Urol Res (2012) 40:415–423
DOI 10.1007/s00240-011-0405-7

Selective Rac1 inhibition protects renal tubular epithelial cells from oxalate-induced NADPH oxidase-mediated oxidative cell injury

Vijayalakshmi Thamilselvan · Mani Menon · Sivagnanam Thamilselvan

Received: 23 June 2011 / Accepted: 14 July 2011 / Published online: 4 August 2011

Abstract Oxalate-induced oxidative cell injury is one of the major mechanisms implicated in calcium oxalate nucleation, aggregation and growth of kidney stones. We previously demonstrated that oxalate-induced NADPH oxidase-derived free radicals play a significant role in renal injury. Since NADPH oxidase activation requires several regulatory proteins, the primary goal of this study was to characterize the role of Rac GTPase in oxalate-induced NADPH oxidase-mediated oxidative injury in renal epithelial cells. Our results show that oxalate significantly increased membrane translocation of Rac1 and NADPH oxidase activity of renal epithelial cells in a time-dependent manner. We found that NSC23766, a selective inhibitor of Rac1, blocked oxalate-induced membrane translocation of Rac1 and NADPH oxidase activity. In the absence of Rac1 inhibitor, oxalate exposure significantly increased hydrogen peroxide formation and LDH release in renal epithelial cells. In contrast, Rac1 inhibitor pretreatment, significantly decreased oxalate-induced hydrogen peroxide production and LDH release. Furthermore, PKC α and δ inhibitor, oxalate exposure did not increase Rac1 protein translocation, suggesting that PKC resides upstream from Rac1 in the pathway that regulates NADPH oxidase. In conclusion, our data demonstrate for the first time that Rac1-dependent activation of NADPH oxidase might be a crucial mechanism responsible for oxalate-induced oxidative renal cell injury. These findings suggest that Rac1 signaling plays a key role in oxalate-induced renal injury, and may serve as a potential therapeutic target to prevent calcium oxalate crystal deposition in stone formers and reduce recurrence.

Keywords NADPH oxidase · Rac1 · Oxalate · Calcium oxalate · Free radical · Kidney stone

V. Thamilselvan · M. Menon · S. Thamilselvan (✉)
Vattikuti Urology Institute, Suite 2D-34, Henry Ford Health System, One Ford Place, Detroit, MI 48202, USA
e-mail: STHAMIL1@HFHS.ORG

Introduction

Oxalate is an end product of glycolate metabolism that is primarily excreted by the kidney and is the most common constituent of kidney stones. Hyperoxaluria is one of the major risk factors for kidney stone formation and approximately 70–80% of kidney stones are composed of calcium oxalate crystals [1]. Since, cell injury is the predisposing factor for calcium oxalate crystal nucleation, aggregation, and stone formation, several studies have shown that oxalate-induced free radical generation leads to oxidative cell injury in renal epithelial cells in culture and in the kidney of hyperoxaluria-induced rats [2–4]. In addition, oxalate exposure alters DNA synthesis, changes cell morphology, induces immediate early gene, redistributes phosphatidylserine to the surface of the cell membrane, lowers viability, reduces antioxidative enzymes and induces apoptosis in renal cells [5–10].

Although a variety of cellular sources of reactive oxygen species (ROS) have been demonstrated, NADPH oxidase has been shown to modulate redox status of the kidney during renal diseases [11]. However, the potential role of NADPH oxidase in hyperoxaluria-induced kidney stone formation is not well known until recently. We were the first to demonstrate in 2004 that oxalate induces ROS generation through the activation of NADPH oxidase, which plays a major role in renal proximal tubular injury [12]. Following completion of our study, Umekawa et al

[13] demonstrated in 2005 that involvement of NADPH oxidase in oxalate and calcium oxalate monohydrate crystal induced ROS generation in rat kidney epithelial cells. Since then, research has been focused on controlling the NADPH oxidase-mediated cell injury to prevent hyperoxaluria-induced kidney stone formation [14–18]. The NADPH oxidase is a multicomponent enzyme complex that consists of the membrane-bound cytochrome b558, which contains gp91phox and p22phox, the cytosolic regulatory subunits p47phox and p67phox, and the small guanosine triphosphate-binding protein Rac. On stimulation, the cytosolic subunits translocate to the membrane and associate with cytochrome b558, resulting in activation of the NADPH oxidase [19]. Formation and activation of NADPH oxidase allow electrons to be passed from the cofactor NADPH to molecular oxygen, producing superoxide radicals [20]. In view of the fact that, NADPH oxidase activity is noticeably increased in renal cells exposed to oxalate, focusing on mechanisms leading to NADPH oxidase activation could unveil further molecular details involved in oxalate-induced renal injury.

Rac1, a small G protein, is a signaling molecule that coordinates the intracellular transduction pathways which activate NADPH oxidase [21]. Once activated, Rac1 migrates from the cytosol to the plasma membrane where its attachment favors assembly of the various NADPH oxidase subunits [22, 23]. While many investigations, including recent animal models, have implicated Rac1 as a central mediator in cardiac and vascular hypertrophy and leukocyte migration [24–27], its role in oxalate-induced renal cell injury is not known. We previously showed that oxalate induces oxidative injury via PKC alpha and delta-mediated activation of NADPH oxidase in renal proximal tubular epithelial cells [15]. However, no direct evidence is available on how NADPH oxidase is activated by oxalate in renal tubular epithelial cells. To determine the signaling component downstream of PKC that regulate NADPH oxidase activation, we focused on Rac1. We determined the impact of Rac1 on oxalate-induced NADPH oxidase activation, ROS generation; and investigated the role of Rac1 in oxalate-induced cell injury in renal epithelial cells.

