PKC-α mediates flow-stimulated superoxide production in thick ascending limbs

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1Department of Internal Medicine, Hypertension and Vascular Research Division, Henry Ford Hospital, Detroit; 2Department of Physiology, Wayne State University, Detroit, Michigan; 3J. Robert Cade Foundation, Cordoba; and 4Mons. Carlos V. Crucellier Foundation, San Juan, Argentina

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Hong NJ, Silva GB, Garvin JL. PKC-α mediates flow-stimulated superoxide production in thick ascending limbs. Am J Physiol Renal Physiol 298: F885–F891, 2010. First published January 6, 2010; doi:10.1152/ajprenal.00543.2009.—We showed that luminal flow increased net O2 production via NADPH oxidase in thick ascending limbs. Protein kinase C (PKC) activates NADPH oxidase activity in phagocytes, cardiomyocytes, aortic endothelial cells, vascular smooth muscle cells, and renal mesangial cells. However, the flow-activated pathway that induces NADPH oxidase activity in thick ascending limbs is unclear. We hypothesized that PKC mediates flow-stimulated net O2 production by thick ascending limbs. Initiation of flow (20 nl/min) increased net O2 production from 4 ± 1 to 61 ± 12 AU/s (P < 0.007; n = 5). The NADPH oxidase inhibitor apocynin completely blocked the flow-induced increase in net O2 production (2 ± 1 ± 1 AU/s; P > 0.05; n = 5). Flow-stimulated O2 was also blocked in p47phox-deficient mice. We measured flow-stimulated PKC activity with a fluorescence resonance energy transfer (FRET)-based membrane-targeted PKC activity reporter and found that the FRET ratio increased from 0.87 ± 0.02 to 0.96 ± 0.04 AU (P < 0.05; n = 6). In the absence of flow, the PKC activator phorbol 12-myristate 13-acetate (200 nM) enhanced net O2 production from 5 ± 2 to 92 ± 6 AU/s (P < 0.001; n = 6). The PKC-α and β-selective inhibitor Gö 6976 (100 nM) decreased flow-stimulated net O2 production from 54 ± 15 to 2 ± 1 AU/s (P < 0.04; n = 5). Flow-induced net O2 production was inhibited in thick ascending limbs transduced with dominant-negative (dn)PKC-α but not dnPKCβI or LacZ (Δ = 11 ± 3 AU/s for dnPKCα, 55 ± 7 AU/s for dnPKCβI, and 63 ± 7 AU/s for LacZ; P < 0.001; n = 6). We concluded that flow stimulates net O2 production in thick ascending limbs via PKC-α-mediated activation of NADPH oxidase.

The protein kinase C (PKC) family is comprised of 12 related serine/threonine kinases (5). PKC has been shown to activate NADPH oxidase in several types of cells, including phagocytes (6), cardiomyocytes (42), aortic endothelial cells (13, 18), vascular smooth muscle cells (12, 37), and renal mesangial cells (41). Various PKC isoforms have been shown to stimulate O2 production, including α (41), β (23), δ (2), ε (42), and ζ (4). In addition, several PKC isoforms are activated in response to cellular stretch (34) and shear stress (35), which are mechanical stimuli associated with increased luminal flow.

The thick ascending limb expresses several PKC isoforms, including α, β, γ, δ, ε, θ, λ, ι, and ζ (38). PKC has been shown to affect several different transporters in the thick ascending limb. This kinase mediates the stimulatory effect of angiotensin II on Na+/K+2Cl- cotransporter activity (1) and stimulates NaHCO3 absorption (11). In addition, a PKC-dependent pathway mediates inhibition of apical K+ channel activity caused by high concentrations of prostaglandin E2 (26). However, the role of PKC in flow-enhanced O2 production as well as the isoform(s) involved are unknown.

We hypothesized that luminal flow stimulates net O2 production in the thick ascending limb via activation of PKC-α and consequently NADPH oxidase. To test this hypothesis, we examined the effect of acutely increasing luminal flow on net O2 production and PKC activity in isolated thick ascending limbs in the absence and presence of NADPH oxidase and PKC inhibitors. Dominant-negative (dn) mutants of specific PKC isoforms were also utilized. Our findings indicate that PKC-α mediates flow-stimulated net O2 production in the thick ascending limb.

