Oxidative Stress Causes Renal Angiotensin II Type 1 Receptor Upregulation, \( \text{Na}^+ / \text{H}^+ \) Exchanger 3 Overstimulation, and Hypertension

Anees A. Banday, Mustafa F. Lokhandwala

Abstract—Oxidative stress modulates angiotensin (Ang) II type 1 receptor (AT\(_1\)R) expression and function. Ang II activates renal \( \text{Na}^+ / \text{H}^+ \) exchanger 3 (NHE3) to increase sodium reabsorption, but the mechanisms are still elusive. In addition, the upregulation of AT\(_1\)R during oxidative stress could promote sodium retention and lead to an increase in blood pressure. Herein, we investigated the mechanism of Ang II–mediated, AT\(_1\)R-dependent renal NHE3 regulation and effect of oxidative stress on AT\(_1\)R signaling and development of hypertension. Male Sprague-Dawley rats received tap water (control) or 30 mmol/L of \( \text{L} \)-buthionine-sulfoximine, an oxidant, with and without 1 mmol/L of Tempol, an antioxidant, for 3 weeks. \( \text{L} \)-Buthionine-sulfoximine–treated rats exhibited oxidative stress and high blood pressure. Incubation of renal proximal tubules with Ang II caused significantly higher NHE3 activation in \( \text{L} \)-buthionine-sulfoximine–treated rats compared with control. The activation of NHE3 was sensitive to AT\(_1\)R blocker and inhibitors of phospholipase C, tyrosine kinase, janus kinase 2 (Jak2), Ca\(^{2+}\)–dependent calmodulin (CaM), and Ca\(^{2+}\) chelator. Also, incubation of proximal tubules with Ang II caused Jak2-dependent CaM phosphorylation, which led to Jak2-CaM complex formation and increased Jak2-CaM interaction with NHE3. The activation of these signaling molecules was exaggerated in \( \text{L} \)-buthionine-sulfoximine–treated rats, whereas Tempol normalized the AT\(_1\)R signaling. In conclusion, Ang II activates renal proximal tubular NHE3 through novel pathways that involve phospholipase C and an increase in intracellular Ca\(^{2+}\), Jak2, and CaM. In addition, oxidative stress exaggerates Ang II signaling, which leads to overstimulation of renal NHE3 and contributes to an increase in blood pressure. (Hypertension. 2011;57:452-459.)

Key Words: angiotensin II ■ Ca\(^{2+}\)-dependent calmodulin ■ hypertension ■ janus kinase ■ phospholipase C

Reactive oxygen species play an important role as signaling molecules in a variety of cellular responses, and sustained perturbations in redox homeostasis can result in oxidative stress leading to cardiovascular damage and cellular injury. It is reported that oxidative stress can modulate angiotensin (Ang) II type 1 receptor (AT\(_1\)R) expression and function.\(^1\)–\(^3\) Ang II, a potent vasoconstrictor and sodium retaining hormone, via AT\(_1\)Rs, is crucial for the regulation of sodium transport in kidney and blood pressure.\(^4\)–\(^6\) Therefore, the upregulation of AT\(_1\)Rs during conditions like oxidative stress could promote sodium retention and lead to development of hypertension. Consequently, normalization of AT\(_1\)R function by antioxidant supplementation could be a useful therapeutic approach to lower blood pressure.

The AT\(_1\)R is responsible for the \( \text{Na}^+ \) retaining effects of Ang II in the kidney.\(^4\)–\(^6\) The renal proximal tubules express AT\(_1\)R on both the apical and basolateral membranes, and Ang II is delivered via the general circulation and filtration or could be locally synthesized.\(^4\)–\(^6\) Ang II increases proximal tubular sodium transport through elevation in the activity of an amiloride-sensitive sodium hydrogen exchanger (NHE), and Ang-converting enzyme inhibitors or AT\(_1\)R blockers have been shown to decrease proximal tubular sodium and water reabsorption.\(^7\)–\(^11\) The NHE3 isoform is the main transporter mediating sodium reabsorption in proximal tubules, and a wide range of evidence shows that NHE3 is regulated by Ang II.\(^8\)\(^,\)\(^10\)\(^,\)\(^12\) Ang II stimulates multiple signaling pathways, including mitogen-activated protein kinases, Src family kinases, phospholipase D, and Janus kinases, which could modulate NHE3 activity.\(^1\)\(^,\)\(^13\)–\(^15\) Ang II has been shown to stimulate NHE3 activity in proximal tubules but does not appear to increase the steady-state levels of NHE3 mRNA.\(^1\)\(^6\) Although there is evidence for involvement of protein kinase C (PKC)–dependent and PKC-independent pathways, as well as tyrosine kinase–dependent pathways, the precise mechanism of NHE3 activation by Ang II remains unclear.\(^1\)\(^7\)\(^,\)\(^1\)\(^8\) A role for p38 and extracellular signal–regulated kinase (ERK)1/2 in Ang II–mediated regulation of NHE3 in vascular...
smooth muscle cells has also been suggested. It is reported that activation of various isoforms of NHE by mitogens including Ang II could involve Ca<sup>2+</sup>-dependent calmodulin (CaM) binding to NHE. Interestingly, Ang II could phosphorylate CaM and subsequently increase its binding to NHE via activation of several protein kinases, including Janus kinase 2 (Jak2). Therefore, the present study was designed to identify the role of Jak2 and CaM in Ang II–induced renal proximal tubular NHE3 activation.

