Loss of Biphasic Effect on Na/K-ATPase Activity by Angiotensin II Involves Defective Angiotensin Type 1 Receptor–Nitric Oxide Signaling

Anees Ahmad Banday, Mustafa F. Lokhandwala

Abstract—Oxidative stress causes changes in angiotensin (Ang) type 1 receptor (AT1R) function, which contributes to hypertension. Ang II affects blood pressure via maintenance of sodium homeostasis by regulating renal Na+ absorption through its effects on Na/K-ATPase (NKA). At low concentrations, Ang II stimulates NKA; higher concentrations inhibit the enzyme. We examined the effect of oxidative stress on renal AT1R function involved in biphasic regulation of NKA. Male Sprague-Dawley rats received tap water (control) and 30 mmol/L of L-buthionine sulfoximine (BSO), an oxidant, with and without 1 mmol/L of Tempol (antioxidant) for 2 weeks. BSO-treated rats exhibited increased oxidative stress, AT1R upregulation, and hypertension. In proximal tubules from control rats, Ang II exerted a biphasic effect on NKA activity, causing stimulation of the enzyme at picomolar and inhibition at micromolar concentrations. However, in BSO-treated rats, Ang II caused stimulation of NKA at both of the concentrations. The effect of Ang II was abolished by the AT1R antagonist candesartan and the mitogen-activated protein kinase inhibitor U0126, whereas the Ang type 2 receptor antagonist PD-123319 and NO synthase inhibitor N'G-nitro-L-arginine methyl ester had no effect. The inhibitory effect of Ang II on NKA was sensitive to candesartan and N'G-nitro-L-arginine methyl ester, whereas PD-123319 and U0126 had no effect. In BSO-treated rats, Ang II showed exaggerated stimulation of NKA, mitogen-activated protein kinase, proline-rich-tyrosine kinase 2, and NADPH oxidase but failed to activate NO signaling. Tempol reduced oxidative stress, normalized AT1R signaling, unmasked the biphasic effect on NKA, and reduced blood pressure in BSO-treated rats. In conclusion, oxidative stress–mediated AT1R upregulation caused a loss of NKA biphasic response and hypertension. Tempol normalized AT1R signaling and blood pressure. (Hypertension. 2008;52:1099-1105.)

Key Words: L-buthionine sulfoximine ■ MAP kinase ■ NKA ■ Na/H-exchanger 3 ■ Tempol

Angiotensin (Ang) II plays an important role in the regulation of body fluid and sodium balance through the modulation of renal tubular functions.1,2 In particular, Ang II acting via the Ang type 1 receptor (AT1R) stimulates the net sodium reabsorption in the renal proximal tubules, and an exaggerated effect of Ang II on this process contributes to the development of hypertension.1,2 In this regard, the ability of Ang II to stimulate Na/K-ATPase (NKA) activity in the renal proximal tubules is an important regulatory component of sodium absorption.3 It is recognized that Ang II exerts a biphasic effect on sodium transport in the kidney.4 Harris and Young5 have shown that Ang II added into peritubular fluid stimulates sodium and water absorption from rat proximal tubules at low (picomolar to nanomolar) concentrations and inhibits at high (nanomolar to micromolar) concentrations. Subsequent studies confirmed this unique biphasic sodium regulation by Ang II in different experimental conditions.6 These studies are particularly relevant because Ang II is generally reported to work as a stimulator of proximal tubular sodium transport; however, in situ proximal tubular fluid is reported to contain markedly high concentrations of Ang II.7 Therefore, inhibition by high concentrations of Ang II could also have some physiological relevance to the regulation of proximal tubular functions in vivo. The biphasic effects of Ang II on sodium absorption are, at least in part, contributed by NKA regulation.6 The mechanism behind the biphasic effect of Ang II on the NKA is unclear; however, the stimulation by Ang II has been traditionally attributed to the activation of protein kinase C and/or the decrease in the intracellular cAMP level.6,8 It has been shown that Ang II, via AT1R, activates NO signaling in renal proximal tubules.9,10 We and others have reported that NO and sodium nitroprusside can decrease the NKA activity in proximal tubules and cultured opossum kidney cells.11,12 Because NO and Ang II are known to antagonize each other at many physiological functions, such as vascular relaxation, tubuloglomerular feedback, sodium absorption, and blood pressure (BP) regulation,9 it is possible that NO signaling may be a physiological modulator for Ang II–mediated NKA regulation.

