THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 28, pp. 21323–21328, July 9, 2010

Angiotensin II Stimulates Thick Ascending Limb Superoxide Production via Protein Kinase Cα-dependent NADPH Oxidase Activation*

Received for publication, January 29, 2010, and in revised form, April 22, 2010 Published, JBC Papers in Press, May 6, 2010, DOI 10.1074/jbc.M110.109157

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Angiotensin II (Ang II) stimulates thick ascending limb (TAL) O_2^- production, but the receptor(s) and signaling mechanism(s) involved are unknown. The effect of Ang II on O_2^- is generally attributed to the AT_1 receptor. In some cells, Ang II stimulates protein kinase C (PKC), whose α isoform (PKCα) can activate NADPH oxidase. We hypothesized that in TALs, Ang II stimulates O_2^- via AT_1 and PKCα-dependent NADPH oxidase activation. In rat TALs, 1 nM Ang II stimulated O_2^- from 0.76 ± 0.17 to 1.97 ± 0.21 nmol/min/mg ($p < 0.001$). An AT_1 antagonist blocked the stimulatory effect of Ang II on O_2^- (0.87 ± 0.25 nmol/min/mg; $p < 0.006$), whereas an AT_2 antagonist had no effect (2.16 ± 0.133 nmol/min/mg; $p < 0.05$ *versus* vehicle). Apocynin, an NADPH oxidase inhibitor, blocked Ang II-stimulated O_2^- by 90% ($p < 0.01$). Ang II failed to stimulate O_2^- in TALs from $p47^{phox-/-}$ mice ($p < 0.02$). Monitored by fluorescence resonance energy transfer, Ang II increased PKC activity from 0.02 ± 0.03 to 0.13 ± 0.02 arbitrary units ($p < 0.03$). A general PKC inhibitor, GF109203X, blocked the effect of Ang II on O_2^- (1.47 ± 0.21 *versus* 2.72 ± 0.47 nmol/min/mg with Ang II alone; $p < 0.03$). A PKCα- and β-selective inhibitor, Gö6976, also blocked the stimulatory effect of Ang II on O_2^- (0.59 ± 0.15 *versus* 2.05 ± 0.28 nmol/min/mg with Ang II alone; $p < 0.001$). To distinguish between PKCα and PKCβ, we used tubules expressing dominant-negative PKCα or -β. In control TALs, Ang II stimulated O_2^- by 2.17 ± 0.44 nmol/min/mg ($p < 0.011$). In tubules expressing dominant-negative PKCα, Ang II failed to stimulate O_2^- (change: −0.30 ± 0.27 nmol/min/mg). In tubules expressing dominant-negative PKCβ1, Ang II stimulated O_2^- by 2.08 ± 0.69 nmol/min/mg ($p < 0.002$). We conclude that Ang II stimulates TAL O_2^- production via activation of AT_1 receptors and PKCα-dependent NADPH oxidase.

The reactive oxygen species superoxide (O_2^-) plays an important role in the regulation of kidney function (1–3). O_2^- decreases renal blood flow by constricting renal vessels (4), reduces glomerular filtration rate by enhancing tubuloglomerular feedback (5) and also promotes salt reabsorption along the nephron (6, 7). Excessive O_2^- generation within the kidneys contributes to the development of hypertension (8), renal damage (9, 10), and atherosclerosis (11, 12). Thus clarifying the mechanisms that regulate O_2^- production within the kidney may help us understand the etiology and pathophysiology of many diseases and develop new targets for treatment.

O_2^- can be generated by several types of cells within the kidney (13, 14); however, it is primarily produced by the medullary thick ascending limb of the loop of Henle (TAL)[2] (14). In the TAL, O_2^- production can be stimulated by several factors, including Ang II (15, 16). Ang II can activate two types of receptors: AT_1 and AT_2. Activation of AT_1 is associated with the salt-retaining and pro-hypertensive actions of Ang II (17, 18). In the TAL, Ang II acutely stimulates O_2^- production (15), but neither the receptor nor the signaling cascade involved has been identified.

O_2^- can be produced by NADPH oxidase, xanthine oxidase, and the mitochondria (19). In the absence of Ang II stimulation, NADPH oxidase appears to be the main source in the renal medulla (20), particularly the TAL (14, 21, 22); however, the source of O_2^- in the TAL during Ang II stimulation is still unknown.