The major purpose of this study is to delineate key components of a signal transduction pathway that leads to the overstimulation of proximal tubular NHE3 by Ang II. Oxidative stress is induced by treating male Sprague-Dawley rats with L-buthionine-sulfoximine (BSO) for 3 weeks, and animals were studied for involvement of the Jak2-CaM pathway in Ang II–induced renal proximal tubule NHE3 stimulation.

**Methods**

Adult male Sprague-Dawley rats were divided into the following 4 groups: (1) control, where animals were provided with tap water; (2) BSO, where animals were provided with 30 mmol/L of BSO (Sigma); Tempol, where animals were provided with 1 mmol/L of Tempol (Sigma); and BSO plus Tempol, where animals were provided with BSO plus Tempol in tap water for 3 weeks. At the end of the experiment, rats were anesthetized with Inactin (100 mg/kg IP), and blood pressure and heart rate were recorded as detailed in the supplemental Methods section (available in the online Data Supplement). Pretreatment with the AT<sub>1</sub>R antagonist candesartan blocked Ang II–mediated PLC stimulation in control and BSO-treated rats (please see Figure S1B). However, the response to Ang II was significantly higher in BSO-treated rats compared with control rats (please see Figure S1B). Tempol alone had no effect on PLC activity, but it normalized Ang II response in BSO-treated rats (please see Figure S1B). Pretreatment of tubules with the AT<sub>1</sub>R antagonist candesartan blocked Ang II–mediated PLC stimulation in control and BSO-treated rats (please see Figure S1C), as well as in Tempol and BSO plus Tempol–treated rats (data not shown). The basal PLC activity (counts per minute per milligram of protein) was similar in all of the experimental groups (control: 203.3 ± 17.4, BSO: 223.9 ± 22.1; Tempol: 209.1 ± 16.7, BSO plus Tempol: 214.5 ± 19.8).

**Statistical Analysis**

Differences between means were evaluated using ANOVA with Newman-Keuls multiple test, as appropriate. P<0.05 was considered statistically significant.

**Results**

Animals treated with BSO for 3 weeks exhibited high blood pressure and oxidative stress (please see Table S1 in the online Data Supplement). The mean blood pressure in BSO-treated rats was 27 mm Hg higher than control rats (please see Table S1). Tempol had no effect on blood pressure, but it attenuated the rise in blood pressure in BSO-treated rats (please see Table S1). The levels of oxidative markers, such as proximal tubular malondialdehyde and urinary 8-isoprostanate, were significantly higher in BSO-treated rats compared with control rats (please see Table S1). Similar to blood pressure, Tempol had no effect on oxidative markers but rescued BSO-treated rats from oxidative stress (please see Table S1). Both BSO and Tempol had no effect on food and water intake (data not shown), body weight, or glomerular filtration rate (please see Table S1).
Ang II+D609
AngII+AG490

-9
-8
-10
-13

II signaling (Figure 2B). Similar effects of genistein were
Daidzein, an inactive genistein analog, had no effect on Ang
II–induced NHE3 stimulation in control, BSO-treated (Figure
PKC inhibitor GF109203X also had no effect on Ang
II-mediated NHE3 stimulation, we first used PLC inhibitors, D609 and ET-18-
three kinase inhibitor okadaic acid and the tyrosine kinase
ways, we tested the role of ERK1/2 and Jak2 in the stimula-
tion of NHE3 by Ang II. Incubation of tubules from control
and BSO-treated rats with Jak2 inhibitor AG490 blocked Ang
II–mediated Na⁺ uptake, whereas the ERK1/2 inhibitor
UO126 had no effect (Figure 2C). Similar effects were
observed in Tempol and BSO plus Tempol-treated rats (data
not shown). It is worth noting that Ang II activated tubular
EKR1/2 in all of the groups, and stimulation was higher in
BSO-treated rats (data not shown). Incubation of tubules with
these pharmacological compounds did not change the basal
NHE3 activity in control (²²Na⁺ uptake, in nanomoles of
²²Na⁺ per milligram of protein per minute: basal, 4.0±0.3;
okadaic acid, 3.8±0.2; genistein, 4.2±0.4; daidzein, 4.0±
0.3; AG490, 4.5±0.05; UO126, 3.9±0.4; GF109203X,
4.3±0.3), BSO, Tempol, and BSO plus Tempol-treated rats
(data not shown).