Received June 9, 2008; first decision June 28, 2008; revision accepted October 3, 2008.
From the Heart and Kidney Institute, College of Pharmacy, University of Houston, Tex.
Correspondence to Anees Ahmad Banday, Heart and Kidney Institute, College of Pharmacy, University of Houston, 4800 Calhoun, S & R-2 Building, Houston, TX 77204. E-mail abanday@uh.edu
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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.108.117911
Compelling experimental evidence indicates that oxidative stress plays an important role in AT1R signaling. It has been shown that, in experimental models of hypertension, the increase in oxidative stress coexists with AT1R upregulation.\(^{11,14}\) Conversely, Ang II via AT1R can activate NADPH oxidase and increase the production of superoxide (\(O_2^{-}\)).\(^{15}\) On the other hand, the increased oxidative stress is associated with NO bioinactivation, because the reaction of \(O_2^{-}\) with NO causes generation of peroxynitrite.\(^{16}\) Taken together, these data indicate that, whereas oxidative stress can positively modulate Ang II signaling via AT1R, it can also disrupt the function of its physiological antagonist NO and, thus, exaggerate AT1R signal transduction. Therefore, the purpose of this study was to examine the effect of oxidative stress on biphasic regulation of NKA activity by Ang II in renal proximal tubules with particular emphasis on the role of NO signaling.

### Methods

#### Animals
Male Sprague-Dawley rats (Harlan, Indianapolis, Ind) were fed a normal rat diet and divided into following 4 groups: control (C), animals that were maintained on tap water; l-buthionine sulfoximine (BSO), animals that were provided with 30 mmol/L of BSO (Sigma); Tempol (T), animals that were provided with 1 mmol/L of Tempol (Sigma); and BSO+T, animals that were provided with BSO plus Tempol. All of the experiments were performed in compliance with Institutional Animal Care and Use Committee–approved protocol.

#### Preparation of Renal Proximal Tubular Suspension
Renal proximal tubular suspension was prepared as described previously.\(^{11}\) Experiments were performed with freshly prepared proximal tubules, and protein was determined by bicinchoninic acid method (Pierce).

#### Indices of Oxidative Stress
Production of malondialdehyde and \(O_2^{-}\) was determined as described previously.\(^{11}\) Superoxide dismutase activity, nitrotyrosine, and 8-isoprostate were determined by commercially available kits.

### NKA, Na/H-Exchanger 3, and Mitogen-Activated Protein Kinase Assay
NKA, Na/H-exchanger 3 (NHE3), and mitogen-activated protein (MAP) kinase were determined by routine laboratory procedures (please see the data supplement available at http://hyper.ahajournals.org).

### Statistical Analyses
Differences between means were evaluated using the unpaired Student \(t\) test or ANOVA with Newman-Keul’s multiple test, as appropriate. \(P<0.05\) was considered statistically significant.

### Results

#### Oxidative Stress and BP
Treatment of Sprague-Dawley rats with BSO, Tempol, and BSO plus Tempol for 2 weeks had no effect on body weight and food intake (Table). Glomerular filtration rate was also significantly increased BP, plasma, and urinary 8-isoprostane levels; renal malondialdehyde; and nitrotyrosine content (Table). These rats also had decreased levels of renal glutathione and superoxide dismutase activity (Table). Tempol supplementation reduced oxidative stress and BP in BSO-treated rats. There were no significant differences in BP or oxidative/antioxidative markers between control and rats treated with Tempol alone (Table).

#### AT1R Expression in Renal Proximal Tubules
BSO treatment caused a significant increase in AT1R number (C: 301.2 ± 29.8, BSO: 535.1 ± 49.6, T: 330.6 ± 30.2, and BSO+T: 382.3 ± 39.1 fmol/mg of protein) and protein content (Figure S1A). Supplementation of Tempol normalized AT1R expression in BSO-treated rats (Figure S1A). Western blot showed a single protein band of \(\approx 42\) kDa in proximal tubules from control rats, which was markedly reduced by an AT1R-selective blocking peptide (Figure S1B). Equal protein loading was confirmed by stripping and reprobing the membranes with GAPDH antibodies, which showed similar band intensity in both lane 1 and lane 2 (Figure S1B).

### Table. Food and Water Intake, Oxidative Markers, and BP in BSO and Tempol-Treated Rats

<table>
<thead>
<tr>
<th>General Parameters</th>
<th>C</th>
<th>BSO</th>
<th>T</th>
<th>BSO+T</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>230.0±23.0</td>
<td>249.0±18.0</td>
<td>245.0±21.0</td>
<td>253.0±27.0*</td>
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<td>Food intake, g/d</td>
<td>17.9±4.2</td>
<td>19.4±3.3</td>
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<td>8-Isoprostone urine, pg/mg of creatinine</td>
<td>0.81±0.02</td>
<td>1.33±0.02*</td>
<td>0.79±0.06†</td>
<td>0.84±0.04†</td>
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<tr>
<td>8-Isoprostone serum, fg/mg of creatinine</td>
<td>40.4±3.1</td>
<td>58.0±4.2*</td>
<td>37.9±3.2</td>
<td>42.3±5.3†</td>
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<tr>
<td>Malondialdehyde, pmol/mg of protein</td>
<td>83.9±6.3</td>
<td>124.9±9.3*</td>
<td>80.9±7.6†</td>
<td>88.9±8.3†</td>
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<tr>
<td>Nitrotyrosine, arbitrary units</td>
<td>105.6±11.3</td>
<td>172.2±15.3*</td>
<td>99.9±8.9†</td>
<td>117.3±10.6†</td>
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<tr>
<td>Glutathione, nmol/mg of protein</td>
<td>2.5±0.3</td>
<td>1.0±0.1*</td>
<td>2.7±0.3†</td>
<td>1.8±0.2**†</td>
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<tr>
<td>Superoxide dismutase, EU/mg of protein</td>
<td>2.2±0.2</td>
<td>1.3±0.1*</td>
<td>2.3±0.3†</td>
<td>2.0±0.2†</td>
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<tr>
<td>Glomerular filtration rate, mL/min</td>
<td>1.43±0.14</td>
<td>1.36±0.2</td>
<td>1.52±0.17</td>
<td>1.49±0.22</td>
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<tr>
<td>Mean blood pressure, mm Hg</td>
<td>105.6±4.3</td>
<td>128.3±6.2*</td>
<td>100.6±5.5†</td>
<td>111.3±6.6†</td>
</tr>
</tbody>
</table>