Materials and methods

Materials

DMEM was purchased from Invitrogen (Gaithersburg, MD) Lucigenin, NADPH, and the anti-Na/K-ATPase antibody was obtained from Sigma (St. Louis, MO). NSC23766 and rottlerin from EMD (Gibbstown, NJ). PKC α inhibitor peptide and anti-Rac1 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Cultures of LLC-PK1 cells, an epithelial cell line from pig kidney with properties of proximal tubular cells (CRL 1392, ATCC, Rockville, MD) were maintained as sub confluent monolayers in 75-cm^2 Falcon T-flasks in DMEM containing 10% fetal bovine serum, streptomycin (0.20 mg/ml) and penicillin (1.0×10^2 IU/ml), pH 7.4, at 37°C in a 5% CO_2–95% air atmosphere. Experiments were carried out with serum- and pyruvate-free MEM. Oxalate was prepared as a stock solution of 10 mM sodium oxalate in normal sterile PBS and diluting it to 0.75 mM in the medium [15].

Inhibitor and oxalate treatments

Thirty minutes before the addition of 0.75 mM oxalate, confluent monolayers of LLC-PK1 cells were exposed to a PKCα-selective inhibitor (2.5 μg/ml inhibitor peptide), a PKCδ-selective inhibitor (7.5 μM rottlerin), a Rac1 inhibitor (50 μM NSC23766). Control cells were treated with vehicle (0.1% DMSO). The cells treated with or without oxalate along with inhibitors for various time periods were used for the assays as described below.

LDH assay

At the end of the experimental period, lactate dehydrogenase (LDH) was measured in the medium using a kit from Roche Diagnostics (Indianapolis, IN) [15]. All determinations were made against appropriate reagent blanks. The reaction product was read at 490 nm and expressed as percent release. The values of treated samples were normalized to the untreated controls.

H_2O_2 assay

Hydrogen peroxide in the medium was measured with a kit from Assay Designs (Ann Arbor, MI) [15]. This assay is based on the reaction of xylenol orange with sorbitol and ammonium iron sulfate in an acidic solution, producing a purple color proportional to the concentration of H_2O_2 in the medium. The reaction product was quantified at 550 nm and expressed as micromolar H_2O_2 released. The values of treated cells were normalized to control.

NADPH oxidase assay

NADPH oxidase activity was determined using an assay based on the chemiluminescence of lucigenin (bis-*N*-methylacridinium nitrate; CL) as described in our previous studies [15]. 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 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 [28], 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.

Sub cellular fractionation and Western blot

At the end of the experimental period, cells were resuspended in hypotonic lysis buffer with 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, incubated for 30 min on ice, and cytosolic and membrane fractions isolated as we described previously [15]. Equal amounts of membrane protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk and incubated with an anti-Rac1 antibody followed by a horseradish peroxidase-conjugated secondary antibody at room temperature. The blots were washed with Tris-buffered saline and 0.1% Tween-20. Immunoreactive bands were visualized with an enhanced chemiluminescence Western blot kit (GE Healthcare Bio-Sciences, Piscataway, NJ) and analyzed with a densitometer using Kodak imaging software. Finally, the membranes were reprobed for Na^+/K^+-ATPase as a loading control for the membrane fraction.

Statistical analysis

Results are expressed as mean ± SE. Student's *t* test was used to evaluate differences between treated and untreated cells using Sigma-Stat software, taking $p < 0.05$ as significant.

Results

Oxalate induces NADPH oxidase activity in renal epithelial cells

As NADPH oxidases are a major source of ROS in renal epithelial cells, we examined the effect of oxalate on

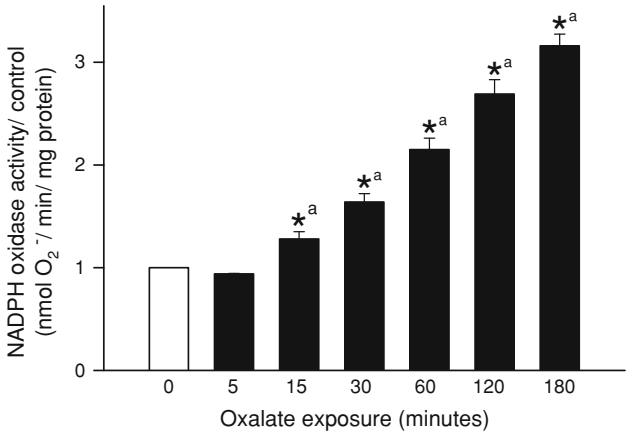

Fig. 1 Oxalate time dependently increases NADPH oxidase activity in LLC-PK1 cells. LLC-PK1 cells were treated with or without 0.75 mM oxalate for different time periods. NADPH oxidase activity was determined as described in "Materials and methods". Data are normalized to control, and values are expressed as mean ± SE. Comparisons shown: *a* significant compared with control. $*p < 0.05$; $n = 6$

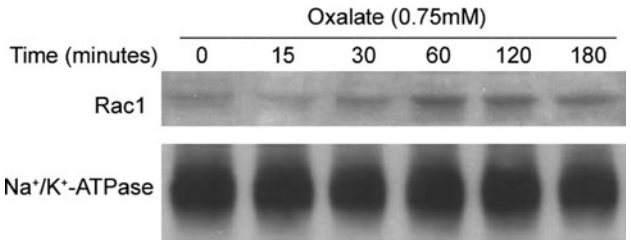

Fig. 2 Oxalate time dependently activates Rac1 in LLC-PK1 cells. LLC-PK1 cells were treated with or without 0.75 mM oxalate for different time periods. Lysates of membrane factions were analyzed for Rac1 expression by Western blotting. A typical western blot from one of three experiments was shown. Na^+/K^+-ATPase was used as a membrane loading control

NADPH oxidase activity in LLC-PK1, a renal epithelial cells. Oxalate (0.75 mM) significantly increased NADPH oxidase activity in renal epithelial cells in a time-dependent manner (15–180 min) (Fig. 1; $n = 6$; $p < 0.05$). A significant increase was observed as early as 15 min and sustained for 180 min compared with control cells.

