Superoxide Stimulates NaCl Absorption in the Thick Ascending Limb Via Activation of Protein Kinase C

Guillermo B. Silva, Pablo A. Ortiz, Nancy J. Hong, Jeffrey L. Garvin

Abstract—Abnormal production of superoxide (O$_2^-$) contributes to hypertension, in part because of its effects on the kidney. The thick ascending limb absorbs 20% to 30% of the filtered load of NaCl. O$_2^-$ stimulates NaCl absorption by the thick ascending limb by enhancing Na$^+$/K$^+$/2Cl$^-$ cotransporter activity; however, the signaling mechanism is unknown. We hypothesized that O$_2^-$ stimulates NaCl absorption by activating protein kinase C (PKC). To test this, we measured the effect of O$_2^-$ on: (1) Cl$^-$ absorption in the presence and absence of PKC inhibitors, (2) total PKC activity, and (3) activation of specific PKC isoforms. Isolated perfused medullary thick ascending limbs were exposed to O$_2^-$ generated by xanthine oxidase (1 mU/mL) and hypoxanthine (0.5 mmol/L). O$_2^-$ increased Cl$^-$ absorption by 42% (from 76.2±3.6 to 108.2±11.9 pmol/min per millimeter; n=5; P<0.05). After treatment with the general PKC inhibitor staurosporine (10 nmol/L), O$_2^-$ did not stimulate Cl$^-$ absorption (Δ=−5.7±8.6%; n=6). In thick ascending limb suspensions, O$_2^-$ increased total PKC activity by 33% (from 66±11 to 88±12 μU/mg protein; n=5; P<0.05) and increased PKC-α and PKC-β activity by 1.75- and 0.37-fold, respectively. The PKC-α/β-selective inhibitor Go976 (100 nmol/L) blocked the ability of O$_2^-$ to stimulate Cl$^-$ absorption by isolated perfused medullary thick ascending limbs (Δ4.5±15.0%; n=5). The role of PKC-δ could not be studied because of cell necrosis caused by the selective inhibitor rotterlin. We conclude that PKC-α is required for O$_2^-$-stimulated NaCl absorption in the thick ascending limb. (Hypertension. 2006;48:467-472.)

Key Words: ion transport ■ kidney

Superoxide (O$_2^-$) is a free radical produced by the 1-electron reduction of molecular oxygen, which has been reported to increase blood pressure. O$_2^-$ has been shown to decrease urinary volume and urinary sodium excretion. These effects can be mediated by increases in tubular sodium reabsorption without changing renal blood flow and glomerular filtration rate. In the thick ascending limb, O$_2^-$ has been shown to increase sodium reabsorption. This effect is primarily because of activation of the Na$^+$/K$^+$/2Cl$^-$ cotransporter, but the signaling mechanism is unknown.

In other cell types, O$_2^-$ stimulates protein kinase C (PKC). In mesangial cells, O$_2^-$ activates PKC in response to high glucose. In the vasculature, O$_2^-$ causes vasoconstriction via activation of PKC. This effect may be used by generation of isoprostanates produced by the nonenzymatic oxidation of arachidonic acid by O$_2^-$.

In addition, in the central nervous system, many effects of O$_2^-$ are mediated by PKC.

PKC activation decreases urinary volume at least partially via its actions on sodium absorption along the nephron. In the proximal tubule, PKC increases sodium reabsorption by enhancing Na$^+$/K$^+$/ATPase activity. In the thick ascending limb, the stimulatory effect of angiotensin II on Na$^+$/K$^+$/2Cl$^-$ cotransporter activity is mediated by PKC. In addition, activation of PKC stimulates NaHCO$_3$ absorption in this segment after it has been reduced by vasopressin. PKC is a family of enzymes with at least 10 members, many of which are expressed in the thick ascending limb. However, it is unclear whether PKC is required for O$_2^-$-stimulated NaCl absorption by the thick ascending limb and which isoform is involved.

Methods

Animals

This study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee. All studies were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague–Dawley rats weighing 75 to 100 g (Charles River Breeding Laboratories, Kalamazoo, MI) were fed a diet containing 0.22% sodium and 1.1% potassium (Purina) for at least 7 days before anesthesia.