P<0.05 vs C.
†P<0.05 vs BSO.
‡P<0.05 vs T.
Figure 1. Effect of Ang II on renal Na/K-ATPase activity. Proximal tubules were challenged with the indicated doses of Ang II for 10 minutes. The data are expressed as a percentage of stimulation produced by the indicated concentration of Ang II. A single experiment is shown, representative of 6 individual experiments performed in triplicate.

Effect of Ang II on NKA and NHE3 Activity

Incubation of renal proximal tubules from control rats with Ang II produced a biphasic response of NKA, stimulation at picomolar concentration, and inhibition at micromolar concentration (Figure 1). However, the biphasic response of NKA to peptide was lost in rats treated with BSO (Figure 1). In BSO-treated rats, Ang II caused a concentration-dependent stimulation of NKA activity, which reached a plateau at $10^{-11}$ mol/L Ang II and remained unchanged through micromolar peptide concentrations (Figure 1). Ang II–mediated NKA stimulation was significantly higher in BSO-treated rats compared with control (Figures 1, S2A, and S2B). In addition, Ang II also caused significantly higher stimulation of NHE3 in BSO-treated rats compared with other experimental groups (Figure S2C). Tempol supplementation of BSO-treated rats normalized the Ang II–induced NKA and NHE3 overstimulation and restored the biphasic response of NKA (Figures 1 and S2A through S2C). The basal activities of NKA (nmol Pi/µg of protein per hour) and NHE3 (nmol $^{32}$Na/mg of protein per minute) were similar in all 4 of the groups: NKA: C, 13.6±0.8; BSO, 14.3±0.9; T, 12.9±0.7; BSO+T, 14.4±1.1; NHE3: C, 4.3±0.2; BSO, 4.6±0.3; T, 4.1±0.4; BSO+T, 4.5±0.3. The immunoreactivity of NKA-α1 subunit and NHE3 was also similar in all of the experimental groups (Figure S2D and S2E).

Role of AT1R on NKA Activity

Incubation of proximal tubules with AT1R antagonist candesartan (1 µmol/L) blocked the Ang II–mediated NKA biphasic response in control (Figure 2A), Tempol, and BSO plus Tempol–treated (data not shown) rats. Candesartan also blocked the NKA stimulation induced by high or low Ang II concentrations in BSO-treated rats (Figure 2B). Ang type 2 receptor antagonist PD-123319 (1 µmol/L) failed to show any effect on NKA regulation in response to Ang II. These concentrations of candesartan and PD-123319 had no effect on basal NKA activity (basal: 13.9±0.4; candesartan: 13.6±0.5; PD-123319: 14.2±0.6 nmol Pi/mg of protein per hour).

We tested various inhibitors to investigate the pathway involved in Ang II–mediated NKA regulation. The MAP kinase inhibitor UO126 (10 µmol/L) antagonized the stimulatory effect of Ang II in both control and BSO-treated rats.

Figure 2. Effect of Ang II on renal Na/K-ATPase activity. Proximal tubules (PT) from control and BSO-treated rats were challenged with the indicated doses of Ang II for 10 minutes in the presence or absence of following pharmacological compounds: A and B: 1 µmol/L of candesartan (Can) or 1 µmol/L of PD-123319 (PD); C and D: 1 µmol/L of UO126 (U) or 10 µmol/L of amiloride (A); E and F: 1 mmol/L of L-NAME or 50 µmol/L of ODQ. The data are expressed as a percentage of stimulation or inhibition produced by the indi-
Ang II–Mediated MAP Kinase Activation