Oxalate increases membrane-associated Rac1 protein expression

Since Rac1 regulates superoxide generation in many cell types, we first tested whether oxalate induces Rac1 activation in cultured renal proximal tubule cells. We have analyzed Rac1 protein expression levels in the membrane fraction, because studies have shown that Rac1 is normally found in the cytosolic compartment, and translocated to the plasma membrane upon activation [22, 23]. LLC-PK1 cells were exposed to 0.75 mM oxalate for 15 to 180 min, and a

membrane-associated Rac1 was assessed by Western analysis. As shown in Fig. 2, oxalate increased membrane-associated Rac1 protein in a time-dependent manner ($n = 3$).

Inhibition of Rac1 attenuates oxalate-induced ROS production and cell injury

To test whether Rac1 activation is required for oxalate-induced ROS generation, we measured hydrogen peroxide generation in the presence and absence of the Rac1 selective inhibitor, NSC23766 [29]. We found that, in the absence of a Rac1 inhibitor, oxalate exposure at a concentration of 0.75 mM for 3 h significantly increased hydrogen peroxide generation compared with vehicle-treated control cells.

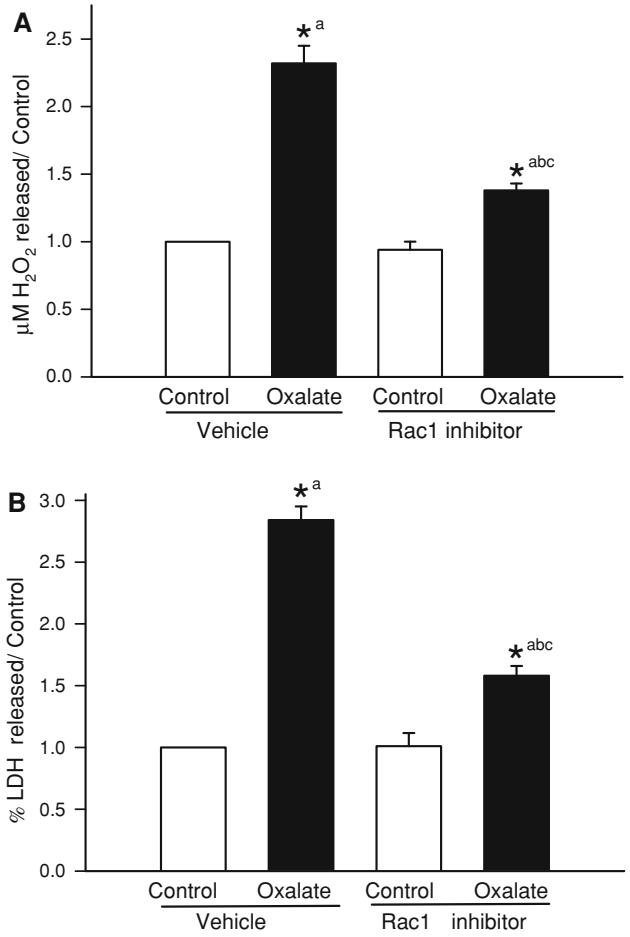

Fig. 3 Inhibition of Rac1 activation inhibits oxalate-induced reactive oxygen species production and cell injury in renal epithelial cells. LLC-PK1 cells were pretreated with a Rac1 inhibitor, NSC23766 (50 μM), for 30 min and then treated with or without 0.75 mM oxalate along with inhibitors for 3 h. Hydrogen peroxide production, and LDH release as a marker of cell injury were determined. DMSO was used as a vehicle control. Data are normalized to control, and values are expressed as mean ± SE. Comparisons shown: *a* significant compared with DMSO treated control, *b* significant compared with oxalate, *c* significant compared with inhibitor-treated control. *$p < 0.05$; $n = 6$

This oxalate induced increases in H_2O_2 generation was markedly attenuated in cells treated with the Rac1 specific inhibitor, NSC23766 (Fig. 3a; $n = 6$; $p < 0.05$).

To investigate the role of Rac1 in oxalate-induced cell injury in cultured proximal tubular epithelial cells, we assessed LDH release in the media. As shown in Fig. 3b oxalate exposure at a concentration of 0.75 mM for 3 h, significantly increased LDH release in LLC-PK1 cells compared with vehicle-treated control cells. In contrast, oxalate-induced increase in LDH release was significantly decreased in cells treated with the Rac1 inhibitor ($n = 6$; $p < 0.05$). The data indicate that activation of Rac1 is required for oxalate-induced cell injury via NADPH oxidase-mediated ROS generation in LLC-PK1 cells.

