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Role of *c-SRC* and ERK in acid-induced activation of NHE3

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Role of *c-Src* and ERK in acid-induced activation of NHE3.

Background. In the renal proximal tubule, chronic acidosis causes increases in apical membrane NHE3 activity, which serve to increase transepithelial H^+ secretion and return systemic pH to normal levels. Incubation of cultured renal epithelial cells in acid media activates c-Src.

Methods. OKP cells were incubated in control (pH 7.4) or acid (7.0) media, and NHE3 activity measured as cytoplasmic pH (pHi) recovery from an acid load using BCECF. c-Src, ERK, and JNK kinase activities were measured by immune complex kinase assays with enolase, MBP, and GST-c-Jun, respectively, as substrates in the in vitro assays. To determine the role of c-Src in acid-induced NHE3 activation, cells were transfected with vector alone or a dominant negative c-Src ($c\text{-}Src^{K295M}$).

Results. Expression of dominant negative $c\text{-}src^{K295M}$ in OKP cells prevented acid-induced activation of NHE3. Incubation of OKP cells in acid media increased ERK activity and *c-fos* expression, but did not increase JNK activity. Acidosis in vivo also activated renal cortical c-Src and ERK kinases, whereas incubation of 3T3 cells in acid media activated c-Src but not ERK kinase. Expression of $c\text{-}src^{K295M}$ did not affect ERK or *c-fos* activation by acid incubation. Inhibition of MEK with PD98059 inhibited activation of NHE3 by acid incubation.

Conclusions. These studies suggest that acidosis activates c-Src and MEK/ERK/*c-fos*. While both pathways are necessary for activation of NHE3, they are activated independently.

Chronic decreases in blood pH induce a series of cellular adaptations that serve to return blood pH to normal. An important component of this response occurs in the renal proximal tubule cell and involves increases in transepithelial H^+ secretion, citrate absorption, and ammonia synthesis [1]. Increases in H^+ secretion occur secondary to parallel increases in the activities of the apical membrane Na^+/H^+ antiporter encoded by NHE3, and the basolateral membrane $Na^+/HCO_3^-/CO_3^{2-}$ cotransporter [2–5]. The effect of acidosis on NHE3 can be reproduced by incubating OKP cells, a cultured renal tubular epithelial cell line, in acidic media [6].

Incubation of renal epithelial cells in acidic media (pH 7.0) causes transcriptional activation of *c-fos*, *c-jun*, *junB*, and *egr-1* [7]. Activation of these immediate early genes is dependent on tyrosine kinase pathways, but the specific pathways responsible have not been defined. In two proximal tubule cell lines, OKP and MCT cells, incubation in acidic media leads to a twofold increase in c-Src activity [8, 9]. An important role for c-Src in the regulation of NHE3 by acid has been suggested by the findings that: (*1*) acid-induced increases in NHE3 activity are inhibited by herbimycin A, a tyrosine kinase inhibitor; and (*2*) acid-induced increases in NHE3 activity are inhibited by overexpression of *csk*, an endogenous inhibitor of Src family kinases [8]. However, herbimycin A and *csk* overexpression may have nonspecific effects on cells. The purpose of the present studies was to further define the signaling pathways activated by acid and to examine the role of c-Src.

METHODS

Materials

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise noted as follows: penicillin and streptomycin from Whittaker MA Bioproducts (Walkersville, MD, USA); culture media and G-418 from Gibco BRL (Grand Island, NY, USA); monoclonal anti-Src antibody 327 from Oncogene Science (Cambridge, MA, USA); polyclonal anti-c-Src antibody SRC 2, polyclonal anti-ERK1 (C-16), and polyclonal anti-JNK1 antibody (C-17) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-ERK1 (CT) antibody, myelin basic protein, and c-Jun (1-169)-GST from Upstate Biotechnology, Inc (Lake Placid, NY, USA); Triton X-100 and protein G agarose from Calbiochem (La Jolla, CA, USA); horseradish peroxidase labeled anti-mouse IgG and ECL kit from Amersham (Arlington Heights, IL, USA); pCI-neo and Tfx-50

Key words: Na/H antiporter, renal acidification, Src kinase, MAP kinase, JNK, c-fos, PD98059, OKP cells, NIH 3T3 cells.

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from Promega (Madison, WI, USA); and BCECF-AM (acetoxymethyl derivative of (2′7′)bis(2-carboxyethyl)-5,6-carboxyfluorescein) from Molecular Probes (Eugene, OR, USA).

Cell culture

OKP cells [10] were passaged in high glucose (450 mg/dL) Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). For study, cells were grown in a 1:1 mixture of low glucose (100 mg/dL) DMEM and Ham's F12. When confluent, cells were rendered quiescent for 48 hours prior to study by removal of serum. NIH 3T3 cells were cultured similarly except that cells were grown in 7% calf serum and maintained in 0.5% calf serum for two days prior to study.

Dominant negative *c-src*^{K295M} was synthesized by site directed mutagenesis as described by the manufacturer (Sculptor™ in vitro mutagenesis system, Amersham Life Science), cloned into a cytomegalovirus (CMV) promoter-driven pCI-neo expression vector, and transfected into OKP cells using Tfx-50, as described by the manufacturer. Neomycin-resistant cells were selected by growth in 400 μg/mL G-418 and maintained in 200 μg/mL G-418. Clonal cell lines were isolated by limiting dilution, and screened by Northern blot for maximal expression. For experimentation, G-418 was removed at the time of the last passage prior to study. Control cells were transfected with vector alone, cloned, and treated similarly to experimental cells.

To study the effects of media acidification, cells were incubated in control (pH 7.4) or acid (pH 7.0) media. To study the effects of glucocorticoids, cells were incubated in 0.1 μmol/L dexamethasone or vehicle (ethanol) × 24 h. To study the role of G proteins, cells were pretreated with 200 ng/mL pertussis toxin × 6 h prior to control or acid incubation, and then during incubation. To study the role of MEK, cells were treated with 50 μmol/L PD98059 × 2 h prior to and then during acid incubation [11].

