁷ U/mg, respectively. The ED₅₀ and the specific activity of IL-6 were 0.1 ng/ml and 10⁻⁷ U/mg, respectively. The ED₅₀, as determined by the dose-dependent induction of resistance to vesicular stomatitis virus by WISH cells, and the specific activity of INF-γ were 0.15 ng/ml and 6.7 × 10⁻⁷ U/mg, respectively. The ED₅₀ of TGF-β, as determined by the inhibition of the murine IL-4-dependent thymidine incorporation by HT-2 cells, was 0.02 to 0.06 ng/ml. Anti-TGF-β antibody purified from egg yolk was specific for neutralizing the biologic activity of recombinant human TGF-β1. This antibody showed less than 10% cross-reactivity with TGF-β5, less than 2% cross-reactivity with TGF-β2 and TGF-β3, and no cross-reactivity with other cytokines. The ND₅₀ of anti-TGF-β antibody, as defined by the concentration required to yield one half of the maximal inhibition of the activity of a supramaximal concentration (0.25 ng/ml) of recombinant human TGF-β1 on HT-2 cells, as described earlier here, was 0.2 to 0.6 μg/ml.

**Mesangial cell culture**

Kidneys were removed from male Sprague-Dawley rats weighing 150 to 200 g, and the cortex was dissected. The glomeruli were isolated from minced cortex by passing through 250, 149, and 53 μm mesh sieves. Mesangial cells were cultured from isolated glomeruli after the treatment of 0.25% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA). Cells were grown in RPMI 1640 medium containing 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and ITS-Supplement® at 37°C in 5% CO₂ and 95% air. The outgrowing cells were identified as mesangial cells by spindle-shaped morphology, positive immunohistochemical staining for rat Thy-1.1 antigen, smooth muscle cell actin, desmin, and vimentin and were negative for factor VIII. The cells were studied between passages 3 and 5. Confluent mesangial cells were incubated in phenol red-free test medium supplemented with 0.1% bovine serum albumin and ITS-Supplement® for 48 hours before the experiments. They were then incubated in the test medium containing IL-1β with or without the test compounds for 24 hours.

**Nitrite analysis**

The levels of nitrite, a stable metabolite of NO, in the medium were used as an index of NO production [20–23]. The medium was mixed with an equal volume of Griess reagent containing 1% sulfanilamide, 0.1% naphthylethylendiamine dihydrochloride, and 2% phosphoric acid for 10 minutes. The absorbance at 540 nm was measured triplicate with a 96-well plate reader (Immunoreader; Intermed, Tokyo, Japan), and the nitrite concentration was determined using a calibration curve with sodium nitrite standards.

**Isolation of RNA and Northern blot analysis**

The levels of NOS-II mRNA were determined by Northern blot analysis, as described previously [4, 5, 7]. Mesangial cells were washed twice with ice-cold 10 mM phosphate-buffered saline (pH 7.4), and total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method [24]. Twenty micrograms of RNA were loaded on 1% agarose gel, size fractionated, and transferred to nylon membrane filters (Gene Screen Plus; DuPont-NEN, Boston, MA, USA). The membranes were hybridized with ³²P-random-labeled rat NOS-II cDNA probe (10² cpm/ml) [25] for 24 hours at 60°C. The membranes were then washed twice with 2 × standard saline citrate (SSC) containing 1% sodium dodecyl sulfate (SDS) for 30 minutes at 60°C and twice with 0.1 × SSC for 15 minutes at room temperature. Radioactive signals of the hybridized membranes were analyzed using a bioimaging analyzer FUJIX BAS-2000 (Fuji Film, Tokyo, Japan). The NOS-II probe was stripped with 0.15 M NaOH and then rehybridized with a ³²P-labeled 18S ribosomal RNA probe.

**Statistical analysis**

Data are expressed as means ± se, and statistical significance was determined by unpaired t-test, linear regression analysis, or analysis of covariance. P values less than 0.01 were considered statistically significant.

**RESULTS**

**Nitric oxide formation and NOS-II gene expression stimulated by interleukin-1β**

As shown in Figure 1, the incubation of cultured mesangial cells with IL-1β for 24 hours resulted in dose-dependent increases in NO production and NOS-II gene expression. The highest dose (100 U/ml) of IL-1β produced approximately sixfold increases in the levels of nitrite and NOS-II mRNA. In our experiments, other cytokines did not influence NO formation or NOS-II gene expression (Table 1).
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Fig. 1. Dose-dependent increase in nitrite accumulation (A) and nitric oxide synthase (NOS)-II mRNA expression (B) stimulated by interleukin (IL)-1β in cultured rat mesangial cells. Values are expressed as means ± sem from four experiments. *P < 0.01 vs. nonstimulated control.

