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## Oxidative signaling in renal epithelium: Critical role of cytosolic phospholipase A<sub>2</sub> and p38<sup>SAPK</sup>

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### Abstract

Previous studies from this laboratory have demonstrated a critical role of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and arachidonic acid in angiotensin II (Ang II) AT<sub>2</sub> receptor-mediated signal transduction in renal epithelium. In primary proximal tubular epithelial cells exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), both the selective cPLA<sub>2</sub> inhibitors and the cPLA<sub>2</sub> antisense oligonucleotides significantly attenuated H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid liberation and activation of p38<sup>SAPK</sup>, ERK1/2, and Akt1. This H<sub>2</sub>O<sub>2</sub>-induced kinase activation was significantly attenuated by a Src kinase inhibitor PP2, or by transient transfection of carboxyl-terminal Src kinase (CSK) that maintained Src in the dormant form. Under basal conditions, Src coimmunoprecipitated with epidermal growth factor receptor (EGFR), while H<sub>2</sub>O<sub>2</sub> increased EGFR phosphorylation in the complex. We observed that inhibition of EGFR kinase activity with AG1478 significantly attenuated H<sub>2</sub>O<sub>2</sub>-induced p38<sup>SAPK</sup> and ERK1/2 activation, but did not inhibit Akt1 activation. Furthermore, it seems that p38<sup>SAPK</sup> is upstream of ERK1/2 and Akt1, since a p38<sup>SAPK</sup> inhibitor SB203580 significantly blocked H<sub>2</sub>O<sub>2</sub>-induced activation of ERK1/2 and Akt1. Interestingly, overexpression of the dominant-negative p38<sup>SAPK</sup> isoform  $\alpha$  inhibited ERK1/2 but not Akt1 activation. Our observations demonstrate that in these nontransformed cells, activation of cPLA<sub>2</sub> is a converging point for oxidative stress and Ang II, which share common downstream signaling mechanisms including Src and EGFR. In addition, p38<sup>SAPK</sup> provides a positive input to both growth and antiapoptotic signaling pathways induced by acute oxidative stress.

### Keywords

Proximal tubular epithelial cells; Oxidative stress; Growth factor; Phospholipase A<sub>2</sub>; Signal transduction; Protein kinase; Free radical

### Introduction

Oxidative stress has a major contributory role in the pathophysiology of various forms of renal disease. As a stable reactive oxygen species (ROS), H<sub>2</sub>O<sub>2</sub> has been shown to be the main initiator of proteinuria associated with acute glomerulonephritis caused by anti-

glomerular basement membrane antibody, complements, and phorbol ester acetate [1-3]. Exposure of freshly isolated rat renal cortical cells to H<sub>2</sub>O<sub>2</sub> produced significant cell death [4]. H<sub>2</sub>O<sub>2</sub> also increased the transepithelial electrical conductance of cultured MDCK cells, as the result of an increase of the paracellular pathway permeability associated with cytoskeleton reorganization, particularly in the area of cell-to-cell junctions [5]. It is clear that at least a portion of ROS is generated by infiltrated phagocytes in disease states [6,7]; however, the mechanisms of de novo ROS production and oxidative signaling pathways within the renal epithelium have not been clarified.

Oxidative stress has been linked to activation of members of the MAP kinase superfamily in various cell types. ERK1/2 are ubiquitously expressed and generally considered to be responsible for mediating the mitogenic and differential effects of growth factors, while p46/p54<sup>SAPK</sup> and p38<sup>SAPK</sup> respond to environmental stress and play an important role in apoptosis [8]. Particularly, p38<sup>SAPK</sup> mediates Ang II- and ROS-induced hypertrophy which was only partially dependent on ERK activation in vascular smooth muscle cells [9]. Moreover, p38<sup>SAPK</sup> has been found to inhibit apoptosis, presumably via Akt1 activation by forming a PDK2-like complex upon stimulation [10]. Therefore, we hypothesize that p38<sup>SAPK</sup> plays an essential role in facilitating survival in renal epithelium in the presence of oxidative stress.

Previous studies from this laboratory and others have shown that Ang II activates ERK1/2 in primary proximal tubular cells. Transactivation of the EGFR tyrosine kinase is an upstream element of shc/grb2/sos-mediated ras activation in our system [11,12]. Recently, we have demonstrated that arachidonic acid and/or its metabolites mediate all downstream effects of Ang II in activation of members of the MAPK, i.e., the ERK1/2, p46/p54<sup>SAPK</sup>, and p38<sup>SAPK</sup> [13,14]. Arachidonic acid also induces ROS production in these proximal tubular epithelial cells [15], which provides mechanism for endogenous ROS generation [16]. However, ERK, but not p38<sup>SAPK</sup>, was suggested to be responsible for Ang II-induced and ROS-mediated hypertrophy in MCT and LLCPK1 cells, cell lines with proximal tubular phenotype [17]. Therefore, the current studies were designed to determine the regulation of oxidative signaling in primary proximal tubular cells based on the hypothesis that arachidonic acid is a common second messenger for oxidative stress and Ang II receptor activation.

## Materials and methods

### Materials

Cell culture medium, additives, and LipofectAmine for transfection were from Gibco BRL (Gaithersburg, MD). [<sup>3</sup>H] Arachidonic acid was obtained from Dupont/NEN (Boston, MA). Rabbit polyclonal anti-phospho antibodies recognizing activated p38<sup>SAPK</sup>, ERK1/2, Akt1, and cPLA<sub>2</sub> were from Cell Signaling (Beverly, MA). Polyclonal anti-p38<sup>SAPK</sup>, anti-ERK1/2, anti-Akt1, anti-p110 $\alpha$ , and anti-cPLA<sub>2</sub> antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-c-Src and anti-EGFR goat polyclonal, anti-ras, anti-vSrc, anti-phosphotyrosine, and anti-phospho-EGFR monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). SB203580, PD98059, LY294002, arachidonyltrifluoromethyl ketone (AACOCF<sub>3</sub>), methyl arachidonyl fluorophosphate (MAFP), oleyloxyethyl phosphorylcholine (OPC), and PP2 were from CalBiochem (San Diego, CA).