Animal studies

To study the effect of acute metabolic acidosis, male Sprague-Dawley rats were gavaged with 2 mol/L NH_4Cl (1 mL/100 g) or an equal volume of water. Water, rather than NaCl, was selected for the control gavage because NaCl administration leads to volume expansion, while NH_4Cl administration does not change volume status. The administration of NH_4Cl causes addition of Cl^-, but removal of a similar amount of HCO_3^- from the body, resulting in no net change in extracellular solute. At the indicated times, rats were anesthetized with Inactin (100 mg/kg), the kidney removed, and renal cortex dissected.

Measurement of intracellular pH and Na/H antiporter activity in OKP cells

Continuous measurement of cytoplasmic pH (pHi) was accomplished in OKP cells grown on coverslips using BCECF as previously described [6]. pHi was estimated from the ratio of fluorescence with excitation wavelengths of 500 and 450 nm and emission wavelength 530 nm (SLM 8000C, Rochester, NY, USA), and calibrated using nigericin, as described [6].

Na^+/H^+ antiporter activity was assayed as the initial rate of Na^+-dependent pHi recovery from an acid load in the absence of CO_2/HCO_3, as previously described [6]. Cells were acidified with 13 μmol/L nigericin in Na^+-free solution. Na^+/H^+ antiporter activity was measured as the initial rate of pHi increase in response to replacement with Na^+-containing solution. Incubation in acidic media has no effect on buffer capacity in OKP cells [6]. Na^+/H^+ antiporter activity therefore is expressed as dpHi/dt.

c-Src kinase activity

Cells were washed in phosphate buffered saline (PBS) × 3, scraped in RIPA buffer [in mmol/L: 150 NaCl, 50 Tris HCl (pH 7.4), 2.5 ethylenediaminetetraacetic acid (EDTA), 5 egtazic acid (EGTA), 50 β-glycerophosphate, 50 NaF, 1 Na orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 0.5 dithiothreitol (DTT), 1% Triton X-100, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 μg/mL pepstatin, 5 μg/mL leupeptin, 5 μg/mL aprotinin], incubated at 4°C × 45 minutes, and centrifuged at 10,000 × *g* × 15 minutes at 4°C. Supernatants were diluted with RIPA buffer to 1 mg protein/ml (Bradford method; Bio-Rad), and 300 μL of cell extract mixed with 2 μg anti-Src monoclonal antibody 327, rocked × 2 h at 4°C, mixed with 10 μL protein G agarose, rocked × 1 h, pelleted at 10,000 × *g* for 30 seconds, and washed × 4 with RIPA buffer, and then with a NaCl solution containing 150 mmol/L NaCl and 20 mmol/L Na HEPES, pH 7.4 × 2. The pellet was then suspended in 400 μL of the NaCl solution, divided into two aliquots and pelleted.

To assay c-Src kinase activity, one aliquot was brought to room temperature × 1.5 minutes and then suspended in 10 μL reaction buffer [150 mmol/L NaCl, 20 mmol/L Na HEPES (pH 7.4), 30 mmol/L $MgCl_2$ 0.01 μmol/L adenine 5′-triphosphate (ATP), 0.3 mg/mL acid treated enolase, and 5 μCi [^{32}P]-γATP] at room temperature × 5 minutes with rotation. The reaction was stopped by addition of 10 μL 2× SDS loading buffer (1 mmol/L Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol) and the sample boiled × 5 minutes. The sample was then size fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 13% gel), and the dried gel subjected to autoradiography. To determine the total

amount of c-Src in the precipitate, the remaining pellet was suspended in 50 μL 1× SDS loading buffer, and subjected to SDS-PAGE (8% gel) and Western blotting with 1 μg/mL of rabbit anti-Src antibody SRC 2. Secondary antibody was horseradish peroxidase labeled anti-rabbit IgG (1:5000 dilution). Bands were visualized by enhanced chemiluminescence (ECL). Enolase phosphorylation was quantitated by densitometry, and corrected for differences in c-Src abundance on western blot. All experiments were performed at least in triplicate.

For animal studies, approximately 1/5 of a kidney cortex was homogenized in 8.4 mL RIPA buffer (with 10% glycerol) using a Polytron × 30 seconds, incubated at 4°C × 60 minutes, and centrifuged at 20,000 rpm × 30 minutes at 4°C. Supernatant was then treated as above.

ERK kinase activity

Proteins were solubilized as described above with a modified RIPA buffer [in mmol/L: 150 NaCl, 50 Tris HCl (pH 7.4), 2.5 EDTA, 5 EGTA, 10% glycerol, 50 β-glycerophosphate, 50 NaF, 1 Na orthovanadate, 1 PMSF, 0.5 dithiothreitol, 1% Triton X-100, 0.25% SDS, 2 μg/mL pepstatin, 5 μg/mL leupeptin, 5 μg/mL aprotinin], rocked × 30 minutes at 4°C, and centrifuged at 50,000 rpm × 30 minutes at 4°C. ERK1 and ERK2 were then immunoprecipitated from the supernatant with 2 μg anti-ERK1(CT or C-16) antibody and protein G agarose as described above, and the pellet divided into two aliquots.

To assay ERK1/2 kinase activity, one pellet was brought to room temperature × 1.5 minutes and then suspended in 20 μL reaction buffer {25 mmol/L Na HEPES (pH 7.4), 25 mmol/L $MgCl_2$, 25 mmol/L β-glycerophosphate, 2 mmol/L DTT, 100 μmol/L orthovanadate, 3 μg myelin basic protein, and 5 μCi [^{32}P]-γATP} at room temperature × 5 minutes with rotation. The reaction was stopped by addition of 20 μL 2× SDS loading buffer and the sample boiled × 10 minutes. The sample was then size fractionated by SDS-PAGE (13% gel), and the dried gel subjected to autoradiography. Kinase activity was measured by densitometry and normalized for the abundance of ERK1/2 measured by Western blot on the second aliquot as described above, using anti-ERK1(CT) antisera. For studies of ERK kinase activity in rat renal cortex, proteins were solubilized as described above for the in vivo c-Src assay except that detergents were 1% Triton X-100 and 0.25% SDS. ERK was then immunoprecipitated and activity assayed as above.

