\(\alpha_2\)-Adrenoceptors Enhance Angiotensin II–Induced Renal Vasoconstriction

Role for NADPH Oxidase and RhoA

Edwin K. Jackson, Delbert G. Gillespie, Chongxue Zhu, Jin Ren, Lefteris C. Zacharia, Zaichuan Mi

Abstract—\(\alpha_2\)-Adrenoceptors potentiate renal vascular responses to angiotensin II via coincident signaling at phospholipase C. This leads to increased activation of the phospholipase C/protein kinase C/c-src pathway. Studies suggest that c-src activates the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase/superoxide system, and reactive oxygen species stimulate the RhoA/Rho kinase pathway. Therefore, we hypothesized that NADPH oxidase/superoxide and RhoA/Rho kinase are downstream components of the signal transduction pathway that mediate the interaction between \(\alpha_2\)-adrenoceptors and angiotensin II on renal vascular resistance. In rat kidneys, both in vivo and in vitro, intrarenal infusions of angiotensin II increased renal vascular resistance, and UK14,304 (\(\alpha_2\)-adrenoceptor agonist) enhanced this response. Intrarenal Tempol (superoxide dismutase mimic) or Y27632 (Rho kinase inhibitor) abolished the interaction between UK14,304 and angiotensin II both in vivo and in vitro. The interaction was also blocked by inhibitors of NADPH oxidase (in vivo using chronic gp91ds-tat administration and in vitro with diphenyleneiodonium). In cultured preglomerular vascular smooth muscle cells, UK14,304 enhanced angiotensin II–induced intracellular superoxide (2-hydroxyethidium production) and potentiated activation of RhoA (Western blot of activated RhoA bound to the binding domain of rhotekin). The interaction between angiotensin II and UK14,304 on superoxide generation and RhoA activation was blocked by inhibitors of phospholipase C (U73312), protein kinase C (GF109203X), c-src (PP1), NADPH oxidase (diphenyleneiodonium), or superoxide (Tempol). We conclude that NADPH oxidase/superoxide and RhoA/Rho kinase are involved in the interaction between \(\alpha_2\)-adrenoceptors and angiotensin II on renal vascular resistance by mediating signaling events downstream of the phospholipase C/protein kinase C/c-src pathway. (Hypertension. 2008;51:1-9.)

Key Words: phospholipase C • protein kinase C • c-src • NADPH oxidase • superoxide • RhoA • Rho kinase

Renal \(\alpha_2\)-adrenoceptors enhance angiotensin (Ang) II–induced increases in renal vascular resistance (RVR), particularly in animals with genetic hypertension.\(^2\) Previous studies suggest that the signal transduction pathway mediating \(\alpha_2\)-adrenoceptor–induced enhancement of Ang II–induced renal vasoconstriction involves coincident signaling (convergence of signaling) at the level of phospholipase C (PLC), followed by activation of protein kinase C (PKC). The evidence for this conclusion is that U73122 (PLC inhibitor) and GF109203X (PKC inhibitor) abrogate \(\alpha_2\)-adrenoceptor–induced enhancement of Ang II–induced renal vasoconstriction.\(^2,3\) Moreover, activation of G\(_q\) by \(\alpha_2\)-adrenoceptors releases \(\beta\gamma\) subunits,\(^4\) and stimulation of Ang II type 1 receptors releases \(\alpha_4\) from G\(_q\).\(^5\) As reviewed by Selbie and Hill,\(^6\) \(\beta\gamma\) subunits released by Gi-coupled receptors can synergize with \(\alpha_i\) subunits released by Gq-coupled receptors to a cause a more robust activation of PLC (most likely PLC-\(\beta\) isoforms) and its downstream transducer molecules, including PKC. Although in reconstituted systems\(^7\) or over-expressing systems\(^8\) \(\beta\gamma\) directly stimulates PLC-\(\beta\), in tissues and cells under physiological conditions, Gi-coupled receptors generally exert minimal "direct" PLC activation, per se, but rather significantly enhance the effects of Gq-coupled receptors on PLC activation via a mechanism that is inhibited by pertussis toxin and \(\beta\gamma\) subunit scavengers.\(^8\) The mechanism of this interaction may in part be because of \(\beta\gamma\) subunits inhibiting the ability of PLC-\(\beta\) to activate the GTPase activity of \(\alpha_q\).\(^9\)

