Oxalate-induced activation of PKC-α and -δ regulates NADPH oxidase-mediated oxidative injury in renal tubular epithelial cells

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NADPH oxidase is the most important source of receptor-mediated ROS generation in nonphagocytic cells (43). Once thought to be present only in neutrophils (36), it has since been found in diverse sites including the kidney (20, 36). NADPH oxidase activity is significantly elevated in atherosclerotic lesions, leading to increased superoxide production (29). Our own studies as well as those of Umekawa et al. (51–65) have demonstrated that NADPH oxidase-mediated ROS generation plays a major role in injury of renal epithelial cells. Protein kinase C (PKC) is a critical component of intracellular signal transduction pathways and has been implicated in homeostasis, migration, proliferation, apoptosis, remodeling of the actin cytoskeleton, and modulation of ion channels (5, 25, 46). PKC is a family of serine/threonine kinases that includes at least 12 known isoforms, in turn grouped into 3 subfamilies based on differences in sequence homology and cofactor requirements (44). While activation of PKC has been associated with increased production of superoxide anions in phagocytes as well as vascular cells (17, 37), the cellular mechanisms that integrate these signaling events in response to oxalate toxicity in renal tubular epithelial cells are not well understood.

It is clear from our previous in vitro and in vivo studies (62, 63) that oxalate-induced free radical injury is involved in nucleation and aggregation of calcium oxalate and resultant development of kidney stones. We have now investigated the mechanisms by which oxalate induces production of ROS and leads to peroxidative injury via PKC signaling in renal epithelial cells. Our results clearly demonstrate that PKC-α and -δ play a role in oxalate-induced ROS production via activation of NADPH oxidase followed by peroxidative injury in renal epithelial cells and suggest that oxalate-mediated PKC signaling is one of the underlying molecular mechanism involved in the development of calcium oxalate kidney stones.

MATERIALS AND METHODS

Cell culture. Serial cultures of LLC-PK₁ cells of proximal tubular origin (CRL 1392, ATCC, Rockville, MD) were maintained as subconfluent monolayers in 75-cm² Falcon T-flasks in DMEM containing 10% fetal bovine serum, streptomycin (0.20 mg/l), and penicillin (1.0 × 10⁶ IU/ml), pH 7.4, at 37°C in a 5% carbon dioxide-95% air atmosphere. Confluent monolayers of LLC-PK₁ cells were used, and experiments were carried out with serum-free, pyruvate-free DMEM. Oxalate was prepared as we described previously (62). Briefly, a stock solution of 10 mM sodium oxalate was prepared in normal sterile PBS and diluted to 0.75 mM in defined medium.

Experiments with PKC and NADPH oxidase inhibitors. Thirty minutes before the addition of 0.75 mM oxalate, LLC-PK₁ cells were
treated with the PKC inhibitor calphostin C (50 nm-1 µM), G60976 (5–20 µM), and chelerythrine chloride (1–5 µM), a PKC-α selective inhibitor, inhibitor peptide (1–10 µg/ml), the PKC-8 selective inhibitor rottlerin (1–20 µM), or the NADPH oxidase inhibitors DPI (0.5 µM) and apocynin (0.5 mM). Cells treated with oxalate along with inhibitors for various time periods were examined as described below.

**Light microscopy.** Calcium oxalate crystal formation was monitored continuously using a Leica DM IRB inverted microscope.

**Transfection of LLC-PK1 cells with a PKC-α small interfering RNA.** LLC-PK1 cells were transfected with a PKC-α stealth small interfering RNA (siRNA). 5'-UUGUUCACAAGAGGGGUCAUGAGUU/AACCUAGGGACCUCUUGUGAACCA-3' directed toward the PKC-α mRNA target, 5'-GGUUCACAAGAGGGGUCAUGAGUU-3' (Invitrogen Life Technology, Carlsbad, CA) using Lipofectamine 2000 according to the manufacturer’s protocol ( Gibco, Gaithersburg, MD). Cells were transfected with a nonsilencing stealth siRNA duplex (control stealth siRNA, Invitrogen) (3, 38), using nontransfected cells as a second negative control (mock). Oxalate experiments were carried out 48 h after transfection. We confirmed target gene silencing by the PKC-α siRNA using Western blotting. Knockdown of PKC-α expression was determined by densitometry of the PKC-α band relative to its GAPDH loading control and comparison to cells transfected with the nonsilencing siRNA control.