Role of CaM in Ang II–Induced Apical
NHE3 Stimulation
Incubation of tubules with Ang II significantly increased the
Ca²⁺ accumulation in tubules from control and BSO-treated
rats (Figure 3A). However, the accumulation was more robust
in tubules from BSO-treated rats (Figure 3A). Tempol-treated
rats showed a response similar to controls, but Tempol
mitigated the effect of BSO in BSO plus Tempol-treated rats
(Figure 3A). Pretreatment of tubules with BAPTA-AM, a
cell-permeable Ca²⁺ chelator, prevented Ang II–mediated
stimulation of NHE3 in control and BSO-treated rats (Figure
3B), as well as in Tempol and BSO plus Tempol-treated rats
(data not shown). Interestingly, CaM inhibitors chlorproma-
zine, fluphenazine, W-7, ophiobolin, and calmidazolium also
prevented Ang II–mediated stimulation of NHE3 in control
and BSO-treated rats (Figure 3C). Similar effects were
observed in Tempol and BSO plus Tempol-treated rats (data
not shown), suggesting the involvement of Ca-CaM signal-
ing. Next, to test the direct involvement of Ca²⁺, tubules were
treated with calcium ionophore A23187. Elevation of Ca²⁺
with A23187 increased NHE3 activity to a similar extent in

Ang II+Genistein

observed in Tempol and Tempol plus BSO-treated rats (data
not shown). To further follow on the tyrosine kinase path-
ways, we tested the role of ERK1/2 and Jak2 in the stimula-
tion of NHE3 by Ang II. Incubation of tubules from control
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²²Na⁺ per milligram of protein per minute: basal, 4.0±0.3;
okadaic acid, 3.8±0.2; genistein, 4.2±0.4; daidzein, 4.0±
0.3; AG490, 4.5±0.05; UO126, 3.9±0.4; GF109203X,
4.3±0.3), BSO, Tempol, and BSO plus Tempol-treated rats
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ing. Next, to test the direct involvement of Ca²⁺, tubules were
treated with calcium ionophore A23187. Elevation of Ca²⁺
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Ang II+D609
Ang II+ET-18-OCH3

Figure 1. Ang II–induced apical NHE3 stimulation in proximal
tubules from control (C), BSO, and Tempol (T)-treated rats. A
single experiment expressed as a percentage of NHE3 stimula-
tion is shown.

Signaling Molecules Involved in Ang II–Mediated
Apical NHE3 Stimulation
To identify the signaling pathway for Ang II–mediated NHE3
stimulation, we first used PLC inhibitors, D609 and ET-18-
OCH3. As shown in Figure 2A, PLC inhibitors blocked the
hormonal response in control and BSO-treated rats. Similar
effects were seen in Tempol and Tempol plus BSO–treated
rats (data not shown). Because PLC can activate various
kinase pathways, we used the broad-spectrum serine/threo-
ine kinase inhibitor okadaic acid and the tyrosine kinase
inhibitor genistein. Preincubation of tubules with okadaic
acid had no effect on Ang II–mediated NHE3 stimulation in
control or BSO-treated rats (Figure 2B). Okadaic acid also
failed to inhibit Ang II–induced Na⁺ uptake in Tempol or
BSO plus Tempol–treated rats (data not shown). In addition,
PKC inhibitor GF109203X also had no effect on Ang
II–induced NHE3 stimulation in control, BSO-treated (Figure
2B), or Tempol and BSO plus Tempol-treated rats (data
not shown). However, incubation of tubules with genistein, a
tyrosine kinase inhibitor, abolished Ang II–mediated NHE3
stimulation in control and BSO-treated rats (Figure 2B),
suggesting the involvement of tyrosine kinase pathways.
Daidzein, an inactive genistein analog, had no effect on Ang
II signaling (Figure 2B). Similar effects of genistein were

Figure 2. Ang II–mediated apical NHE3 stimulation in proximal tubules from control (C) and BSO-treated rats. Ang II (10 μM)–induced
brush border membrane vesicle NHE3 stimulation in the presence of (A) PLC inhibitors D609 (100 μM/L) and ET-18-OCH3 (50 μM/L);
serine/threonine kinase inhibitor okadaic acid (1 μM/L), PKC inhibitor GF109203X (1 μM/L), tyrosine kinase inhibitor genistein
(10 μM/L), inactive genistein analog daidzein (50 μM/L); and (C) janus kinase 2 inhibitor AG490 (10 μM/L) and ERK1/2 inhibitor
UO126 (10 μM/L). Data represent mean±SE from 6 to 8 animals performed in triplicate. *P<0.05 vs basal and #P<0.05 vs respective
control, using 1-way ANOVA followed by Newman-Keuls post hoc test.
proximal tubules from all experimental groups (please see Figure S3).