The ability of \(\alpha_2\)-adrenoceptor activation to enhance Ang II–induced renal vasoconstriction also involves c-src as evidenced by the fact that a c-src inhibitor (CGP77675) blocks the interaction.\(^3\) Moreover, in cultured preglomerular vascular smooth muscle cells, activation of \(\alpha_2\)-adrenoceptors potentiates the ability of Ang II to increase the phosphorylation of c-src on tyrosine 416 (associated with c-src activation\(^10\)) via a mechanism that is blocked by an inhibitor of either PLC or PKC.\(^3\) Thus, it seems that PKC induces activation of c-src and causes c-src phosphorylation on tyrosine 416; however,
of PKC on tyrosine 416 phosphorylation must be indirect, because PKC is a serine/threonine kinase rather than a tyrosine kinase. Importantly, Moyers et al. report that PKC activation of c-src may be mediated by direct phosphorylation by PKC of serine-12 and serine-48 on c-src, thus possibly permitting autophosphorylation of c-src on tyrosine 416; however, Brandt et al. suggest that PKC may phosphorylate and activate a phosphatase which then dephosphorylates the inhibitory 527 phosphotyrosine on c-src, thus allowing auto-phosphorylation of c-src at tyrosine 416. Regardless of the mechanistic details, it is likely that PKC activates c-src via a phosphorylation mechanism that indirectly induces autophosphorylation of c-src on tyrosine 416 and, thus, activates the kinase.

The signaling components that participate downstream of c-src in the interaction between α2-adrenoceptors and Ang II on RVR are less clear. Touyz et al. suggest that c-src activates NADPH oxidase and increases superoxide production, and studies by Jin et al. demonstrate that reactive oxygen species activate RhoA/Rho kinase. These findings suggest that NADPH oxidase/superoxide and RhoA/Rho kinase are downstream participants in the coincident signaling mechanism by which renal α2-adrenoceptors enhance Ang II-induced increases in RVR. As discussed by Touyz et al., the mechanism by which c-src activates NADPH oxidase could involve the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and Bourguignon et al. report that RhoA activates PI3K. Therefore, the hypothesis that NADPH oxidase and RhoA are downstream participants in the coincident signaling mechanism is strengthened by the observation that inhibition of PI3K also blocks α2-adrenoceptor–induced enhancement of Ang II–induced renal vasconstriction.3

The purpose of the present study was to examine the role of NADPH oxidase/superoxide and RhoA/Rho kinase in the interaction between α2-adrenoceptors and Ang II on RVR changes in kidneys from spontaneously hypertensive rats (SHRs). We used SHRs because previous studies demonstrate that the interaction between α2-adrenoceptors and Ang II on vasconstriction is especially large in the SHR kidney, therefore maximizing the size of the phenomenon under study.

Methods

Animals

Male SHRs (16 to 20 weeks of age; 338 ± 3 g) were obtained from Taconic Farms (Germantown, NY). Mean arterial blood pressure (MABP) after anesthesia but before major surgical interventions was 210 ± 1 mm Hg.

Renovascular Responses In Vivo

Rats (n = 82) were anesthetized (Inactin; 90 mg/kg IP, Sigma), and the trachea, jugular vein, and carotid artery (MABP) were cannulated. Body temperature was maintained at 37°C. The effects of endogenous catecholamines on α2-adrenoceptors were reduced by bilateral adrenalectomy and denervating the left kidney, and adrenal steroids were replaced by infusing aldosterone (20 mg/min, Sigma) and hydrocortisone (20 μg/min, Sigma) intravenously.

A flow probe was placed on the left renal artery to monitor renal blood flow (RBF), and a 32-gauge needle was inserted into the renal artery and attached to a 4-way connector. Three lines linked this connector to 3 infusion pumps. Line 1 infused of dimethyl sulfoxide (Sigma) at 5 μL/min, and lines 2 and 3 infused of 0.9% saline at 25 μL/min. A converting enzyme inhibitor (captopril, 30 mg/kg, Sigma) was given to minimize the influence of endogenous Ang II, and 0.9% saline (20 mL/kg) was administered.