In many types of cells, including TAL cells, activation of protein kinase C (PKC) has been shown to stimulate O_2^- production in response to different stimuli, including Ang II (23, 24, 25). The PKC family of serine/threonine kinases is composed of many isoforms, some of which are expressed in the TAL, including PKCα, -β, -δ, -ϵ, and -ξ (26, 27). Yang *et al.* have shown that in the TAL PKCα mediates the enhanced O_2^- levels observed during diabetes (24). However, to our knowledge there have been no studies investigating whether PKC mediates the stimulatory effect of Ang II on TAL O_2^- production or the isoform(s) involved. We hypothesized that Ang II binds to the AT_1 receptors, activating PKCα, which in turn stimulates NADPH oxidase activity, enhancing O_2^- production by the TAL.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats (Charles River, Kalamazoo, MI) were fed a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 7 days. Wild-type and $p47^{phox}$ knock-out mice (Jackson Laboratories, Bar Harbor, ME), were fed regular chow for at least 7 days. On

* This work was supported, in whole or in part, by National Institutes of Health Grants HL-70985 and HL-028982 (to J. L. G.).

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[2] The abbreviations used are: TAL, thick ascending limb of the loop of Henle; Ang II, angiotensin II; PKC, protein kinase C; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; CMV, cytomegalovirus; HA, hemagglutinin; dn, dominant negative.

the day of the experiment, animals were anesthetized with ketamine (100 mg/kg body weight, intraperitoneally) and xylazine (20 mg/kg body weight, intraperitoneally). All protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Medullary TAL Suspensions—TAL suspensions were obtained from rats weighing 150–220 g as described previously (28). This procedure yields a suspension of TALs that is >90% pure (29), so that contamination by other types of cells in our preparation was minimal or absent.

Measurement of O_2^- Production—200-μl aliquots of rat TAL suspensions were placed in glass tubes, and HEPES-buffered physiological saline (130 mM NaCl, 2.5 mM NaH_2PO_4, 4 mM KCl, 1.2 mM $MgSO_4$, 6 mM alanine, 1 mM Na_3 citrate, 5.5 mM glucose, 2 mM $Ca^{2+}(lactate)_2$, and 10 mM HEPES (pH 7.4)) was added for a final volume of 1 ml. The whole suspension was used when TALs were obtained from mice. *N*,*N*′-Dimethyl-9,9′-biacridinium dinitrate (Lucigenin, Sigma-Aldrich) was added to the suspensions to give a final concentration of 5 μM. When investigating the effects of apocynin (4-hydroxy-3-methoxyacetophenone, 10 μM, Sigma-Aldrich), GF109203X (100 nM, Enzo Life Sciences, Plymouth Meeting, PA), or Gö6976 (100 nM, Enzo Life Sciences), these were added to the tube, and the volume of physiological saline was adjusted accordingly. When using the Ang II receptor antagonists PD123319 (1 μM, Parke-Davis, Ann Arbor, MI) and losartan (1 μM, Merck, Rahway, NJ), these were added to the tubules 5 min before Ang II (1 nM, Bachem, Torrance, CA). Tubules were incubated for 10 min at 37 °C and then placed in a luminometer (model FB12/Sirius, Zylux Co., Oak Ridge, TN) and maintained at 37 °C. The average of the last 3 of 10 consecutive measurements was calculated for each sample. The O_2^- scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron, Sigma-Aldrich) was added to the tube to give a final concentration of 10 mM, and measurements were repeated. The difference in average luminescence between samples with and without Tiron was used to calculate the luminescence produced by O_2^-. Measurements were normalized for protein content.

Protein Content Determination—Total protein content was measured using Coomassie Plus reagent (Pierce), based on Bradford's colorimetric method.

PKC Reporter—PKC activity was measured using a fluorescence resonance energy transfer (FRET)-based PKC reporter, CKAR (generously provided by Dr. Alexandra C. Newton, Howard Hughes Medical Institute, University of California-San Diego) (30). This probe consists of the consensus sequence for PKC phosphorylation with cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP) at either end. Under basal conditions CFP emission excites YFP due to its close proximity (FRET signal). Upon PKC activation, CKAR becomes phosphorylated and the probe "opens," resulting in an increased CFP signal and decreased FRET. Thus an increase in the CFP/YFP ratio indicates heightened PKC activity. The probe was subcloned into the adenoviral shuttle vector pVQ Ad5CMV K-NpA, which contains the CMV promoter, and sent to ViraQuest (North Liberty, IA) for viral production.

Dominant Negative PKC Isoforms—The HA (hemagglutinin)-tagged dominant negative PKCα and PKCβ1 plasmids (dn-PKCα and dn-PKCβ1) were kindly provided by Dr. Jae-Won Soh, Biomedical Research Center for Signal Transduction Networks, Incheon, Korea. The dominant negatives consist of kinase-dead PKC isoforms, generated by single amino acid substitution within the kinase domain. Sequences encoding for both proteins were subcloned into a shuttle vector containing the CMV promoter. Plasmids were sent to ViraQuest for adenoviral production.