Role of Jak2 and CaM Interaction

Because inhibitors for both Jak2 and CaM blocked Ang II–induced NHE3 stimulation, we wanted to test whether Jak2 interacts with CaM. Proximal tubules were incubated with Ang II, and homogenates were used to coimmunoprecipitate the JAK2-CaM complex with CaM antibodies, followed by immunoblotting for Jak2. Ang II significantly increased the Jak2-CaM interaction in control and BSO-treated rats, but interaction was much higher in BSO-treated rats (Figure 4A). Tempol alone had no effect, but it normalized the Ang II–induced Jak2-CaM interaction when given to BSO-treated rats (data not shown). The Jak2-CaM interaction was abolished by BAPTA-AM, suggesting the role of Ca2\^{2+} accumulation (Figure 4A). Next we determined whether AT \textsuperscript{1}R activation could increase tyrosine phosphorylation of Jak2 and CaM proteins. Interestingly, exposure of tubules to Ang II increased Jak2 and CaM tyrosine phosphorylation in control and BSO-treated rats (Figure 4B and 4C). The phosphorylation was higher in BSO-treated compared with control-treated rats (Figure 4B and 4C). Tempol and BSO plus Tempol-treated rats showed a response similar to the control group (data not shown). To identify the exact pathways for Jak2-CaM phosphorylation and interaction, we used inhibitors for these proteins. Preincubation of tubules with Jak2 inhibitor abolished Ang II–induced CaM phosphorylation, whereas CaM inhibitors failed to block Ang II–mediated Jak2 phosphorylation (Figure 4B and 4C). We failed to detect Jak2 and CaM phosphorylation or coimmunoprecipitation in tubular homogenates without Ang II exposure (data not shown), suggesting that phosphorylation is required for complex formation. Interestingly, BAPTA-AM blocked phosphorylation of both Jak2 and CAM in response to Ang II (Figure 4D). To further test the role of Ca2\^{2+} in the Jak2-CaM interaction, proximal tubules were incubated with A23187, a Ca2\^{2+} ionophore. A23187 significantly increased the Jak2 and CaM phosphorylation and their interaction in tubules from control and BSO-treated rats (please see Figure S4A through S4C). Basal Jak2 and CaM protein expressions were similar in control and BSO-treated rats (please see Figure S4D). A similar effect of A23187 was seen in Tempol and Tempol plus BSO-treated rats (data not shown).

Jak2, CaM, and NHE3 Interaction

To test whether phosphorylated CaM interacts with NHE3, proximal tubules were incubated with Ang II, and brush border membrane was immunoprecipitated with NHE3 antibodies followed by immunoblotting for Jak2 and CaM proteins. As shown in Figure 5A and 5B, NHE3 coimmunoprecipitated with both Jak2 and CaM proteins in both control and BSO-treated rats, but the complex formation was significantly higher in BSO-treated rats. The response to Ang II in Tempol and BSO plus Tempol-treated was similar to control rats (data not shown). Preincubation of tubules with candesartan abolished this interaction (Figure 5A and 5B). We failed to detect the NHE3-Jak 2-CaM interaction in brush...
border membranes from any experimental group without previous exposure of tubules to Ang II (data not shown).

**Discussion**

Proximal tubular NHE3, which is responsible for 60% of transcellular sodium reabsorption in the proximal tubule, is regulated by both natriuretic and antinatriuretic hormones to maintain the sodium homeostasis and blood pressure. Ang II, a potent antinatriuretic hormone, activates NHE3 to increase sodium reabsorption and maintain a positive sodium balance, but the mechanisms are still elusive. We now demonstrate that, in Sprague-Dawley rats, Ang II activates proximal tubular NHE3 through novel pathways that involve PLC and an increase in intracellular Ca$^{2+}$, Jak2, and CaM. In addition, during oxidative stress, which upregulates renal AT1Rs, Ang II signaling is exaggerated, which leads to overstimulation of NHE3 and contributes to an increase in blood pressure.