Table 1. Effect of cytokines on nitrite concentrations in the medium in cultured rat mesangial cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Nitrite levels μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>IL-1β 100 U/ml</td>
<td>4</td>
<td>33.4 ± 0.85a</td>
</tr>
<tr>
<td>IL-6 100 U/ml</td>
<td>4</td>
<td>5.8 ± 0.1b</td>
</tr>
<tr>
<td>INF-γ 100 U/ml</td>
<td>4</td>
<td>5.6 ± 0.1b</td>
</tr>
<tr>
<td>IL-1β 100 U/ml + IL-6 100 U/ml</td>
<td>4</td>
<td>34.2 ± 1.2a</td>
</tr>
<tr>
<td>IL-1β 100 U/ml + INF-γ 100 U/ml</td>
<td>4</td>
<td>36.1 ± 2.5a</td>
</tr>
</tbody>
</table>

Values are means ± sem. Abbreviations are: IL, interleukin; INF, interferon. *P < 0.01 vs. vehicle-treated control
bP < 0.01 vs. IL-1β alone

Effect of angiotensin II on interleukin-1β–stimulated nitric oxide formation

When mesangial cells were coincubated with angiotensin II, IL-1β–stimulated nitrite levels were significantly decreased (Fig. 2A). The highest concentration (10⁻⁶ M) of angiotensin II reduced the mean nitrite level by approximately 40%. This angiotensin II-mediated inhibition of NO production was prevented by 10⁻⁵ M of the angiotensin type 1 (AT1) receptor antagonist CV11974 (Fig. 2A), whereas the same concentration of PD123319, an angiotensin type 2 (AT2) receptor antagonist, did not alter the effect of angiotensin II (data not shown). CV11974 alone did not affect NO formation.

To examine whether angiotensin II-mediated inhibition of NO production was associated with changes in NOS-II gene expression, Northern blot analysis was performed. As shown in Figure 2B, angiotensin II or CV11974 did not significantly influence NOS-II mRNA levels.

Effect of transforming growth factor-β and anti-transforming growth factor-β antibody on nitric oxide formation

Transforming growth factor-β inhibited IL-1β–stimulated NO production and NOS-II gene expression in a dose-dependent manner (Fig. 3). When 5 ng/ml of TGF-β was applied, the mean nitrite concentration and the NOS-II mRNA level were decreased by 80% and 60%, respectively. This inhibitory effect of TGF-β was abolished by TGF-β–neutralizing antibody. The treatment of this antibody alone did not affect NO production or NOS-II gene expression.

It has been suggested that angiotensin II induces TGF-β secretion from mesangial cells. To examine whether the inhibitory effect of angiotensin II on NO production was mediated by an autocrine action of TGF-β, IL-1β–stimulated mesangial cells were coincubated with angiotensin II and TGF-β–neutralizing antibody. As shown in Figure 4, this antibody did not reverse the effect of angiotensin II.

Effect of angiotensin II and transforming growth factor-β on the stability of NOS-II gene

After the stimulation of IL-1β (100 U/ml) for 24 hours, mesangial cells were treated with vehicle, angiotensin II,
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Fig. 2. Effect of angiotensin II on interleukin (IL)-1β-stimulated nitric oxide (NO) formation (A) and nitric oxide synthase (NOS)-II mRNA levels (B). Glomerular mesangial cells were stimulated with 100 U/ml IL-1β in the presence of test medium or its vehicle. Values are expressed as means ± sem from six to eight experiments. *P < 0.01 vs. vehicle-treated control.

or TGF-β under the presence of 5 μg/ml of actinomycin D. NOS-II mRNA levels divided by 18S rRNA levels were determined at one, two, four, and eight hours (Fig. 5). The half-life of NOS-II mRNA was approximately 2.3 hours in the vehicle-treated group. Angiotensin II did not alter the half-life of NOS-II mRNA, whereas TGF-β significantly reduced it to approximately 1.5 hours. An analysis of covariance indicated the probability that the slopes from vehicle- and TGF-β-treated groups were the same at less than 0.01.

DISCUSSION

In glomerulonephritis, inflammatory cytokines are secreted locally in glomeruli and affect mesangial cell functions [26, 27]. As demonstrated in this study, mesangial cells express NOS-II gene and produce large amounts of NO in response to IL-1β. It has been demonstrated that NOS-II-derived NO is regulated by several of vasoconstrictors and cell growth factors. Angiotensin II and TGF-β are known to inhibit cytokine-induced NO production in vascular smooth muscle cells, macrophages, and astroglial cells [12, 13, 20–22]. In contrast, angiotensin II was shown to augment NO synthesis in cardiac myocytes [23]. This study demonstrated that in mesangial cells, angiotensin II and TGF-β significantly decreased IL-1β-stimulated NO formation. This angiotensin II-mediated inhibition of NO production was reversed by CV11974, thereby indicating that the AT1 receptor is responsible for the effect of angiotensin II. The local levels of angiotensin II in the glomerular space may be sufficient to affect NO release in vivo [28]. The inhibitory effect of TGF-β on NO production is consistent with the results of an earlier study [29]. These results suggest that angiotensin II and TGF-β negatively regulate cytokine-stimulated NO production in mesangial cells.