**Subcellular fractionation and Western blotting.** At the end of the experiments, cells were harvested and resuspended in hypotonic lysis buffer with 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin and incubated for 30 min on ice. Cytosolic and membrane particulate fractions of the lysates were isolated using a FractionPREP cell fractionation system (BioVision, Mountain View, CA) following the manufacturer’s instructions. Protein content in each fraction was measured using a BCA protein assay kit (Pierce, Rockford, IL). We estimated the degree of contamination using tracking enzymes, measuring GAPDH in the cytosolic fraction and Na⁺-K⁺-ATPase in the membrane fraction. As expected, contamination was low, comprising 3–10% of total protein expression (GAPDH: membrane 10%, cytosol 90%; Na⁺-K⁺-ATPase: membrane 97%, cytosol 3%), confirming earlier studies (23, 47). Accordingly, relative amounts of PKC in the membrane fraction were corrected for cytotoxic contamination using densitometric analysis of Na⁺-K⁺-ATPase and GAPDH.

Equal protein aliquots of cytosol and membrane fractions were subjected to SDS-PAGE, and the separated proteins were transferred to a nitrocellulose membrane. Blots were probed with antibodies specific for PKC isoenzymes (Cell Signaling Technology, Boston, MA; Santa Cruz Biotechnology, Santa Cruz, CA). Specific reactive bands were detected using goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with horseradish peroxidases. The immunoreactive bands were visualized using an enhanced chemiluminescence Western blot detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ) and analyzed by densitometric scanning using Kodak imaging software. After stripping, the membranes were reprobed with antibodies to GPDH (cytosolic marker, Bioscience International, Saco, ME) or Na⁺-K⁺-ATPase (membrane marker, Sigma-Aldrich, St. Louis, MO) to ensure equal protein loading.

**Determination of PKC activity.** PKC activity in the cytosolic and membrane fractions was measured using a nonradioactive ELISA that utilizes a synthetic PKC pseudosubstrate and a monoclonal antibody that recognizes the phosphorylated form of the peptide, according to the manufacturer’s instructions (Calbiochem, EMB Biosciences, San Diego, CA). Protein concentration was determined with a BCA assay kit. PKC activity was expressed as optical density (OD) per milligram protein, and the data were normalized to control.

**Determination of NADPH oxidase activity.** NADPH oxidase activity was determined using an assay based on the chemiluminescence of lucigenin (bis-N-methylacridinium nitrate; CL) as described previously (22). Briefly, control cultures or cultures exposed to oxalate with or without inhibitors were washed with 5 ml ice-cold PBS and scraped from the plate into 5 ml of the same solution. Samples were transferred to a 50-ml tube and centrifuged at 750 g for 10 min at 4°C. The pellet was resuspended (0.5–1.0 ml/dish) in lysis buffer containing protease inhibitors (20 mM monobasic potassium phosphate, pH 7.0, 1 mM EGTA, 10 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was then disrupted using a dounce homogenizer on ice, and the homogenate was stored on ice until use. Protein content was measured in a homogenate aliquot by Lowry’s method (39), and NADPH oxidase activity was assessed by luminescence assay in 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 500 µM lucigenin as the electron acceptor, and 100 µM NADPH as the substrate. Enzyme activity was expressed as nanomoles superoxide produced per minute per milligram protein, and the data were normalized to control. To confirm the validity of the CL method, specific NADPH oxidase activity was also measured by SOD-inhibitable cytochrome c reduction using NADPH as a substrate and expressed as nanomoles superoxide produced per minute per milligram protein (58).

**Determination of apoptosis.** Apoptosis was detected using an ELISA Plus cell death detection kit (Roche Applied Science, Indianapolis, IN). This technique is based on a quantitative sandwich enzyme immunoassay that allows specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates, using mouse monoclonal antibodies directed against DNA and histones. Nucleosome enrichment was quantified based on absorbance at 405 nm. ODs in the treated samples were normalized to control.

**Determination of superoxide anions.** At the end of the experiments, intracellular superoxide anions were measured by a nitroblue tetrazolium (NBT) reduction assay as we described previously (60). The amount of reduced NBT was determined based on absorbance at 630 nm. Values were expressed as OD at 630 nm, and ODs in the treated samples were normalized to control.

**Determination of H₂O₂ release.** Hydrogen peroxide in the medium was measured with an assay kit according to the manufacturer’s instructions (Assay Designs, Ann Arbor, MI). This assay is based on the reaction of xylene orange with sorbitol and ammonium iron sulfate in an acidic solution, producing a purple color proportional to the concentration of H₂O₂ in the medium. The reaction product was quantified at 550 nm and expressed as micromolar H₂O₂ released. H₂O₂ production in treated cells was normalized to control.