### Cell culture

Proximal tubule epithelial cells were isolated from New Zealand White rabbits as previously described [15]. They were maintained for 9–14 days in modified DMEM:F12 (1:1) media supplemented with 5% fetal calf serum, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 0.5  $\mu$ M

hydrocortisone, 350 µg/ml L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin. Subcon-fluent monolayers of first-passage cells were employed for experiments.

### Arachidonic acid liberation

Cells grown on 12-well plates to subconfluence were labeled with 0.5 µCi/ml/well [<sup>3</sup>H]arachidonic acid for 3 h prior to treatment. They were washed 3 times with DMEM medium containing 1 mg/ml fatty acid-free bovine serum albumin to remove free labels. After treatment of cells, the medium was removed and the released arachidonic acid was determined by scintillation counting. Each data point is the average from at least three wells.

### Rabbit cPLA<sub>2</sub> antisense

The amount of 5 µM of each of the two phosphorothioate modified antisense oligonucleotides was introduced to cells in serum-free medium 24 h prior to experiment, with control cells treated with corresponding sense oligonucleotides. The antisense oligonucleotides were: 5'-GATCTATAAATGACATTTTGGT-3' and 5'-ATCCGGGGTGTC AAGCATGTCA-3'. They were designed to target the sequences 1–22 and 114–135 that contain the two possible translation starting codons.

### Immunoblot and immunoprecipitation

Cells were serum-deprived for 20–24 h prior to experiments. Following experimental treatments, the cells were washed twice with ice-cold Dubecco's phosphate-buffered saline. They were then lysed on ice with lysis buffer (50 mM Tris, pH 7.2, 1% (vol/vol) Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 25 µg/ml leupeptin, and 10 µg/ml aprotinin). The samples were centrifuged at 14,000g for 10 min. Protein content in the supernatants was determined by the BCA assay according to the manufacturer's instructions (Pierce, Rockford, IL). Fifteen micrograms of total cell lysate protein was subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane by electroblotting at 200 mA for 1.5 h. The membrane was immunoblotted according to Western blot protocol furnished by the manufacturer, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech Inc., Piscataway, NJ). All experiments had at least one membrane reprobed with antibodies recognizing nonphosphorylated kinases to confirm equal protein loading. The exposure autoradiograph was analyzed by the OS-Scan image analysis system to obtain the densitometry data. For immuno-precipitation, each cell lysate containing 1 mg protein was mixed with 10 µg antibody and 40 µl protein A + G agarose bead suspension. The bead pellet was washed 3 times and then heated in Laemmli's sample buffer. The supernatant was resolved by 10% SDS-PAGE. The associated protein was detected by immunoblot.

### Src GST-SH2 fusion protein and pull-down experiment

Src SH2 sequence (amino acids 140–250) was retrieved from chicken c-Src cDNA plasmid by PCR and cloned into pGEX-2T vector and transformed into BL21 *Escherichia coli*. The sequence was verified by sequencing both strands at Cleveland Genomic LT with ABI PRISM, Model 377. Standard protocol (Pharmacia) was employed to prepare the GST fusion protein conjugated with glutathione Sepharose 4B beads. The preparation was washed and resuspended to 1:1 slurry in PBS containing 0.1% azide, 0.1% bovine serum albumin. Twenty microliters GST fusion protein slurry was mixed with 1 mg cell lysate prepared in SDS/RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 100 µM NaVO<sub>4</sub>, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 25 µg/ml leupeptin, 0.1% SDS) at 4°C for 2 h. The samples were centrifuged and washed for 3 times in lysis buffer to remove nonspecific bound

protein. The final pellets were boiled in Laemmli's sample buffer and resolved by 8% SDS-PAGE. The associated EGF receptor was detected by anti-EGF receptor immunoblot.

### cDNA transient transfection

Cells grown on 6-well plates were transfected using LipofectAmine 2000 with 2  $\mu$ g/well of CSK plasmid or p38<sup>SAPK</sup>  $\alpha$  (TGY  $\rightarrow$  AGF) plasmid or control plasmid for mock transfection. These cells were employed 48–72 h following transfection.

### Data analysis

The obtained densitometry arbitrary units varied from blot to blot depending on the exposure time of ECL. Therefore, data were converted to percentage of the resting value (set as 100%). The basal effect of a putative inhibitor was first compared with resting value, and then the effect on H<sub>2</sub>O<sub>2</sub>-stimulated kinase activation was calculated as (change of kinase activity in the presence of inhibitor/change of kinase activity in the absence of inhibitor)  $\times$  100. The significance was determined by the paired two-tailed Student *t* test, with  $p \leq 0.05$  designated as significant. The effects of putative inhibitors or antisense on arachidonic acid release were calculated as above, except that the raw data are the mean of released radioactive counts (percentage of total) from three wells.

## Results

We used H<sub>2</sub>O<sub>2</sub> as the model molecule to initiate intracellular oxidative stress, as this molecule mediates renal pathologies and is readily permeable to the plasma membrane. We have previously employed immune complex kinase assay and demonstrated in primary proximal tubular epithelial cells that H<sub>2</sub>O<sub>2</sub> dose dependently activates p46/p54<sup>SAPK</sup> [15]. In the current studies, activation of p38<sup>SAPK</sup>, ERK1/2, and Akt1 was determined by immunoblot with anti-phospho-p38<sup>SAPK</sup> (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), or anti-phospho-Akt1 (Ser473) antibodies which recognize the activated enzymes. Therefore, we refer to the phosphorylation as enzyme activation in the text. We have chosen 0.5 mM H<sub>2</sub>O<sub>2</sub> to stimulate cells according to a dose-dependent activation of Akt1 in preliminary studies (not shown). As shown in Fig. 1, when cells were exposed to 0.5 mM H<sub>2</sub>O<sub>2</sub>, the activation of p38<sup>SAPK</sup> peaked at 5 min ( $8.9 \pm 5$ -fold) and subsided, which was followed by a second activation that peaked at 60 min. At 2 and 4 h, p38<sup>SAPK</sup> activation was  $118 \pm 37$  and  $51 \pm 16\%$  of the 60 min value, respectively. The activation of ERK1, ERK2, and Akt1 was significant at 5 min ( $2.5 \pm 0$ -fold,  $2.3 \pm 0$ -fold, and  $7.8 \pm 2$ -fold, respectively) and was sustained for 1 h. During this time frame, there was no change of protein expression level (bottom panel) for p38<sup>SAPK</sup>.