MATERIALS AND METHODS

Chemicals and solutions. Dihydroethidium was obtained from Molecular Probes (Eugene, OR). Apocynin (acetoxyaniline), hypoxanthine, xanthine oxidase, N,N’-dimethyl-9,9’-biancridium dinitrate (lucigenin), 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO). Gö 6976 was purchased from Biomol International (Plymouth Meeting, PA). The physiological saline used to perfuse and bathe the thick ascending limbs contained (in mM) 130 NaCl, 2.5 NaH2PO4, 4 KCl, 1.2 MgSO4, 6 L-alanine, 1 trisodium citrate, 5.5 glucose, 2 calcium citrate, and 10 HEPES, pH 7.4 at 37°C. The osmolarity of this solution should be 290 ± 3 mosM; slight adjustments (±3 mosM), when necessary, were made by adding either H2O or NaCl.

Isolation and perfusion of thick ascending limbs. All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilming-
ton, MA) were maintained on a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 5 days. Male C57BL/6J and p47phox-deficient mice were from the Jackson Laboratory (Bar Harbor, ME). On the day of the experiment, rats weighing 120–150 g or 5- to 6-week-old mice were anesthetized intraperitoneally with ketamine and xylazine (100 and 20 mg/kg body wt, respectively). The abdominal cavity was opened and the left kidney was bathed in ice-cold saline and removed. Coronal slices were placed in oxygenated physiological saline. Medullary thick ascending limbs were dissected from the outer medulla under a stereomicroscope at 4–10°C. Tubules measuring 0.5–1.0 mm were transferred to a temperature-regulated chamber (37 ± 1°C) and perfused using concentric glass pipettes as described previously (9). We perfused tubules at a physiological flow rate of 20 nl/min using a nanoliter syringe pump (Harvard Apparatus, Holliston, MA).

Adenovirus construction. MyrPalm-CKAR (Addgene plasmid 14862), a membrane-targeted fluorescence resonance energy transfer (FRET)-based PKC activity reporter developed by Dr. A. Newton at the University of California at San Diego (40), was obtained from Addgene (Cambridge, MA). The dnPKCa and βl plasmids were kindly provided by Dr. J.-W. Soh at the Biomedical Research Center for Signal Transduction Networks, Incheon, Korea (www.pkclab.org). These genes were subcloned into the adenoviral shuttle vector pVQAAd5CMV K-NpA, which contains the cytomegalovirus (CMV) promoter, and sent to ViraQuest (North Liberty, IA) for adenovirus production. VQAAd5 CMV ntLacZ purchased from ViraQuest served as a negative control.

Adenoviral gene delivery. Rat medullary thick ascending limbs were transduced in vivo with an adenovirus carrying 1) MyrPalm-CKAR, 2) dnPKCa, 3) dnPKCB, or 4) lacZ as reported previously (32, 33). Briefly, the left kidney of a rat weighing 85–105 g was exposed via a flank incision, and then the left renal artery and vein were clamped. Four 20-μl injections of virus (2–5 × 1010 PFU/ml) at a rate of 20 μl/min were made by inserting the needle perpendicular to the renal capsule, parallel to the medullary rays and directed toward the medulla. Injection points were along the longitudinal axis of the kidney separated by 2.5 mm. The clamp was released and the kidney was returned to the abdominal cavity. The muscle was sutured and the skin was closed with staples. Medullary thick ascending limbs were dissected 3–5 days after injection.

Measurement of O2\textsubscript{3} using dihydroethidium. Isolated thick ascending limbs were loaded with 10 μM dihydroethidium in physiological saline for 15 min and then washed in dye-free solution for 20 min. O2\textsubscript{3} converts dihydroethidium to oxyethidium (7, 46). Oxyethidium and dihydroethidium were excited using 488- and 365-nm light, respectively. Emitted fluorescence between 520 and 600 nm (oxyethidium) and 400 and 450 nm (dihydroethidium) was imaged digitally with an image intensifier connected to a CCD camera and recorded with the Metafluor system (Universal Imaging, West Chester, PA). Fluorescence was measured from regions of interest every 5 s for 12 measurements. Regression analysis of the fluorescence ratios for each measurement period was performed and differences in slopes were evaluated.