**Determination of LDH release.** Cellular injury was assessed by release of lactic dehydrogenase (LDH). The medium from control and the experiment was centrifuged to remove crystals and cellular debris. LDH activity was determined using a commercial kit (Roche Diagnostics). All determinations were made against appropriate reagent blanks. The reaction product was read at 490 nm and expressed as percent release. Values in treated samples were normalized to control.

**Determination of lipid hydroperoxide.** Cells were harvested in HPLC-grade water. Lipid hydroperoxide (LHP) was assayed immediately after sonication, according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). Briefly, cells were extracted with 0.5 ml Extract-R-saturated methanol and vortexed for 15 s; 1 ml deoxygenated chloroform was added to each test tube, vortexed for 15 s, and the mixture was centrifuged at 1,500 g for 5 min at 0°C. The lower chloroform phase was transferred to clean tubes and stored on ice. LHP was prepared, and the chromogenic reaction was assayed according to the manufacturer’s protocol. After color development, the samples were pipetted into a 96-well glass plate and absorbance was measured at 492 nm. Values were expressed as nanomoles LHP formed per milligram protein, and the experimental data were normalized to control.

**Statistical analysis.** All data are expressed as means ± SE. Data were analyzed by ANOVA followed by Tukey’s multiple comparisons test. Student’s t-test was used for comparison between the two groups. A P value of < 0.05 was considered significant.
RESULTS

Inhibition of PKC activation attenuates oxalate-induced ROS production and cell injury. We determined the effects of PKC inhibitors on oxalate-induced ROS production and LDH release by exposing LLC-PK₁ cells to 0.75 mM oxalate for 3 h in the presence or absence of a PKC inhibitor, specifically, calphostin C (100 nM) or chelerythrine chloride (5 μM). Following 3-h exposure to oxalate, there was a significant increase in superoxide (Fig. 1A) and hydrogen peroxide (Fig. 1B) compared with control. Both calphostin C and chelerythrine chloride significantly attenuated this increase, indicating that PKC activation is required for oxalate-mediated ROS production.

The effect of PKC inhibitors on oxalate-induced cell injury was examined by measuring LDH release into the medium (Fig. 1C). Following exposure to oxalate for 3 h, there was a significant increase in LDH release by oxalate-treated cells compared with control (2.18 ± 0.10-fold increase; n = 6; P < 0.05). In the presence of a PKC inhibitor, LDH release was significantly lower compared with cells exposed to oxalate for 3 h (calphostin C: 1.32 ± 0.03-fold increase; chelerythrine chloride: 1.45 ± 0.06-fold increase; n = 6; P < 0.05). Thus oxalate toxicity is dependent on PKC activation.

Effect of oxalate on subcellular distribution of PKC isoenzymes. We tested whether oxalate exposure activates PKC in LLC-PK₁ cells by determining PKC activity using ELISA. Under control conditions, PKC activity was found mainly in the cytosolic compartment and translocated to the membrane compartment upon activation, but total PKC activity in the homogenate remained unchanged. This shows that the increase in the ratio of membrane to cytosol activity accurately reflects activation of PKC (11). PKC activity from cytosolic and particulate membrane fractions was quantified after oxalate treatment to LLC-PK₁ cells for 3 h. Oxalate exposure significantly increased PKC activity in the particulate fraction (control: 0.305 ± 0.06; oxalate: 0.657 ± 0.06; n = 6; P < 0.05), whereas activity was decreased in the cytosol (control: 1.0 ± 0.1; oxalate: 0.78 ± 0.03; n = 6; P < 0.05) (Fig. 2A).

Since PKC activity was increased in the membrane fraction, we further quantified the subcellular distribution of the PKC isoenzymes in control cells and cells exposed to oxalate. PKC distribution in the particulate fraction was examined in oxalate-treated cells using Western blotting. We observed significant translocation of PKC-α and -δ to the membrane fraction in oxalate-treated cells (Fig. 2B); however, the membrane distribution of other PKC isoenzymes remained unaltered (Fig. 2C). PMA, a known activator of PKC isoenzymes that translocates PKC from the cytosol to the membrane, was used as a positive control for PKC activation. Figure 2D suggests that PMA and oxalate differ markedly in their ability to translocate PKC. While both PMA and oxalate induced significant redistribution of PKC-α and -δ to the plasma membrane, PMA translocated PKC-ε and -β₁ to the membrane whereas oxalate did not. Thus oxalate has a unique profile of PKC isoenzyme activation, inducing free radical-mediated injury in LLC-PK₁ cells (Fig. 2D).