JNK kinase activity

JNK1 was solubilized and immunoprecipitated as described for c-Src except that 600 μL cell lysate was precipitated with 3 μg JNK1 (C-17) antibody. The kinase reaction was performed in 20 μL reaction buffer {20 mmol/L Na HEPES (pH 7.4), 20 mmol/L $MgCl_2$, 20 mmol/L β-glycerophosphate, 100 μM Na_3VO_4, 2 μg c-Jun (1-169)-GST, and 5 μCi [^{32}P]-γATP} at 30°C × 20 minutes. Kinase activity was quantitated by densitometry and normalized for JNK1 abundance by western blot on the second aliquot using an anti-JNK1 (C-17) antibody.

RNA blotting

RNA was extracted using RNeasy (Qiagen). Fifteen micrograms of total RNA was size fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes. The radiolabeled *c-fos* and 18S probes were synthesized from a full length rat *c-fos* cDNA [12] and from a 752 bp SphI/BamHI fragment of the mouse 18S rRNA (ATCC #63178) by the random hexamer method. Prehybridization, hybridization, and washing were as previously described [13]. Filters were exposed to film overnight at −70°C and labeling quantitated by densitometry.

RESULTS

Acid activation of c-Src

We previously found that incubation of rabbit proximal tubule primary cultures and MCT cells in acid media caused an increase in Na^+/H^+ antiporter activity, while acid incubation of cultured human foreskin fibroblasts and NIH 3T3 cells in acid media caused a decrease in Na^+/H^+ antiporter activity [13, 14]. This tissue specificity was not attributable to differences in the Na^+/H^+ antiporter isoform studied in that acid incubation increased NHE1 mRNA abundance in MCT cells and decreased NHE1 mRNA abundance in NIH 3T3 cells [13]. Incubation of OKP and MCT cells in acid media (pH 7.0) causes a twofold increase in c-Src activity [8, 9].

To determine whether the tissue specificity of acid regulation resides in regulation of c-Src, we examined the effect of incubating 3T3 cells in acid media. Incubation of 3T3 cells in acid media caused a twofold increase in c-Src activity at 1.5 min, the time point of maximal c-Src activation in MCT cells (Fig. 1, $P < 0.01$) [9]. Thus, tissue specificity of the effects of acid cannot be attributed to tissue specific regulation of c-Src by acid.

To confirm that regulation of c-Src by acid in cultured renal epithelial cells is relevant to the intact kidney, we measured the effect of in vivo metabolic acidosis on renal cortical c-Src activity. Rats were gavaged with 1 mL of 2 mol/L NH_4Cl/100 g body weight, which caused plasma [HCO_3] to decrease from 24.6 ± 0.3 to 18.9 ± 1.0 mEq/L at 15 minutes. c-Src activity was then assayed in renal cortical homogenates. Metabolic acidosis caused a 39% increase in c-Src activity at 15 minutes, which remained slightly increased at 30 minutes but returned to baseline levels at 50 minutes (Fig. 2). The increase was seen in four of four experiments ($P < 0.005$). While this increase

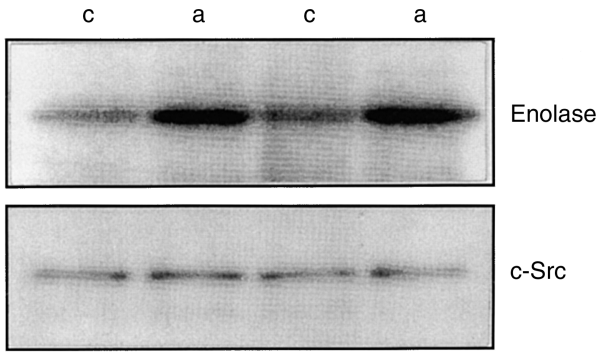

Fig. 1. Incubation of 3T3 cells in acid media activates c-Src. 3T3 cells were incubated in control (pH 7.4) or acid media (pH 7.0) for 1.5 minutes. c-Src was then immunoprecipitated and activity measured by immune complex assay using enolase as the kinase substrate. ^{32}P incorporation into enolase (upper bands) and c-Src abundance in the precipitate (lower bands; Western blot using anti-c-Src antibody) are shown. Abbreviations are: c, control; a, acid. $N = 3$.

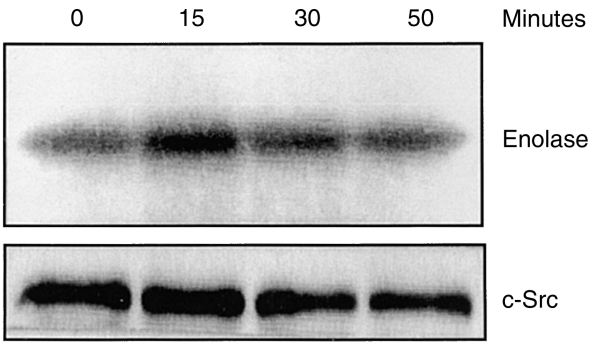

Fig. 2. Metabolic acidosis activates renal cortical c-Src. Rats were gavaged with NH_4Cl to induce an acute metabolic acidosis. At the indicated times, renal cortex was harvested, c-Src immunoprecipitated, and activity measured by immune complex assay using enolase as the kinase substrate. ^{32}P incorporation into enolase (upper bands) and c-Src abundance in the precipitate (lower bands; Western blot using anti-c-Src antibody) are shown ($N = 4$).

is smaller than that seen in cultured cells, the pH changes are also significantly smaller in vivo.

c-Src activation is required for acid activation of NHE3

The Na^+/H^+ antiporter in OKP cells, under control conditions and following acid treatment, is encoded by NHE3, the amiloride-resistant isoform that encodes the apical membrane Na^+/H^+ antiporter of the renal proximal tubule [6, 13, 15, 16]. In OKP cells, incubation in acid media leads to NHE3 activation [6]. A role for c-Src in this process has been suggested, based on the findings that acid-induced NHE3 activation is inhibited by herbimycin A and overexpression of *csk* in OKP cells [8]. However, herbimycin A is a nonspecific inhibitor of many tyrosine kinases. *Csk* inhibits Src family kinases by phosphorylating a key carboxy-terminal tyrosine, and is likely more specific [17, 18]. However, overexpression of *csk* has profound effects on the cytoskeleton and cell morphology, and thus, its effects on acid regulation could be indirect [19].