Because angiotensin II induces secretion of TGF-β from mesangial cells [18, 19], it could be argued that the inhibitory effect of angiotensin II on NO production was mediated through TGF-β secretion. As demonstrated in this study, TGF-β is a potent inhibitor of NOS-II expression. TGF-β–neutralizing antibody, however, did not alter the effect of angiotensin II. This antibody completely reversed TGF-β–mediated inhibition of NO production. Therefore, an autocrine action of TGF-β on mesangial cells is unlikely to contribute to the effect of angiotensin II.

Angiotensin II and TGF-β have been implicated in
Fig. 3. Effect of transforming growth factor-β (TGF-β) and TGF-β-neutralizing antibody (α-TGF-β, anti-TGF-β antibody) experiments on interleukin (IL)-1β-stimulated nitric oxide (NO) formation (A) and NOS-II mRNA levels (B). Mesangial cells were stimulated with 100 U/ml IL-1β in the presence of the test medium or its vehicle. Values are expressed as means ± sem from four experiments. *P < 0.01 vs. vehicle-treated control.

Fig. 4. Effect of anti-TGF-β antibody (α-TGF-β) on angiotensin II-mediated inhibition of interleukin (IL)-1β-stimulated nitric oxide (NO) formation. Mesangial cells were stimulated with 100 U/ml IL-1β in the presence of test medium or its vehicle. Values are expressed as means ± sem from four experiments. *P < 0.01 vs. vehicle-treated control.

the progression of glomerular diseases by stimulating mesangial cell growth and extracellular matrix protein synthesis [19, 30]. In addition, angiotensin II is a major cause of glomerular hypertension, thereby promoting glomerulosclerosis [14–16]. In contrast, NO has been shown to prevent glomerular thrombosis and ischemia during the course of glomerulonephritis [8–10]. In cultured mesangial cells, NO inhibits mesangial proliferation and extracellular matrix protein synthesis [11]. The results of this study suggest that decreased NO production might be one of the mechanisms by which angiotensin II and TGF-β accelerate glomerular injury. Selective modulation of this mechanism will find potential applications in therapy in glomerular diseases.

In our study, angiotensin II failed to affect NOS-II mRNA levels in mesangial cells, whereas TGF-β markedly reduced them. Earlier studies have demonstrated that angiotensin II and TGF-β regulate NOS-II gene expression in a tissue-specific manner. In vascular smooth muscle cells and astroglial cells, angiotensin II decreases NO production by inhibiting NOS-II gene expression [12, 13]. On the other hand, in the renal tubular cells, angiotensin II suppresses INF-γ-stimulated NO formation without affecting gene transcription or steady-state levels of NOS-II mRNA [31]. TGF-β was shown to decrease NO production in macrophages by three distinct mechanisms, including decreased stability and translation of NOS-II mRNA and increased degradation of NOS-II protein [20]. Although TGF-β decreases stimulated NO production in vascular smooth muscle cells, it remains controversial how TGF-β regulates NOS-II gene expression [21, 22]. In our study, in glomerular mesangial cells, NOS-II mRNA levels and the half-life of the mes-
Fig. 5. Effect of vehicle (●), angiotensin II (■) and transforming growth factor-β (TGF-β; △) on the stability of nitric oxide synthase (NOS)-II mRNA. After the stimulation of 100 U/ml interleukin (IL)-1β for 24 hours, mesangial cells were treated with vehicle, 10−6 M angiotensin II, or 5 µg/ml TGF-β in the presence of 5 µg/ml actinomycin D. The radioactivity of NOS-II mRNA was divided by that of 18S rRNA. The normalized radioactivity was plotted as a percentage of 0-hour control against time. The data are expressed as means of three experiments.

sage were constant during the angiotensin II treatment. The results suggest that angiotensin II regulates NO production at the levels after gene expression in these cells. On the other hand, TGF-β markedly decreased the half-life and steady-state levels of NOS-II mRNA. Therefore, TGF-β may inhibit NO production by decreasing the stability of NOS-II mRNA in mesangial cells. Beck, Mohaupt and Sterzel postulated this mechanism in an earlier study [29].

Recently, Marrero et al demonstrated that angiotensin II directly activates the JAK/STAT pathway via AT1 receptors [32]. JAK/STAT is known as a protein tyrosine kinase system activated by the receptors for cytokines of IL-6 family. IL-6 is secreted from mesangial cells, for example, by the stimulation of IL-1 and angiotensin II, and plays an important role in the progression of glomerulonephritis [33–35]. In our study, however, IL-6 did not modify IL-1β-stimulated nitrite accumulation or NOS-II gene expression.

In conclusion, this study demonstrated that angiotensin II and TGF-β inhibit IL-1β-stimulated NO production in cultured rat mesangial cells. An autocrine action of TGF-β on the mesangial cell is unlikely to contribute to angiotensin II-mediated inhibition of NO release. Although clinical relevance of this mechanism is currently unclear, it is conceivable that inadequate NO production in mesangial cells is involved in the process of angiotensin II- and TGF-β-induced glomerular injury during the course of glomerular inflammatory diseases.

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