Effect of PKC-α- and -δ-specific inhibitors on oxalate-induced ROS generation and cell injury. Since oxalate activated PKC-α and -δ in LLC-PK₁ cells, we next studied whether activation of PKC-α or -δ plays a role in oxalate-induced ROS production using isoenzyme-specific inhibitors. To determine the optimum concentration of inhibitors of PKC-α (“inhibitor peptide” and G06976) and PKC-δ (rotterlin), LLC-PK₁ cells were exposed to increasing concentrations of the inhibitor for 30 min and then coincubated with 0.75 mM oxalate for 3 h, measuring superoxide generation. Inhibitor peptide at 1–10 μg/ml significantly decreased superoxide generation in a concentration-dependent manner (P < 0.05; n = 6) (Fig. 3A). Similar effects were observed with another PKC-α inhibitor, G06976 (data not shown). Similarly, the...
PKC-δ inhibitor rolleterin (2.5–10 μM) had a concentration-dependent effect on cells exposed to oxalate for 3 h, resulting in a significant decrease in superoxide production ($P < 0.05; n = 6$). At higher concentrations, rolleterin (10 and 20 μM) induced superoxide production both at baseline and following oxalate treatment (Fig. 3B).

PKC-α and -δ are involved in superoxide production in LLC-PK1 cells following oxalate exposure. Studies were carried out using PKC-α and -δ-selective inhibitors. Treatment with 0.75 mM oxalate significantly increased LHP whereas rolleterin or inhibitor peptide pretreatment significantly inhibited LHP formation following oxalate exposure (Fig. 3C).

Effect of PKC-α and -δ inhibitors on induction of apoptosis and necrosis following oxalate treatment. Apoptosis was quantified after exposure of LLC-PK1 cells to oxalate, using an ELISA that measures histone-associated DNA fragmentation (Fig. 5). In the early stages of cell death, endogenous endo-
nucleases cleave double-stranded DNA at the most accessible internucleosome-linked regions, generating mono- and oligo-nucleosomes. We found that apoptosis of LLC-PK1 cells increased following oxalate exposure, as evidenced by accumulation of nucleosomal DNA. In the presence of a PKC-α/H9251 or -δ/H9254 inhibitor, enrichment of nucleosomal DNA was significantly lower compared with oxalate exposure for 3 h. Oxalate also induced necrosis as indicated by increased release of LDH (Fig. 1C). Thus our results demonstrated that renal epithelial cells can undergo both apoptosis and necrosis following oxalate exposure.

Effects of downregulating PKC-α expression on oxalate-induced ROS production and cell injury. The effectiveness of siRNA transfection in reducing PKC-α expression was determined using semiquantitative Western blotting. We found that by 48 h after transfection, the siRNA had significantly reduced PKC-α protein expression (70%) compared with cells transfected with a nonsilencing siRNA or mock (Fig. 6A; n = 4; P < 0.05). To determine the possible off-target effect of the PKC-α siRNA sequences, we probed the same blot with a PKC-δ antibody but found that reduction of PKC-α protein expression by siRNA transfection did not reduce PKC-δ protein expression (Fig. 6A).

To further examine the ability of PKC-α-downregulated cells to generate hydrogen peroxide upon oxalate challenge, we exposed cells to oxalate for 3 h and measured hydrogen peroxide in the medium. Cells transfected with the nonsilencing siRNA (control stealth siRNA) showed a significant increase in hydrogen peroxide generation (Fig. 6B) and LDH release (Fig. 6C) following oxalate exposure. When cells transfected with the PKC-α stealth siRNA were exposed to oxalate for 3 h, both hydrogen peroxide and LDH were significantly decreased compared with cells transfected with the nonsilencing siRNA.

Since PKC-δ siRNA transcripts of porcine origin (LLC-PK1 cells) were not available in GenBank, we used a PKC-δ siRNA which is specific to the human PKC-δ transcript. Although an antibody specific to the human PKC-δ transcript recognized PKC-δ of porcine origin (LLC-PK1 cells), the siRNA targeted to human PKC-δ did not reduce PKC-δ protein expression in LLC-PK1 cells (data not shown). Consistent with our findings, other studies have shown that a siRNA targeted to mammalian