After a 1-hour stabilization period, animals were assigned to 4 groups according to the infusion via line 1: no-inhibitor group continued to receive dimethyl sulfoxide; the Tempol-pretreated group received Tempol (1 mg/min; superoxide dismutase mimetic26; Sigma); the Y27632-pretreated group received Y27632 (10 μg/min; Rho kinase inhibitor31; Tocris); and the gp91ds-tat–pretreated group received gp91ds-tat (5 μg/min; peptide that transverses cell membranes and inhibits NADPH oxidase by preventing association of NADPH oxidase subunits18; synthesized by the University of Pittsburgh Peptide Synthesis Core). Inhibitors were dissolved in dimethyl sulfoxide and infused at 5 μL/min. Also, the gp91ds-tat–pretreated group received an intraperitoneal infusion of gp91ds-tat (10 mg/kg per day) for 1 week before the acute experiments using minipumps. Ten minutes after starting the infusions of inhibitors, RBF and MABP were recorded just before and during the last minute of a 5-minute intrarenal infusion of Ang II (10 ng/kg per minute, Sigma) administered via line 2 (period 1). RVR was calculated by dividing RBF into the MABP. While the infusions of inhibitors continued, an intrarenal infusion of UK14,304 (3 μg/kg per minute; α2-adrenoceptor agonist30; Sigma) was initiated via line 3 in some animals. After 20 minutes, RVR responses to Ang II were redetermined in all of the animals (period 2).

Renovascular Responses in Isolated Kidneys

Left kidneys from 21 rats were isolated and perfused (single pass; 5 mM/L/min) with Tyrode’s solution as described previously.2 Kidneys were assigned to 4 groups: no-inhibitor group, Tempol-pretreated group (3 mmo/L), Y27632-pretreated group (3 μm/L, and diphenyleneiodonium-pretreated group (0.1 mmo/L; inhibitor of NADPH oxidase). After a 30-minute rest period, vasoconstrictor responses (changes in perfusion pressure) to Ang II (0.3 to 3 nmol/L) were obtained by infusing Ang II for 2 minutes, with a 10-minute rest period between each concentration of Ang II (period 1). After another 30-minute rest period, UK14,304 (1 μm/L) was infused for 10 minutes, and vasoconstrictor responses to Ang II were repeated while the UK14,304 infusion was maintained (period 2). Inhibitors were added to the perfusate throughout the protocol beginning with initial perfusion.

Superoxide Production and RhoA Activation in Preglomerular Vascular Smooth Muscle Cells

Preglomerular vascular smooth muscle cells (PGSMCs) were cultured from freshly isolated SHR microvessels as described previously.23 Our previous studies indicate that SHR PGSMCs are responsive to Ang II with regard to changes in intracellular calcium,20 phospholipase D activity,21 extracellular signal–regulated kinase 1/2 and mitogen-activated protein kinase phosphorylation,22 and RhoA activation23 and that Ang II signaling is mediated predominantly by the Ang II type 1 receptor.21,24

To examine the interaction between Ang II and UK14,304 on superoxide production, PGSMCs were grown to 80% to 90% confluent in a 75-cm2 flask, serum-starved for 48 hours, and then treated for 30 minutes with PBS, Ang II (0.1 μm/L), UK14,304 (1.0 μm/L), or Ang II plus UK14,304. Diethylthiocarbamate (100 μm/L, Sigma) was also added to the cells to inhibit superoxide dismutase25,26 and increase the superoxide signal. Also, dihydroethidium (25 μm/L, Sigma) was added to the cells along with the other treatments. Some PGSMCs also received cotreatment with U73122 (1 μm/L; PLC inhibitor27; Tocris), GF109203X (3 μm/L; PKC inhibitor28; Tocris), PP1 (0.1 μm/L; c-src inhibitor29; Tocris), diphenyleneiodonium (10 μm/L; NADPH oxidase inhibitor30; Sigma), or Tempol (3 mm/L).

After 30 minutes, cells were harvested and stored at −80°C until assayed. After thawing, cells were lysed in a 0.5-mL lysis buffer (PBS with 0.1% Triton X-100 [pH 7.4]). The solution was mixed with 0.5 mL of 1-butanol, vortexed, and centrifuged. The 1-butanol
Duo ion trap mass spectrometer (Thermo Electron Corp) in the 1 minute. The analytes were detected on a ThermoFinnigan LCQ another 2 minutes before changing to initial conditions (70:30) over 1 minute. This composition was kept constant for composition of water:methanol (%/%): 70:30 for 3 minutes, changing was water:methanol containing 0.1% formic acid at the following 4.6 cm Luna C-182 column (Phenomenex). The mobile phase phase was separated, taken to dryness by centrifugation under vacuum, and then reconstituted in 70% water/30% methanol. Dihydroethidium and 2-hydroxyethidium were resolved on a sample, the higher the ratio for 2-hydroxyethidium versus dihydroethidium. 2-Hydroxyethidium production is the “gold standard” for detecting superoxide levels in cardiovascular cells.31