The current study expands our previous work, which shows that oxidative stress upregulates AT1Rs, causing overstimulation of sodium transporters, and subsequently contributes to

**Figure 4.** Ang II–mediated Jak2 and CaM interaction in proximal tubules from control (C) and BSO-treated rats. Proximal tubules were incubated with 10 pM Ang II and immunoprecipitation (IP) was performed with CaM or JAK antibodies (Abs), followed by immunoblotting for Jak2 or CaM. A, IP with CaM Abs and immunoblotting (IB) for Jak2. B, IP with Jak2 Abs and IB for phosphorylated Jak2 (P-Jak2). Respective blots were stripped and reprobed with Abs for total Jak2 and CaM to ensure equal loading of protein samples (data not shown). C, IP with CaM Abs and IB for phosphorylated CaM (P-CaM). D, IP with Jak2 and CaM Abs and IB for P-JAK2 and P-CaM, respectively. Top bands are representative Western blots. Data represent mean ± SE from 6 to 8 animals performed in triplicate. *P < 0.05 vs basal and #P < 0.05 vs respective control, using 1-way ANOVA followed by Newman-Keuls post hoc test.

**Figure 5.** Ang II–mediated Jak2, CaM, and NHE3 interaction in proximal tubules from control (C) and BSO-treated rats. Proximal tubules were incubated with 10 pM Ang II, and immunoprecipitation (IP) was performed with NHE3 antibodies (Abs), followed by immunoblotting for Jak2 or CaM in the absence and presence of AT1R antagonist candesartan (1 μmol/L). A, IP with NHE3 Abs and IB for Jak2 and (B) IP with NHE3 Abs and IB for CaM. Top bands are representative Western blots. Data represent mean ± SE from 6 to 8 animals performed in triplicate. *P < 0.05 vs basal and #P < 0.05 vs respective control, using 1-way ANOVA followed by Newman-Keuls post hoc test.
The novel aspect of this study is that it elucidates the mechanism for Ang II–mediated NHE3 stimulation. We demonstrate that, in BSO-treated rats, which exhibit oxidative stress and high blood pressure, the Ang II–induced NHE3 stimulation is significantly higher than normotensive rats. The overstimulation of NHE3 is AT1R specific, because it is blocked by candesartan, and also the basal activity and expression of NHE3 are similar in hypertensive and normotensive rats. In addition, Tempol, which mitigates oxidative stress and high blood pressure, also normalizes AT1R expression and Ang II–induced NHE3 activation. However, Tempol significantly reduced both oxidative stress and blood pressure, but it failed to completely normalize the BSO effects, probably because of its inability to normalize glutathione levels, in BSO-treated rats. Nevertheless, these data confirm previous findings that oxidative stress could be an independent risk factor to the development of hypertension by exaggerating AT1R expression, response, and stimulation of renal sodium transporters.1,30,32–34

Ang II via AT1Rs activates serine/threonine and tyrosine kinase pathways, both of which can regulate NHE3 activity.1,15,20,28 The serine/threonine kinases are activated via PLC stimulation, and our data show that D609 and ET-18-OCH3 (PLC inhibitors) abolished Ang II–induced NHE3 stimulation, indicating the involvement of PLC pathways. However, the stimulation of NHE3 in response to Ang II was insensitive to the nonspecific serine/threonine kinase inhibitor or PKC-specific inhibitor, thus excluding the involvement of these kinases. The role of serine/threonine kinase in Ang II–mediated renal NHE is controversial. Although some studies suggest the inhibition of protein kinase A, others have reported that PKC stimulation is involved in NHE stimulation.17,35 The discrepancies could be because of failure of these studies to discriminate between various NHE isoforms. The present study, which focuses primarily on proximal tubular NHE3, shows that serine/threonine kinases are not involved in Ang II–mediated regulation of this isoform. Because PLC could increase intracellular Ca2+ and activate CaM, various inhibitors were used to identify their role in NHE3 stimulation. Pretreatment of cells with Ca2+-chelator BAPTA-AM abolished NHE3 stimulation in response to Ang II. Similar effects were observed when proximal tubules were pretreated with various CaM inhibitors, suggesting the involvement of the PLC-Ca2+-CaM pathway in Ang II–mediated NHE3 stimulation. This is further supported by data showing that Ca2+ accumulation in response to Ang II was higher in BSO-treated rats, which also showed greater NHE3 stimulation.