Our previous studies demonstrated that cPLA<sub>2</sub> activation is critical for Ang II-induced MAP kinase activation in these cells. We decided to investigate if cPLA<sub>2</sub> serves as a converging point of oxidative stress- and growth hormone-induced signaling. We observed that H<sub>2</sub>O<sub>2</sub> time dependently induced cPLA<sub>2</sub> activation, by phosphorylation at Ser505 [18], in these proximal tubular cells (Fig. 2A). We then measured H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid liberation from [<sup>3</sup>H]arachidonic acid-labeled cells. A significant  $135 \pm 6\%$  increase was observed within 5 min, and reached  $230 \pm 15\%$  of the control level at 60 min (Fig. 2B). Our experiments have revealed that the presence of 3 mM EGTA or pretreatment of cells with a nonspecific PLA<sub>2</sub> inhibitor mepacrine significantly blocked above H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid liberation (not shown). We then pretreated cells with two structurally unrelated specific cPLA<sub>2</sub> inhibitors arachidonyl trifluoromethyl ketone and methyl arachidonyl fluorophosphate [19–21]. Both inhibitors blocked H<sub>2</sub>O<sub>2</sub>-induced p38<sup>SAPK</sup>, ERK1/2, and Akt1 activation (Fig. 2C). AACOCF<sub>3</sub> also blocked H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid release by 66–80% in two independent experiments (data not shown). In contrast,

H<sub>2</sub>O<sub>2</sub>-induced activation of kinases was not inhibited by oleyloxyethyl phosphorylcholine, an inhibitor of secretory PLA<sub>2</sub> (Fig. 2C).

To further investigate the role of cPLA<sub>2</sub> in the signal transduction, we cloned the full-length rabbit cPLA<sub>2</sub> cDNA (GenBank Accession No. AF204923) by RT-PCR of total RNA extracted from heart tissue of New Zealand White rabbit. We verified the presence of cPLA<sub>2</sub> in rabbit renal epithelial cells. As shown in Fig. 3A, a 999-bp fragment was amplified from renal epithelial cell total RNA employing the primers designed with the heart tissue cPLA<sub>2</sub> sequence. With introduction of the cPLA<sub>2</sub> antisense oligonucleotides that target the translation starting sites of cPLA<sub>2</sub>, we observed a decreased expression of cPLA<sub>2</sub> protein by 50% as compared to cells treated with sense oligonucleotides (Fig. 3B). As expected, expression of another protein, Akt1, which is also involved in the H<sub>2</sub>O<sub>2</sub> signaling, was not affected by this antisense treatment. The antisense treatment attenuated arachidonic acid release induced by H<sub>2</sub>O<sub>2</sub> and Ang II to  $23 \pm 7$  and  $31 \pm 22\%$ , respectively, as compared to those treated with sense oligonucleotides (Fig. 3C). Furthermore, introduction of the cPLA<sub>2</sub> antisense oligonucleotides significantly attenuated activation of p38<sup>SAPK</sup>, ERK1/2, and Akt1 to  $37 \pm 27$ ,  $12 \pm 8$  (ERK1),  $19 \pm 14$  (ERK2), and  $66 \pm 14\%$ , respectively, as compared to levels in cells exposed to sense oligonucleotides (Fig. 3D). Therefore, we have demonstrated that H<sub>2</sub>O<sub>2</sub>-induced kinase activation is mediated by a cPLA<sub>2</sub>-dependent release of arachidonic acid in these cells. It indicates that oxidative stress (represented by H<sub>2</sub>O<sub>2</sub>) and growth hormones (represented by Ang II) share a common signaling mechanism in these cells.

We have previously demonstrated that arachidonic acid mimics Ang II in activation of Src tyrosine kinase, induction of Src and EGFR association via SH2 domain, transactivation of the EGFR tyrosine kinase, activation of small GTPase ras, and activation of downstream ERK1/2 [11,12,14,22]. Experiments were designed to test whether Src and EGFR tyrosine kinases are responsible for mediating H<sub>2</sub>O<sub>2</sub>-induced signaling in these epithelial cells. As shown in Fig. 4A, a specific Src-family kinase inhibitor PP2 significantly attenuated p38<sup>SAPK</sup> activation to  $38 \pm 14\%$ , blocked ERK1/2 activation to  $2 \pm 3$  and  $1 \pm 2\%$ , and decreased Akt1 activation to  $79 \pm 6\%$ , at a submaximum dose of 5  $\mu$ M (effective range 1–50  $\mu$ M, not shown). Alternatively, transient transfection of CSK phosphorylates Tyr529 in Src C-terminus and keeps the Src SH2 domain intramolecularly bound [22,23]. This manipulation also significantly attenuated H<sub>2</sub>O<sub>2</sub>-induced ERK1/2 and Akt1 activation to  $59 \pm 5$ ,  $51 \pm 1$ , and  $24 \pm 19\%$ , respectively. Immunoprecipitation with anti-v-Src antibody and blotting with anti-total EGFR antibody indicated a constitutive association of Src and EGFR in these cells (Fig. 4B, middle panel). However, H<sub>2</sub>O<sub>2</sub> induced tyrosine phosphorylation of the Src-associated EGFR, which peaked at 10 min (Fig. 4B, top panel). In parallel, H<sub>2</sub>O<sub>2</sub> induced the association of phosphorylated EGFR with ras, suggesting induction of downstream ERK1/2 signaling as seen with Ang II stimulation. The induction of Src-phospho-EGFR association was confirmed with Western blot analysis using anti-phospho-EGFR antibody (Fig. 4B), as well as with in vitro pull-down experiments employing immobilized Src-SH2 protein (Fig. 4C). Further, an EGFR kinase inhibitor AG1478 [23] dose dependently attenuated H<sub>2</sub>O<sub>2</sub>-induced kinase activation (Fig. 5A). But AG1478 was obviously more effective in blocking ERK1/2 activation ( $5 \pm 6$  and  $5 \pm 7\%$ , respectively) than p38<sup>SAPK</sup> ( $62 \pm 28\%$ ) and Akt1 ( $67 \pm 37\%$ ) activation (Fig. 5B). Taken together, our data confirmed the existence of Src/EGFR-dependent ERK1/2 pathway induced by acute oxidative stress.