Table 1. Baseline RVR in In Vivo Experiments

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Period 1</th>
<th>Period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-inhibitor group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time control (n=22)</td>
<td>No UK: 20±1</td>
<td>No UK: 19±1</td>
</tr>
<tr>
<td>UK14,304 (n=21)</td>
<td>No UK: 17±1</td>
<td>Plus UK: 22±2*</td>
</tr>
<tr>
<td>Tempol group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time control (n=6)</td>
<td>No UK: 21±3</td>
<td>No UK: 21±3</td>
</tr>
<tr>
<td>UK14,304 (n=6)</td>
<td>No UK: 23±1</td>
<td>Plus UK: 24±2</td>
</tr>
<tr>
<td>Y27632 group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time control (n=6)</td>
<td>No UK: 20±3</td>
<td>No UK: 19±2</td>
</tr>
<tr>
<td>UK14,304 (n=6)</td>
<td>No UK: 13±1</td>
<td>Plus UK: 20±1*</td>
</tr>
<tr>
<td>gp91ds-tat group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time control (n=7)</td>
<td>No UK: 21±3</td>
<td>No UK: 21±3</td>
</tr>
<tr>
<td>UK14,304 (n=6)</td>
<td>No UK: 14±1</td>
<td>Plus UK: 15±1</td>
</tr>
</tbody>
</table>

Values represent means±SEMs for indicated No. Data are in millimeters of mercury per milliliter per minute per gram of kidney weight. UK indicates UK14,304.

Results

Renovascular Responses In Vivo

Three of the 4 main groups received 1 of 3 inhibitors (Tempol, Y27632, or gp91ds-tat), and 1 main group did not receive an inhibitor (no-inhibitor group). The inhibitors were given beginning before period 1 and continuing through period 2. Each of the 4 main groups contained 2 subgroups, ie, a positive ion mode with tandem mass spectrometry (for dihydroethidium: 316 and 287 m/z; for 2-hydroxyethidium: 330 and 301 m/z). The greater the generation of intracellular superoxide in the sample, the higher the ratio for 2-hydroxyethidium versus dihydroethidium. 2-Hydroxyethidium production is the “gold standard” for detecting superoxide levels in cardiovascular cells.31

To examine the interaction between Ang II and UK14,304 on RhoA activity, PGSMCs were serum starved for 48 hours and treated for 15 minutes with PBS, Ang II (3.0 nmol/L), UK14,304 (0.3 nmol/L), or Ang II+UK14,304. Some PGSMCs also received cotreatment with U73122 (1.0 μmol/L), GF109203X (3.0 μmol/L), PP1 (0.1 μmol/L), diphenyleneiodonium (10.0 μmol/L), or Tempol (3.0 mmol/L). Activated RhoA was isolated from cells with glutathione S-transferase-rhotekin-rhotekin binding domain (RBD) and analyzed by Western blotting (EZ-Detect Rho Activation Kit, Pierce).

Superoxide Production in Isolated Kidneys

Kidneys were isolated and perfused as described above. Dihydroethidium (10 μmol/L) complexed to salmon DNA (25 mg/L) was added to the perfusate. Samples of venous perfusate were obtained 10 minutes after initiating the dihydroethidium and again after sequential infusions of Ang II (0.3 nmol/L), UK14,304 (1.0 μmol/L), and Ang II+UK14,304 (10 minutes per treatment). The perfusate was analyzed for 2-hydroxyethidium by liquid chromatography-tandem mass spectrometry as described above.

Figure 1. Effects of an intrarenal artery infusion of Ang II (10 ng/kg per minute) on RVR (millimeters of mercury per milliliter per minute per gram of kidney weight) in the absence (−) or presence (+) of an intrarenal artery infusion of UK14,304 (3 μg/kg per minute). Some animals were pretreated with an intrarenal infusion of Tempol (1 mg/min; C and D) or Y27632 (10 μg/min; E and F). Other animals were pretreated for 1 week with gp91ds-tat (10 mg/kg per day) using an osmotic minipump in the peritoneal cavity (G and H). In some animals, UK14,304 was not administered during either periods 1 or 2 (time control; A, C, E, and G). In other animals, UK14,304 was administered during period 2 but not during period 1 (UK14,304; B, D, F, and H). Numbers of animals are indicated in parentheses. P values over bars indicate comparisons between period 1 and period 2 within same group by Wilcoxon signed-rank test. A Mann–Whitney U test was used to compare period 2 values in the Time control versus UK14,304 groups. Values are means±SEMs.
subgroup that did not (time-control subgroup) and did receive UK14,304 during period 2. Table 1 lists the basal RVRs for experimental periods 1 and 2 for each of the 8 subgroups. Basal RVRs in period 1 were not significantly different from basal RVRs in period 2 in all of the 4 time-control groups. However, in the subgroups that did receive UK14,304 during period 2, basal RVR was modestly higher in period 2 than in period 1 in the no-inhibitor group and the group pretreated with Y27632 but was not greater in the groups pretreated with either Tempol or gp91ds-tat. These data indicate that basal RVR was stable from period 1 to period 2, that UK14,304 modestly increased basal RVR, and that Tempol and gp91ds-tat, but not Y27632, blocked this effect of UK14,304.