Thick Ascending Limb Isolation and Perfusion

Rats were anesthetized with ketamine (100 mg/kg body weight IP) and xylazine (20 mg/kg body weight IP). The abdominal cavity was opened, and the left kidney was bathed in ice-cold physiological saline and removed. Coronal slices were placed in HEPES-buffered physiological saline gassed with 100% O$_2$ (pH 7.40). The composi-
tion of the HEPES-buffered physiological saline was (in mmol/L): NaCl 130, NaH₂PO₄ 2.5, KCl 4, MgSO₄ 1.2, L-alanine 6, Na₂-citrate 1, glucose 5.5, Ca-lactate 2, HEPES 10. Thick ascending limbs were dissected from the medulla under a stereomicroscope to 4°C, and tubules ranging from 0.5 to 1.0 mm were transferred to a temperature-regulated chamber and perfused using concentric glass pipettes at 37±1°C. Tubules were bathed and perfused with HEPES-buffered saline. The luminal perfusion rate was 5 to 10 nl/min per millimeter, and the flow rate of the basolateral bath was 0.5 ml/min. Xanthine oxidase (1 mU/ml; Sigma-Aldrich and Calbiochem) and hypoxanthine (0.5 mmol/L; Sigma-Aldrich) were added to the bath. The enzyme and its substrate were mixed and warmed to 37°C seconds before passing into the perfusion chamber through a continuous flow system.

**Measurement of Cl⁻ Absorption**

After initial perfusion, thick ascending limbs were equilibrated for 20 minutes, and 3 to 4 measurements were made to calculate basal Cl⁻ absorption. Tubules were then treated with O₂⁻, and after a 20-minute re-equilibration period, 3 to 4 additional collections were made. Cl⁻ concentrations in the perfusate and collected fluid were measured by microfluorimetry. All of the data were recorded and stored using data acquisition software (DATAQ Instruments). Data analysis was performed with software specifically designed for voltage spike analysis. Because water is not reabsorbed by the thick ascending limb, Cl⁻ absorption (JCl⁻) was calculated as follows: JCl⁻=C.R (C₀⁻/C₋Cl⁻) where C.R is the concentration rate normalized per tubule length, C₀⁻ is the Cl⁻ concentration in the perfusion solution, and C₋Cl⁻ is the Cl⁻ concentration in the collected fluid. Control experiments showed no significant change in Cl⁻ absorption over time.

**Medullary Thick Ascending Limb Suspensions**

Medullary thick ascending limb suspensions were prepared as described previously.²¹ Briefly, kidneys were perfused retrograde via the aorta with 40 ml of HEPES-buffered physiological saline plus 0.1% collagenase A (Sigma-Aldrich) and 100 U heparin. The inner stripe of the outer medulla was cut from coronal slices of the kidney, minced, and incubated at 37°C for 30 minutes in 0.1% collagenase A, agitating and gassing with 100% O₂ every 5 minutes. Tissue was pelleted by centrifugation at 100g for 2 minutes, resuspended in cold HEPES-buffered physiological saline, and stirred on ice for 30 minutes to release the tubules. The suspension was filtered through a 250-μm nylon mesh and centrifuged at 100g for 2 minutes. The pellet was washed, centrifuged again, and resuspended in 1 ml of cold HEPES-buffered physiological saline.

**PKC Activity Assay**

Xanthine oxidase (1 mU/ml) and hypoxanthine (0.5 mmol/L) or vehicle were added to medullary thick ascending limb suspensions and incubated for 3 minutes. Catalase (100 U/ml; Oxis Research) was also added to avoid generation of hydrogen peroxide. Tubes were spun at 100g and resuspended in lysis buffer. Total PKC activity was measured using a Pierce SpinZyme colorimetric PKC assay kit.

**PKC Isoform Activation**

Suspensions of thick ascending limbs were treated with vehicle or O₂⁻ as described above. Tubules were resuspended in 250 μl of homogenization buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, and a protease inhibitor mixture; 5 μg/ml antipain, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 4 mmol/L benzamidine, 5 μg/ml chymostatin, 5 μg/ml aprotinin A, and 0.105 mol/L 4(2-aminoethyl)benzene sulfon fluoride (Sigma-Aldrich). The suspensions were homogenized using 10 manual strokes in a glass homogenizer and then sonicated on ice for 2 cycles of 20 s each at output 2 of an ultrasonic processor (W-385 sonicator, Ultrasonics). The homogenate was centrifuged at 1200g for 10 minutes at 4°C to pellet cell debris and nuclei, and the supernatant was subjected to ultracentrifugation at 100 000g for 60 minutes at 4°C. The resulting supernatant was considered the soluble fraction. Triton X-100 was added to obtain a final concentration of 0.1%. The pellet was then resuspended in homogenization buffer containing 0.1% Triton X-100 and the protease inhibitor mixture. This solution was ultracentrifuged at 100 000g for 60 minutes at 4°C, and the resulting supernatant was considered the particulate fraction.