To confirm a role for c-Src in NHE3 regulation, we examined the effect of acid incubation in OKP cells expressing dominant negative *c-src*^{K295M} [20, 21]. Cells were incubated in acid (pH 7.0) or control media for 24 hours and NHE3 activity assayed as the Na^+-dependent increase in cell pH following an acid load. In wild-type OKP cells, acid incubation caused a 33% increase in NHE3 activity (Fig. 3). Similarly, in two clones of vector transfected cells, acid incubation caused a 32% and a 36% increase in NHE3 activity (Fig. 3). By contrast, in three clones expressing *c-src*^{K295M}, acid incubation had no significant effect on NHE3 activity (Fig. 3).

To examine whether pertussis toxin-sensitive G proteins play a role in acid regulation of NHE3, OKP cells were pretreated with 200 ng/mL pertussis toxin $\times$ 6 h prior to control or acid incubation, and then during incubation. Treatment of OKP cells with 200 ng/mL pertussis toxin $\times$ 4 h inhibits all subsequent ribosylation of G proteins in vitro [22]. As shown in Figure 4, acid incubation caused a 27% increase in NHE3 activity in pertussis toxin treated cells, similar to the 31% increase seen in vehicle treated cells. Thus, pertussis toxin-sensitive G proteins do not play a key role in acid regulation of NHE3.

Acid incubation activates ERK1,2

We previously found that incubation of MCT cells in acid media leads to transcriptional activation of immediate early genes including *c-fos*, *c-jun*, *junB*, and *egr-1* [7, 23]. Transcriptional activation of *c-fos* is frequently mediated by phosphorylation of ternary complex factor by MAP kinases [24]. To address this, the effects of acid incubation were measured on ERK and JNK, two members of the MAP kinase family. ERK1 and 2 were immunoprecipitated and their activity measured by immune complex kinase assay using myelin basic protein as the kinase substrate. OKP cells were found to express significantly more ERK1 than ERK2. Figure 5 shows a typical experiment. In four experiments, incubation in acid media caused a fourfold increase in ERK1,2 activity that peaked at 5 to 15 minutes. ERK1,2 activity was increased 2.3-fold at 5 minutes ($P < 0.05$), 4.0-fold at 10 minutes ($P < 0.005$), 2.9-fold at 15 minutes ($P = 0.053$), and 2.5-fold at 30 minutes (NS).

Similar results were found in renal cortical ERK1,2 activity when metabolic acidosis was induced in rats by NH_4Cl gavage. NH_4Cl lavage caused serum [HCO_3] to decrease from 24.0 $\pm$ 0.3 to 18.7 $\pm$ 0.9 mEq/L at 30 minutes. This degree of acidosis induced a 77% increase in renal cortical ERK1,2 activity at 30 minutes ($P <$ 0.001, Fig. 6). To determine the cell specificity of this effect, studies were performed in 3T3 cells. Unlike OKP

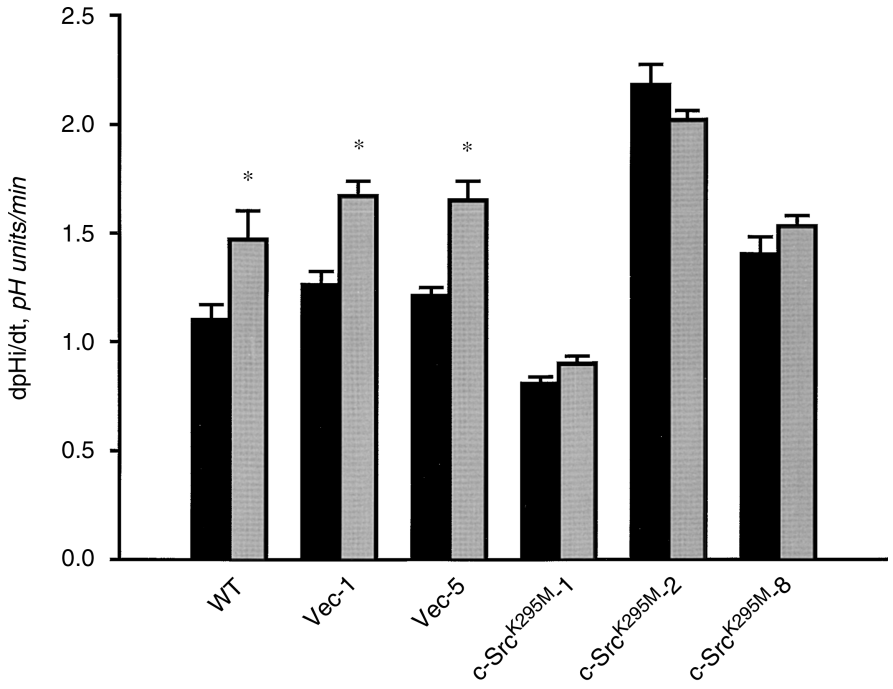

Fig. 3. Expression of *c-src*^{K295M} in OKP cells prevents media acidification induced-NHE3 activation. Wild-type (WT) or clonal cell lines stably expressing *c-src*^{K295M} or vector alone (Vec-1, Vec-5) were incubated at pH 7.4 (control; ■) or 7.0 (acid; ▩) × 24 h, and Na/H antiporter activity measured as the initial rate of Na^+-dependent cell pH recovery from an acid load (dpHi/dt). *$P < 0.05$ vs. control; $N = 5$ for WT, $N = 15$ for Vec-1, $N = 9$ for Vec-5, $N = 11$ for c-Src^{K295M}-1, $N = 10$ for c-Src^{K295M}-2, and $N = 8$ for c-Src^{K295M}-8.