We analyzed the phosphorylation of extracellular signal–regulated kinase (ERK)1/2 in renal proximal tubules. The Western blotting experiments showed that 10 pM (Figure 3) and 1 µmol/L (data not shown) of Ang II caused a significant increase in ERK1/2 phosphorylation, without affecting the ERK1/2 protein expression, in renal proximal tubules from both control and BSO-treated rats (Figure 3). However, the Ang II–mediated phosphorylation of ERK1/2 was markedly higher in BSO-treated rats compared with control rats (Figure 3). Tempol abolished the increased phosphorylation of ERK1/2 in BSO-treated rats, while showing no effect when provided alone (Figure 3). The Ang II (10 pM)–mediated ERK1/2 activation in control rats was blocked by candesartan and UO126, a MAP kinase inhibitor, whereas PD-123319 had no effect (Figure S4A). Similarly, Ang II (1 µmol/L) caused a significant increase in nitrate/nitrite production in control rats but had no effect in BSO-treated rats (Figure S4B). The nitrate/nitrite production was also sensitive to candesartan and l-NAME (Figure S4C). Interestingly, at similar concentrations, Ang II (1 µmol/L) caused significant cGMP accumulation in control rats but not in BSO-treated rats (Figure S4D). Preincubation of proximal tubules with candesartan, l-NAME, or ODQ inhibited the Ang II–mediated cGMP accumulation, whereas PD-123319 had no effect (Figure S4E). Tempol supplementation of BSO-treated rats restored the NO and cGMP production in response to high Ang II (1 µmol/L) concentration (Figures 4, S4B, and S4D). The basal NO (data not shown) and cGMP levels were similar in all of the experimental groups (C: 0.72 ± 0.08; BSO: 0.67 ± 0.07; T: 0.78 ± 0.09; BSO + T: 0.70 ± 0.06 pmol/mg of protein).

Ang II–Mediated NO and cGMP Accumulation

Incubation of proximal tubules with 10 pM Ang II failed to increase NO or cGMP levels in all of the experimental groups (data not shown). However, at micromolar concentration, Ang II caused a significant increase in L-[14C]citrulline accumulation in control rats but not in BSO-treated rats (Figure 4). The L-[14C]citrulline production was inhibited by l-NAME and candesartan but not by PD-123319 (Figure S4A). Similarly, Ang II (1 µmol/L) caused a significant increase in nitrate/nitrite production in control rats but had no effect in BSO-treated rats (Figure S4B). The nitrate/nitrite production was also sensitive to candesartan and l-NAME (Figure S4C). Interestingly, at similar concentrations, Ang II (1 µmol/L) caused significant cGMP accumulation in control rats but not in BSO-treated rats (Figure S4D). Preincubation of proximal tubules with candesartan, l-NAME, or ODQ inhibited the Ang II–mediated cGMP accumulation, whereas PD-123319 had no effect (Figure S4E). Tempol supplementation of BSO-treated rats restored the NO and cGMP production in response to high Ang II (1 µmol/L) concentration (Figures 4, S4B, and S4D). The basal NO (data not shown) and cGMP levels were similar in all of the experimental groups (C: 0.72 ± 0.08; BSO: 0.67 ± 0.07; T: 0.78 ± 0.09; BSO + T: 0.70 ± 0.06 pmol/mg of protein).

Ang II–Mediated O2•− Production

Incubation of tubular homogenates, from Ang II–exposed proximal tubules, with NADPH oxidase substrate reduced nicotinamide-adenine dinucleotide (0.1 mmol/L) caused an increase in O2•− production in all of the experimental groups (Figure S5A). However, the generation of O2•− in response to Ang II and in the presence of reduced nicotinamide-adenine dinucleotide was significantly higher in BSO-treated rats compared with control, Tempol, or BSO plus Tempol–treated rats (Figure S5A). In proximal tubules from control rats, the O2•− production was inhibited by candesartan and NADPH oxidase inhibitor diphenylene iodonium chloride (0.1 mmol/L), whereas PD-123319 had no effect (Figure S5A).

Incubation of homogenates, after the exposure of tubules to Ang II, with NOS substrate L-arginine (1 mmol/L) failed to produce O2•− in control, Tempol, or BSO plus Tempol–treated rats but caused significant O2•− production in BSO-treated rats (Figure 5B). The O2•− production in BSO-treated rats was sensitive to the NOS inhibitor l-NAME (1 mmol/L;...
Ang II–Induced Proline-Rich Tyrosine Kinase 2 Activation

As illustrated in Figure 6, incubation of proximal tubules with Ang II caused a significant increase in proline-rich tyrosine kinase 2 (PyK2) phosphorylation in both control and BSO-treated rats. The Ang II–mediated PyK2 phosphorylation was higher in BSO-treated rats compared with control (Figure 6). Tempol normalized the Ang II–induced PyK2 phosphorylation in BSO-treated rats, while having no effect when given alone (Figure 6). The phosphorylation of PyK2 in response to peptide was blocked by candesartan and not by PD-123319 (Figure S6). PyK2 basal protein levels were similar in all of the groups (data not shown) and remained unchanged by Ang II exposure (Figure 6).