SDS-polyacrylamide gels (5%) were loaded with equal amounts of protein from control and O₂⁻-treated soluble (8 to 20 μg) and particulate (20 to 40 μg) fractions. Proteins were separated by electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore) at 160 mA. The membrane was incubated in blocking buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, 5% nonfat dried milk, and 0.1% Tween-20 for 60 minutes and the appropriate PKC isoform-specific monoclonal antibody (BD Transduction Laboratories) in blocking buffer for 60 minutes at room temperature. The membrane was washed in a buffer containing 50 mmol/L Tris, 500 mmol/L NaCl, and 0.1% Tween-20 and incubated with a 1:1000 dilution of secondary antibody against the appropriate IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). The reaction products were detected with a chemiluminescence kit (Amersham Pharmacia Biotech). The signal was detected by exposure to Fuji RX film and quantified by densitometry. All of the PKC isoforms we studied have been shown to translocate to membrane-enriched fractions under different conditions in other cell types.²²⁻²³ A decrease in the amount of individual PKC isoforms from the cytosolic fraction and, consequently, an increase in particulate fraction was taken as activation of the enzyme. Band intensities were normalized to protein loading.

**Determination of Protein Content**

Total protein content was determined using Coomassie Plus reagent (Pierce), based on Bradford’s colorimetric method.

**Statistics**

Data are reported as mean±SEM. Differences in means were analyzed using an unpaired t test. Statistical analysis was performed by the Department of Biostatistics and Epidemiology of Henry Ford Hospital.

**Results**

O₂⁻ increases thick ascending limb sodium reabsorption.⁸⁻¹¹ In other tissues, O₂⁻ acts via PKC, and this enzyme stimulates Cl⁻ absorption in the thick ascending limb.⁹ Consequentially, we first measured the ability of PKC inhibitors to block O₂⁻-stimulated JCl⁻ in isolated perfused thick ascending limbs. O₂⁻ was produced by xanthine oxidase (1 μmol/L) and its rate was raised thick ascending limb JCl⁻ from 76.2±3.6 to 108.2±11.9 pmol/min per millimeter, a 42% increase (P<0.05; n=5; Figure 1A). In the presence of staurosporine (10 nmol/L), adding xanthine oxidase and hypoxanthine to the bath decreased JCl⁻ by 5.7±8.6% (n=6; Figure 1B). To demonstrate that the ability of staurosporine to block O₂⁻-induced stimulation of NaCl absorption was not unique, we tested whether a chemically different PKC inhibitor could block the effect of O₂⁻. In the presence of calphostin C (500 nmol/L), JCl⁻ showed no significant change (Δ4.5±15%; n=4) after O₂⁻ treatment. These data suggest that O₂⁻ stimulates thick ascending limb JCl⁻ by activating PKC.

Because PKC inhibitors blocked the effect of O₂⁻ on JCl⁻, we next measured the effect of O₂⁻ on total PKC activity. We found that basal activity in untreated tubules was 66±11 mU/mg of protein. After O₂⁻ treatment, PKC activity in-
increased by 33% to 88±12 mU/mg protein (P<0.05; n=5; Figure 2).

The PKC family is composed of several isoforms.24 To determine which isoform mediates the effect of O$_2^-$, we measured activation of individual isoforms. First we studied the effect of O$_2^-$ on PKC-$\alpha$, -$\beta$, and -$\gamma$. Under basal conditions, PKC-$\alpha$ activity was 4.8±1.1 A.U. Three minutes after O$_2^-$ treatment, it increased by 175% to 13.2±1.8 A.U. (P<0.01; n=5; Figure 3A). In contrast, we found no significant effect of O$_2^-$ on PKC-$\beta$ or -$\gamma$. Next, we measured the effect of O$_2^-$ on the novel PKC isoforms $\delta$, $\varepsilon$, $\eta$, and $\theta$. Under basal conditions, PKC-$\delta$ activity was 12.6±1.0. After 3 minutes of incubation with O$_2^-$, PKC-$\delta$ activity was 17.3±1.4 A.U, a 37% increase (P<0.05; n=5; Figure 3B). We found no significant effect of O$_2^-$ on PKC-$\varepsilon$ or -$\theta$.

Although we increased the amount of protein loading and decreased the antibody dilution, we could not find PKC-$\eta$. Finally, we tested the effect of O$_2^-$ on the atypical PKC isoforms $\lambda$, $\iota$, and $\xi$. Although they were present in the thick ascending limb, we found no increase in activity.