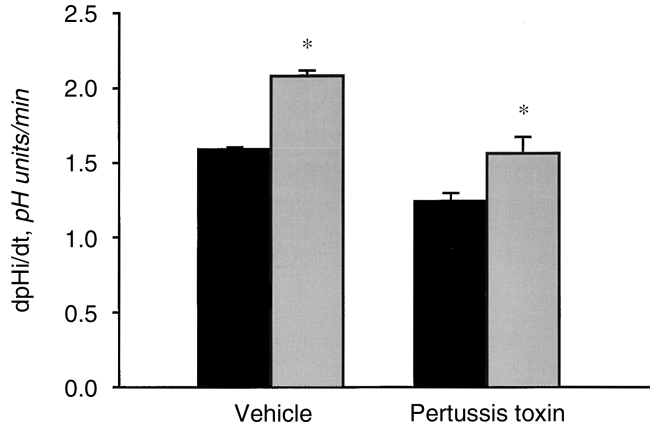

Fig. 4. Pertussis toxin-sensitive G proteins are not required for activation of NHE3 by acid incubation. OKP cells were pretreated with 200 ng/mL pertussis toxin × 6 h prior to and then during 24 h incubation at pH 7.4 (control; ■) or 7.0 (acid; ▩). Na/H antiporter activity was then measured as the initial rate of Na^+-dependent cell pH recovery from an acid load (dpHi/dt). *$P < 0.05$; $N = 5$ for vehicle and $N = 6$ for pertussis toxin experiments.

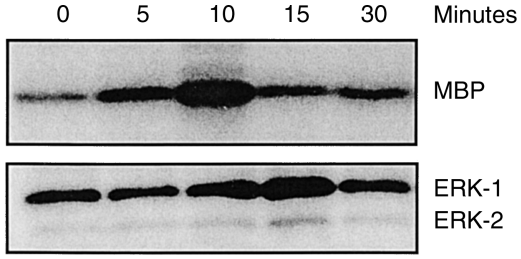

Fig. 5. Incubation of OKP cells in acid media activates ERK. OKP cells were incubated in acid media (pH 7.0) for the indicated times. ERK1,2 were then immunoprecipitated and activity measured by immune complex assay using myelin basic protein (MBP) as the kinase substrate. ^{32}P incorporation into MBP (upper bands) and ERK abundance in the precipitate (lower bands; Western blot using anti-ERK1/2 antibody) are shown ($N = 4$).

cells, in these fibroblasts incubation in acid media did not regulate ERK1,2 activity (Fig. 7). These results agree with our previous results that demonstrated that acid incubation did not increase *c-fos* expression in 3T3 cells, independent of changes in protein synthesis (**Discussion** section) [7]. Thus, while acid activation of c-Src is not cell specific, acid activation of ERK1,2, *c-fos* expression, and Na^+/H^+ antiporter activity is cell specific and does not occur in fibroblasts.

JNK was assayed in OKP cells by an immune complex kinase assay, in which JNK was immunoprecipitated and its ability to phosphorylate a GST-Jun fusion protein measured. Incubation of OKP cells in acid media caused a consistent 27% decrease in JNK activity ($P < 0.001$; Fig. 8, left 6 lanes).

c-Src activation is not required for acid activation of ERK1,2 or *c-fos* expression

To determine if acid induced activation of ERK1,2 is dependent on c-Src, studies were performed in cells expressing dominant negative *c-src*^{K295M}. In V1 and V5 vector expressing cells, incubation in acid media induced a 4.8-fold and 4.3-fold increase, respectively, in ERK1,2 activity at 5 min ($P < 0.05$; Fig. 9 A, B). Similarly, in c-Src^{K295M}-2 cells and c-Src^{K295M}-8 cells, acid incubation

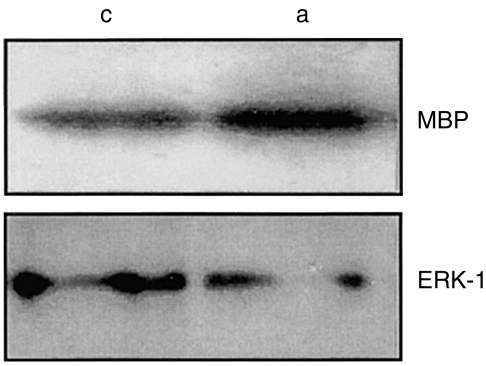

Fig. 6. Metabolic acidosis activates renal cortical ERK. Rats were gavaged with NH_4Cl to induce an acute metabolic acidosis. After 15 minutes, renal cortex was harvested, ERK was immunoprecipitated, and ERK activity measured by immune complex assay using myelin basic protein (MBP) as the kinase substrate. ^{32}P incorporation into MBP (upper bands) and ERK abundance in the precipitate (lower bands; Western blot using anti-ERK1/2 antibody) are shown. Abbreviations are: c, control; a, acid. $N = 3$.

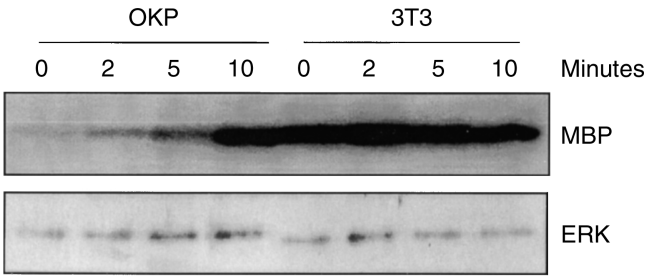

Fig. 7. Incubation of 3T3 cells in acid media does not activate ERK. 3T3 cells were incubated in acid media (pH 7.0) for the indicated times. ERK1,2 were then immunoprecipitated and activity measured by immune complex assay using myelin basic protein (MBP) as the kinase substrate. ^{32}P incorporation into MBP (upper bands) and ERK abundance in the precipitate (lower bands; western blot using anti-ERK1/2 antibody) are shown. Studies in 3T3 cells are the right set of bands; For comparison, a study in OKP cells, where ERK is activated by acid incubation, are shown in the left set of bands. For 3T3 cells, $N = 5$ for 0 min, $N = 3$ for 2 min, $N = 5$ for 5 min, and $N = 5$ for 10 min.

increased ERK1,2 activity 3.0-fold and 4.6-fold, respectively ($P < 0.05$; Fig. 9 C, D). Thus, acid stimulated ERK activity is not dependent on c-Src.