Discussion

The results of this study show that, in Sprague-Dawley rats, oxidative stress increased BP, which was associated with renal AT1R upregulation and exaggerated signaling. In BSO-treated rats, Ang II caused significantly higher NKA stimulation and failed to cause inhibition of NKA activity, as seen in control rats. Our data shows that AT1R-mediated NKA stimulation by low Ang II concentration involves MAP kinase, whereas the inhibition at high Ang II concentration involves NO-cGMP signaling. Interestingly, whereas picomolar concentration of Ang II showed robust stimulation of MAP kinase in BSO-treated rats, it failed to activate NO-cGMP signaling and inhibit NKA activity at micromolar concentrations. In control rats, Ang II stimulated NADPH oxidase, but the activation of this enzyme was much higher in BSO-treated rats. In addition, Ang II–mediated, AT1R-dependent O$_2^-$ production was also contributed by NOS in BSO-treated rats. Furthermore, Ang II–mediated Pyk2 stimulation was also higher in BSO-treated rats compared with control animals. Supplementation of BSO-treated rats with Tempol abolished the oxidative stress, normalized AT1R expression and signaling, and reduced BP.

Oxidative stress contributes, at least in part, to the development and maintenance of hypertension, and antioxidant treatment lowers BP in hypertensive animal models. Also, AT1Rs are upregulated in central and peripheral sites involved in BP regulation in hypertension. Our results are consistent with these finding regarding the increased oxidative stress and AT1R function in hypertension. We found that BSO-treated rats exhibited high BP and showed marked oxidative stress and AT1R upregulation. Antioxidant supplementation restored redox status and normalized AT1R expression and BP. Although we did not elucidate the mechanisms for AT1R upregulation, evidence to date indicates that the AT1R gene possess the binding sites for nuclear factor κB (NF-κB). Cowling et al have shown that NF-κB activation is necessary for cytokine-induced AT1 mRNA upregulation in cardiac fibroblasts. NF-κB–dependent reporter constructs demonstrated rapid activation of NF-κB with interleukin 1β, which was paralleled by increased AT1R mRNA levels. Recently, we showed that oxidative stress caused increased nuclear translocation of NF-κB in BSO-treated rats. These data suggest that oxidative stress via NF-κB activation upregulates AT1R and contributes to the development of hypertension, thus elucidating the existence of novel cross-talk between oxidative stress and AT1R function in hypertension.
Ang II, via AT1R, stimulates net sodium absorption in renal proximal tubules and, thus, affects the BP regulation.23,24 Interestingly, Ang II exerts a biphasic regulation of NKA in proximal tubules.6,25 At picomolar concentrations, Ang II activates NKA, whereas at higher concentration an inhibitory effect is observed.6 We also found a biphasic response of Ang II on NKA activity in control rats. In BSO-treated rats the stimulatory response to NKA at picomolar Ang II was markedly higher than in control rats, but there was no inhibitory effect at higher concentrations. Tempol restored the biphasic Ang II response on NKA in BSO-treated rats. Physiologically, Ang II maintains body sodium and fluid balance and BP homeostasis by stimulating tubular sodium and fluid reabsorption. However, sustained increases in intrarenal Ang II levels because of local formation and/or uptake of circulating Ang II by proximal tubules may contribute to sodium retention and hypertension if sodium transporter stimulation is maintained at higher concentrations. Therefore, the lack of NKA biphasic response may contribute to increased sodium reabsorption and hypertension in BSO-treated rats. Ang II is coupled to a variety of signaling cascades depending on the cell type, and both stimulatory and inhibitory roles of ERK have been suggested in the regulation of proximal tubular transport of sodium by Ang II.26 Also, conflicting data have been reported as to the identity of the receptor subtype involved in mediating biphasic response of Ang II. Some studies suggest the involvement of AT1R in stimulation and Ang type 2 receptor in inhibition.27 Our study indicates that, in normo-tensive rats, the biphasic peptide response on renal NKA is mediated by AT1 and not by Ang type 2 receptor subtype. With regard to MAP kinase pathways, the role of ERK1/2 activation in Ang II–mediated NKA was assessed using UO126. This inhibitor blocked the stimulatory effect of Ang II, while having no effect on inhibitory response in control rats. However, in BSO-treated rats, which lack the inhibitory response at higher Ang II concentration, UO126 blocked the NKA stimulation at both low and high concentrations. These data suggest that, in renal proximal tubules, the Ang II–mediated NKA stimulation involves MAP kinase pathways and is independent of Ang II concentration.