Fig. 3. Effect of PKC-α- or -δ-specific inhibitors on oxalate-induced ROS production and cell injury. A and B: both the PKC-α inhibitor inhibitor peptide (A) and the PKC-δ inhibitor rottlerin (B) attenuated oxalate-induced superoxide production. LLC-PK1 cells were pretreated with different concentrations of inhibitor peptide (1–10 μg/ml) or rottlerin (2–5–10 μM) for 30 min and then treated with 0.75 mM oxalate along with inhibitor for 3 h, after which superoxide production was determined. C and D: effect of PKC-α and -δ inhibitors on oxalate-induced H_2O_2 production (C) and LDH release (D). LLC-PK1 cells were treated with PKC-α (2.5 μg/ml) or -δ inhibitor (7.5 μM) for 30 min and then exposed to 0.75 mm oxalate along with inhibitors for 3 h, after which H_2O_2 and LDH were determined. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. Comparisons shown: a, significant compared with vehicle-treated control; b, significant compared with oxalate; c, significant compared with rottlerin-treated control; d, significant compared with inhibitor peptide-treated control. *P < 0.05; n = 6.
PKC-δ does not suppress PKC-δ protein expression in cells derived from rats (27). Therefore, we were unable to perform oxalate experiments with PKC-δ siRNA. While the data described here confirm the role of PKC-α in oxalate-induced oxidative cell injury, the fact that the PKC-δ inhibitor attenuated oxalate-induced ROS and cell injury suggests that the role of PKC-δ in oxalate toxicity cannot be disregarded.

In addition, LLC-PK1 cells transfected with a PKC-α siRNA or a nonsilencing siRNA were treated with oxalate for 3 h, fractionated, and protein expression was determined by Western blotting. Significant accumulation of PKC-α was observed in the membrane fraction of the cells transfected with the nonsilencing siRNA. PKC-α siRNA transfection significantly decreased PKC-α protein expression in the cytosol. Although oxalate translocated PKC-α to the membrane fraction in the cells transfected with the PKC-α siRNA (Fig. 6D), the level of expression in the membrane fraction was not sufficient to induce free radical production or LDH release compared with oxalate treatment (Fig. 6, B and C).

Effect of inhibitors on oxalate-induced membrane translocation of PKC isoenzymes. Given that PKC-α or -δ inhibitors attenuated oxalate-induced ROS and cell injury, we questioned whether this effect was due to inhibition of PKC-α or -δ translocation from the cytosol to the membrane, which is necessary for PKC function. This supposition proved to be correct (Figs. 7, A and B), as oxalate treatment significantly increased PKC-α and -δ protein expression in the membrane fraction. Inhibitor peptide and rottlerin significantly reduced PKC-α and -δ protein expression, respectively, in the cells exposed to oxalate.

Oxalate-induced ROS formation and cell injury are dependent on NADPH oxidase activity. Exposure of LLC-PK1 cells to oxalate for 3 h significantly increased production of hydrogen peroxide (Fig. 8A) and LDH release (Fig. 8B). Treatment with a NADPH oxidase inhibitor, either 0.5 μM DPI or 0.5 mM apocynin, significantly blocked oxalate-induced hydrogen peroxide formation or LDH release, suggesting that NADPH oxidase is involved in oxalate-mediated ROS generation.

Effect of PKC-α and -δ-selective inhibitors on oxalate-induced NADPH oxidase activity. Although we used DPI and apocynin to demonstrate that oxalate-induced activation of NADPH oxidase is required for ROS generation, a recent review indicates these are not specific inhibitors (1). Therefore, as an alternate approach, we used PKC-α- and -δ-specific inhibitors to demonstrate the crucial role of NADPH oxidase in oxalate-induced oxidative injury by measuring enzyme activity, as adding exogenous NADPH to assess NADPH oxidase activity has been performed by several groups. We also assessed NADPH oxidase activity in cell homogenates by SOD-inhibitable cytochrome c reduction assay. Using this method, NADPH oxidase-dependent superoxide production in LLC-PK1 cells exposed to oxalate was still significantly increased compared with control (data not shown). Comparison of lucigenin-amplified CL and cytochrome c reduction showed excellent correlation. NADPH oxidase activity was significantly increased in LLC-PK1 cells treated with oxalate for 3 h (Fig. 9). To investigate the potential role of PKC-α and -δ in regulating NADPH oxidase activity, we examined the effects of selective inhibitors of PKC-α and -δ. As shown in Fig. 9, inhibitor peptide and rottlerin significantly blocked the oxalate-induced increase in NADPH oxidase activity, indicating that PKC-α and -δ are the isoenzymes involved in activation of oxalate-induced NADPH oxidase. NADPH oxidase activity was also significantly abolished by DPI or apocynin in cells treated with or without oxalate.