The pyridinyl imidazole compound SB203580 has been reported to inhibit p38<sup>SAPK</sup> isoforms [24]. Pretreatment of renal proximal tubular cells with SB203580 dose dependently inhibited H<sub>2</sub>O<sub>2</sub>-induced early and sustained p38<sup>SAPK</sup> activation at 5 and 30 min, respectively, while ERK1/2 and Akt1 activation was attenuated as well (Fig. 6A). Fig. 6B shows that 10  $\mu$ M SB203580 pretreatment decreased 5 min H<sub>2</sub>O<sub>2</sub> stimulation of p38<sup>SAPK</sup> to



14 ± 7% as compared to values in the absence of the inhibitor, while activation of ERK1/2 and Akt1 was decreased to 3 ± 3, 0 ± 0, and 25 ± 16%, respectively. In addition, we employed the dominant-negative p38<sup>SAPK</sup> α (TGY → AGF) plasmid to transiently transfect the cells. Interestingly, expression of the dominant-negative p38<sup>SAPK</sup>αAGF only significantly attenuated p38<sup>SAPK</sup> and ERK1/2 activation following a 30 min H<sub>2</sub>O<sub>2</sub> exposure (51 ± 16, 64 ± 6, and 69 ± 7%, respectively). Akt1 activation was not affected. With these data, we conclude that p38<sup>SAPK</sup> differentially regulates the ERK1/2 and Akt1 pathways in renal epithelial cells.

The relationship between the ERK1/2 and Akt1 pathways was next examined. As shown in Fig. 7A, the specific ERK1/2 pathway inhibitor PD98059 did not affect p38<sup>SAPK</sup> activation (72 ± 27%), while it completely blocked ERK1/2 activation (3 ± 2 and 2 ± 3%, respectively). PD98059 also attenuated Akt1 activity to 58 ± 28%. On the other hand, the specific inhibitor of the PI3-kinase/Akt1 pathway LY20094 completely blocked Akt1 activation (1 ± 1%) and decreased ERK1/2 activation to 24 ± 41 and 25 ± 22%, respectively. By contrast, LY20094 significantly potentiated p38<sup>SAPK</sup> activation to 199 ± 50%. With immunoprecipitation employing anti-ras antibody, we observed that H<sub>2</sub>O<sub>2</sub> stimulated association of the PI3-kinase subunit p110α with ras, which peaked at 10 min. The association of p110α with Src was also found increased in a similar pattern (Fig. 7B).

## Discussion

The current work establishes a signal transduction pathway whereby H<sub>2</sub>O<sub>2</sub> induced activation of cPLA<sub>2</sub>, Src, EGFR, and p38<sup>SAPK</sup> that link two downstream kinases, i.e., ERK1/2 and Akt1 in kidney epithelial cells. The conclusion that cPLA<sub>2</sub> is a key upstream enzyme which mediates the oxidative signaling is based on observations that (1) cPLA<sub>2</sub> is activated by H<sub>2</sub>O<sub>2</sub>; (2) specific cPLA<sub>2</sub> inhibitors (AACOCF<sub>3</sub> and MAFP) and the cPLA<sub>2</sub> antisense oligonucleotides blocked H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid release and activation of all kinases in the signaling pathway; and (3) other PLA<sub>2</sub> family members such as the secretory PLA<sub>2</sub> play a negligible role as we could not detect their existence in these cells by immunoblot, and inhibitors such as OPC [25] and sPLA<sub>2</sub>-IIA Inhibitor I (CalBiochem) [26] were not effective (L.D. Alexander, unpublished observations). To our knowledge this is the first study in nontransformed cells to show that oxidative stress induces cPLA<sub>2</sub> activation and shares a common signaling mechanism of Ang II.

Arachidonic acid is a polyunsaturated fatty acid with significant importance in membrane structural integrity and function [27]. It is also a regulator of oxidative status documented by its role in activation of NADPH oxidase to generate ROS in the epithelial cells used herein and other cell types [15,28,29]. Recent studies demonstrate that exposure of these renal epithelial cells to exogenous arachidonic acid and/or its metabolites differentially activate ERK1/2, p46/p54<sup>SAPK</sup>, and p38<sup>SAPK</sup> [13]. On the other hand, both ERK2 and p38<sup>SAPK</sup> have been shown to activate cPLA<sub>2</sub> in various cell systems [18,30,31]. Therefore, our work provides a potential mechanism for feed forward of the oxidative signaling at multiple levels. That is, a combined effect of arachidonic acid and H<sub>2</sub>O<sub>2</sub> could yield significant damage to the kidney.

The Src family of nonreceptor tyrosine kinases have been reported to be activated by ROS in various cell types [32,33]. Environmental stress, including ROS, has also been shown to induce MAP kinase pathway activation via activation of receptor tyrosine kinases [34,35]. A number of mechanisms have been proposed for ROS-induced EGFR activation [36]. In some cell lines, Src is responsible for direct activation of the EGFR through phosphorylating essential tyrosine residues on EGFR [37,38]. In renal epithelium, Src tyrosine kinase activation and transactivation of EGFR were found to activate the ERK1/2 pathway in

responding to Ang II or arachidonic acid stimulation [11,14]. A physical association of Src and EGFR was observed in these cells under basal conditions. However, H<sub>2</sub>O<sub>2</sub> induces Src-bond EGFR phosphorylation, that may be via new formation through the Src SH2 domain. It seems that Src-dependent oxidative signaling in these cells is complex. Inhibition of Src activation with PP2 or CSK transient transfection significantly decreased kinase activation, however, with different profiles. PP2 was more efficient in ERK1/2 inhibition and CSK was more efficient in Akt1 inhibition (Fig. 4A). It suggests that the Src and EGFR kinase activities are essential for ERK1/2 activation, while Akt1 might be regulated by conformational change of Src or perhaps Src association with proximal Akt1 effectors, rather than being an EGFR kinase target.