AT1R can activate tyrosine kinases, which can regulate renal sodium transporters.1,15 We found that tyrosine kinase inhibitor genistein, but not its inactive analog daidzein, blocked the Ang II–induced NHE3 stimulation. To identify more specific molecules involved in Ang II signaling, we used ERK1/2 inhibitor UO126 and Jak2 inhibitor AG490, both of which have been shown to modulate NHE signaling. The selective Jak2 inhibitor effectively blocked NHE3 stimulation, whereas UO126 had no effect, suggesting the involvement of Jak2, although both Jak2 and EKR1/2 were activated by Ang II. Further studies showed that incubation of proximal tubules with Ang II increased Jak2 tyrosine phosphorylation. Interestingly, Ang II also increased CaM phosphorylation, which led to complex formation between Jak2 and CaM. Tyrosine phosphorylation was essential for Jak2-CaM interaction, because nonphosphorylated Jak2 and CaM failed to coimmunoprecipitate. Next, we tested whether the phosphorylated Jak2 and CaM and the subsequent complex formation modulate the NHE3 function. Immunoprecipitation and immunoblotting studies revealed that Ang II treatment of proximal tubules causes complex formation of phosphorylated Jak2 and CaM with NHE3, and this complex formation is AT1-dependent, because it is blocked by candesartan. Taken together, these data show that, in renal proximal tubules, Ang II causes Jak2 and CaM complex formation, which subsequently interacts with NHE3 and stimulates its activity.

To determine the exact pathway for the phosphorylation of Jak2 and CaM, the cells were treated with Jak2 and CaM inhibitors before Ang II exposure. CaM inhibitor W7 failed to block the Jak2 phosphorylation, but Jak2 inhibitor AG490 abolished CaM phosphorylation. Interestingly, the cell-permeable Ca2+-chelator BAPTA-AM abolished the Ang II–mediated phosphorylation of both Jak2 and CaM. These data suggest that, although Ca2+ accumulation leads to phosphorylation of both Jak2 and CaM, Jak2 activation is required for CaM phosphorylation. This is a novel finding, because the role of Ca2+ in Jak2 or CaM tyrosine phosphorylation is not clear. There are reports that show that Ca2+ can increase CaM and Jak2 phosphorylation in vascular smooth muscle cells.39 However, these data are contrary to a report by Fukami et al.,37 which shows that Ca2+ could decrease CaM phosphorylation in Rous sarcoma–transformed cells. Also, Ca2+ via PLC-γ has been shown to inhibit NHE3 in fibroblasts.38 These discrepancies could be either because of a difference in the cell system used or in response to various hormones used to increase Ca2+ signaling and/or isoform-specific PLC activation. Our data suggest that, in proximal tubules, Ang II increases intracellular Ca2+, causing tyrosine phosphorylation/activation of Jak2, which, in turn, phosphorylates CaM.

In addition to an increase in oxidative stress and blood pressure, the BSO-treated rats showed increased AT1R signaling and NHE3 stimulation in response to Ang II. The enhanced response to Ang II could be because of AT1R upregulation, as receptor normalization by Tempol also led to normal Ang II response in BSO-treated rats. However, it is also possible that higher oxidative stress in BSO rats directly enhanced or inhibited the activity of intermediary kinases or phosphatases, respectively. The modulation of these signaling molecules could lead to increased hormonal response and enhanced NHE3 stimulation. Therefore, in the present study we bypassed the receptor stimulation by using Ca2+ ionophore A23187, which increased proximal tubular intracellular Ca2+ concentration to a similar extent in both control and BSO-treated rats. It is worth noting that the basal Ca2+ concentration is similar among all of the experimental groups, and only Ang II–dependent Ca2+ accumulation is higher in BSO-treated rats compared with other groups. Our data show that an increase in intracellular Ca2+ by A23187 caused similar activation of NHE3 in all of the groups. More
importantly, incubation of tubules with A23187 increased Jak and CaM phosphorylation and complex formation to a similar extent in control and BSO-treated rats. Because these molecules are directly involved in Ang II–induced NHE3 stimulation, our data provide direct evidence that exaggerated response to Ang II during oxidative stress is because of AT1R upregulation. It also provides further support that increases in intracellular Ca2+ are responsible for AT1R-dependent Jak2 and CaM phosphorylation and NHE3 stimulation in response to Ang II.

In conclusion, we have identified a novel pathway for Ang II–mediated NHE3 activation in renal proximal tubules. Ang II via AT1R activates PLC and increases intracellular Ca2+, which phosphorylates and activates Jak2. The activated Jak2 phosphorylates CaM, which leads to a complex formation between Jak2 and CaM. The Jak2/CaM complex interacts which phosphorylates and activates Jak2. The activated Jak2 provides insights that oxidative stress, associated with many hypertensive tissues by thiobarbituric acid test. 2219–2226.