Oxalate-induced PKC activation leading to NADPH oxidase-mediated ROS production and cell injury. Since activation of PKC by oxalate induced ROS production in LLC-PK1 cells, we also tested whether ROS formation is mediated via PKC-dependent NADPH oxidase activation, using PMA, a known activator of PKC, as a positive control. Adding PMA increased ROS production (Fig. 10A) or LDH release (Fig. 10B) in the absence of DPI. However, ROS levels and LDH release...
remained unchanged when cells were first treated with DPI and then exposed to PMA, indicating that activation of PKC is required for NADPH oxidase-mediated ROS generation or LDH release. Similarly, when NADPH oxidase was inhibited with DPI and oxalate was added, ROS and LDH were reduced while PKC activity increased (Fig. 10C). These results suggest that oxalate-induced ROS production and cell injury involve PKC-mediated activation of NADPH oxidase.

Next, we examined whether NADPH oxidase activity is required for oxalate-induced PKC activation. As shown in Fig. 10C, PKC activity in the membrane fraction increased significantly when the cells were treated with oxalate, but when we...
inhibited NADPH oxidase with DPI and exposed the cells to oxalate PKC activity remained increased, indicating that PKC activation was upstream from NADPH oxidase. Even though PKC activity was increased by oxalate in the DPI-treated cells, ROS levels and LDH release remained low (Fig. 10, A–D). Moreover, inhibitor peptide (PKC-α inhibitor) and rottlerin (PKC-δ inhibitor) suppressed oxalate-induced NADPH oxidase activity (Fig. 9), ROS levels, and cell injury (Fig. 3, A–D). Taken together, these data indicate that oxalate-induced PKC activation is required for NADPH oxidase activity.

**DISCUSSION**

The new findings of this study address the signaling mechanism upstream from NADPH oxidase-mediated oxidative injury and demonstrate that oxalate increases ROS production and peroxidative injury in renal epithelial cells via PKC-α- and -δ-mediated activation of NADPH oxidase. We found that 1) oxalate increases PKC activity, translocates PKC-α and -δ to the membrane particulate fraction, and increases NADPH oxidase activity, superoxide, and H₂O₂ generation, apoptosis, and necrosis, and peroxidative injury in renal epithelial cells; 2) inhibition of PKC-α or -δ decreases oxalate-induced NADPH oxidase activity, superoxide, H₂O₂, apoptosis, and necrosis, and peroxidative injury; and 3) treatment with DPI or apocynin prevented oxalate-induced ROS generation and cell injury, indicating that oxalate-induced activation of PKC-α and -δ plays a significant role in NADPH oxidase-mediated peroxidative injury in renal epithelial cells. Peroxidative injury stemming from the oxalate-induced activation

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**Fig. 7.** A and B: effect of PKC isoenzyme-specific inhibitors on oxalate-induced membrane translocation of PKC-α (A) and PKC-δ (B) protein expression in LLC-PK₁ cells. LLC-PK₁ cells were pretreated with inhibitor peptide (2.5 μg/ml) or rottlerin (7.5 μM) for 30 min and then exposed to 0.75 mM oxalate along with inhibitors for 3 h. Lysates of the membrane fractions were analyzed for PKC-α (A) and PKC-δ (B) expression by Western blotting. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. A typical blot from 1 of 3 experiments is shown. The graph represents the ratio of densitometric analysis of PKC-α or PKC-δ protein expression to Na⁺/K⁺-ATPase. Comparisons shown: a, significant compared with vehicle-treated control; b, significant compared with oxalate-treated cells; c, significant compared with inhibitor peptide- or rottlerin-treated cells. *P < 0.05; n = 3.

**Fig. 8.** A and B: effect of DPI and apocynin on oxalate-induced H₂O₂ generation (A) and LDH release (B). LLC-PK₁ cells were pretreated with 0.5 μM DPI or 0.5 mM apocynin for 30 min and then exposed to oxalate along with inhibitors for 3 h, after which H₂O₂ production and LDH release were determined. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. Comparisons shown: a, significant compared with control; b, significant compared with oxalate; c, significant compared with DPI-treated control; d, significant compared with apocynin-treated control. *P < 0.05; n = 6.
PKC signaling might result in calcium oxalate nucleation and aggregation and lead to development of kidney stones.