In Vivo Gene Delivery of CKAR and dn-PKC Isoforms—TALs were transduced *in vivo* with recombinant replication-deficient adenoviruses expressing the dn-PKCα, dn-PKCβ1, or CKAR sequence as we reported previously (31, 32). Briefly, kidneys of a 95- to 105-g rat were exposed via a flank incision, and the renal artery and vein were clamped. Four 20-μl virus injections (1×10^{12} particles/ml) were made along the longitudinal axis at a flow rate of 20 μl/min. The renal vessels were unclamped; kidneys were returned to the abdominal cavity, the muscle incision was sutured, and the skin was clipped. Because we previously found that maximum expression occurred 3–5 days after injection of the adenovirus (32, 33), all experiments were performed within these time points. Expression of the dominant negatives was confirmed by Western blots.

Expression of dn-PKCα and -β—Western blots were performed as routinely done in our laboratory (28, 29). Briefly, 40 μg of TAL suspension homogenates was loaded onto an 8% polyacrylamide gel, and electrophoresis was performed for 2 h at 92 mV. After an overnight transfer, the polyvinylidene difluoride membrane was blocked in a buffer containing 20 mM Tris, 137 mM NaCl, 5% nonfat dried milk, and 0.1% Tween 20 (TBS-T) and 5% milk for 1 h at room temperature and then incubated with either a 1:1,000 dilution of a mouse monoclonal anti-HA antibody (Abgent, San Diego, CA), 1:1,000 dilution of a mouse anti-PKCα antibody (BD Biosciences, San Jose, CA) or a 1:250 dilution of a mouse anti-PKCβ antibody (BD Biosciences) for 1 h at room temperature. The membrane was washed using TBS-T and incubated for another hour with a 1:1,000 dilution of the appropriate IgG conjugated to horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. The reaction products were detected using a chemiluminescence kit (Amersham Biosciences) and by exposure to Fuji RX film.

Measurements of PKC Activity by FRET—On the day of the experiment, TAL suspensions were obtained from CKAR-transduced kidneys as indicated above and 1/5 of the suspension was seeded in a temperature-controlled chamber and warmed to 37 °C. The flow rate of the bath was 0.3 ml/min. During the 30-min equilibration period, images were acquired (100× oil objective, numerical aperture: 1.3) by alternately exciting CFP (442 nm) and YFP (514 nm) and monitoring YFP emission at 540 nm to determine expression of the FRET sensor and highlight regions of interest. During the control period, CFP/YFP emission ratios were measured by exciting CFP at 442 nm once a minute for 5 min and simultaneously monitoring CFP and YFP emissions at 440–480 (CFP) and 540–545 nm (YFP). At the end of the control period, Ang II was added to the bath and CFP/YFP monitored once every minute for 15 min. The averages corresponding to the 5-min control period and the last 5 min of the experimental period were compared. To

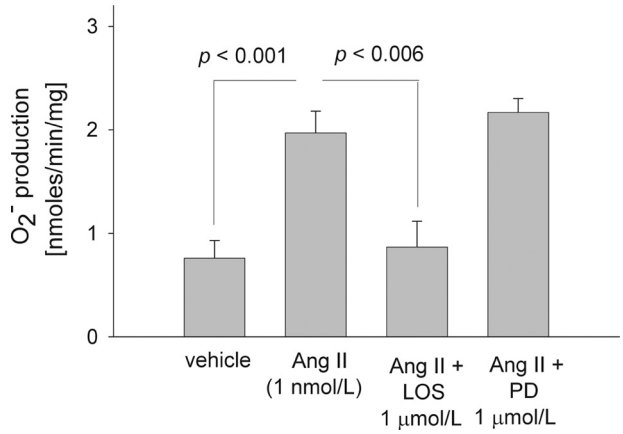

FIGURE 1. **Effect of 1 nM angiotensin II for 10 min on rat thick ascending limb O_2^- production in the absence and presence of 1 μM losartan (an AT_1 receptor blocker) or 1 μM PD123319 (an AT_2 receptor blocker).** *Ang II*, angiotensin II; *LOS*, losartan; *PD*, PD123319. $n = 11$ for Ang II, 5 for vehicle and 6 for Ang II plus LOS and Ang II plus PD.

confirm that the YFP signal was due to FRET, control experiments were performed by photobleaching CFP and measuring the decrease in YFP emission. Images were acquired using the same settings (laser intensity, detector gain and offset, resolution, and exposure time).