The role of NO-cGMP signaling in Ang II–mediated NKA was assessed using NOS and soluble guanylyl cyclase inhibitors l-NAME and ODQ, respectively. Treatment of proximal tubules from control rats with l-NAME or ODQ abolished the inhibitory effect of Ang II while having no effect on stimulation of NKA in response to the peptide. Because NKA from BSO-treated rats lacks the biphasic response to Ang II, l-NAME or ODQ failed to show any effect on Ang II–mediated stimulation at both lower and higher peptide concentrations. The effect of Ang II on NO signaling was further investigated by examining the NO production and cGMP accumulation in response to Ang II. Interestingly, the incubation of proximal tubules from control rats with 1 μmol/L of Ang II caused a significant increase in NO and cGMP levels but failed to show response in BSO-treated rats. The Ang II–mediated NO and cGMP production in control rats was inhibited by l-NAME and candesartan, whereas PD-123319 had no effect. Tempol was able to restore the Ang II–mediated NO signaling, as well as the biphasic response of NKA in BSO-treated rats. These data confirm that the inhibitory response of NKA by Ang II is mediated by NO-cGMP signaling. The NO-cGMP signaling is activated by the AT1R subtype, whereas the Ang type 2 receptor has no effect.

Another important signal molecule, NADPH oxidase, was studied because activation of NADPH oxidase by Ang II and subsequent O2•− generation can reduce the NO bioavailability.15,16 We found that incubation of proximal tubules with Ang II stimulated NADPH oxidase in all of the experimental groups, but the effect of Ang II on O2•− generation was much higher in BSO-treated rats. Tempol supplementation of BSO-treated rats normalized the Ang II–mediated NADPH oxidase activation, suggesting that excessive Ang II–mediated O2•− production could be because of AT1R upregulation and may, therefore, be responsible for NO inactivation. Also, in BSO-treated rats, Ang II–mediated, NOS-dependent O2•− production was mediated by AT1R and was abolished by Tempol, suggesting the role of oxidative stress in NOS uncoupling. In the present study we cannot distinguish whether the NOS uncoupling was because of BSO-induced oxidative stress or AT1R upregulation and subsequent O2•− production. However, irrespective of the cause for NOS uncoupling, these data show that NO inactivation may lead to sustained and exaggerated AT1R signaling, which, in turn, may contribute to hypertension.

The intracellular signaling events that link AT1R stimulation to an increase in NADPH oxidase activity and O2•− production are unclear at present. Indirect evidence suggests that PyK2 might be upstream of oxidase activation.15,28,29 The time course of PyK2 phosphorylation by Ang II parallels closely that of NADPH oxidase activation, and PyK2 and Rac-1 form a complex in response to Ang II in smooth muscle cells.15,28,29 We found that Ang II–mediated PyK2 phosphorylation paralleled O2•− production in BSO-treated rats, because both were significantly higher than in other groups, and Tempol normalized the Ang II–mediated PyK2 phosphorylation and NADPH oxidase activation in BSO-treated rats. These data show that upregulation of AT1R could lead to overactivation of PyK2, which, in turn, caused excessive O2•− production via NADPH oxidase activation in BSO-treated rats.

In conclusion, we have shown that oxidative stress–mediated renal AT1R upregulation causes loss of biphasic effect of Ang II on NKA regulation and hypertension. The upregulated AT1R not only caused overstimulation of NKA at lower Ang II concentration but failed to show inhibition at higher concentration. The loss of inhibition was because of failure of Ang II to stimulate NO-cGMP signaling. Most importantly, Ang II via AT1R caused exaggerated PyK2 activation, which, in turn, led to robust O2•− generation that may have contributed to NO inactivation and NOS uncoupling and subsequently disrupted Ang II–AT1-NO-cGMP pathways. Antioxidant Tempol reduced oxidative stress and normalized AT1R signaling and reduced BP.
Perspectives
The biphasic effect of Ang II on NKA is quite unique and potentially relevant, because it relates to the control of proximal tubular sodium reabsorption under normal and pathophysiological conditions. In the present study we used the pharmacological inhibitor of the glutathione pathway to produce oxidative stress in Sprague-Dawley rats and studied the mechanism by which the effect of Ang II on NKA was altered. These studies provide an insight as to how oxidative stress may lead to AT1R upregulation, disturb sodium homeostasis, and contribute to the development of hypertension.

Sources of Funding
This study was supported by Scientist Development grant 0835428N from American Heart Association (to A.A.B.) and National Institutes of Health grant AG-25056 from the National Institute on Aging (M.F.L.).

Disclosures
None.

References
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Hypertension. 2008;52:1099-1105; originally published online October 27, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.117911
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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/52/6/1099

Data Supplement (unedited) at:
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Defective AT1 Receptor—Nitric Oxide signaling Causes Loss of Biphasic effect on Na/K-ATPase Activity by Angiotensin II

Supplement Material and Methods and Figures
Methods

Animals

Male Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN) were fed a normal rat diet and divided into following four groups: C—control, animals that were maintained on tap water; BSO—L-buthionine sulfoximine, animals that were provided with 30 mM BSO (Sigma, St. Louis, MO); T—tempol, animals that were provided with 1 mM tempol (Sigma); and BSO+T, animals that were provided with BSO plus tempol. BSO, a glutamate cysteine ligase inhibitor, and tempol, a superoxide dismutase (SOD) mimetic compound, were provided in drinking water for 2 weeks. The BSO dosage and duration is based on previous studies which show a significant depletion of glutathione without toxicity.\textsuperscript{1,2} All experiments were performed in compliance with Institutional Animal Care and Use Committee (IACUC) approved protocol. BP and glomerular filtration rate (GFR) were measured as detailed earlier.\textsuperscript{3}