O2\textsubscript{3} concentration drives the reaction between dihydroethidium and O2\textsubscript{3}. However, O2\textsubscript{3} determination is not only by both production and degradation. An increased rate of change of the oxyethidium/ dihydroethidium fluorescence ratio is a measure of increased "net O2\textsubscript{3} production" (production – degradation), although it is commonly referred to as O2\textsubscript{3} production. Given that net O2\textsubscript{3} production represents production – degradation, it may increase when degradation is reduced or production is enhanced without indicating whether the former, latter, or both has/have occurred. Therefore, we will refer to O2\textsubscript{3} measurements in this study as net O2\textsubscript{3} production.

The general format for all protocols was that O2\textsubscript{3} was measured in a tubule in the absence of luminal flow (period 1) and then again 5 min after luminal flow was initiated (period 2). For Fig. 6, an additional set of measurements was performed. After period 2, flow was stopped. In the same tubule, the PKC-α- and βl-selective inhibitor Gö 6976 was added. After 15 min, O2\textsubscript{3} measurements were recorded again in the absence and presence of flow.

Measurement of O2\textsubscript{3} production in a cell-free system. To generate O2\textsubscript{3}, a 1-mM solution of xanthine oxidase (10 μU; 0.9 U/mg) and 5 μM lucigenin in physiological saline was placed in a glass tube and incubated for 10 min at 37°C. Hypoxanthine (0.5 mM final concentration) was added, and the solution was incubated for 5 min. The tube was then placed in a luminometer (model FB12/Sirius, Zylux) and maintained at 37°C. Luminescence was measured for a 5-min control period, and then apocynin (10 μM final concentration) was added and measurements were taken for 5 min. Then, the O2\textsubscript{3} scavenger tiron was added at a final concentration of 10 mM, and the measurements were repeated. The difference in average luminescence between periods with and without tiron was used to calculate the luminescence produced by O2\textsubscript{3}.

Measurement of PKC activity using MyrPalm-CKAR. PKC activity was measured using a FRET-based membrane-targeted PKC activity reporter, MyrPalm-CKAR (40), which contains the FRET pair cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Isolated tubules from rat kidneys transduced with MyrPalm-CKAR were equilibrated for 20 min. Using a laser-scanning confocal microscope system (VisiTech International), MyrPalm-CKAR was excited at 442 nm (CFP); CFP and YFP emissions were measured at 480 ± 20 and 540 ± 20 nm, respectively, and the CFP/YFP emission ratio was calculated. Data were obtained once every minute for 5 min (control period). Then, flow (20 nl/min) was initiated and measurements were taken once every minute for 15 min (experimental period). The mean of the 5-min control period was compared with that of five consecutive measurements during the peak of response in the experimental period. Increases in CFP/YFP ratio were taken as a measure of increases in PKC activity. Control experiments were performed to show that emissions from YFP were due to FRET (14). Time control experiments were also performed. The same microscope settings (laser intensity, contrast, brightness, resolution, and exposure time) were used for all data.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed by the Henry Ford Hospital Department of Biostatistics and Epidemiology. Data were evaluated using Student’s t-test for paired experiments (see Figs. 1–5), Student’s t-test of paired differences (see Fig. 6), or ANOVA of the three differences (see Fig. 7), taking P < 0.05 as significant.

RESULTS

To begin to test our hypothesis, we first subjected isolated thick ascending limbs to enhanced luminal flow and measured the net rate of O2\textsubscript{3} production using the dye dihydroethidium. Figure 1A shows that in tubules in which there was no luminal flow, net O2\textsubscript{3} production was 4 ± 1 AU/s. Flow (20 nl/min) increased net O2\textsubscript{3} production to 61 ± 12 AU/s (P < 0.007; n = 5).

To study whether this increase was dependent on activation of NADPH oxidase, we used apocynin (Fig. 1B). In the presence of 10 μM apocynin and in the absence of flow, net O2\textsubscript{3} production was 2 ± 1 AU/s. After flow was started, it was 1 ± 1 AU/s (P > 0.05; n = 5).