It is interesting that H<sub>2</sub>O<sub>2</sub> induces p38<sup>SAPK</sup> activation with different kinetics (Fig. 1). At present we are unsure whether the biphasic activation of p38<sup>SAPK</sup> is a result of activation of two different isoforms of p38<sup>SAPK</sup> or a consequence of simultaneous activation of protein phosphatases that dephosphorylates and inactivates p38<sup>SAPK</sup> after the immediate activation phase. Protein tyrosine phosphatase 2C has been reported to directly interact with p38<sup>SAPK</sup> [39], and MKP-1 is another potential candidate [40]. It is worth noting that the p38<sup>SAPK</sup> isoforms have been reported to differentially regulate cell functions such as cardiac myocyte hypertrophy and apoptosis [41]. In addition, a p38<sup>SAPK</sup> $\alpha$  activation mechanism that depends on interaction with transforming growth factor- $\beta$ -activated protein kinase 1, rather than MEK, has been elucidated [42]. In human kidney,  $\beta$  and  $\alpha$  isoforms of the p38<sup>SAPK</sup> were identified by Northern blot [43,44]. Indeed, we have amplified two p38<sup>SAPK</sup>-related fragments from the rabbit epithelial cells employing RT-PCR (data not shown). The current data favor activation of distinct p38<sup>SAPK</sup> isoforms for the following reasons: (1) although SB203580 blocked both ERK1/2 and Akt1 activation which suggests that both kinases are regulated by p38<sup>SAPK</sup>, the dominant-negative p38 $\alpha$  construct blocked the delayed phase of p38<sup>SAPK</sup> and the ERK1/2 pathway rather than the Akt1 pathway; (2) inhibition of the EGF receptor kinase with AG1478 or the Src kinase with PP2 blocked ERK1/2 activation profoundly, but had moderate effect on Akt1 activation. As a matter of fact, in all our experiments, Akt1 inhibition would not be obvious if p38<sup>SAPK</sup> inhibition were not deep or complete. Therefore, we propose that the two activation phases of p38<sup>SAPK</sup> may have distinct physiological roles. Although the current study focused more on the acute phase of oxidative stress, it is important to further study the mechanism of p38<sup>SAPK</sup> activation starting with cloning the p38<sup>SAPK</sup> isoforms.

There are apparent interactions of the ERK1/2 and Akt1 pathways because the inhibitors of either pathway were partially effective on each other. We realize that what we observed here could be cell type specific. For example, in opossum kidney proximal tubular cells, both kinases mediate the proliferating effect of lysophosphatidic acid without converging to each other [45]. However, PI3-kinase-dependent ERK activation has been shown to mediate insulin-induced protein synthesis in renal epithelial cells [46]. At the upstream level, ras-dependent activation of PI3-kinase has been elucidated both in vivo and in vitro [47,48]. H<sub>2</sub>O<sub>2</sub> induces association of the p110 $\alpha$  subunit of PI3-kinase with ras and Src in the renal epithelial cells. It is then possible that Src, EGFR, ras, and PI3-kinase form a dynamic protein complex that is regulated by acute oxidative input. These data also reveal the complexity of the signaling pathways and suggest, again, regulation at multiple levels.

Thus in summary, our work provides the novel observation that in renal epithelial cells, cPLA<sub>2</sub> activation and arachidonic acid release are critical mediators of oxidative stress signaling in a manner similar to Ang II AT2 receptor signaling. The current studies also demonstrated that p38<sup>SAPK</sup> is an essential oxidative mediator, as it transduces the signal to at least two downstream pathways, i.e., ERK1/2 and Akt1. It extends our previous observations wherein arachidonic acid-dependent ROS generation via NADPH oxidase was

responsible for activation of p46/p54<sup>SAPK</sup> in these same kidney epithelial cells [15]. Together, the data provide a signaling paradigm for ROS-induced renal pathology.

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## Abbreviations

H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
ROS	reactive oxygen species
Ang II	angiotensin II
cPLA <sub>2</sub>	cytosolic phospholipase A <sub>2</sub>
EGFR	epidermal growth factor receptor
SAPK	stress-activated protein kinase
CSK	Src carboxyl-terminal kinase
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
AACOCF <sub>3</sub>	arachidonyltrifluoromethyl ketone
MAFP	methyl arachidonyl fluorophosphates
OPC	oleoyloxyethyl phosphorylcholine

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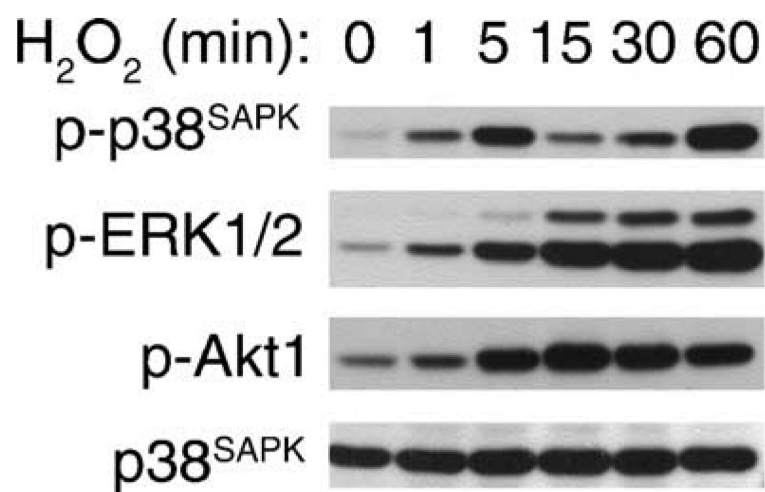
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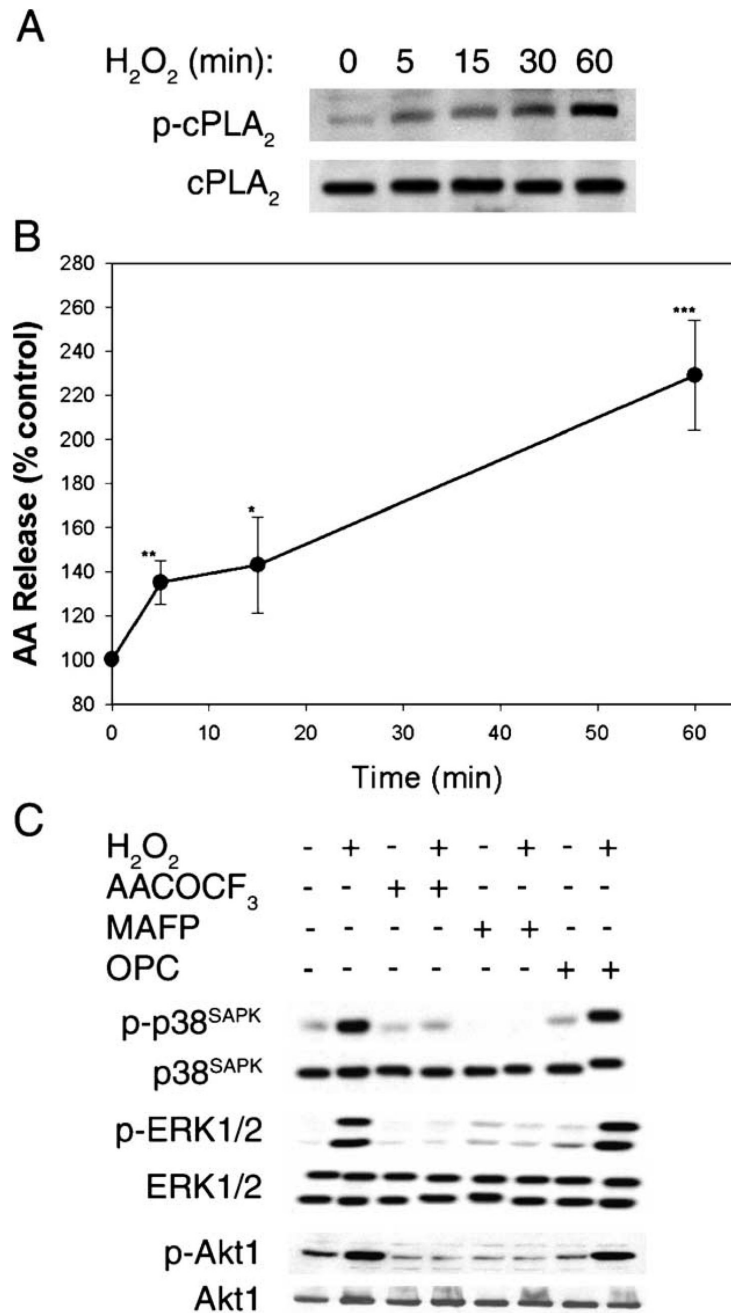
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**Fig. 1.**