Effect of NSC23766 on Rac1 translocation

As Rac1 protein expression level was increased in the membrane fraction of oxalate exposed cells, we next demonstrated the specificity of NSC23766 on the inhibition of Rac1 translocation from cytosol to membrane in cells treated with oxalate. In the absence of Rac1 inhibitor, oxalate exposure at a concentration of 0.75 mM for 3 h, increased Rac1 protein expression levels in the membrane fraction. In contrast, in the presence of Rac1 inhibitor, oxalate exposure at a concentration of 0.75 mM for 3 h did not increase Rac1 protein expression levels in the membrane fraction. The data suggest that the membrane translocation of Rac1 was effectively blocked by the Rac1 inhibitor, NSC23766 (Fig. 4; $n = 3$).

Role of Rac1 in oxalate-induced NADPH oxidase activation

The activity of NADPH oxidase was determined in LLC-PK1 cells treated with oxalate in the absence or presence of

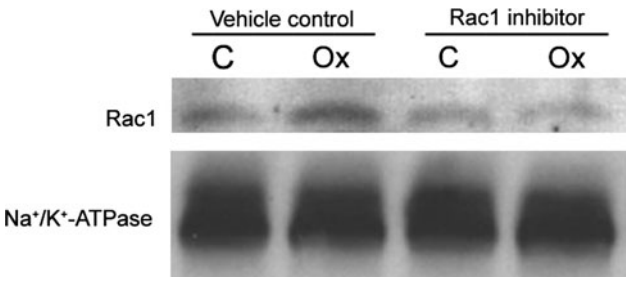

Fig. 4 NSC23766 inhibits oxalate-induced Rac1 translocation in renal epithelial cells. LLC-PK1 cells were pretreated with a Rac1 inhibitor, NSC23766 (50 μM) for 30 min and then treated with 0.75 mM oxalate along with inhibitor for 3 h. Lysates of membrane factions were analyzed for Rac1 expression by Western blotting. DMSO was used as a vehicle. A typical western blot from one of three experiments was shown. Na^+/K^+-ATPase was used as a membrane loading control

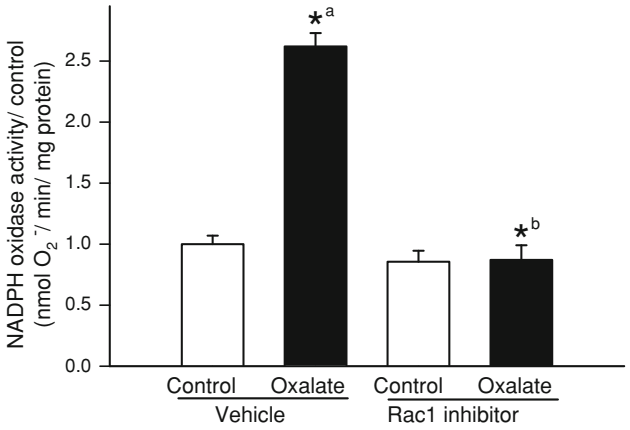

Fig. 5 Inhibition of Rac1 activation inhibits oxalate-induced NADPH activity in renal epithelial cells. LLC-PK1 cells were pretreated with a Rac1 inhibitor, NSC23766 (50 μM) for 30 min and then treated with or without 0.75 mM oxalate for 3 h. NADPH oxidase activity was determined as described in "Materials and methods". DMSO was used as a vehicle. Data are normalized to control, and values are expressed as mean ± SE. Comparisons shown: *a* significant compared with DMSO treated control, *b* significant compared with oxalate (*$p < 0.05$; $n = 6$)

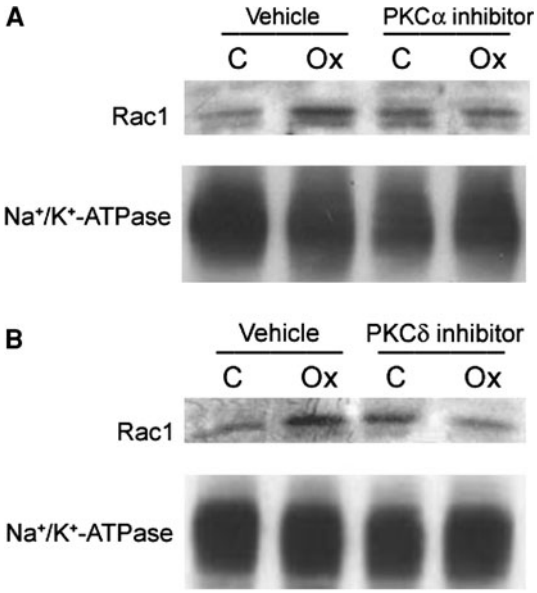

Fig. 6 Inhibition of PKC α and δ blocks oxalate-induced Rac1 activation in renal epithelial cells. LLC-PK1 cells were pretreated with a PKC α inhibitor, inhibitor peptide (2.5 μg/ml) or PKC delta inhibitor, rottlerin (7.5 μM) for 30 min and then treated with 0.75 mM oxalate for 3 h. Lysates of membrane factions were analyzed for Rac1 expression by Western blotting. A typical western blot from one of three experiments was shown. Na^+/K^+-ATPase was used as a membrane loading control

Rac inhibitor, NSC23766. As shown in Fig. 5, oxalate exposure at a concentration of 0.75 mM for 3 h significantly increased the NADPH oxidase specific activity in the homogenates of renal epithelial cells. In the presence of NSC23766, oxalate-induced increase in NADPH oxidase activity was completely blocked. These results support the hypothesis that oxalate-stimulated ROS production in renal tubular cells involves a Rac1-dependent NADPH oxidase activation ($n = 6$; $p < 0.05$).