To elucidate which of the 2 O$_2^-$-activated PKC isoforms ($\alpha$ or $\delta$) is involved in the stimulation of sodium transport by O$_2^-$ in the thick ascending limb, we studied the effect of the PKC-$\alpha$ and -$\beta$-selective inhibitor G6976 (100 nmol/L) on $J_{\text{Cl}}$. G6976 is a selective inhibitor for PKC-$\alpha$ and -$\beta$ with IC$_{50}$s of 2.3 and 6.2 nmol/L, respectively, and has no effect on the other isoforms.25 We found that in the presence of the inhibitor, O$_2^-$ treatment had no significant effect (Δ4.5±15.0%; n=5; Figure 4). Because O$_2^-$ increased PKC-$\alpha$ and not -$\beta$, these data indicate that O$_2^-$ increases $J_{\text{Cl}}$ by activating PKC-$\alpha$ in the thick ascending limb.

Because isoprostanes mediate O$_2^-$-induced vasoconstriction of the vasculature by activating PKC,14 we tested the...
Figure 4. Effect of the PKC-α/β-selective inhibitor Gö6976 on the ability of O$_2^-$ to stimulate NaCl absorption in the thick ascending limb (n=5). O$_2^-$ was generated by adding xanthine oxidase (XO; 1 μM/mL) and hypoxanthine (HX; 0.5 mM/mL) to the bath.

Effect of the isoprostane 8-iso prostaglandin (PG)F$_2$-α on J$_{cl}$ at 3 different concentrations: 100 mM/L, 250 mM/L, and 5 μM/L. However, we found that it did not enhance, but rather inhibited, NaCl absorption. These data suggest that 8-iso-PGF$_2$-α does not mediate the effects of O$_2^-$ on PKC or NaCl reabsorption by the thick ascending limb.

Discussion
O$_2^-$ is a free radical known to stimulate sodium reabsorption by the thick ascending limb via activation of the Na$^+$/K$^+$/2Cl$^-$ cotransporter, but the mechanism involved is unknown. We found that O$_2^-$ increases NaCl absorption by the thick ascending limb via activation of PKC. Blockade of PKC with calphostin C or staurosporine inhibited O$_2^-$-stimulated NaCl absorption, and treating tubule suspensions with O$_2^-$ increased total PKC activity.

Our data are consistent with other investigators who showed that the effects of O$_2^-$ are mediated by PKC. In the proximal tubule, O$_2^-$ induces acute expression of c-fos. The PKC inhibitor staurosporine blocked the O$_2^-$-induced increase in c-fos, indicating that PKC mediates this effect. In the vasculature, treatment of pulmonary arteries with O$_2^-$ induces vasoconstriction. This effect can be abolished by PKC inhibitors, indicating that O$_2^-$ enhances the PKC signaling pathway. High-frequency stimulation of hippocampal neurons induces long-term potentiation. O$_2^-$ dismutase attenuates this effect and decreases PKC activity. These data indicate that O$_2^-$ induces long-term potentiation via PKC. In amyotrophic lateral sclerosis, a mutation in O$_2^-$ dismutase that enhances O$_2^-$ reduces glutamate metabolism and increases aspartate uptake. The effects of this mutation are also prevented by PKC inhibition.

Our data showing that PKC stimulates thick ascending limb NaCl absorption are consistent with other reports showing that this kinase is necessary for the effects of other factors that enhance transport in this segment. In medullary thick ascending limb suspensions, angiotensin II stimulates Na$^+$/K$^+$/2Cl$^-$ cotransporter activity. This effect was blocked by the PKC inhibitor staurosporine, suggesting that PKC enhances cotransporter activity. Because angiotensin II augments O$_2^-$ production in other cell types, it is possible that the ability of this hormone to stimulate Na$^+$/K$^+$/2Cl$^-$ cotransport is a result of O$_2^-$ increasing PKC activity. Insulin also increases NaCl absorption in the thick ascending limb. This effect was shown to be sensitive to staurosporine and calphostin C, suggesting that PKC is a mediator in this process. Finally, phorbol esters stimulate NaHCO$_3$ absorption in this segment. This effect can be blocked by chelerythrine or staurosporine, inhibitors of PKC, indicating that PKC is also required for this process.