Statistics—All statistical analyses were performed by the Biostatistics Department of Henry Ford Hospital. Results are expressed as mean ± S.E. Data were analyzed using Student's *t*-tests. A version designed for unequal standard deviations was used when necessary. Some comparisons were studied using contrast statements. When multiple testing was involved, Hochberg's method was used.

RESULTS

We first investigated the effect of Ang II on TAL O_2^- production. When rat TAL suspensions were incubated for 10 min in vehicle (0.005% acetic acid in water), O_2^- production was 0.76 ± 0.17 nmol/min/mg. However, in TALs treated with Ang II (1 nM for 10 min) it increased to 1.97 ± 0.21 nmol/min/mg ($p < 0.001$; $n = 11$), 159% stimulation (Fig. 1). These data suggested that Ang II stimulates O_2^- production by TALs.

To investigate which angiotensin receptor mediates the effect of Ang II on O_2^-, we used pharmacological inhibitors of AT_1 and AT_2. In the presence of losartan, an AT_1 receptor antagonist (1 μM), Ang II (1 nM) failed to stimulate O_2^- production (0.87 ± 0.25 nmol/min/mg; $p < 0.006$ *versus* Ang II alone; $n = 6$) by rat TALs. However, when we used PD 123319, an AT_2 receptor antagonist (1 μM), Ang II raised O_2^- production to 2.16 ± 0.13 nmol/min/mg ($p < 0.05$ *versus* vehicle; $n = 5$) (Fig. 1). In different sets of experiments, neither losartan nor PD123319 changed basal O_2^- levels (0.78 ± 0.38 nmol/min/mg for baseline *versus* 0.80 ± 0.06 nmol/min/mg for losartan alone; $n = 3$ and 1.04 ± 0.09 nmol/min/mg for baseline *versus* 1.05 ± 0.12 nmol/min/mg for PD123319 alone; $n = 3$). These data indicated that Ang II binds the AT_1 receptor to stimulate O_2^- production by TALs.

Next we tested whether NADPH oxidase is the source of Ang II-stimulated O_2^- in TALs using the NADPH oxidase inhibitor apocynin and TALs isolated from $p47^{phox}$ knock-out ($^{-/-}$) mice. When rat TAL suspensions were incubated with vehicle (0.005% acetic acid), O_2^- production was 1.54 ± 0.31nmol/min/mg. With Ang II (1 nM for 10 min) it increased to 4.10 ± 0.69 nmol/min/mg ($p < 0.001$ *versus* vehicle; $n = 4$). However, when we added apocynin (10 μM), Ang II failed to stimulate O_2^- production (1.49 ± 0.32 nmol/min/mg; $p < 0.01$ *versus* Ang II alone; $n = 5$) (Fig. 2*A*). In a different set of experiments, apocynin alone significantly reduced basal O_2^- production by 80% ($p < 0.005$; $n = 5$). To make sure the effect of apocynin was due to specific inhibition of NADPH oxidase, we performed experiments using tubules isolated from $p47^{phox-/-}$ mice. In the presence of Ang II, O_2^- production was 1.45 ± 0.12 nmol/min/mg in wild-type controls but undetectable in TALs from $p47^{phox-/-}$ (0.00 ± 0.32 nmol/min/mg) ($p < 0.02$; $n = 4$ for each group) (Fig. 2*B*). These data indicate that NADPH oxidase is the primary source of O_2^- in the TAL under both basal and Ang II-stimulated conditions.

To test whether Ang II directly enhances PKC activity in the rat TAL, we measured the effect of Ang II on PKC activity using FRET. In tubules expressing CKAR and incubated with vehicle (0.005% acetic acid) the CFP/YFP ratio was 0.02 ± 0.03 arbitrary unit. Upon adding Ang II (1 nM) to the same tubules,