Oxalate induces NADPH oxidase activation via PKC-dependent Rac1 signaling

Given that the inhibition of PKC α and δ attenuated oxalate-induced NADPH oxidase-mediated ROS and cell injury was shown in our previous studies [15], we questioned whether this effect was due to the inhibition of small GTP-binding protein Rac1. Therefore, we examined the effect of PKC α and δ inhibitors on Rac1 protein expression in the membrane fraction of LLC-PK1 cells. As shown in Fig. 6a and b, in the absence of PKC α and δ inhibitors, oxalate exposure at a concentration of 0.75 mM for 3 h significantly increased the Rac1 protein expression levels in the membrane fraction of LLC-PK1 cells. In contrast, in the presence of PKC α and δ inhibitors, oxalate exposure did not increase Rac1 protein expression levels. These results demonstrate that oxalate induces ROS production in renal epithelial cells via a PKC-dependent activation of Rac1 and Rac1-mediated activation of NADPH oxidase.

Discussion

Our previous studies indicated that oxalate significantly increases NADPH oxidase activity via PKC signaling pathway, which stimulates superoxide production and resultant injury in renal epithelial cells [15]. In the present study, we performed a series of experiments to explore the mechanism by which oxalate enhances NADPH oxidase activity in these cells. The major findings of this study was that (1) oxalate stimulates NADPH oxidase activity in renal epithelial cells; (2) exposing renal epithelial cells to oxalate increases membrane translocation of Rac1; (3) inhibition of Rac1 signaling attenuates oxalate-induced Rac1 translocation, NADPH oxidase activity, ROS production and cell injury; and (4) in particular, blockade of PKC signaling by PKC α and δ inhibitors attenuates Rac1 activation in oxalate-treated cells suggesting that PKC resides upstream of Rac1 in the pathway that regulates NADPH oxidase. We believe this is the first demonstration that Rac1 signaling plays a crucial role in oxalate-induced NADPH oxidase-mediated renal tubular cell injury.

The role of oxidative stress in kidney stone formation has received increasing attention in recent years [30, 31]. We have already shown that oxalate-induced oxidative injury is a major promoter of calcium oxalate crystal attachment to renal tubules [4, 15, 32]. Integrity of the

renal epithelium is necessary to maintain normal kidney function, which includes secretion and reabsorption of various solutes. However, when oxidation products greatly overwhelm the capacity of endogenous cellular antioxidants, ionic homeostasis across the renal cell membrane is disrupted, increasing membrane permeability to ions including oxalate and calcium ions, and crystals formed which can bind to the damaged renal tubular membrane [32]. Several studies have shown that oxalate and calcium oxalate crystals acting independently increase free radical injury in a concentration (0.5–2 mM) and time-dependent manner [15, 33–35]. However, the pathophysiologically relevant source of increased ROS production in hyperoxaluria remains to be further characterized.

The NADPH oxidase is a multicomponent enzyme complex, and has been proved important in the pathogenesis of renal damage. Moreover, activation of NADPH oxidase contributes to increased ROS generation in the kidney of diabetic, hypertensive and Dahl salt-sensitive rats [11, 36]. Studies also have shown that the component proteins for a NADPH oxidase system, including p47phox, p67phox and Rac1, were found in the kidney [37, 38]. An important step for the assembly and function of this multicomponent NADPH oxidase complex is the heterodimerization of gp91phox with p67phox, which is mediated by Rac [39].

The small G protein Rac is the one of the major adapter protein [40, 41] which regulate oxidase activity. Three isoforms of Rac have been identified to date. Rac1 is ubiquitously expressed, while Rac2 is primarily expressed by hematopoietic cells [42, 43] and Rac3 by the brain, nervous system and mammary glands [44, 45]. Rac1 regulates gene expression, cell cycle progression, cell spreading, rearrangement of the actin cytoskeleton, and activation of nonphagocytic NADPH oxidase has been implicated in ROS generation [46–48]. Studies have reported that Rac1 is required to anchor cytosolic p67phox to the membrane for the assembly of active NADPH oxidase protein complex, leading to superoxide generation [49]. Consistent with our findings, other studies have shown increased ROS production mediated by Rac1-regulated NADPH oxidase in a variety of disease conditions including hypoxia/reoxygenation [50], elevated shear stress [51] and cyclosporine-A treatment [52].

The nature and mechanism of action of the active NADPH oxidase complex formed have not yet been defined in stone forming condition. Rac must bind GTP to promote superoxide formation and undergo regulated cycles of GTP binding, which is mediated by a guanine nucleotide exchange factor [21, 53–56]. Rac must be in a GTP-bound form for oxidase activation to occur and GTP binding to Rac may precede the translocation event [53, 57, 58]. Rac dissociates from guanosine diphosphate dissociation inhibitor (GDI), allowing GTP-bound Rac to translocate to the plasma membrane [59, 60]. At the plasma membrane, Rac in the GTP-bound state directly interacts with p67phox via binding to the N-terminal domain that harbors tetratricopeptide repeat (TPR) motifs [61, 62], and thus the Rac-p67phox complex supports NADPH oxidase activity, leading to superoxide production [63, 64].

Since activated Rac1 migrates from the cytosol to the plasma membrane [59], we tested whether oxalate increases NADPH oxidase activity by stimulating this process. The increased membrane translocation of Rac1 accompanies enhanced NADPH oxidase activity following oxalate exposure suggesting that the sequence of molecular events leading to ROS generation in oxalate toxicity involves Rac1 translocation. Consistent with our findings, membrane translocation of Rac1, an important event of NADPH oxidase activation, has been reported in a variety of cells, including macrophages, kidney, vascular smooth muscle, and endothelium [65–68].