A high concentration of apocynin (1 mM) has been shown to reduce O2\textsubscript{3} by acting as a scavenger (15). Therefore, the effect of apocynin we observed (Fig. 1B) could be due to scavenging rather than inhibition of NADPH oxidase. To rule out this possibility, we used a lucigenin-based assay to measure O2\textsubscript{3} generated by xanthine oxidase/hypoxanthine to study the effect of apocynin at the concentration used in our study. In the absence of apocynin, net O2\textsubscript{3} production was 4.46 ± 0.33.4 on October 21, 2017 http://ajprenal.physiology.org/ Downloaded from
change significantly after 10 μM apocynin was added (4.50 ± 0.44 nmol O₂·min⁻¹·mg⁻¹ xanthine oxidase; P > 0.05; n = 5; Fig. 2).

To further demonstrate that NADPH oxidase is the source of flow-induced O₂, we used a nonpharmacological approach in which we examined the effect of flow on O₂ in wild-type and p47phox-deficient mice. Figure 3A shows that in wild-type C57BL/6J mice, net O₂ production in the absence of flow was 7 ± 2 AU/s. An increase in flow caused net O₂ production to rise to 108 ± 28 AU/s (P < 0.04; n = 4). In mice deficient in p47phox, net O₂ production in the absence of flow was 3 ± 1 AU/s, and it did not change significantly when flow was increased (11 ± 6 AU/s; P > 0.05; n = 4; Fig. 3B). Taken together, these data indicate that luminal flow stimulates net O₂ production by NADPH oxidase.

To study whether flow-induced increases in net O₂ production in the thick ascending limb involve PKC, we examined the effect of flow on PKC activity in isolated thick ascending limbs from rat kidneys transduced with the membrane-targeted FRET-based PKC activity reporter MyrPalm-CKAR (40). A representative experiment (Fig. 4A) shows that in the absence of flow, basal PKC activity was 0.96 AU. After flow was initiated, it rose to an average of 1.03 AU. Increasing flow raised mean PKC activity from 0.87 ± 0.02 to 0.96 ± 0.04 AU (P < 0.05; n = 6; Fig. 4B).

PKC stimulates NADPH oxidase activity and thus O₂ production in several types of cells. To study whether activation of PKC alone can stimulate net O₂ production in thick ascending limbs, we used the PKC activator PMA in the absence of flow (Fig. 5). Adding 200 nM PMA in the absence of flow caused an increase in net O₂ production from 5 ± 2 to 92 ± 6 AU/s (P < 0.001; n = 5). Thus, PKC activation stimulates net O₂ production in the thick ascending limb.

Changes in PKC activity measured by MyrPalm-CKAR are primarily due to diacylglycerol-activated and Ca²⁺-dependent PKCs (the conventional PKCs α, βI, βII, and γ). Therefore, we next examined the specific PKC isoform(s) involved in flow-stimulated O₂ production by using the PKC-α, βI-selective inhibitor Gö 6976 (Fig. 6). In the absence of flow, net O₂ production was 6 ± 1 AU/s. Initiation of flow caused net O₂ production to increase to 60 ± 15 AU/s. In the presence of 100 nM Gö 6976 but no flow, net O₂ production was 5 ± 1 AU/s. After flow was started in the presence of 100 nM Gö 6976, net O₂ production was 7 ± 1 AU/s. Thus, Gö 6976 blocked
flow-induced net \(O_2^-\) production (\(\Delta = 54 \pm 15\) vs. 2 \(\pm 1\) AU/s; \(P < 0.04; n = 5\)). These data indicate that virtually all of the net \(O_2^-\) production stimulated by flow is mediated by PKC-\(\alpha\) and/or PKC-\(\beta\).