References


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Oxidative Stress Causes Renal AT1 Receptor Upregulation, \( \text{Na}^+ / \text{H}^+ \) Exchanger 3 Overstimulation and Hypertension

Anees Ahmad Banday, PhD and Mustafa F. Lokhandwala, PhD

Heart and Kidney Institute, College of Pharmacy, University of Houston, Houston TX

Short Title: Ang II Mediated NHE3 Regulation

Supplemental Data: Methods, Table S1 and Figures S1-S4
Methods

Adult male SD rats were divided into following four groups: C (control), animals were provided with tap water; BSO (L-buthionine sulfoximine), animals were provided with 30 mM BSO (Sigma, St. Louis, MO); T (tempol), animals were provided with 1 mM tempol (Sigma); and BSO + T, animals were provided with BSO plus tempol. BSO, a glutamate cysteine ligase inhibitor, and tempol, a superoxide dismutase mimetic compound, were provided in tap water for 3 weeks. At the end of the treatment rats were anesthetized with Inactin (100 mg/kg IP) and tracheotomy was performed to facilitate breathing, the left carotid artery was catheterized with PE-50 tubing, connected to a Statham P23AC pressure transducer, and blood pressure and heart rate were recorded on a Grass polygraph (model 7D, Grass Instrument, Quincy, MA). Renal proximal tubular suspension was prepared as described previously. Briefly, enrichment of proximal tubules was carried out using 20% Ficoll gradient in Krebs buffer. The band at Ficoll water interface was collected and washed by centrifugation at 250g for 5 min. Cell lysate, cytosol (35,000g supernatant), and membrane fractions (35,000g pellet) were isolated from proximal tubules by differential centrifugation as detailed previously. Plasma and urinary creatinine levels were measured by creatinine analyzer (model 2, Beckman, CA) and glomerular filtration rate (ml/min) was calculated based on the creatinine clearance. 8-isoprostane was measured by RIA kit (516351; Cayman, Ann Arbor, MI), and malondialdehyde was determined by the method of Uchiyama and Mihara. Protein was determined by using a bicinchoninic acid method (Thermo Fisher, Houston TX). The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

Phospholipase (PL) C and Na+/H+ Exchanger (NHE) 3 Assay

PLC activity was determined by using an exogenous source of [3H]phosphatidylinositol 4,5-bisphosphate ([3H]PIP2; PerkinElmer, Shelton, CT) as the primary substrate for PLC and monitoring the release of [3H] inositol trisphosphate ([3H]IP3) as detailed previously. Briefly, membranes were preincubated with 10 pM Ang II (Sigma) in presence and absence of AT1 receptor antagonist candesartan (1 µM) and the reaction was started by adding [3H]PIP2 (0.005 µCi/assay tube) and phosphatidylserine. After 10 min, the reaction was terminated by addition of lipid extraction medium and radioactive decay was counted in a liquid scintillation spectrophotometer. Inositol trisphosphate (IP3) accumulation was measured by IP3 Biotrak Assay System (TRK1000, GE Healthcare, Piscataway, NJ). NHE3 activity was determined by measurement of 5-(N-methyl-N-isobutyl)-amiloride (MIA) -sensitive 22Na+ uptake in brush border membrane vesicles. Tubules were incubated with Ang II (10 pM) for 10 min in absence and presence of candesartan (1 µM), PLC inhibitors (100 µM D609 and 50 µM ET-18-OCH3), serine/threonine kinase inhibitor (1 µM okadaic acid), tyrosine kinase inhibitor (10 µM genistein), protein kinase C inhibitor (1 µM GF109203X), CaM inhibitor (100 µM W7, 50 µM fluphenazine, 100 µM chlorpromazine, 10 µM ophiobolin, 10 µM calmidazolium), Jak2 inhibitor (10 µM AG490), ERK1/2 inhibitor (10 µM U0126), NHE1 inhibitor (1 µM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA)) and 100 µM daidzein, an inactive genistein analogue. It is worth mentioning that okadaic acid is also a phosphatase inhibitor and could modulate AT1 signaling. However, in present study a more specific PKC inhibitor was used to identify the role of PKC in Ang II-mediated NHE3 stimulation. Following the treatment, proximal tubules were homogenized to prepare brush border membrane vesicles and perform 22Na+ uptake as detailed previously.
125I-sar-Ang II binding
Membranes binding of 125I-sar-Ang II (PerkinElmer) was performed as described earlier.3 The membranes (50 µg protein) were incubated with 30 pM 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 a gamma counter. Nonspecific binding was determined by performing the binding assay in the presence of 1µM unlabeled Ang II.