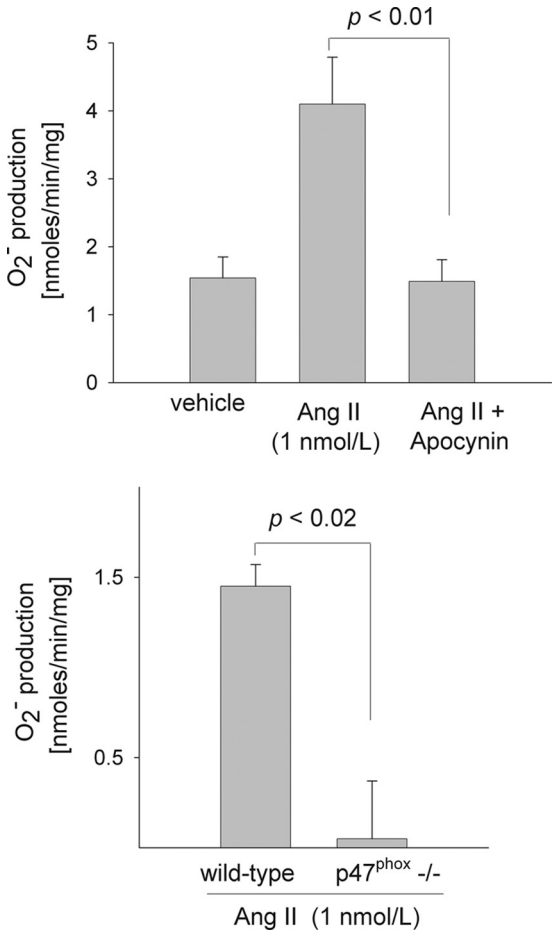

FIGURE 2. *Top*, effect of 10 μM apocynin (an NADPH oxidase inhibitor) on the stimulatory effect of angiotensin II on O_2^- production by rat thick ascending limbs. Rat thick ascending limbs were incubated with 1 nM angiotensin II for 10 min in the presence or absence of apocynin and superoxide production measured. $n = 5$ per group. *Bottom*, effect of 1 nM angiotensin II for 10 min on O_2^- production by thick ascending limbs isolated from wild-type and $p47^{phox}$ knock-out mice ($p47^{phox-/-}$). $n = 4$. *Ang II*, angiotensin II.

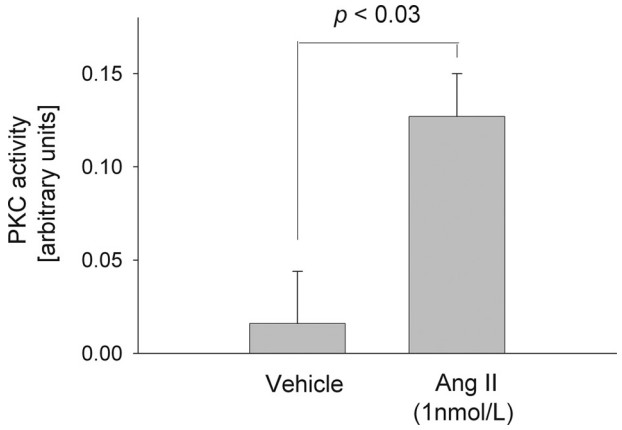

FIGURE 3. **Acute effect of 1 nM Ang II on total PKC activity by FRET.** Rat thick ascending limbs expressing a PKC activity reporter were used. $n = 6$.

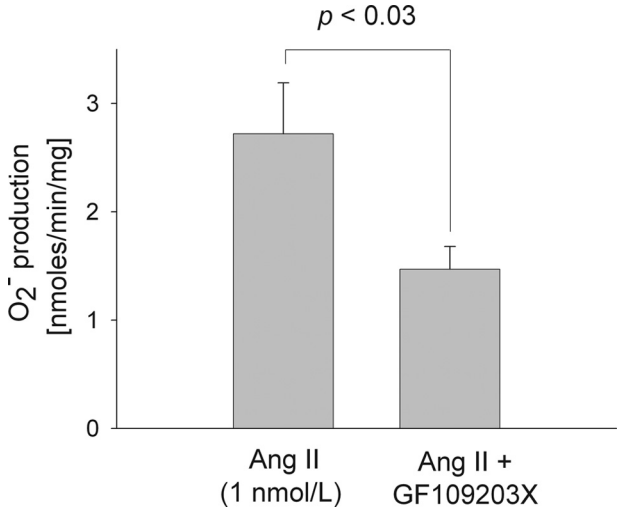

FIGURE 4. **Effect of 100 nM GF109203X (a general PKC inhibitor) on the stimulatory effect of 1 nM Ang II for 10 min on O_2^- production by rat thick ascending limbs.** $n = 5–6$.

CFP/YFP increased to 0.13 ± 0.02 arbitrary unit ($p < 0.03$; $n = 6$) (Fig. 3). These data suggested that Ang II activates PKC activity in the TAL.

To determine whether activation of PKC is required for the stimulatory effect of Ang II on O_2^- production, we used a general PKC inhibitor, GF109203X. In rat TALs 1 nM Ang II stimulated O_2^- production to 2.72 ± 0.47 nmol/min/mg. However, with 100 nM GF109203X the effect of Ang II was significantly reduced (1.47 ± 0.21 nmol/min/mg; $p < 0.03$ *versus* Ang II alone; $n = 6$) (Fig. 4). In a different set of experiments, GF109203X did not change basal O_2^- production (1.13 ± 0.15 nmol/min/mg for baseline *versus* 0.83 ± 0.12 nmol/min/mg for GF109203X alone; $n = 3$). These data indicate that Ang II stimulates TAL O_2^- production by activating PKC.