Oxalate may cause precipitation of calcium oxalate, especially when inhibitors of calcium oxalate crystallization are low. When we treated LLC-PK1 cells with oxalate and monitored them for 30 min, we did not find calcium oxalate crystals under high-power magnification; however, superoxide production (data not shown), translocation of PKC-α, and PKC-δ were increased. After 1 h, we detected a few calcium oxalate crystals, while superoxide continued to increase. Finally, by 2–3 h we observed significant crystal accumulation along with further increases in superoxide production and cell injury. Clearly, when oxalate-induced free radical generation overwhelms cellular defense mechanisms, cells become damaged, as we reported previously (61). Our study reveals that oxalate itself can initiate ROS generation and peroxidative injury to renal tubular cells.

Concentration-dependent oxalate studies involving renal cell cultures have shown that many birefringent calcium oxalate crystals are formed in 1 h at oxalate concentrations of 1.0 mM or greater (4). It has been shown that both oxalate and calcium oxalate crystals independently increase free radical generation in a time- and concentration-dependent manner (8, 21, 56) and that oxalate alone increases oxidative cell injury while calcium oxalate crystals potentiate injury (60). There is evidence that exposure to elevated levels of oxalate leads to progressive as well as dose- and time-dependent changes in the morphology and viability of LLC-PK1 and HK2 cells. Morphological changes accompanying oxalate treatment of renal epithelial cells led to an increase in both cytoplasmic vacuolization and pyknotic nuclei as well as the appearance of dysmorphic cells and condensation and disintegration of the nuclei (41, 55).

Intracellular reactive oxidants are generated by both enzymatic and nonenzymatic sources, including the mitochondrial electron transport system (9), NADPH oxidase (35), 5-lipoxygenase (52), several oxidases located in subcellular compartments such as peroxidases and mono- and dioxygenases, and isoenzymes of the cytochrome P-450 superfamily, including nitric oxide synthase (67), xanthine oxidase (50), and cyclooxygenase (52). Because PKC is a critical component of

![Graph](image-url)

**Fig. 9.** Effect of PKC isoenzyme-specific inhibitors and NADPH oxidase inhibitors on oxalate-induced NADPH oxidase activity in LLC-PK1 cells. LLC-PK1 cells were pretreated with 0.5 μM DPI, 0.5 mM apocynin, 7.5 μM rottlerin or 2.5 μg/ml inhibitor peptide for 30 min and then exposed to 0.75 mM oxalate along with inhibitors for 3 h, after which NADPH oxidase activity was determined. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. Comparisons shown: a, significant compared with control; b, significant compared with oxalate-treated; c, significant compared with inhibitor peptide-treated. *P < 0.05; n = 4.

![Graph](image-url)

**Fig. 10.** Effect of the NADPH oxidase inhibitor DPI on oxalate- or PMA-induced H2O2 production, LDH release, and PKC activity. A and B: LLC-PK1 cells were pretreated with 0.5 μM DPI for 30 min and then exposed to 0.75 mM oxalate or 0.75 mM PMA along with DPI for 3 h, after which H2O2 production (A) and LDH release (B) were determined. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. Comparisons shown: a, significant compared with control; b, significant compared with oxalate-treated; c, significant compared with PMA-treated; d, significant compared with DPI-treated control. *P < 0.05; n = 6. C: effect of DPI on oxalate-induced PKC activity in the membrane fraction. LLC-PK1 cells were pretreated with 0.5 μM DPI for 30 min and then exposed to 0.75 mM oxalate along with DPI for 3 h, after which PKC activity was measured. DMSO was used as a vehicle. Data are normalized to control, and values are expressed as means ± SE. Comparisons shown: a, significant compared with control; b, significant compared with DPI-treated control. *P < 0.05; n = 3.
intracellular signal transduction pathways, the studies presented here were designed to extend our earlier work on oxalate-induced NADPH oxidase activation (51). We still need to evaluate the effect of oxalate exposure on other free radical sources.

A number of specific pharmacological PKC inhibitors have been synthesized and used to study the role of PKC in signaling. In the present study, oxalate-induced ROS generation in renal epithelial cells was effectively inhibited by the PKC inhibitors calphostin C and chelerythrine chloride, suggesting that oxalate-induced ROS generation is PKC dependent. Many renal diseases are known to be associated with activation of the PKC pathway, and consistent with our findings PMA-, ANG-, and glucose-stimulated ROS generation in BAECs were all reportedly inhibited by calphostin C and chelerythrine chloride (40). Increased production of ROS by PKC activation has also been demonstrated in mononuclear cells in patients with multiple sclerosis (68), endothelial cells treated with cyclic strain (13), and mesangial cells treated with high glucose (34).