Preparation of renal proximal tubular suspension

Renal proximal tubular suspension was prepared as described earlier.\textsuperscript{3} Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and after a midline abdominal incision, the aorta was cannulated below the kidneys and an in situ digestion was accomplished by perfusing an enzyme solution of collagenase and hyaluronidase. Enrichment of proximal tubules was carried out using 20% ficoll gradient in Krebs buffer. Experimental were performed with freshly prepared proximal tubules. Cell lysate, cytosol, and membrane fractions were isolated from proximal tubules by centrifugation.\textsuperscript{3} Protein was determined by bicinchoninic acid method (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.
Indices of oxidative stress

Malondialdehyde was determined by the method of Mihara and Uchiyama. Superoxide dismutase (SOD) activity was determined as described previously. Nitrotyrosine was determined by immunoblotting kit (Upstate, Charlottesville, VA), renal glutathione levels were assayed by colorimetric assay kit with sensitivity of 5 μmol/L and standard error of the mean value (SEM) <2% (21023; OXIS, Foster City, CA). 8-isoprostane was measured by RIA kit with detection limit of 2.7 pg/ml and intra- and inter-assay variability of 12.6% and 10.5% respectively (516351; Cayman, Ann Arbor, MI). For nicotinamide-adenine dinucleotide phosphate oxidase [NAD(P)H] activity, fluorescence spectrometric assay of O₂⁻ production was performed as detailed by Satoh et al. Briefly, proximal tubules were incubated with and without Ang II and oxidation of dihydroethidium (DHE) to ethidium (Eth) was used as a measure of O₂⁻ production. Conversion of DHE to Eth in absence of Ang II and other pharmacological compounds was considered as basal O₂⁻ production.

NKA and Na/H-exchanger 3 (NHE3) assay

NKA activity was determined by the method of Quigley and Gotterer with slight modification as reported earlier. To determine AT1R-mediated Na/K/ATPase regulation, proximal tubular suspensions (1 mg protein/ml) were incubated with or without Ang II (10⁻¹⁴-10⁻⁶ M) at 37°C for 15 min. The tubules were lysed by flash freezing in liquid nitrogen. Tubular suspension (0.1 mg protein/ml) was used immediately to assay ouabain (4 mM)-sensitive NKA activity, using end-point phosphate hydrolysis of ATP (4 mM). The inorganic phosphate released was determined colorimetrically. NHE3 activity was determined by measurement of 5-(N-methyl-N-isobutyl)-amiloride–sensitive ²²Na⁺ uptake.
**Immunoblotting and MAP kinase activity**

Proteins were solubilized in Laemmli buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. The membranes were blocked, incubated with antisera directed against AT1R (Santa Cruz Biotechnology, Santa Cruz, CA), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ERK1/2 (Calbiochem, La Jolla, CA), and nonreceptor protein-tyrosine kinase/proline-rich tyrosine kinase 2 (Pyk2) (Santa Cruz) in 0.1% Tris-buffered saline followed by incubation with horseradish peroxidase-conjugated secondary antibodies. For extracellular signal-regulated kinases 1 and 2 (ERK1/2) and Pyk2 activation, the blots were incubated for 12h with antiphosphospecific ERK1/2 and Pyk2 respectively or antibodies that recognize ERK1/2 and Pyk2. GAPDH was used as loading control for whole cell homogenates/lysates.

**125I-sar-Ang II binding**

Membrane binding of 125I-sar-Ang II (PerkinElmer) was performed as described earlier. The membranes (50 µg protein) were incubated with 30 pM of 125I-sar-Ang II at 30°C for 60 min in a shaking water bath. The assay was terminated by rapid filtration on GF/C filters under vacuum. The radioactivity on the filters was counted in gamma counter. Nonspecific binding was determined by performing the binding assay in the presence of 1 µM unlabeled Ang II.

**Determination of NO and cGMP Level**

Renal proximal tissue was incubated with Ang II and tissues were snap frozen in liquid nitrogen, followed by homogenization. Nitrate/nitrite levels were measured by colorimetric assay kit with the detection limit of 2 nmol/ml and inter- and intra-assay variation of 3.4 % and 2.7% respectively. L-[14C]citrulline was determined by nitric oxide synthase (NOS) assay kit and
cGMP content was determined by an enzyme immunoassay kit with detection limit of 1 pmol/ml and inter- and intra-assay variation of 5.7 % and 11.3% respectively. All the assay kits were obtained from Cayman chemical, Ann Arbor, Michigan.