Figure 1A and 1B illustrate RVR responses to Ang II in the no-inhibitor group. In the time-control subgroup, RVR responses to Ang II decreased slightly from period 1 to period 2 (Figure 1A). In the subgroup that received UK14,304 during period 2 beginning 20 minutes before the Ang II infusion, RVR responses to Ang II were significantly increased from period 1 to period 2 (Figure 1B). This indicated that UK14,304 potentiated RVR responses to Ang II (ie, the response to Ang II in the presence of UK14,304 was greater than the response to Ang II in the absence of UK14,304).

Figure 1C and 1D illustrate RVR responses to Ang II in the Tempol-pretreated group. RVR responses to Ang II did not change from period 1 to period 2 in this group regardless of whether vehicle (Figure 1C) or UK14,304 (Figure 1D) was administered during period 2. This indicated that Tempol completely blocked UK14,304-induced potentiation of RVR responses to Ang II.

Figure 1E and 1F illustrate RVR responses to Ang II in the Y27632-pretreated group. RVR responses to Ang II did not change from period 1 to period 2 in this group regardless of whether vehicle (Figure 1E) or UK14,304 (Figure 1F) was administered during period 2. This indicated that Y27632 completely blocked UK14,304-induced potentiation of RVR responses to Ang II.

Because RVR is the ratio of MABP:RBF, absolute values of RBF are of limited value in assessing vasoconstriction in vivo. Accordingly, the analysis provided above is based on RVR changes. Nonetheless, Tables 2 and 3 list absolute RBFs and MABPs, respectively. As shown in Table 2, Ang II significantly decreased RBF in all 8 of the subgroups during both periods 1 and 2. An analysis of the percentage of change in RBF showed that, in the time-control subgroup of the no-inhibitor group, Ang II decreased RBF by 27% during period 1 (no UK14,304) and by 24% during period 2 (UK14,304 present). The effect of Ang II on the percentage reduction in RBF was significantly greater in the presence of UK14,304, regardless of whether the comparison was between period 1 versus period 2 of the UK14,304 subgroup or period 2 of the time-control subgroup versus period 2 of the UK14,304 subgroup. However, in the presence of Tempol, Y27632, or gp91ds-tat, UK14,304 did not significantly alter the percentage of reduction in RBF induced by Ang II. Table 3 demonstrates that the intrarenal infusion of Ang II had little, whether vehicle (Figure 1E) or UK14,304 (Figure 1F) was administered during period 2. This indicated that Y27632 completely blocked UK14,304-induced potentiation of RVR responses to Ang II.
if any, effect on MABP and shows that intrarenal infusions of Tempol, Y27632, and UK14,304 significantly decreased MABP, whereas the gp91ds-tat-group had higher blood pressures (perhaps because of the presence of an osmotic minipump in the peritoneal cavity).

Renovascular Responses in Isolated Kidneys
In the in vivo studies described above, Tempol, Y27632, and UK 14,304 affected MABP, the MABP was higher in the gp91ds-tat-group, and these systemic effects could have confounded the results. Accordingly, we repeated the studies in the isolated, perfused rat kidney, a model in which systemic effects are avoided because the kidney is isolated from the animal’s body.

Table 4 lists the basal perfusion pressures in the 4 groups before and after administration of UK14,304. UK14,302 had little effect on basal perfusion pressure. Pilot studies demonstrated that concentration-response curves to Ang II were stable and reproducible for the duration of the experiment.

As shown in Figure 2A, Ang II concentration-dependently (0.3 to 3.0 nmol/L) increased perfusion pressure (period 1), and this response was enhanced by UK14,304 (period 2). The other panels of Figure 2 illustrate the concentration-dependent effects of Ang II on perfusion pressure in the absence (period 1) and presence (period 2) of UK14,304 and in the presence of Tempol (Figure 2B), Y27632 (Figure 2C), or diphenyleneiodonium (Figure 2D). Although both Tempol and Y27632 significantly attenuated Ang II-induced changes in perfusion pressure, responses to Ang II were nonetheless clearly observable. As in the in vivo experiments, both Tempol and Y27632 blocked the ability of UK14,304 to potentiate Ang II-induced changes in perfusion pressure, as did diphenyleneiodonium.