We found that oxalate, a potent inducer of free radicals, also triggers cytosol-to-membrane translocation of PKC-α and -δ but not other PKC isoenzymes (δ, ε, θ, η, ι, β1, ε1, ε2, and μ). Chang and Beezhold (10) have shown that cytosol-to-membrane translocation of isoenzymes reflects their enzymic activity and that PMA-triggered cytosol-to-membrane translocation of PKC-α is accompanied by a shift of PKC enzymatic activity from the cytosol to the membrane. As a positive control, we confirmed our findings using PMA, which also induces free radical formation and triggers translocation of PKC isoenzymes. Different PKC isoenzymes have been shown to be involved in diverse responses of various types of cells (59). For example, in LLC-PK1 cells PKC involvement has been demonstrated in tight junction barrier function, H2O2-induced cell injury, regulation of sodium metabolism, and cell growth, although each function is regulated by a different PKC isoenzyme(s) (18, 45, 49, 53). Blockade of activation and translocation of PKC by PKC inhibitors reduces 4-hydroxy-2-nonenal-induced ROS generation in airway smooth muscle cells (57). In addition to the classic PKCs and PKC-δ, researchers have reported that PKC-ε and PKC-ζ also play a role in oxidative stress in different types of cells (28, 59). However, in our study neither PKC-ε nor PKC-ζ was altered by oxalate treatment.

Although PKC inhibitors such as calphostin C and chelerythrine chloride have been shown to inhibit oxalate-induced ROS generation and cell injury, they are not specific for PKC isoenzymes. To determine PKC isoenzyme specificity in oxalate-induced ROS generation, we used the PKC-α and -δ-specific blockers, inhibitor peptide and rottlerin, respectively. PKC-δ is reportedly a key signaling molecule in the ROS-induced apoptotic pathway through generation of active catalytic fragments by proteolytic cleavage (19), and consistent with our findings rottlerin also inhibited agonist-induced translocation of PKC-δ in rat skeletal muscle and glutathione-depleted neuroblastoma cells (6, 14). PKC-δ has also been shown to be important for a number of other cell functions such as stimulation of the Na+/K+ antiporter in C8 glioma cells (12).

G06976 is a potent inhibitor of the Ca2+/-dependent PKC isoenzymes α, β1, βII, and γ (42). Consistent with our findings, reduction of ROS generation by G06976 has been correlated with KCN-induced LDH release in PC12 cells (26). When we used specific inhibitors of PKC-α and a PKC-α siRNA to evaluate the role of PKC-α in oxalate-induced oxidative stress, we found that both inhibitor peptide and PKC-α siRNA transfection significantly reduced oxalate-induced ROS production and cell injury. Adenoviral overexpression of a dominant negative mutant PKC-α has also been shown to modulate oxidative stress-mediated signaling pathways in cardiac myocytes by decreasing superoxide production (16). Furthermore, inhibition of PKC-α activity by either siRNA transfection or overexpression of a dominant negative mutant decreased nitric oxide production in primary aortic endothelial cells (48). Our results convincingly demonstrate the essential role of PKC-α and -δ in oxalate-induced ROS production and cell injury.

Not only oxalate-induced PKC activation but also ROS-mediated cell injury was mimicked by PMA, a PKC activator, in renal epithelial cells. The present findings also demonstrate that PMA-induced ROS production and cell injury were prevented by the NADPH oxidase inhibitors DPI and apocynin, providing further evidence that NADPH oxidase-dependent ROS generation requires PKC activation in LLC-PK1 cells. NADPH oxidase is accepted as the most important mechanism of receptor-stimulated ROS generation in nonphagocytic cells (43). Furthermore, recent studies have suggested that PKCs activate NADPH oxidase by phosphorylating the NADPH oxidase subunit p47Phox, and PKC-β was shown to increase NADPH oxidase activity in diabetic glomeruli and HL60 cells (15, 33). PKC-δ has also been implicated as a regulator of NADPH oxidase and appears to be required for assembly of this complex enzyme’s components (7).

In conclusion, we believe this is the first demonstration that the mechanism of oxalate-induced free radical production involves activation of NADPH oxidase in renal tubular cells and is mediated by PKC signaling. We also provide evidence that among the PKC isoenzymes studied, PKC-α and -δ play a particularly important role in oxalate-induced free radical production. These findings strongly suggest that PKC-dependent activation of NADPH oxidase may be one of the essential mechanisms responsible for peroxidative cell injury in hyperoxaluria. As a result of oxalate exposure, the injured renal tubular membrane plays a significant role in calcium oxalate adhesion, aggregation, and growth of kidney stones. These novel findings support PKC signaling as a significant therapeutic target in the prevention of renal tubular calcium oxalate adhesion and retention in recurrent stone formers.

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REFERENCES

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