The selective Rac1 blockade we achieved with NSC23766 showed that the treated cells were protected against oxidative stress and cell injury, leading us to conclude that Rac1 signaling plays a crucial role in oxalate-induced ROS-mediated renal cell injury. Moreover, Rac1 inhibitor fails to activate NADPH oxidase by inhibiting the Rac1 translocation from cytosol to membrane thereby prevented oxalate-induced ROS production in renal epithelial cells. It was reported that a higher NADPH-oxidase-dependent superoxide generation was present in renal cortex and outer medulla than in the papilla [69]. We have shown oxalate exposure significantly increased NADPH oxidase activity in the renal proximal tubular cells. Consistent with our findings others have shown that NADPH oxidase is abundantly localized in proximal convoluted tubule cells in kidney [38]. Therefore, our results indicate that Rac1-dependent NADPH oxidase is a major source of oxalate-stimulated ROS production in renal proximal tubular cells, and deregulation of NADPH oxidase in the proximal tubule may be involved in the pathogenesis of hyperoxaluria-induced kidney stone formation.

In our previous studies, when NADPH oxidase was inhibited by DPI or apocynin, oxalate-induced superoxide and H_2O_2 production was eliminated [15]. However, the present study establishes a sequential link between oxalate-induced Rac1 activation and NADPH oxidase-mediated increase in intracellular ROS production. PKC α and δ isoforms have been linked to Rac1, because inhibiting them suppressed oxalate-mediated Rac1 signaling. Our observation that the inhibitors prevented PKC from inducing translocation of Rac1 and interaction between Rac1 and NADPH oxidase in renal cells exposed to oxalate supports the view that PKC acts upstream of Rac1 in this system and together with our previous evidence [15] suggests that

oxalate could induce renal oxidative stress via a PKC/Rac1 signaling pathway.

In conclusion, the present study demonstrates that oxalate increases ROS production in renal epithelial cells via Rac1-regulated NADPH oxidase activation and suggesting that Rac1 plays a key role in oxalate-mediated oxidative renal cell injury. The injury as a result of oxalate exposure plays a significant role in calcium oxalate adhesion, aggregation, and growth of kidney stones. Inhibition of Rac1 results in decreased ROS production and a reduction in cell injury. In light of our results, we suggest that Rac1-mediated oxidative stress could be a potential therapeutic target to prevent renal injury in calcium oxalate kidney stone formers and reduce recurrence rates. However, further studies will be needed in animal models to better define this possibility.

Acknowledgment This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant NIH RO1 DK056249. This work was orally presented at the 11th International Symposium on Urolithiasis, Nice, France, September 2–5, 2008 and published in abstract form [Urol Res 36(3–4): 203, 2008].