In c-Src^{K295M}-8 cells, acid incubation inhibited JNK activity by 20% ($P < 0.05$; Fig. 8, right 6 lanes). A similar degree of inhibition was seen in c-Src^{K295M}-2 cells (39% inhibition, $N = 3$, $P < 0.002$, data not shown). Both of these effects are similar in magnitude to that seen in wild-type OKP cells (Fig. 8 left, 6 lanes) and in V1 vector expressing cells (35% inhibition, $N = 6$, $P < 0.001$, data not shown). Thus, regulation of MAP kinase pathways by acid incubation is not dependent on c-Src.

Similar studies were performed examining acid-induced increases in *c-fos* expression. In wild-type OKP cells, acid incubation induced a 3.8-fold increase in *c-fos* mRNA abundance ($N = 7$, $P < 0.02$, Fig. 10), similar to our

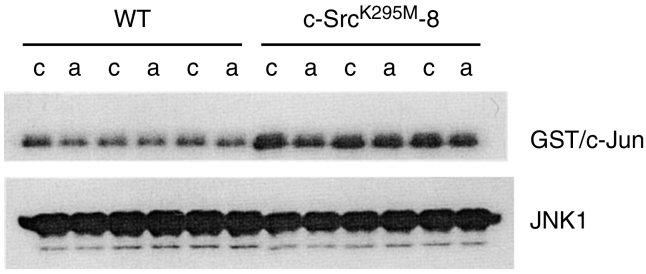

Fig. 8. Incubation of wild type and c-Src^{K295M}-8 OKP cells in acid media inhibits JNK. Cells were incubated in control (pH 7.4) or acid media (pH 7.0) × 10 min. JNK was then immunoprecipitated and activity measured by immune complex assay using a GST-Jun fusion protein as the kinase substrate. ^{32}P incorporation into GST/c-Jun (upper bands) and JNK1 abundance in the precipitate (lower bands; Western blot using anti-JNK antibody) are shown. Abbreviations are: c, control; a, acid. $N = 6$.

previous report [7]. In c-Src^{K295M}-8 cells, acid incubation caused a 9.4-fold increase in *c-fos* expression ($P < 0.05$; Fig. 10). Thus, acid-induced increases in ERK1,2 and *c-fos* expression are not dependent on c-Src activation.

Acid activation of NHE3 is dependent on MEK

MEK phosphorylates and activates ERK. To address whether the MEK/ERK pathway plays a role in acid activation of NHE3, we used the MEK inhibitor, PD98059 [11]. In the absence of PD98059, acid incubation caused a 34% increase in NHE3 activity ($P < 0.02$), while in its presence (50 μm PD98059) there was no effect of acid incubation (P = NS; Fig. 11), consistent with a requirement for ERK kinase activity in acid-induced NHE3 activation.

We have previously shown that dexamethasone increases NHE3 activity, and that this effect is not inhibited by herbimycin A, *csk* overexpression, or expression of dominant negative *c-src*^{K295M} [8, 25]. Thus, it would not be expected to be dependent on the MEK/ERK pathway. In the presence of PD98059, 10^{-7} mol/L dexamethasone induced a 34% increase in NHE3 activity ($P < 0.02$; Fig. 12). This is similar to the effect observed in wild-type OKP cells [26].

DISCUSSION

Chronic metabolic acidosis induces homeostatic responses in many cells, which result in amelioration and correction of the acidotic state. These responses include activation of osteoclastic bone resorption, inhibition of osteoblastic bone deposition, and muscle protein breakdown [1]. In the renal proximal tubule chronic acidosis causes increased transepithelial H^+ secretion, increased ammonia synthesis, increased citrate reabsorption, and growth. These changes in proximal tubule function are associated with changes in the activities of a number of proteins, including the apical membrane Na^+/H^+ anti-

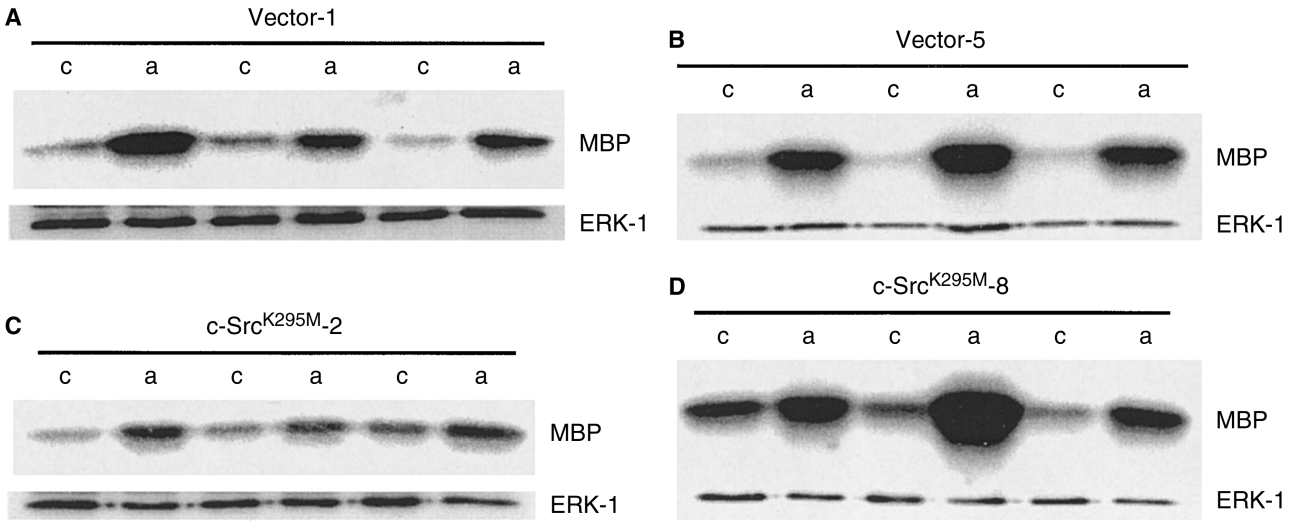

Fig. 9. Expression of *c-src*^{K295M} in OKP cells does not prevent activation of ERK. Clonal cell lines stably expressing *c-src*^{K295M} or vector alone were incubated at pH 7.4 (control) or 7.0 (acid) × 5 min. ERK1,2 were then immunoprecipitated and activity measured by immune complex assay using myelin basic protein (MBP) as the kinase substrate. ^{32}P incorporation into MBP (upper bands) and ERK abundance in the precipitate (lower bands; western blot using anti-ERK1/2 antibody) are shown. (*A*) Vector clone 1; (*B*) Vector clone 5; (*C*) c-Src^{K295M} clone 2; (*D*) c-Src^{K295M} clone 8. Abbreviations are: c, control; a, acid. $N = 6$ for each group.