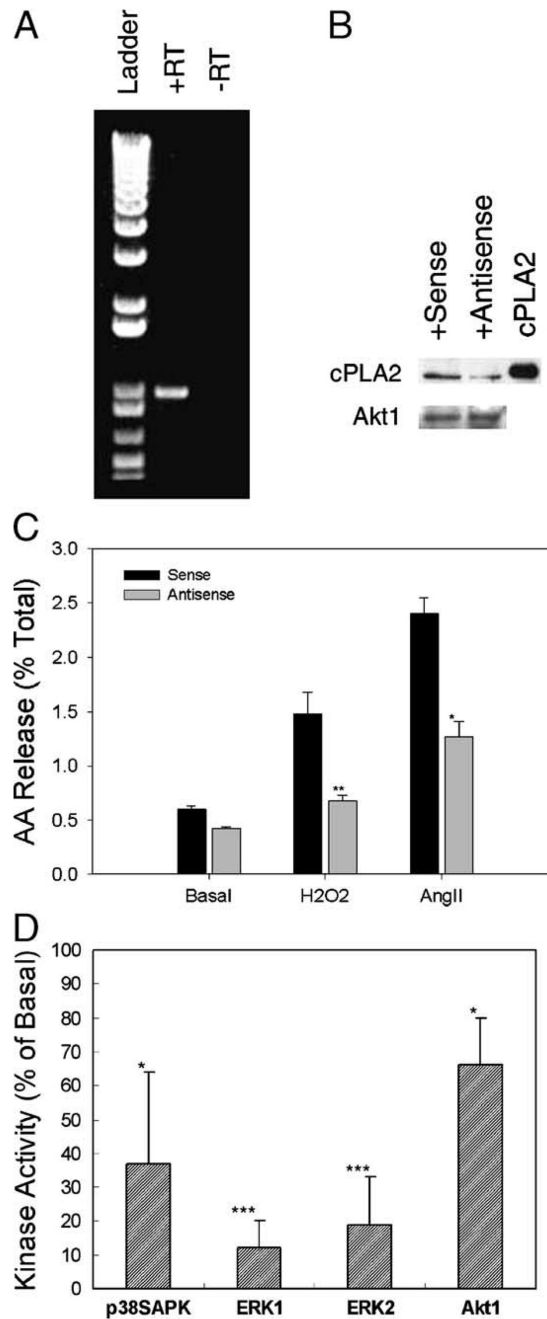
Kinetics of kinase activation: 0.5 mM H<sub>2</sub>O<sub>2</sub> was added to quiescent cells for the time period indicated. Fifteen micrograms of each cell lysate was resolved by 10% SDS-PAGE. Activation of p38<sup>SAPK</sup>, ERK1/2, and Akt1 was detected by immunoblotting with anti-phospho antibodies that recognize the active forms of the kinases. p38<sup>SAPK</sup> protein level and equal protein loading are shown at the bottom.

**Fig. 2.**

(A) Time course of H<sub>2</sub>O<sub>2</sub>-induced cPLA<sub>2</sub> activation: 0.5 mM H<sub>2</sub>O<sub>2</sub> was added to quiescent cells for the time period indicated. Fifteen micrograms of each cell lysate was resolved by 7.5% SDS-PAGE. Activation of cPLA<sub>2</sub> was detected by immunoblotting with anti-phospho-cPLA<sub>2</sub> antibody (top panel). The same blot was stripped and reprobed with control anti-cPLA<sub>2</sub> antibody showing equal loading (lower panel). (B) Time course of H<sub>2</sub>O<sub>2</sub>-induced arachidonic acid release. Cells were labeled with [<sup>3</sup>H]arachidonic acid and were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for the time period indicated. Aliquots of medium were counted in the scintillation fluid and calculated as a percentage of radioactivity released from cells exposed to medium alone for the same length of time. Arachidonic acid release was calculated from