Immunoprecipitation
Proximal tubules were incubated with 100 pM Ang II for 10 min and then lysed in buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail [Complete®, Roche Diagnostics, Indianapolis, IN]) and lysates were precleared by protein A−agarose bead slurry for 2 hr at 4-8 °C. Precleared lysates (1 µg/µL total cell protein) were incubated with anti-Jak2/protein A−agarose, anti-NHE3 antibody, or anti-phosphotyrosine antibody overnight at 4-8°C. Phosphotyrosine and NHE3 immunoprecipitates were incubated with protein A−agarose and beads were collected by centrifugation, washed with buffer A, resuspended in 2× Laemmli buffer, boiled for 5 min and subjected to SDS–PAGE and immunoblotted with monoclonal anti-CaM or anti-phosphotyrosine IgG. The membranes were reprobed with the antibody used for immunoprecipitation to ensure that equal amounts of protein were loaded in each lane.

Phosphorylation of Jak2 in response to Ang II was assessed by using a Jak2 dual phospho-specific antibody. Proximal tubules were treated with 10 nM Ang II, lysed in buffer A and subjected to SDS–PAGE under reducing conditions using 4−20% (Thermo Fisher). The protein was transferred to PDVF membranes and membranes were blocked and incubated with phospho-Jak2 antibody and/or Jak2 antibody and bands were visualized by a chemiluminescent method (Thermo Fisher) as detailed previously.1, 3, 6

References
Table S1. General Parameters

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<th>BSO</th>
<th>T</th>
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<td>Mean Blood Pressure, mm Hg</td>
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<td>107.3 ± 5.5</td>
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<td>103.5 ± 6.5†</td>
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<td>Body weight, g</td>
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<td>258.1 ± 20.2</td>
<td>262.6 ± 18.9</td>
<td>251.3 ± 19.6</td>
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*Data represent mean ± SE from 6-8 animals. C—control; BSO—L-buthionine sulfoximine; T—tempol and GFR—glomerular filtration rate. *P< 0.05 vs. C and †P< 0.05 vs. BSO, using one-way ANOVA followed by Newman-Keul post hoc test.
Figure S1: Effect of oxidative stress on AT1 receptor ligand binding and angiotensin (Ang) II-mediated inositol 1,4,5-trisphosphate (IP3) accumulation in renal proximal tubules from control (C), L-buthionine sulfoximine (BSO) and tempol (T) treated rats. (A) Proximal tubular membrane ligand binding. (B) Ang II (10 pM)-induced IP3 accumulation in proximal tubules. (C) Proximal tubules were preincubated with AT1 receptor antagonist candesartan (1 µM). Data represent mean ± SE from 6-8 animals performed in triplicate. *P<0.05 vs. respective control, #P<0.05 vs. BSO, $P<0.05 vs. basal and $P<0.05 vs. Ang II+Candesartan, using one-way ANOVA followed by Newman-Keuls post hoc test.
Figure S2. Effect of oxidative stress on protein expression and angiotensin (Ang) II-induced stimulation of Na\(^+\)/H\(^+\) exchanger (NHE) 3 in proximal tubular brush border membrane vesicles from control (C), L-buthionine sulfoximine (BSO) and tempol (T) treated rats. (A) Bands are representative western blots for NHE3 protein expression and bars represent NHE3 density. Equal amount of protein (15 µg) was loaded in all lanes. (B) Ang II (10 pM)-mediated NHE3 stimulation in absence and presence of AT1 receptor antagonist candesartan (1 µM). Data represent mean ± SE from 6-8 animals performed in triplicate. *P<0.05 vs. respective basal and #P<0.05 vs. Ang II form C-rats, using one-way ANOVA followed by Newman-Keuls post hoc test.
Figure S3. Effect of oxidative stress on Ca\(^{2+}\) ionophore A23187 (1 µM) -induced proximal tubular NHE3 stimulation from control (C), L-buthionine sulfoximine (BSO) and tempol (T) -treated rats. Data represent mean ± SE from 6-8 animals performed in triplicate. *P<0.05 vs. basal, using one-way ANOVA followed by Newman-Keuls post hoc test.
**Figure S4.** Ca\(^{2+}\) ionophore A23187-mediated janus kinase (Jak) 2 and Ca\(^{2+}\)-dependent calmodulin (CaM) interaction in proximal tubules from control (C) and L-buthionine sulfoximine (BSO) -treated rats. Proximal tubules were incubated with A23187 (1 µM) and immunoprecipitation (IP) was performed with CaM or JAK antibodies (Abs) followed by immunoblotting for Jak2, or CaM. (A) IP with Jak2 Abs and IB for phosphorylated Jak2 (P-Jak2), (B) IP with CaM Abs and IB for phosphorylated CaM (P-CaM), (C) IP with CaM Abs and IB with Jak2 Abs. (D) Basal Jak 2 and CaM expression. Upper bands are representative western blots. Data represent mean ± SE from 6-8 animals performed in triplicate. *P<0.05 vs. basal, using one-way ANOVA followed by Newman-Keuls post hoc test.