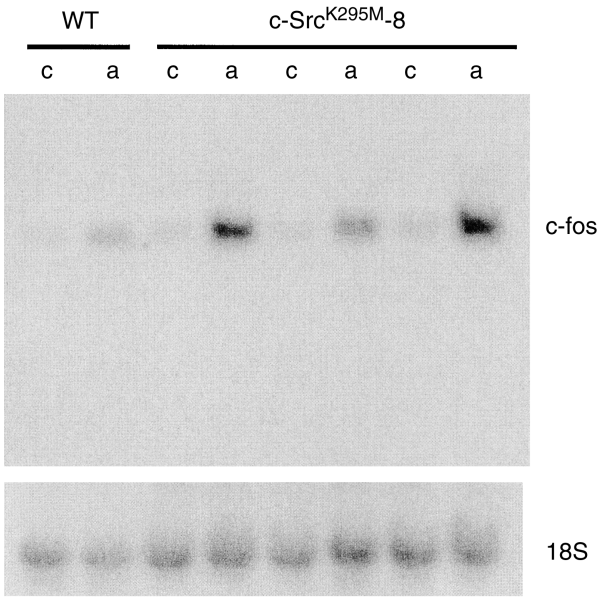

Fig. 10. Expression of *c-src*^{K295M} in OKP cells does not prevent increases in *c-fos* expression induced by incubation in acid media. Wild type and c-Src^{K295M}-8 cells were incubated at pH 7.4 (control) or 7.0 (acid) × 30 min, and *c-fos* expression measured by northern blot. c, control; a, acid. $N = 3$.

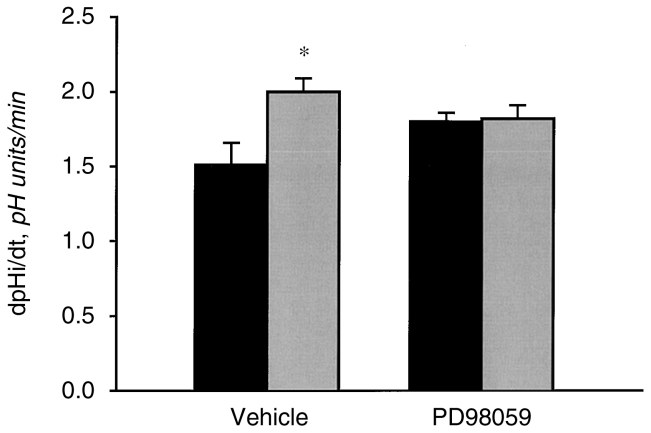

Fig. 11. MEK inhibitor PD98059 inhibits activation of ERK kinase and NHE3 activity by incubation in acid media. OKP cells were treated with 50 μmol/L PD98059 or vehicle × 2 h prior to and then during incubation at pH 7.4 (control) or 7.0 (acid) × 24 h. Na/H antiporter activity was measured as the initial rate of Na^+-dependent cell pH recovery from an acid load (dpHi/dt). *$P < 0.02$ vs. vehicle. $N = 5$ with vehicle and $N = 10$ with PD98059.

porter, the basolateral membrane $Na^+/HCO_3^-/CO_3^{2-}$ cotransporter, glutaminase, glutamate dehydrogenase, phosphoenolpyruvate carboxykinase, the apical membrane Na^+/citrate cotransporter, mitochondrial aconitase, and ATP citrate lyase [1].

Given the broad array of responses to acidosis, the existence of an acid stimulated signaling pathway seems likely. One component of this signaling pathway is c-Src. c-Src is activated by acidosis in OKP and MCT cells (cell lines with characteristics of the renal proximal tubule) [8, 9], renal cortex, and cultured fibroblasts. This effect is mediated by a decrease in intracellular pH. In MCT cells, addition of weak acids, nigericin, or NH_4Cl prepulse, maneuvers that acidify the cell in the absence of extracellular acidification, all activated c-Src [9]. Measurable changes in c-Src activity were elicited by decreases in cell pH smaller than 0.1 pH unit.

The role of c-Src in acid-induced activation of NHE3

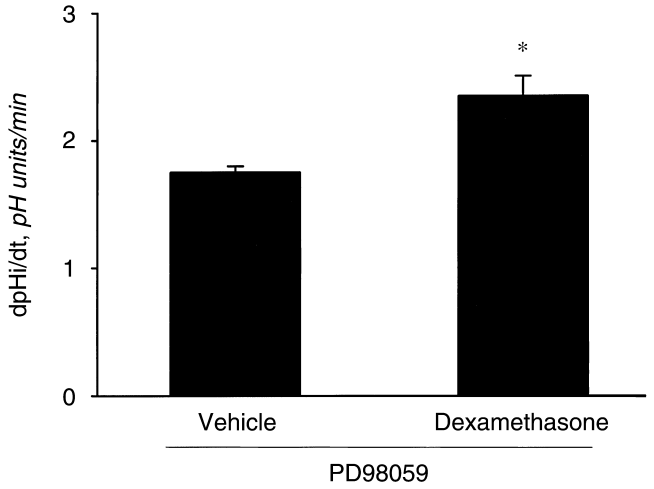

Fig. 12. MEK inhibitor PD98059 does not inhibit activation of NHE3 by glucocorticoids. OKP cells were treated with 50 μmol/L PD98059 × 2 h prior to and then during incubation with 10^{-7} mol/L dexamethasone or vehicle × 24 h. Na/H antiporter activity was then measured as the initial rate of Na^+-dependent cell pH recovery from an acid load (dpHi/dt). *$P < 0.02$ vs. control. $N = 4$.

was investigated using a dominant negative *c-src*, in which lysine 295, which is responsible for ATP binding in the catalytic domain, was mutated to methionine (*c-src*^{K295M}). Courtneidge and coworkers have utilized this dominant negative *c-src* to demonstrate that the proliferative response to platelet-derived growth factor (PDGF), colony stimulating factor (CSF), and epidermal growth factor (EGF) are dependent on c-Src [20, 21]. Similar results were obtained with injection of anti-Src antibodies, confirming that the effect was due to c-Src inhibition. In addition, we've demonstrated that expression of *c-src*^{K295M} inhibited angiotensin II induced increases in NHE3 activity [25].