There are at least 10 members of the PKC family. Consequently, we investigated which PKC isoforms were present and activated in the thick ascending limb after O$_2^-$ stimulation. We found that after treatment with O$_2^-$, PKC-α and PKC-δ activity were increased by 175% and 37%, respectively. To elucidate which isoform is responsible for O$_2^-$-stimulated NaCl absorption in the thick ascending limb, we measured NaCl absorption in the presence of Gö6976, a selective inhibitor of PKC-α and PKC-β. We found that when PKC-α and PKC-β were inhibited, O$_2^-$-stimulated NaCl absorption was blocked. Because O$_2^-$ had no effect on PKC-β activity, we conclude from these data that activation of PKC-α is required for O$_2^-$ to stimulate NaCl absorption by the thick ascending limb.

Our data concerning PKC-α do not address the role of PKC-δ. We could not examine the contribution of PKC-δ to NaCl absorption, because the only commercially available PKC-δ-selective inhibitor, rotterlin, had toxic effects at all concentrations tested. Treating isolated perfused thick ascending limbs with rotterlin caused cell necrosis and detachment of cells from the basement membrane. These data may indicate that PKC-δ is necessary for cell survival.

In the thick ascending limb, PKC-α has been shown to enhance ion transport by stimulating Na$^+$/K$^+$/2Cl$^-$ cotransporters. In microdissected medullary thick ascending limbs, PKC-α activation correlated with Na$^+$/K$^+$/2Cl$^-$ cotransporter activity and increased activity. Although these data tend to support the hypothesis that PKC-α stimulates NaCl by the thick ascending limb, as do our data, we found increased Na$^+$/K$^+$/2Cl$^-$ cotransporter activity but no increase in Na$^+$/K$^+$/2Cl$^-$ cotransport activity after treatment with O$_2^-$.

Our data suggest that PKC-α may directly affect the Na$^+$/K$^+$/2Cl$^-$ cotransporter rather than the Na pump.

Our data showing that PKC-α and possibly -δ are involved in the actions of O$_2^-$ are similar to reports for other tissues. High-frequency field stimuli induce PKC-α translocation to the membrane in hippocampal neurons. After stimulation, PKC-α translocation could be inhibited by O$_2^-$ dismutase mimetics and enhanced with O$_2^-$ dismutase inhibitors, indicating the involvement of O$_2^-$ in this process. In mesangial cells, high doses of glucose stimulated PKC-δ activity. Activation of PKC-δ was suppressed by dipheneleuroidioidum, an inhibitor of NADPH oxidase, suggesting that this effect was caused by increases in O$_2^-$ production. In proximal tubule cells, angiotensin II has been shown to induce activation of PKC-α, but no particular mechanism was studied. This phenomenon may have been caused by O$_2^-$, because chronic angiotensin II induces O$_2^-$ production in this nephron segment.

8-iso-PGF$_2$-α has been proposed to mediate at least some of the effects of O$_2^-$ in addition, 8-iso-PGF$_2$-α induces vasoconstriction via activation of PKC. Consequently, we
tested the effect of 8-iso-PGF2-α on NaCl absorption. Contrary to their hypothesis, transport was inhibited. These data suggest that the effects of O2− on NaCl absorption are not mediated by 8-iso-PGF2-α. Thus, the question remains as to how O2− activates PKC in the thick ascending limb.

If O2− could diffuse across the plasma membrane, it could directly activate PKC by thiol oxidation. In hippocampal homogenates, O2− has been shown to directly increase PKC-α activity by cysteine oxidation of the cysteine-rich domain and release of zinc from the zinc-finger region. However, such a mechanism remains speculative for activation of PKC in the thick ascending limb and raises the additional question of how O2− generated in the extracellular compartment could have such an effect.

In conclusion, we found that O2− stimulates NaCl absorption in the thick ascending limb by enhancing PKC-α. Activation of PKC-α and augmentation of NaCl absorption by the thick ascending limb are not caused by generation of 8-iso-PGF2-α.

Perspectives

In the present study, we demonstrated that activation of PKC-α is required for O2−-stimulated NaCl absorption in the thick ascending limb. Oxidative stress can cause36,37 and may be the result38,39 of several forms of hypertension. The actions of O2− on thick ascending limb NaCl absorption may initiate or contribute to the sodium retention associated with increased blood pressure. PKC-α is activated by O2− in the thick ascending limb and may also enhance O2− production via NADPH oxidase by stimulating phosphorylation of p47phox and assembly of the enzyme complex40 by stimulation of a secondary signaling cascade involving Src, phosphatidylinositol 3-kinase, and Rac. As these authors have proposed, this leads to the possibility of the development of a vicious cycle in which a small increase in blood pressure begets an increase in Na absorption, and this, in turn, leads to a larger increase in blood pressure.

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Disclosures

None.

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