To find out which PKC isoform(s) mediates Ang II-stimulated O_2^- production, we used Gö6976, a PKCα- and β1-selective inhibitor. In rat TAL suspensions, 1 nM Ang II raised O_2^- production to 2.05 ± 0.28 nmol/min/mg. However, when we added Gö6976 (100 nM) to the preparation, Ang II failed to stimulate O_2^- production (0.59 ± 0.15 nmol/min/mg; $p < 0.001$; $n = 6$) (Fig. 5). In a different set of experiments, Gö6976 did not change basal O_2^- production (1.17 ± 0.10 nmol/min/mg for

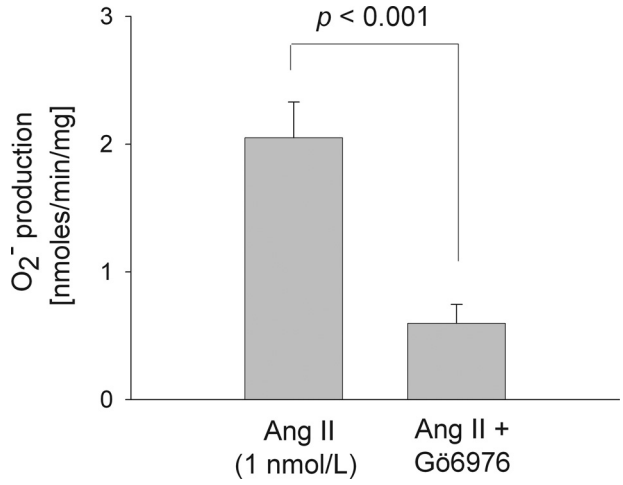

FIGURE 5. **Effect of 100 nM Gö6976 (a selective PKCα and β1 inhibitor) on the stimulatory effect of 1 nM Ang II for 10 min on O_2^- production by rat thick ascending limbs.** $n = 6$.

baseline *versus* 1.10 ± 0.07 nmol/min/mg for Gö6976 alone; $n = 3$). These results suggested that Ang II stimulates TAL O_2^- production by activating PKCα and/or PKCβ1.

To clarify the PKC isoform(s) involved, we transduced rat TALs *in vivo* so that they expressed either control DNA, dominant negative PKCα (dn-PKCα) or dominant negative PKCβ1 (dn-PKCβ1). Expression of the dominant negatives was maximal 3–5 days after adenoviral injection as assessed by Western blots. The dominant negatives are HA-tagged, kinase-dead mutants generated by a single point mutation within the kinase domain. Thus, their expression can be monitored by the presence of HA and also by an increase in total PKCα or -β (because the antibodies used for Western blot also recognize the mutants). We found >500% increase of HA expression compared with the non-injected kidney ($n = 4$ for dn-PKCα and $n = 3$ for dn-PKCβ1). In addition, total PKCα increased by 250% in dn-PKCα-injected *versus* non-injected kidney ($p <$ 004; $n = 4$) and PKCβ by 293% dn-PKCβ1-injected *versus* non-injected kidney ($p < 0.08$; $n = 3$). All experiments were performed 3–5 days after adenoviral transduction. In control rat TALs incubated with vehicle (0.005% acetic acid), O_2^- production was 1.42 ± 0.12 nmol/min/mg, and with 1 nM Ang II it rose to 3.58 ± 0.51 nmol/min/mg of protein ($p < 0.011$, $n = 5$) (Fig. 6). In contrast, in tubules expressing dn-PKCα, Ang II failed to stimulate O_2^- (1.53 ± 0.67 nmol/min/mg; *n.s. versus* vehicle), whereas in tubules expressing dn-PKCβ1 Ang II raised O_2^- production to 3.89 ± 0.37 nmol/min/mg ($p < 0.002$ *versus* vehicle; $n = 6$) (Fig. 6). Neither dn-PKC isoform had any effect on basal O_2^- (vehicle-treated suspensions; *black bars* in Fig. 6). These data indicated that PKCα mediates the stimulatory effect of Ang II on O_2^- production by TALs.