References

1. Kaufman DW, Kelly JP, Curhan GC, Anderson TE, Dretler SP, Preminger GM, Cave DR (2008) Oxalobacter formigenes may reduce the risk of calcium oxalate kidney stones. J Am Soc Nephrol 19:1197–1203
2. Thamilselvan S, Byer KJ, Hackett RL, Khan SR (2000) Free radical scavengers, catalase and superoxide dismutase provide protection from oxalate-associated injury to LLC-PK1 and MDCK cells. J Urol 164:224–229
3. Thamilselvan S, Hackett RL, Khan SR (1997) Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. J Urol 157:1059–1063
4. Thamilselvan S, Menon M (2005) Vitamin E therapy prevents hyperoxaluria-induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status. BJU Int 96:117–126
5. Maroni PD, Koul S, Chandhoke PS, Meacham RB, Koul HK (2005) Oxalate toxicity in cultured mouse inner medullary collecting duct cells. J Urol 174:757–760
6. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J, Honeyman T, Kennington L, Kohli R, Hodapp J, Ayvazian P, Menon M (1996) Oxalate toxicity in LLC-PK1 cells, a line of renal epithelial cells. J Urol 155:1112–1116
7. Khan SR, Byer KJ, Thamilselvan S, Hackett RL, McCormack WT, Benson NA, Vaughn KL, Erdos GW (1999) Crystal–cell interaction and apoptosis in oxalate-associated injury of renal epithelial cells. J Am Soc Nephrol 10(Suppl 14):S457–S463
8. Wiessner JH, Hasegawa AT, Hung LY, Mandel NS (1999) Oxalate-induced exposure of phosphatidylserine on the surface of renal epithelial cells in culture. J Am Soc Nephrol 10(Suppl 14): S441–S445
9. Jonassen JA, Cooney R, Kennington L, Gravel K, Honeyman T, Scheid CR (1999) Oxalate-induced changes in the viability and growth of human renal epithelial cells. J Am Soc Nephrol 10(Suppl 14):S446–S451
10. Koul H, Kennington L, Nair G, Honeyman T, Menon M, Scheid C (1994) Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells. Biochem Biophys Res Commun 205:1632–1637
11. Peixoto EB, Pessoa BS, Biswas SK, Lopes de Faria JB (2009) Antioxidant SOD mimetic prevents NADPH oxidase-induced oxidative stress and renal damage in the early stage of experimental diabetes and hypertension. Am J Nephrol 29:309–318
12. Rashed T, Menon M, Thamilselvan S (2004) Molecular mechanism of oxalate-induced free radical production and glutathione redox imbalance in renal epithelial cells: effect of antioxidants. Am J Nephrol 24:557–568
13. Umekawa T, Byer K, Uemura H, Khan SR (2005) Diphenyleneiodium (DPI) reduces oxalate ion- and calcium oxalate monohydrate and brushite crystal-induced upregulation of MCP-1 in NRK 52E cells. Nephrol Dial Transplant 20:870–878
14. Moriyama MT, Miyazawa K, Noda K, Oka M, Tanaka M, Suzuki K (2007) Reduction in oxalate-induced renal tubular epithelial cell injury by an extract from Quercus salicina Blume/Quercus stenophylla Makino. Urol Res 35:295–300
15. Thamilselvan V, Menon M, Thamilselvan S (2009) Oxalate-induced activation of PKC-alpha and -delta regulates NADPH oxidase-mediated oxidative injury in renal tubular epithelial cells. Am J Physiol Renal Physiol 297:F1399–F1410
16. Yoshioka I, Tsujihata M, Akanae W, Nonomura N, Okuyama A (2011) Angiotensin type-1 receptor blocker candesartan inhibits calcium oxalate crystal deposition in ethylene glycol-treated rat kidneys. Urology 77:1007.e9–1007.e14
17. Tsujihata M, Yoshioka I, Tsujimura A, Nonomura N, Okuyama A (2011) Why does atorvastatin inhibit renal crystal retention? Urol Res. doi:10.1007/s00240-011-0370-1
18. Zuo J, Khan A, Glenton PA, Khan SR (2011) Effect of NADPH oxidase inhibition on the expression of kidney injury molecule and calcium oxalate crystal deposition in hydroxy-L-proline-induced hyperoxaluria in the male Sprague-Dawley rats. Nephrol Dial Transplant 26:1785–1796
19. DeLeo FR, Quinn MT (1996) Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. J Leukoc Biol 60:677–691
20. Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313
21. Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW (1991) Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature 353:668–670
22. Diekmann D, Abo A, Johnston C, Segal AW, Hall A (1994) Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. Science 265:531–533
23. Kinsella BT, Erdman RA, Maltese WA (1991) Carboxyl-terminal isoprenylation of ras-related GTP-binding proteins encoded by rac1, rac2, and ralA. J Biol Chem 266:9786–9794
24. Sussman MA, Welch S, Walker A, Klevitsky R, Hewett TE, Price RL, Schaefer E, Yager K (2000) Altered focal adhesion regulation correlates with cardiomyopathy in mice expressing constitutively active rac1. J Clin Invest 105:875–886
25. Li C, Hu Y, Mayr M, Xu Q (1999) Cyclic strain stress-induced mitogen-activated protein kinase (MAPK) phosphatase 1 expression in vascular smooth muscle cells is regulated by Ras/Rac-MAPK pathways. J Biol Chem 274:25273–25280
26. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK (2002) Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. Circ Res 91:406–413
27. Jones GE, Allen WE, Ridley AJ (1998) The Rho GTPases in macrophage motility and chemotaxis. Cell Adhes Commun 6:237–245