Superoxide Production and RhoA Activation in Preglomerular Vascular Smooth Muscle Cells
To assess the effects of Ang II, UK14,304, and Ang II+UK14,304 on intracellular levels of superoxide and activated RhoA, we performed a series of experiments in cultured PGSMCs. Changes in intracellular superoxide levels were monitored by measuring oxidation of dihydroethidium to 2-hydroxyethidium, a process that is mediated solely by

Table 4. Baseline Renal Perfusion Pressures in In Vitro Experiments

<table>
<thead>
<tr>
<th>Period</th>
<th>Baseline Perfusion Pressure (mm Hg)</th>
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<tbody>
<tr>
<td>Period 1</td>
<td>Period 2</td>
</tr>
<tr>
<td>No inhibitor group (n=5)</td>
<td>No inhibitor group (n=6)</td>
</tr>
<tr>
<td>No UK: 61±5</td>
<td>No UK: 76±4</td>
</tr>
<tr>
<td>Plus UK: 61±6</td>
<td>Plus UK: 84±6</td>
</tr>
<tr>
<td>Tempol group (n=6)</td>
<td>Tempol group (n=6)</td>
</tr>
<tr>
<td>No UK: 76±4</td>
<td>No UK: 82±7</td>
</tr>
<tr>
<td>Plus UK: 84±6</td>
<td>Plus UK: 83±9</td>
</tr>
<tr>
<td>Y27632 group (n=6)</td>
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</tr>
<tr>
<td>No UK: 76±4</td>
<td>No UK: 82±7</td>
</tr>
<tr>
<td>Plus UK: 84±6</td>
<td>Plus UK: 83±9</td>
</tr>
<tr>
<td>Diphenyleneiodonium group (n=6)</td>
<td>Diphenyleneiodonium group (n=6)</td>
</tr>
<tr>
<td>No UK: 58±2</td>
<td>No UK: 58±2</td>
</tr>
<tr>
<td>Plus UK: 58±3</td>
<td>Plus UK: 58±3</td>
</tr>
</tbody>
</table>

Values represent means±SEMs for indicated No. Data are in millimeters of mercury. UK indicates UK14,302.
intracellular superoxide.31 Using the ratio of 2-hydroxyethidium to dihydroethidium corrects for different loading efficiencies and recoveries.

Figure 3 (top panel) shows that neither 0.1 μmol/L of Ang II nor 1 μmol/L of UK14,304 affected superoxide levels in PGSMCs. In contrast, when Ang II and UK14,304 were combined, intracellular levels of superoxide increased by 3-fold. Figure 3 (bottom panel) illustrates that the ability of Ang II/UK14,304 to increase intracellular levels of superoxide was abolished by diphenyleneiodium, GF109203X, PP1, Tempol, and U73122.

Figure 4 (top panel) demonstrates the effects of Ang II (3.0 nmol/L), UK14,304 (UK, 0.3 μmol/L), and Ang II+UK14,304 (Ang II+UK) on levels of activated RhoA in PGSMCs as assessed by binding of activated RhoA to a glutathione S-transferase (GST)-rhotekin-RBD fusion protein followed by Western blotting. Bottom, Y-axis denotes the percentage of increase in activated RhoA in response to a combination of Ang II (3.0 nmol/L)+UK14,304 (UK, 0.3 μmol/L) in PGSMCs in the absence and presence of diphenyleneiodonium (DPI; 10.0 μmol/L), GF109203X (GF; 3.0 μmol/L), PP1 (0.1 μmol/L), Tempol (3.0 mmol/L), or U73122 (1.0 μmol/L). Values are means±SEMs. The indicated P values were generated by Fisher’s least-significant difference test.

Superoxide Production in Isolated Kidneys

In an attempt to determine whether Ang II+UK14,304 increases renovascular superoxide production in the intact kidney, dihydroethidium complexed to DNA (to confine the dihydroethidium to the vascular compartment) was added to the perfusate. In a single pass through the kidney, nearly all of the dihydroethidium was oxidized to ethidium and 2-hydroxyethidium. As shown in Figure 5, the amount of 2-hydroxyethidium recovered in the perfusate was not increased by Ang II or UK14,304, per se; but was increased by the combination.