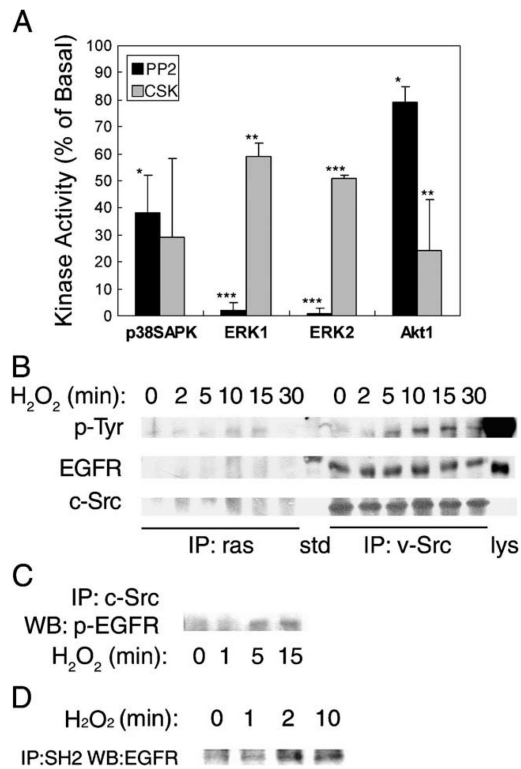


at least 3 determinations of two similar experiments (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). (C) Effects of PLA<sub>2</sub> inhibitors on H<sub>2</sub>O<sub>2</sub>-induced p38<sup>SAPK</sup>, ERK1/2, and Akt1 phosphorylation. Quiescent cells were incubated with or without 10 μM AACOCF<sub>3</sub>, 10 μM MAFP, or 10 μM OPC for 30 min followed by exposure to 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. The cell lysates were subjected to immunoblot analysis as described in Fig. 1 legend. The same blot was stripped and reprobbed with control anti-p38<sup>SAPK</sup>, anti-ERK1/2, and anti-Akt1 antibodies showing equal loading (lower panels).

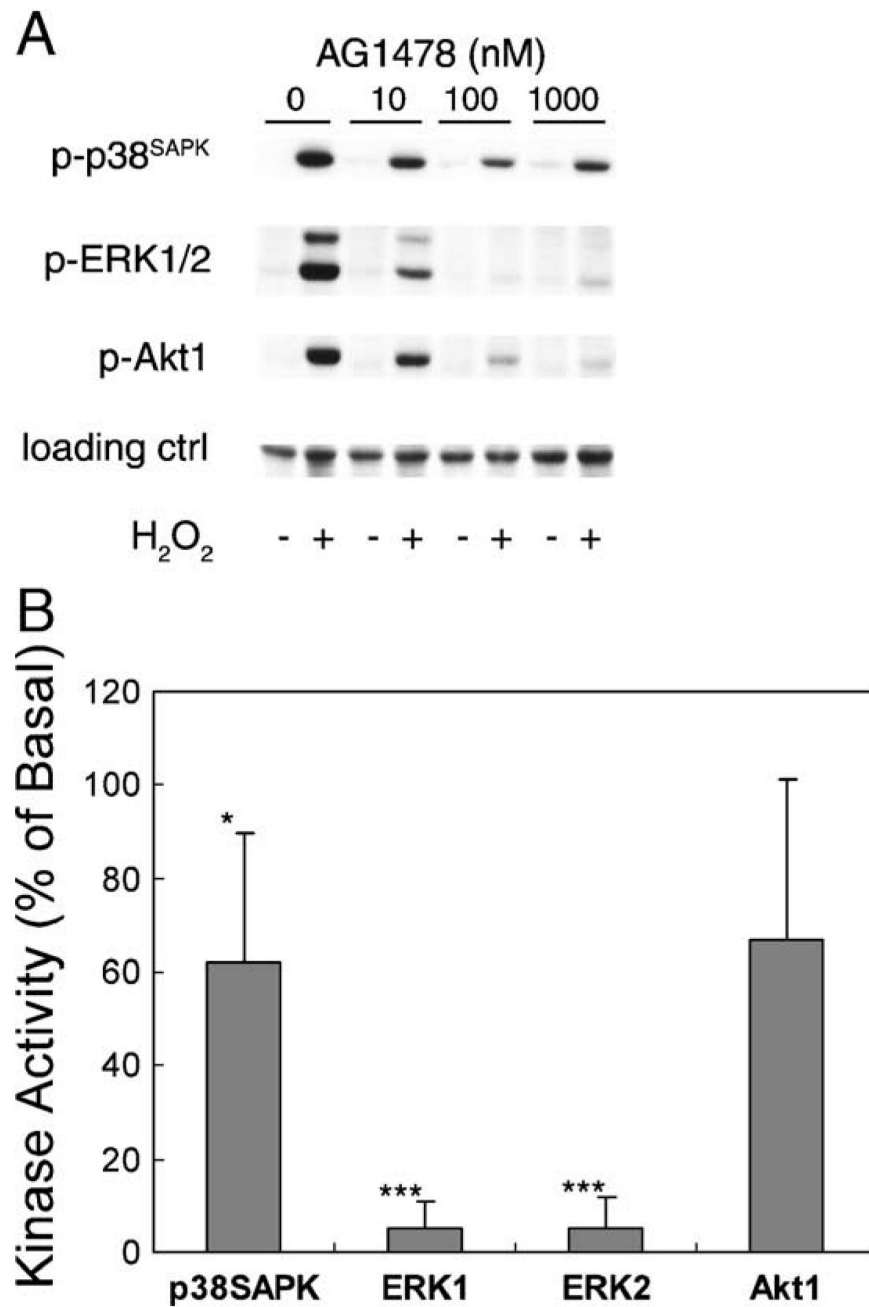
**Fig. 3.**

(A) Amplification of cPLA<sub>2</sub> by RT-PCR. Total RNA was extracted using a Qiagen kit and was reverse-transcribed (RT) with random hexamer primers. The primers used for PCR were 5'-GGAGATCACATTGATGGATGC-3' (forward) and 5'-TCTTCTCCATTGTGGAACC-3' (reverse). (B) Protein expression. The cPLA<sub>2</sub> protein expression was detected by immunoblot of 15 µg lysate from sense or antisense oligonucleotide-treated cells employing anti-mouse cPLA<sub>2</sub> antisera. Mouse cPLA<sub>2</sub> was employed as standard. Membrane of above blot was stripped followed by immunoblotting with anti-Akt1 antibody. (C) Effects of cPLA<sub>2</sub> antisense on H<sub>2</sub>O<sub>2</sub>- and Ang II-induced arachidonic acid release. Cells treated with sense or antisense oligonucleotides were labeled

with [<sup>3</sup>H]arachidonic acid and treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> or 1 μM Ang II for 5 min. Arachidonic acid release was determined as described in Fig. 2B legend and the results are presented as the percentage of released arachidonic acid of total label. The mean of two experiments is shown with 3 replicates in each experiment. (D) Effects of cPLA<sub>2</sub> antisense on H<sub>2</sub>O<sub>2</sub>-induced kinase activation. Cells treated with cPLA<sub>2</sub> sense or antisense oligonucleotides were exposed to 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. Kinase activities were determined as described in Fig. 1 legend (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