28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
29. Hable WE, Reddy S, Julien L (2008) The Rac1 inhibitor, NSC23766, depolarizes adhesive secretion, endomembrane cycling, and tip growth in the fucoid alga, *Silvetia compressa*. Planta 227:991–1000
30. Khan SR (2005) Hyperoxaluria-induced oxidative stress and antioxidants for renal protection. Urol Res 33:349–357
31. Ilbey YO, Ozbek E, Simsek A, Cekmen M, Somay A, Tasci AI (2009) Effects of pomegranate juice on hyperoxaluria-induced oxidative stress in the rat kidneys. Ren Fail 31:522–531
32. Thamilselvan S, Khan SR, Menon M (2003) Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. Urol Res 31:3–9
33. Byer K, Khan SR (2005) Citrate provides protection against oxalate and calcium oxalate crystal induced oxidative damage to renal epithelium. J Urol 173:640–646
34. Greene EL, Farell G, Yu S, Matthews T, Kumar V, Lieske JC (2005) Renal cell adaptation to oxalate. Urol Res 33:340–348
35. Scheid C, Koul H, Hill WA, Luber-Narod J, Kennington L, Honeyman T, Jonassen J, Menon M (1996) Oxalate toxicity in LLC-PK1 cells: role of free radicals. Kidney Int 49:413–419
36. Tojo A, Onozato ML, Kobayashi N, Goto A, Matsuoka H, Fujita T (2002) Angiotensin II and oxidative stress in Dahl Salt-sensitive rat with heart failure. Hypertension 40:834–839
37. Chabrashvili T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, Welch WJ, Wilcox CS (2002) Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. Hypertension 39:269–274
38. Geiszt M, Kopp JB, Varnai P, Leto TL (2000) Identification of renox, an NAD(P)H oxidase in kidney. Proc Natl Acad Sci USA 97:8010–8014
39. Babior BM, Lambeth JD, Nauseef W (2002) The neutrophil NADPH oxidase. Arch Biochem Biophys 397:342–344
40. Bokoch GM, Zhao T (2006) Regulation of the phagocyte NADPH oxidase by Rac GTPase. Antioxid Redox Signal 8:1533–1548
41. Bokoch GM, Diebold BA (2002) Current molecular models for NADPH oxidase regulation by Rac GTPase. Blood 100:2692–2696
42. Gu Y, Jia B, Yang FC, D'Souza M, Harris CE, Derrow CW, Zheng Y, Williams DA (2001) Biochemical and biological characterization of a human Rac2 GTPase mutant associated with phagocytic immunodeficiency. J Biol Chem 276:15929–15938
43. Kim C, Dinauer MC (2001) Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. J Immunol 166:1223–1232
44. Corbetta S, Gualdoni S, Albertinazzi C, Paris S, Croci L, Consalez GG, de Curtis I (2005) Generation and characterization of Rac3 knockout mice. Mol Cell Biol 25:5763–5776
45. Leung K, Nagy A, Gonzalez-Gomez I, Groffen J, Heisterkamp N, Kaartinen V (2003) Targeted expression of activated Rac3 in mammary epithelium leads to defective postlactational involution and benign mammary gland lesions. Cells Tissues Organs 175:72–83
46. Hordijk PL (2006) Regulation of NADPH oxidases: the role of Rac proteins. Circ Res 98:453–462
47. Li SM, Zeng LW, Feng L, Chen DB (2010) Rac1-dependent intracellular superoxide formation mediates vascular endothelial growth factor-induced placental angiogenesis in vitro. Endocrinology 151:5315–5325
48. Moldovan L, Irani K, Moldovan NI, Finkel T, Goldschmidt-Clermont PJ (1999) The actin cytoskeleton reorganization induced by Rac1 requires the production of superoxide. Antioxid Redox Signal 1:29–43
49. Satoh M, Ogita H, Takeshita K, Mukai Y, Kwiatkowski DJ, Liao JK (2006) Requirement of Rac1 in the development of cardiac hypertrophy. Proc Natl Acad Sci USA 103:7432–7437
50. Martin SF, Chatterjee S, Parinandi N, Alevriadou BR (2005) Rac1 inhibition protects against hypoxia/reoxygenation-induced lipid peroxidation in human vascular endothelial cells. Vascul Pharmacol 43:148–156
51. Yeh LH, Park YJ, Hansalia RJ, Ahmed IS, Deshpande SS, Goldschmidt-Clermont PJ, Irani K, Alevriadou BR (1999) Shear-induced tyrosine phosphorylation in endothelial cells requires Rac1-dependent production of ROS. Am J Physiol 276:C838–C847
52. Vetter M, Chen ZJ, Chang GD, Che D, Liu S, Chang CH (2003) Cyclosporin A disrupts bradykinin signaling through superoxide. Hypertension 41:1136–1142
53. Mizuno T, Kaibuchi K, Ando S, Musha T, Hiraoka K, Takaishi K, Asada M, Nunoi H, Matsuda I, Takai Y (1992) Regulation of the superoxide-generating NADPH oxidase by a small GTP-binding protein and its stimulatory and inhibitory GDP/GTP exchange proteins. J Biol Chem 267:10215–10218
54. Gregg D, Rauscher FM, Goldschmidt-Clermont PJ (2003) Rac regulates cardiovascular superoxide through diverse molecular interactions: more than a binary GTP switch. Am J Physiol Cell Physiol 285:C723–C734
55. Grand RJ, Owen D (1991) The biochemistry of ras p21. Biochem J 279(Pt 3):609–631
56. Downward J (1992) Regulatory mechanisms for ras proteins. Bioessays 14:177–184
57. Kwong CH, Malech HL, Rotrosen D, Leto TL (1993) Regulation of the human neutrophil NADPH oxidase by rho-related G-proteins. Biochemistry 32:5711–5717
58. Heyworth PG, Knaus UG, Xu X, Uhlinger DJ, Conroy L, Bokoch GM, Curnutte JT (1993) Requirement for posttranslational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase. Mol Biol Cell 4:261–269
59. Quinn MT, Evans T, Loetterle LR, Jesaitis AJ, Bokoch GM (1993) Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. J Biol Chem 268:20983–20987
60. Abo A, Webb MR, Grogan A, Segal AW (1994) Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. Biochem J 298(Pt 3): 585–591
61. Koga H, Terasawa H, Nunoi H, Takeshige K, Inagaki F, Sumimoto H (1999) Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. J Biol Chem 274:25051–25060
62. Lapouge K, Smith SJ, Walker PA, Gamblin SJ, Smerdon SJ, Rittinger K (2000) Structure of the TPR domain of p67phox in complex with Rac.GTP. Mol Cell 6:899–907
63. Dang PM, Cross AR, Quinn MT, Babior BM (2002) Assembly of the neutrophil respiratory burst oxidase: a direct interaction between p67PHOX and cytochrome b558 II. Proc Natl Acad Sci USA 99:4262–4265
64. Bokoch GM (1995) Regulation of the phagocyte respiratory burst by small GTP-binding proteins. Trends Cell Biol 5:109–113
65. Wu RF, Gu Y, Xu YC, Nwariaku FE, Terada LS (2003) Vascular endothelial growth factor causes translocation of p47phox to membrane ruffles through WAVE1. J Biol Chem 278:36830–36840
66. Silva GB, Garvin JL (2010) Rac1 mediates NaCl-induced superoxide generation in the thick ascending limb. Am J Physiol Renal Physiol 298:F421–F425

67. Patil S, Bunderson M, Wilham J, Black SM (2004) Important role for Rac1 in regulating reactive oxygen species generation and pulmonary arterial smooth muscle cell growth. Am J Physiol Lung Cell Mol Physiol 287:L1314–L1322
68. Lemarie A, Bourdonnay E, Morzadec C, Fardel O, Vernhet L (2008) Inorganic arsenic activates reduced NADPH oxidase in human primary macrophages through a Rho kinase/p38 kinase pathway. J Immunol 180:6010–6017
69. Zou AP, Li N, Cowley A W Jr (2001) Production and actions of superoxide in the renal medulla. Hypertension 37:547–553