To study which of these two isoforms is responsible for flow-induced net \(O_2^-\) production, we transduced tubules with dnPKC-\(\alpha\) or dnPKC-\(\beta\). Tubules transduced with LacZ served as controls and exhibited an increase from 6 \(\pm 2\) to 69 \(\pm 7\) AU/s in net \(O_2^-\) production after flow was applied (Fig. 7). In contrast, this flow-induced increase was attenuated in dnPKC-\(\alpha\)-transduced tubules (7 \(\pm 1\) AU/s in the absence of flow vs. 18 \(\pm 4\) AU/s with flow). Thus, flow-stimulated net \(O_2^-\) production in dnPKC-\(\alpha\)-transduced tubules decreased by 83\% (\(\Delta = 11 \pm 3\) vs. 63 \(\pm 7\) AU/s for LacZ-transduced tubules; \(P < 0.001; n = 6\)). However, in dnPKC-\(\beta\)-transduced tubules flow increased net \(O_2^-\) production from 5 \(\pm 1\) to 60 \(\pm 7\) AU/s (\(\Delta = 55 \pm 7\) AU/s; \(P < 0.001; n = 6\) vs. dnPKC-\(\alpha\); \(P > 0.05\) vs. LacZ). Therefore, flow-induced net \(O_2^-\) production was inhibited in thick ascending limbs transduced with dnPKC-\(\alpha\), but not dnPKC-\(\beta\) or LacZ.

**DISCUSSION**

We hypothesized that flow stimulates net \(O_2^-\) production in thick ascending limbs by activating PKC-\(\alpha\), which in turn activates NADPH oxidase. To test this, we first had to confirm that flow increases net \(O_2^-\) production and that the source of \(O_2^-\) is NADPH oxidase. We found that flow stimulated net \(O_2^-\) production and that apocynin, a NADPH oxidase inhibitor, blocked this effect confirming that the source of flow-induced \(O_2^-\) is NADPH oxidase.

The use of apocynin as an NADPH oxidase inhibitor has come into question, because it has been found to act as a scavenger of reactive oxygen species at concentrations of 1 mM or greater (15). However, we used 10 \(\mu\)M apocynin, a 100 times lower concentration, in the present and previous studies. Thus, it is likely that the effect of apocynin we observed was due to inhibition of the NADPH oxidase rather than scavenging.
ing. To directly address this issue, we tested the effect of 10 µM apocynin on \( O_2^- \) produced in vitro by xanthine oxidase from the substrate hypoxanthine. We found that at this concentration, apocynin did not affect \( O_2^- \) production, indicating that it did not act as a scavenger nor an inhibitor of xanthine oxidase.

Additionally, we used a nonpharmacological approach to confirm that NADPH oxidase is involved in flow-stimulated \( O_2^- \) production: \( p47^{phox} \)-deficient mice. \( p47^{phox} \) is a regulatory subunit of NADPH oxidase and its deletion prevents \( O_2^- \) generation from NADPH oxidase. We found that flow did not change \( O_2^- \) production. Thus, taken together with the apocynin data, we conclude that luminal flow stimulates NADPH oxidase to cause an increase in \( O_2^- \) production.

There are several other sources of \( O_2^- \) in the thick ascending limb, such as mitochondria, xanthine oxidase, and uncoupled nitric oxide synthase (43). However, our data are consistent with our previous study (16) as well as others attributing \( O_2^- \) production in the renal medulla and thick ascending limb to NADPH oxidase (24, 25, 30, 47). We demonstrated that Na-K-2Cl cotransporter activation and mechanical stress stimulated NADPH oxidase-dependent \( O_2^- \) production. Li et al. (25) showed that outward flow of H\(^+\) ions activated NADPH oxidase and \( O_2^- \) production, whereas O’Connor et al. (30) observed that NADPH oxidase-dependent \( O_2^- \) production was enhanced in Dahl salt-sensitive rats.

NADPH oxidase can be activated by several pathways, including PKC. To study whether PKC is involved in flow-induced \( O_2^- \) production, we first used the FRET-based reporter MyrPalm-CKAR to measure PKC activity in the absence and presence of flow. We found that flow stimulated PKC activity. Next, we showed that in the absence of flow, the PKC activator PMA stimulated \( O_2^- \) production, indicating a PKC-dependent pathway.