We previously demonstrated that herbimycin A and *csk* overexpression inhibited acid regulation of NHE3. We now show that inhibition of c-Src by expression of *c-src*^{K295M} also inhibits acid regulation of NHE3. While each of these maneuvers may have nonspecific effects on the cell, the observation that all three methods of inhibiting c-Src have a similar result suggests that c-Src plays an important role in NHE3 regulation by acid. Also against a nonspecific effect is our previous demonstration that expression of *c-src*^{K295M} does not inhibit regulation of NHE3 by glucocorticoids or cAMP [25]. Overexpression of *csk* inhibits a number of c-Src family members. Similarly, expression of *c-src*^{K295M} can inhibit any c-Src family member that utilizes a similar SH2 or SH3 binding domain. Thus, these studies demonstrate a role for c-Src family members.

Incubation of OKP cells in acid media activates ERK and increases expression of *c-fos* mRNA. The increase in ERK activity also is seen in the renal cortex in vivo in response to metabolic acidosis. This is consistent with previous results demonstrating acidosis induced immediate early gene expression in cultured renal cells in vitro and in rat renal cortex in vivo [7]. Of interest, acid activation of ERK is cell specific and is not seen in fibroblasts. This again agrees with previous results in which activation of immediate early genes by acid was cell-specific [7].

In general, growth factors tend to activate ERK more than JNK MAP kinases, while stresses such as UV irradiation and hypertonicity tend to activate JNK more [24]. The present studies find that incubation in acid media does not activate JNK, and in fact causes a consistent small decrease in JNK activity. This suggests that the response to acidosis is more similar to that seen with growth factors than to a stress response. While one may have anticipated that acidosis would be a stress, the changes in cell pH that occur in acidosis are small and unlikely to be stressful to cells. It also should be noted that acidosis causes the kidney to grow [27]. In addition, the effects of acidosis are mediated by endothelin-1, a known growth factor [28].

Of interest, acid regulation of *c-fos* expression is inhibited by the tyrosine kinase inhibitors herbimycin A and tyrphostin A47 [7], but is not inhibited by *c-src*^{K295M} expression. Thus, while tyrosine kinases are required for *c-fos* activation, c-Src activity is not required. Similarly, acid activation of ERK is not inhibited by *c-src*^{K295M} expression. In fibroblasts, acid incubation activates c-Src, but does not activate ERK or increase immediate early gene expression. These results dissociate activation of ERK and immediate early genes from that of c-Src, and imply that activation of ERK and immediate early gene expression are mediated by pathways distinct from c-Src. This conclusion agrees with the observation that inhibition of PDGF-induced cell growth by c-Src inhibition is not rescued by overexpression of *c-fos* or *c-jun*, but is rescued by overexpression of *c-myc* [29]. Incubation of OKP cells in acid caused a transient increase in *c-myc* expression, but this also was not inhibited by *c-src*^{K295M} expression (data not shown).

An important role of the ERK/immediate early gene pathway in the increase in NHE3 activity was demonstrated using PD98059, a specific inhibitor of MEK. MEK is a dual specific kinase that activates ERK by phosphorylation of threonine and tyrosine in the tripeptide motif, Thr-Glu-Tyr. PD98059 inhibits MEK1 with an IC_{50} of 4 μmol/L, and MEK2 with an IC_{50} of 50 μmol/L. In most cells, MEK1 is responsible for activation of ERK [24]. PD98059 inhibited acid activation of NHE3. As a control we showed that PD98059 does not inhibit glucocorticoid-induced NHE3 activation. Thus, the MEK/ERK/immediate early gene pathway is required for acid activation of NHE3.

These studies demonstrate that acidosis activates c-Src and ERK kinases by parallel pathways, both of which

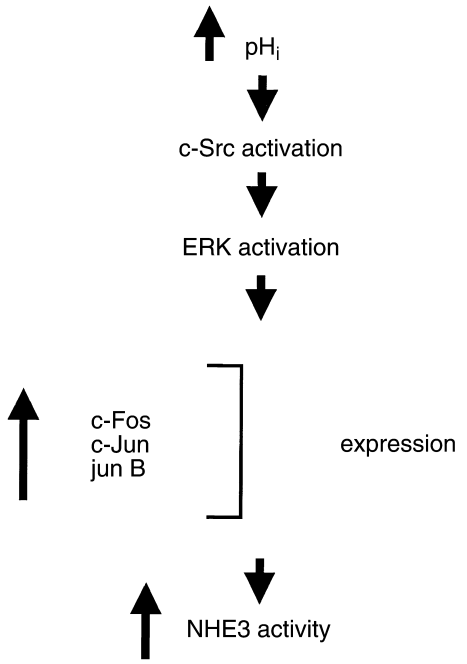

Fig. 13. Proposed signaling pathway mediating activation of NHE-3 by acid. It is important to note that none of the steps denoted by arrows imply direct activation. In addition, other pathways not shown may be involved.

are required for NHE3 activation (Fig. 13). While c-Src may contribute to ERK activation, it is not required. The role of ERK is likely to increase immediate early gene expression and AP-1 transcription factor activity, but ERK may play a key role in NHE3 regulation independent of immediate early genes. In OKP cells, intracellular acidosis increases NHE3 activity by increasing NHE3 mRNA and protein abundance, and inducing trafficking to the plasma membrane [6, 30]. In addition, we have recently shown that acidosis induces expression of preproendothelin-1 mRNA, which plays a key role in NHE3 activation [28]. This is due to activation of the preproendothelin-1 promoter by activated protein-1 (AP-1) [28]. Thus, increases in NHE3 activity likely occur through multiple mechanisms. c-Src and ERK appear to play an integral role in these processes.

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