DISCUSSION

We hypothesized that Ang II acts on the AT_1 receptor to stimulate O_2^- production by the TAL, and that this process involves stimulation of PKCα, which in turn activates NADPH oxidase. We found that: 1) Ang II stimulated rat TAL O_2^- production, and this process was halted by blocking AT_1 but not AT_2; 2) the NADPH oxidase inhibitor apocynin blocked the

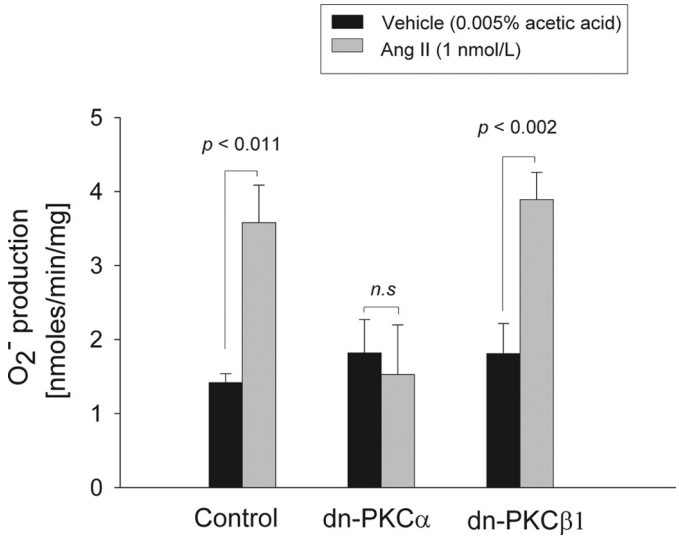

FIGURE 6. **Effect of vehicle or 1 nM Ang II for 10 min on O_2^- production by rat thick ascending limbs expressing control, dn-PKCα, or dn-PKCβ1.** n = 5–6.

stimulatory effect of Ang II on O_2^- production; 3) Ang II-induced O_2^- production was blunted in TALs isolated from p47$^{phox-/-}$ mice; 4) in rat TALs Ang II increased PKC activity as measured by FRET; 5) in rat TALs, Ang II-induced O_2^- production was blocked by a general PKC inhibitor as well as by an inhibitor of both PKC α and β1; and finally 6) Ang II-induced O_2^- production was reduced in rat TALs expressing dn-PKCα but intact in tubules expressing dn-PKCβ1.

We found that AT_1 mediated the stimulatory effect of Ang II on O_2^- production in the rat TAL but AT_2 did not, consistent with several studies conducted with other tissues. Fu *et al.* (33) recently reported that, in freshly isolated macula densa cells, Ang II stimulated O_2^- production, and this effect was blocked by the AT_1 antagonist losartan. Jaimes *et al.* (13) found that Ang II stimulated O_2^- production in cultured mesangial cells, and this effect was blocked by an AT_1 antagonist. Plumb *et al.* (35) reported that Ang II stimulated O_2^- production in human platelets, and this effect was blunted by an AT_1 receptor antagonist. Although we recently reported that Ang II acts on AT_2 receptors to activate other signaling events in the TAL (34), in the present study the AT_2 antagonist PD123319 had no effect on Ang II-stimulated O_2^- production, suggesting that AT_2 receptors do not play a role in AT_1-stimulated O_2^- production in the TAL. Thus it seems likely that activation of each receptor subtype leads to stimulation of independent signaling pathways.

We also questioned whether NADPH oxidase is the source of O_2^- in Ang II-stimulated TALs. NADPH oxidase is an enzymatic complex that comprises five components: p40^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, and NOX (35). Under basal conditions p40^{phox}, p47^{phox} and p67^{phox} are located in the cytosol as a complex. Upon stimulation, p47^{phox} becomes phosphorylated and the cytosolic complex translocates to the cell membrane, where it assembles with p22^{phox} and NOX and generates O_2^- (36). Thus p47^{phox} is essential for activation of NADPH oxidase. We found that apocynin, which inhibits translocation of p47^{phox} to the plasma membrane, completely inhibited Ang II-stimulated O_2^- production, suggesting that in the TAL all Ang II-stimulated O_2^- is generated by NADPH oxidase. In addition, we found that apocynin alone significantly reduced basal levels of O_2^- in the rat TAL suggesting that NADPH oxidase generates basal O_2^- levels. We recognize that the basal level of O_2^- on Fig. 2 is higher than what we found for Fig. 1. The explanation for such discrepancy is unknown; however, it should be mentioned that experiments were done during different times of the year, and this may influence production of O_2^- by the rat TAL.