Discussion

Published results support the following pathway for the interaction between α-adrenoceptors and Ang II on RVR: coincident signaling at PLC leads to activation of PKC, which stimulates c-src.2-3 However, previous work leaves unclear the signaling components downstream of c-src. A recent study shows that c-src strongly increases NADPH oxidase activity by inducing (indirectly) the phosphorylation of p47^phox and causing its translocation to the membrane to assemble an active NADPH oxidase complex.13 NADPH

Figure 3. Top, Effects of Ang II (0.1 μmol/L), UK14,304 (UK, 1.0 μmol/L), and Ang II+UK14,304 (Ang II+UK) on intracellular levels of superoxide in PGSMCs as assessed by the 2-hydroxyethidium:dihydroethidium ratio. Bottom, Y-axis denotes the percentage of increase in superoxide production in response to a combination of Ang II (0.1 μmol/L)+UK14,304 (UK, 1.0 μmol/L) in PGSMCs in the absence and presence of diphenyleneiodonium (DPI; 10.0 μmol/L), GF109203X (GF; 3.0 μmol/L), PP1 (0.1 μmol/L), Tempol (3.0 mmol/L), or U73122 (1.0 μmol/L). Values are means±SEMs. The indicated P values were generated by Fisher’s least-significant difference test.

Figure 4. Top, Effects of Ang II (3.0 nmol/L), UK14,304 (UK, 0.3 μmol/L), and Ang II+UK14,304 (Ang II+UK) on levels of activated RhoA in PGSMCs as assessed by binding of activated RhoA to a glutathione S-transferase (GST)-rhotekin-RBD fusion protein followed by Western blotting. Bottom, Y-axis denotes the percentage of increase in activated RhoA in response to a combination of Ang II (3.0 nmol/L)+UK14,304 (UK, 0.3 μmol/L) in PGSMCs in the absence and presence of diphenyleneiodonium (DPI; 10.0 μmol/L), GF109203X (GF; 3.0 μmol/L), PP1 (0.1 μmol/L), Tempol (3.0 mmol/L), or U73122 (1.0 μmol/L). Values are means±SEMs. The indicated P values were generated by Fisher’s least-significant difference test.

Figure 5. Effects of Ang II (0.3 nmol/L), UK14,304 (UK, 1.0 μmol/L), and Ang II+UK14,304 (Ang II+UK) on superoxide production (assessed by the recovery of 2-hydroxyethidium in the venous perfusate) in perfused kidneys.

n=8

a p<0.05 vs. all other groups

n=11-12

a p<0.05 vs. all other groups

n=3-18

a p<0.05 vs. all other groups

n=9-10

p<0.05 vs. all other groups

p<0.05 vs. all other groups
oxidase increases the production of superoxide (a reactive oxygen species), and another recent study demonstrates that reactive oxygen species activate RhoA. Thus, we propose an expanded hypothesis regarding the mechanism of interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR: PLC → PKC → c-src → NADPH oxidase/superoxide → RhoA/Rho kinase.

The present study shows that Tempol (superoxide dismutase mimetic that penetrates cell membranes) and Y27632 (Rho kinase inhibitor) abrogate the interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR in vivo and in vitro. Moreover, our results demonstrate that in vivo chronic administration of the gp91ds-tat (construct that prevents assembly of an active NADPH oxidase) inhibits the interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR. Finally, additional studies in vitro illustrate that diphenyleneiodonium (inhibitor of NADPH oxidase) abrogates the interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR. These results are consistent with our expanded hypothesis.

The present study demonstrates that the Rho kinase inhibitor Y27632 suppresses basal vascular responses to Ang II in vivo and in vitro. This suggests that Rho kinase participates in vascular responses to Ang II even in the absence of \( \alpha_2 \)-adrenoceptor stimulation. Others have observed that inhibition of Rho kinase attenuates vascular responses to various vasoconstrictors, so our results in this regard are in line with previously published studies. It is also possible that the reduction in basal responses to Ang II “nonspecifically” prevented the potentiation of vascular responses to Ang II by UK14,304. However, this is unlikely, because the interaction between Ang II and UK14,304 was blocked in all of the experiments, yet in the gp91ds-tat experiments in vivo and the diphenyleneiodonium experiments in vitro, basal responses to Ang II were normal.

An important strength of the present study was that the inhibitors blocked the interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation both in vivo and in vitro. The fact that similar conclusions were obtained using 2 approaches provides assurance that our conclusions were not biased by confounding variables in the experiments, because each approach had a different set of confounding variables.

Our hypothesis predicts that the interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation should extend to the production of superoxide and that inhibitors of PLC, PKC, c-src, or NADPH oxidase should interrupt any interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation on superoxide production. Using a gold standard method for detection of intracellular superoxide, we show in the present study in PGSMSCs that indeed Ang II and stimulation of \( \alpha_2 \)-adrenoceptors synergize with respect to superoxide production and that this synergy is abolished by inhibitors of PLC, PKC, c-src, or NADPH oxidase.