References


Figure S1

Figure 1. Effect of oxidative stress on AT1 receptor (AT1R) expression in renal proximal tubules from control—C, L-buthionine sulfoximine—BSO, tempol—T and BSO plus tempol—BSO+T -treated rats. A: Upper blot: AT1R protein expression and lower blot: GAPDH protein in cell homogenates. B: Upper blot, lane 1: AT1R protein (~42 kDa) expression in proximal tubular homogenate from control rats, lane 2: AT1R specific antibodies pre-incubated with selective blocking peptide. Lower blot, GAPDH (~45 kDa) expression. Bars represent mean ± SE from 6-8 animals performed in triplicate. *P<0.05, versus C; #P < 0.05, versus BSO, using one-way ANOVA followed by post hoc Newman-Keuls multiple test and $P < 0.05, versus lane 1, using Student’s t-test.;
Figure 2. Effect of Ang II on Na/K-ATPase and Na/H-exchanger 3 activity in renal proximal tubules from control—C, L-buthionine sulfoximine—BSO, tempol—T and BSO plus tempol—BSO+T -treated rats. A-C : Renal proximal tubules were incubated with indicated doses of Ang II for 10 min. D: Proximal tubular Na/K-ATPase expression: upper blot—α1 subunit protein in cell homogenate/lysate (~90 kDa), middle blot—α1 subunit in cell membrane, lower blot—GAPDH in cell lysate. E: Na/H-exchanger 3 expression in cell lysate/homogenate; upper blot—Na/H-exchanger 3 protein (~87 kDa), lower blot—GAPDH protein. Bars represent means ± SE of 6-8 different experiments performed in triplicate. *P <0.05, versus basal; #P < 0.05 versus C; $P <0.05, versus BSO, using one-way ANOVA followed by post hoc Newman-Keuls multiple test.
Figure S3

Figure 3. Effect of oxidative stress on Ang II-induced MAP kinase activation in renal proximal tubules from control rats. Proximal tubules were incubated with 1 µM candesartan (Can) or 10 µM UO126 (UO) followed by challenge with 10 pM Ang II for 10 min. Bands are representative western blots and bars are means ± SE of 6-8 different experiments performed in triplicate. *P < 0.05, versus basal; $P < 0.05$, versus Ang II; †P < 0.05, versus Ang II + PD, using one-way ANOVA followed by post hoc Newman-Keuls multiple test.
Figure 4. Effect of oxidative stress on Ang II-induced nitric oxide (NO) production and cyclic guanosine monophosphate (cGMP) accumulation in renal proximal tubules from control—C, L-buthionine sulfoximine—BSO, tempol—T and BSO plus tempol—BSO+T-treated rats. Renal proximal tubules were incubated for 10 min with, A: 1 µM candesartan (Can), 1 µM PD-123319 (PD) or 1mM N (G)-nitro-L-arginine methyl ester (L-NAME) followed by challenge with 1 µM Ang II; B: 1 µM Ang II; C: 1 µM candesartan/PD-123319 or 1mM L-NAME followed by challenge with 1 µM Ang II; D: 1 µM Ang II; E: 1 µM candesartan, PD-123319, 1mM L-NAME or 50 µM ODQ followed by challenge with 1 µM Ang II. Bars are means ± SE of 6-8 different experiments performed in triplicate. *P <0.05, versus basal; #P < 0.05, versus BSO; $P < 0.05, versus Ang II; †P < 0.05, versus Ang II + PD, using one-way ANOVA followed by post hoc Newman-Keuls multiple test.
Figure 5. Effect of oxidative stress on Ang II-induced superoxide production in renal proximal tubules (PT) from control—C, L-buthionine sulfoximine—BSO, tempol—T and BSO plus tempol—BSO+T-treated rats. A: Renal proximal tubules from control rats were challenged with 1 μM Ang II for 10 min and homogenate was incubated with 0.1 mM NADH in presence and absence of 0.1 mM diphenylene iodonium chloride (DPI), 1 μM candesartan (Can) or 1 μM PD-123319 (PD). B: Renal proximal tubules from BSO-treated rats were challenged with 1 μM Ang II for 10 min and homogenate was incubated with 1.0 mM L-arginine in presence and absence of 1 mM L-NAME, 1 μM candesartan or PD-123319. C: Renal proximal tubular NAD(P)H oxidase expression in cell lysate/homogenate; upper blot—p22 subunit protein, lower blot—GAPDH protein. D: upper blot—gp91phox and lower blot—GAPDH protein in homogenates. Bars are means ± SE of 6-8 different experiments performed in triplicate. *P < 0.05, versus basal; $P < 0.05, versus Ang II; †P < 0.05, versus Ang II + PD, using one-way ANOVA followed by post hoc Newman-Keuls multiple test.
Figure 6. Effect of oxidative stress on Ang II-induced proline-rich tyrosine kinase 2 (Pyk2) activation in renal proximal tubules from control rats. Proximal tubules were pre-incubated with 1 μM candesartan (Can) or PD-123319 (PD) followed by challenge with 1 μM Ang II for 10 min *$P < 0.05$, versus basal and $^P < 0.05$, versus Ang II + Can, using one-way ANOVA followed by post hoc Newman-Keuls multiple test.