To make sure the effect of apocynin was specifically due to inhibition of NADPH oxidase, we tested TALs isolated from p47$^{phox-/-}$ mice and found that they had low basal levels of O_2^-, which were not stimulated by Ang II, indicating that: 1) p47^{phox} is required for Ang II-stimulated O_2^- production in TALs and 2) it maintains basal TAL levels of O_2^-. We were unable to uncover any compensatory mechanism that enables O_2^- to be generated under both basal and stimulated conditions in these mice. We recognize that the results obtained in mice cannot necessarily be extrapolated to rats. In fact, the degree of Ang II-stimulated O_2^- production was lower in mice compared with rats. However, the p47$^{phox-/-}$ mice were used as a tool to investigate the involvement of NADPH oxidase so that we did not rely only on pharmacological inhibition. These findings are consistent with data from Li *et al.* (14) showing that in unstimulated TALs NADPH oxidase is the major source of O_2^- production. In addition, a recent report from our laboratory demonstrated that luminal flow stimulated TAL O_2^- production via activation of NADPH oxidase (37). Thus both mechanical and humoral factors are capable of activating NADPH oxidase and thereby enhancing O_2^- production by the TAL. Taken together, these data confirm that NADPH oxidase is the main source of O_2^- in the TAL under both basal and stimulated conditions.

To test the involvement of PKC in Ang II-stimulated O_2^- production, we first measured total PKC activation in real-time by FRET. We found that Ang II acutely increased PKC activity within 3 min after adding Ang II. When we applied the general PKC pharmacological inhibitor GF109203X, we found that it inhibited Ang II-induced O_2^- production, indicating that PKC activity is necessary for Ang II to stimulate O_2^- production by TALs. These data are consistent with recent reports from other investigators suggesting that PKC contributes to enhanced O_2^- production in the kidney. Zhang *et al.* (38) showed that Ang II constricted pericytes within the vasa recta via a mechanism requiring activation of PKC. Yang *et al.* reported that PKC activation is responsible for the increased O_2^- in diabetic rat TALs (24). More recently, we have shown that luminal flow stimulates O_2^- production via activation of PKC in isolated perfused TALs (37). Thus activation of PKC appears to be an important mechanism leading to enhanced O_2^- within the kidney.

The PKC protein family is composed of at least eight members, of which five have been shown to be expressed in the TAL: PKCα, -β, -δ, -ϵ, and -ξ (26, 27). To find out which isoform(s) might be involved in Ang II-induced O_2^- production, we used Gö6976, which inhibits both PKCα and -β. We found that Gö6976 completely blocked Ang II-induced O_2^- production. Because we know of no pharmacological inhibitor specific enough to target only PKCα or -β1, we used adenoviral-mediated transduction of dn-PKCα or -β. We found that Ang II stimulated O_2^- production both in controls and in dn-PKCβ1-

transduced TALS; however, it had no effect on TALs transduced with dn-PKCα. Taken together, these data indicate that Ang II stimulates NADPH oxidase-derived O_2^- production in the TAL by activating PKCα.

We conclude that in the TAL, Ang II acts on the AT_1 receptors, activating PKCα, which in turn stimulates first NADPH oxidase and ultimately O_2^- production, although the exact signaling pathway remains unknown. The AT_1 receptors are coupled to G_q and G_i proteins. Activation of G_q enhances diacylglycerol production and stimulates intracellular Ca^{2+} (39, 40), either of which is capable of activating the classic PKC isoforms α and β (41). In other cells, O_2^- stimulation by AT_1 activation has been attributed to increased diacylglycerol generation and subsequent activation of PKC. In addition, AT_1-stimulated O_2^- production is mediated by increases in intracellular Ca^{2+} (42). Because PKC is stimulated by diacylglycerol, and both NADPH oxidase and PKC are sensitive to increases in intracellular Ca^{2+} (42), both of these pathways could mediate AT_1-dependent activation of PKCα in the TAL. In addition, Ang II has been reported to activate the small GTPase Rac (43), whose trafficking and translocation to the plasma membrane play an important role in activation of NADPH oxidase (44). In the TAL, Rac mediates NaCl-induced O_2^- production (45). Thus Rac could also participate in both Ang II-induced NADPH oxidase activation and O_2^- production in the TAL.

In this study we report that activation of PKCα is required for Ang II to stimulate O_2^- production in the TAL. However, we have shown previously that O_2^- activates PKCα in this segment (26). According to our data, PKCα also enhances O_2^- production via NADPH oxidase assembly with the $p47^{phox}$ subunit. Therefore it is possible that Ang II initiates a cycle whereby small increases in Ang II increase O_2^- production, which in turn overstimulates PKCα and ultimately heightens oxidative stress.

In summary, in TALs Ang II acts on the AT_1 receptor to activate PKCα, which in turn stimulates NADPH oxidase and enhances O_2^- production. This could be an important regulatory mechanism whereby Ang II modulates O_2^- levels in the renal medulla under physiological conditions. In addition, defects in the Ang II/PKC/NADPH/O_2^- pathway in the TAL could play a role in the development of hypertension, renal damage, and atherosclerosis.

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