Determining vascular superoxide production in the intact kidney is a challenge because of the presence of multiple cell types that could be a source of superoxide and because of the presence of high concentrations of oxygen. We decided to add dihydroethidium complexed to DNA into the perfusate with the concept that the DNA would help confine the dihydroethidium to the vascular compartment. Thus, any superoxide produced by vascular cells would have only a short distance to diffuse to the complexed dihydroethidium and convert it to 2-hydroxyethidium. However, because the perfusate was gassed with oxygen, nearly all of the added dihydroethidium was oxidized to either ethidium or 2-hydroxyethidium, with a very high level of 2-hydroxyethidium even at baseline. Despite this obstacle, we were able to detect an interaction between Ang II and UK14,304 on 2-hydroxyethidium production in the intact kidney.

Our hypothesis predicts that the interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation should extend to activation of RhoA and that inhibitors of PLC, PKC, c-src, or NADPH oxidase or administration of a superoxide dismutase mimetic should interrupt any interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation on RhoA. Indeed, in a previous study in PGSMSCs, we found that \( \alpha_2 \)-adrenoceptor stimulation potentiated Ang II-induced activation of RhoA. However, in that study, we used high concentrations of both Ang II and UK14,304, and Ang II, per se, caused a 9-fold increase in activated RhoA. In the present study, we show that even low concentrations of UK14,304 will potentiate the RhoA-activating efficacy of low concentrations of Ang II that do not, per se, activate RhoA. Moreover, we demonstrate that inhibitors of PLC, PKC, c-src, and NADPH oxidase or a superoxide dismutase mimetic prevent the interaction between Ang II and \( \alpha_2 \)-adrenoceptor stimulation on activated RhoA.

Although the data are consistent with the signal transduction mechanism of PLC → PKC → c-src → NADPH oxidase/superoxide → RhoA/Rho kinase, it is doubtless that the elucidated mechanism remains incomplete and certainly more complicated. For example, studies by Seshiah et al in rat thoracic aortic vascular smooth muscle cells suggest that PKC directly activates NADPH oxidase and that c-src functions both “upstream” and “downstream” of NADPH oxidase to amplify the effects of Ang II on superoxide production. It is entirely possible that, in SHR PGSMSCs, in addition to the proposed linear pathway, PKC may also directly stimulate NADPH oxidase, and reactive oxygen species could further activate c-src to amplify the vasoconstriction. Also, the current scheme leaves unexplained exactly how PKC induces activation of c-src, how c-src induces activation of NADPH oxidase, and how superoxide induces activation of RhoA. Moreover, our previous work demonstrates the involvement of PI3K in the interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR. It is noteworthy that RhoA activates PI3K and that PI3K participates importantly in Ang II-induced vasoconstriction. Therefore, it is conceivable that PI3K participates downstream of both superoxide and RhoA in the interaction between \( \alpha_2 \)-adrenoceptors and Ang II on RVR. However, additional studies are required to further test this hypothesis, and it is possible that PI3K plays a role at multiple steps in the pathway.

An obvious caveat regarding our studies is the reliance on pharmacological inhibitors to explore the signal transduction pathway, because these inhibitors are not necessarily selective for the intended targets. The concentrations of inhibitors
used in the in vitro studies were selected based on published literature, and the doses used in vivo were based either on published literature or calculations extrapolated from in vivo studies and adjusting for dilution by RBF. Nonetheless, it would be important to confirm our findings with other approaches.

**Perspectives**

The sympathoadrenal system and the renin-angiotensin system interact at multiple levels to regulate renal vascular tone and, hence, renal function and long-term levels of arterial blood pressure. Enhancement of any of these interactions could participate in the pathophysiology of renal disease and high blood pressure. The present experiments, when combined with other previously published data, demonstrate that the robust interaction between α2-adrenoceptors and Ang II on the renal vasculature involves in part the following signaling pathway: PLC→PKC→C-src→NADPH oxidase/superoxide anion→RhoA/Rho kinase. Alterations in this pathway may importantly influence the renal vascular effects of activation of the sympathoadrenal axis and the renin-angiotensin system and, consequently, may contribute to the pathophysiology of renal disease and hypertension.

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**Disclosures**

None.

**References**


α2-Adrenoceptors Enhance Angiotensin II–Induced Renal Vasoconstriction. Role for NADPH Oxidase and RhoA

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