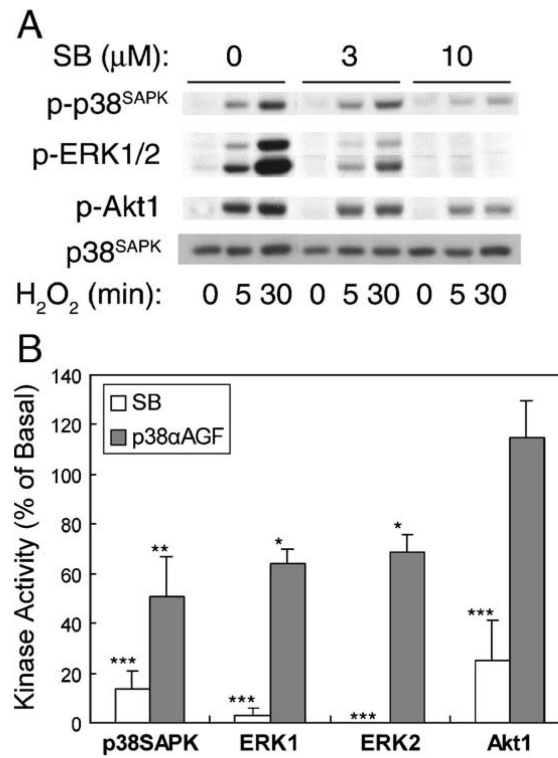


**Fig. 4.** Assessment of the role of Src. (A) Cells pretreated with 5  $\mu$ M PP2 for 30 min or transfected with Csk plasmid were stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. Kinase activities were determined as described in Fig. 1 legend (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001). (B) Cells were lysed following treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> for the time period indicated. One milligram total protein was immunoprecipitated with either anti-ras (left) or anti-v-Src (right) monoclonal antibody followed by blotting with (a) anti-phosphotyrosine antibody (shown are bands that resolve at 180 kDa); (b) anti-EGF receptor antibody; and (c) anti-c-Src antibody; std, molecular weight standard; Lys, lysate of EGF-treated A431 cell as positive control. (C) Cells were lysed following treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 0-15 min. Lysates containing 1 mg total protein were immunoprecipitated with anti-c-Src goat antibody followed by blotting with anti-phospho-EGFR monoclonal antibody. (D) Association of the SH2 domain of Src with the EGF receptor. Cells were lysed following treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> for the time period indicated. Cell lysates were mixed with GST-SH2 fusion protein conjugated with agarose beads. The pull-down products were resolved by SDS-PAGE and immunoblotted with anti-EGF receptor antibody.

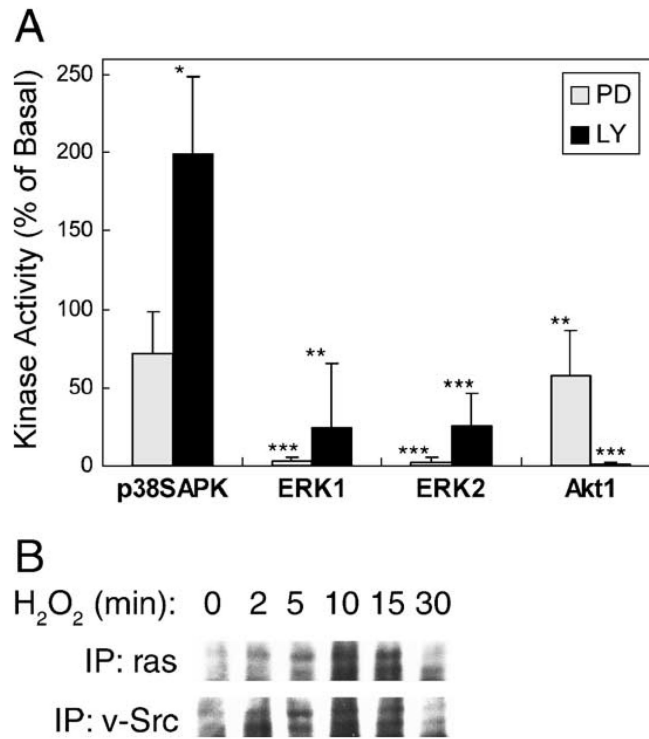


**Fig. 5.** Assessment of the role of EGFR. (A) Cells were exposed to 0–1000 nM AG1478 for 30 min prior to stimulation with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. (B) Cells pretreated with 100 nM AG1478 for 30 min were stimulated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. Kinase activities were detected as described in Fig. 1 legend (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).



**Fig. 6.**

Assessment of the role of p38<sup>SAPK</sup>. (A) Cells were incubated with 0–10  $\mu$ M SB203580 for 20 min prior to stimulation with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 and 30 min, respectively. (B) Cells were pretreated with 10  $\mu$ M SB203580 followed by stimulation with H<sub>2</sub>O<sub>2</sub> for 5 min, or transfected with the dominant-negative p38 $\alpha$ AGF plasmid followed by stimulation with H<sub>2</sub>O<sub>2</sub> for 30 min. Kinase activities were determined as described in Fig. 1 legend (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001).

**Fig. 7.**

Assessment of the crosstalk between the pathways. (A) Cells were incubated with 30  $\mu$ M PD98059 or 50  $\mu$ M LY294002 for 20 min prior to stimulation with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 5 min. Kinase activities were detected as described in Fig. 1 legend (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). (B) Cells were lysed following treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> for the time period indicated. One milligram total protein was immunoprecipitated with either anti-ras or anti-v-Src monoclonal antibody followed by immunoblotting with anti-p110 $\alpha$  antibody. Two protein bands were seen in the blots and in positive control lysate (not shown).