To study the PKC isoform(s) involved in flow-induced \( O_2^- \) production, we utilized the PKC inhibitor Gö 6976 and dnPKCs. It appears that only PKC-\( \alpha \) is involved in flow-induced activation of NADPH oxidase and resultant increased \( O_2^- \) production. Although the thick ascending limb expresses several PKC isoforms, we did not test all of them as possible mediators of flow-stimulated \( O_2^- \) production, since we found that we could block virtually all flow-stimulated \( O_2^- \) production with Gö 6976 which is selective for PKC-\( \alpha \) and -\( \beta I \). Moreover, flow-induced \( O_2^- \) production was reduced by 83% in dnPKC\( \alpha \)-transduced thick ascending limbs, whereas it was not affected in dnPKC\( \beta I \)-transduced tubules. Taken together, our data indicate that PKC-\( \alpha \) is responsible for most if not all flow-stimulated \( O_2^- \) production. To our knowledge, this is the first study to show PKC-mediated \( O_2^- \) production in the thick ascending limb.

Recently, we reported that cellular stretch is involved in flow-stimulated \( O_2^- \) production (10). Others showed that cellular stretch activates PKC. PKC-\( \alpha \), -\( \delta \), and -\( \varepsilon \) are stimulated in cardiac myocytes subjected to various types of stretch (3). PKC-\( \alpha \) and -\( \delta \) mediate Rho GTPase and MAP kinase activation in cyclic stretch-induced hypertrophy (34). In addition, PKC-\( \alpha \) is involved in stretch-induced myogenic tone (45) and hyaluronan secretion in fibroblast-like synoviocytes (29). These findings support our data that suggest PKC plays a role in flow-stimulated \( O_2^- \) production.

Our data are consistent with other studies proposing a PKC-\( \alpha \)/NADPH oxidase/\( O_2^- \) pathway. In cell-free systems, many PKC isoforms, including PKC-\( \alpha \), phosphorylate \( p47^{phox} \), a regulatory subunit of NADPH oxidase (8). PKC-\( \alpha \)-dependent activation of NADPH oxidase via \( p47^{phox} \) phosphorylation has been demonstrated in cultured mesangial cells (41). In addition, knockdown of PKC-\( \alpha \) with siRNA blocks upregulation of NADPH oxidase in human umbilical vein endothelial cells (44). As far as we are aware, \( p47^{phox} \) is the only NADPH oxidase subunit known to be regulated by PKC.

Our findings are also consistent with studies showing that PKC activates NADPH oxidase in other types of cells. PKC-dependent NADPH oxidase-mediated oxidative burst has been characterized in phagocytes (6). A PKC/NADPH oxidase pathway has been implicated in inhibition of the Na\(^+\)/K\(^+\) pump by angiotensin II in cardiomyocytes (42) as well as in the oxidative stress associated with diabetes and hypertension in the vasculature (13, 18).

An increase in net \( O_2^- \) production may be due to enhanced production and/or reduced degradation. Therefore, our data could be interpreted as PKC inhibiting enzymes involved in degradation and a resultant net increase in \( O_2^- \) production. However, given the abundance of data that show that PKC phosphorylates \( p47^{phox} \) to activate NADPH oxidase assembly, and no evidence that PKC inhibits degradation enzymes such as superoxide dismutase, it is most likely that the role of PKC in flow-stimulated \( O_2^- \) production is as an activator of NADPH oxidase.

The experiments in the present study were performed in the absence L-arginine, the substrate for nitric oxide synthase. Under these conditions, it is possible that \( O_2^- \) could be produced from uncoupled nitric oxide synthase. However, previously we found that the nitric oxide synthase inhibitor L-NAME alone did not change net \( O_2^- \) production compared with nontreated tubules (17). These data suggest that the source of flow-stimulated \( O_2^- \) is not nitric oxide synthase.

Under physiological conditions, luminal flow stimulates both \( O_2^- \) and nitric oxide production in the thick ascending limb, creating a delicate balance between these opposing factors to maintain proper renal function. We previously showed that nitric oxide reduces flow-induced \( O_2^- \) (17). In conditions of chronic high flow or reduced nitric oxide production, the resultant increase in net \( O_2^- \) production leads to renal dysfunction and damage associated with diseases such as diabetes and hypertension.

In summary, we found that luminal flow stimulates NADPH oxidase-dependent \( O_2^- \) production by the thick ascending limb via PKC-\( \alpha \). Enhanced \( O_2^- \) in the thick ascending limb may be important in the pathogenesis of hypertension and other diseases associated with oxidative stress.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

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