Regulation of the renal Na-HCO₃ cotransporter. XI. Signal transduction underlying CO₂ stimulation

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Ruiz, Ofelia S., R. Brooks Robey, Yi-Yong Qiu, Long Jiang Wang, Cheng Jin Li, Jianfei Ma, and Jose A. L. Arruda. Regulation of the renal Na-HCO₃ cotransporter. XI. Signal transduction underlying CO₂ stimulation. Am. J. Physiol. 277 (Renal Physiol. 46): F580–F586, 1999.—We have previously shown that CO₂ stimulation of the renal Na-HCO₃ cotransporter (NBC) activity is abrogated by general inhibitors of protein tyrosine kinases. The more selective inhibitor herbimycin also blocked this effect at concentrations known to preferentially inhibit Src family kinases (SFKs). We therefore examined a role for SFKs in CO₂-stimulated NBC activity. To this end, we engineered OK cells to express the COOH-terminal Src kinase (Csk), a negative regulator of SFKs. CO₂ stimulated NBC activity normally in β-galactosidase-expressing and untransfected control cells. In contrast, Csk-expressing cells had normal baseline NBC activity that was not stimulated by CO₂. CO₂ stimulation increased both total SFK activity and specific tyrosine phosphorylation of Src. The specific MEK1/2 inhibitor PD-98059 completely inhibited the CO₂ stimulation of NBC activity as well as the accompanying phosphorylation and activation of ERK1/2. Our data suggest the involvement of both SFKs, probably Src, and the “classic” MAPK pathway in mediating CO₂-stimulated NBC activity in renal epithelial cells.

SRC family kinase; ERK1/2 kinase; phosphorylation

Vectorial HCO₃ transport from the renal proximal tubule cell into the blood is mediated by the basolateral Na-HCO₃ cotransporter, NBC (2). We have shown that NBC is regulated by ambient pH (27), by glucocorticoids (23), and by activators of both G protein-coupled receptors, e.g., carbachol, angiotensin II, parathyroid hormone (21, 22, 24), and receptor protein tyrosine kinases, e.g., insulin and epidermal growth factor (EGF) (19). A number of pH-dependent and pH-independent signal transduction mechanisms, involving G proteins (25), protein kinase A (PKA), PKC, and calcium-calmodulin kinase (26), have been implicated as mediators of these effects. Of relevance to the present work, we have previously shown that general inhibitors of protein tyrosine kinases prevent the adaptive increase in NBC activity in response to acute CO₂ elevation, suggesting that tyrosine phosphorylation is involved in NBC regulation (20). The nonreceptor protein tyrosine kinase c-Src plays a role in the analogous adaptive increase in renal brush border Na/H exchanger activity (NHE3) that accompanies metabolic acidosis (32). Moreover, Src kinase activity is increased by both metabolic acidosis and decreased intracellular pH (pHi) in a murine proximal tubule cell line (34). We therefore sought to examine whether Src family kinase (SK), and Src in particular, may play a similar role in the regulation of NBC by CO₂. To this end, we tested the ability of COOH-terminal Src kinase (Csk), a negative regulator of SFKs, to influence NBC activity when expressed in cultured rabbit proximal tubule cells and OK (American opossum kidney) cells. Since the “classic” MAPK pathway mediates a number of downstream effects of Src activation, we also examined its role in the stimulation of NBC by CO₂.

MATERIALS AND METHODS

Materials. Mycoplasma-free OK cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 37. Mammalian expression vectors containing full-length cDNAs for rat (pLXSH/Csk) and chicken (pcDNA1/Csk) Csk were provided by Drs. Jonathan Cooper (University of Washington, Seattle, WA) and Hidesaburo Hanafusa (Rockefeller University, New York, NY), respectively. Standard DMEM-F12 cell culture medium was purchased from Atlanta Biologicals (Norcross, GA), and all other cell culture reagents, including LipofectAMINE, were obtained from Gibco-BRL (Life Technologies, Gaithersburg, MD). The acetoxymethyl ester of 2',7'-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) was purchased from Molecular Probes (Eugene, OR), amiloride was from Research Biochemicals (Natick, MA), hygromycin was from Calbiochem Novabiochem (San Diego, CA), c-Src and Csk specific antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA), and RC20 anti-phosphotyrosine was from Transduction Laboratories (Lexington, KY). ERK1/2-specific antibodies and protein tyrosine kinase assay kits specific for SFKs and ERK1/2 were obtained from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence detection kit (ECL) used to analyze immunoblots was obtained from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Isolation and culture of rabbit proximal tubule segments and culture of OK cells. Proximal tubules were isolated from New Zealand White rabbits as previously described (4, 27). Electron microscopy confirmed that these preparations were homogenous, with over 98% of the isolated tubules exhibiting morphological characteristics of proximal tubules. We have previously shown that explant cultures from these tubules retain morphological, enzymatic, and transport functions of the original epithelium (19–26). Isolated proximal tubules were routinely suspended and cultured in defined serum-free growth medium: a mixture (50:50) of DMEM-F12 medium containing 24 mM NaHCO₃, 192 IU/ml penicillin, and 200 µg/ml streptomycin, 5 µg/ml bovine insulin, 5 µg/ml human transferrin, and 5 × 10⁻⁸ M hydrocortisone. Cultures were maintained at 37°C in a 5% CO₂ incubator at pH 7.40. Growth
medium was changed regularly every 3–4 days. Confluence was typically achieved within 8–10 days, after which cells were detached by trypsinization and replated on dear plastic coverslips. Confluence was typically achieved 2–3 days later, and cells were then used for fluorometric assays of NBC activity. Cultured proximal tubule cells were rendered quiescent by removal of hormones 48 h prior to experimental procedures.

OK cells were cultured in standard Eagle’s MEM medium supplemented with 10% fetal calf serum and were maintained in 5% CO2 at 37°C. To minimize the effects of phenotypic variation in culture, we routinely tested cells between passages 38 and 43. OK cells were typically serum deprived for 24 h prior to and during any experimental procedures.

Fluorometric assay of pHi and NBC activity in cultured renal epithelial cells. pHi was continuously monitored using the pH-sensitive fluorescent probe, BCECF as previously described (4, 23). In brief, cells grown on coverslips were perfused with a Cl-free solution to minimize contributions by both the Na-dependent Cl/HCO3 exchanger and the HCO3/Cl exchanger. Amiloride, 1 mM, was also included to inhibit Na/H exchanger activity. The cell chamber was perfused prior to assay with a Cl-free solution containing (in mM) 25 NaHCO3, 110 sodium gluconate, 5 potassium gluconate, 9 HEPES (pH 7.40), 2 CaSO4, 1 K2HPO4, 0.5 MgSO4, 10 glucose, and 1 amiloride at a rate of 20 ml/min at 37°C. Extracellular pH was maintained constant at 7.40. Once a stable fluorescence signal was achieved, Na was removed by equimolar substitution of choline for Na. Na removal resulted in a decrease in pHi that rapidly and fully recovered upon Na readdition. NBC activity was assayed as the initial rate of pHi recovery following the addition of NaHCO3 to cells perfused in the absence of Na. pHi recovery was inhibited by DIDS and, in the absence of chlorides, was primarily attributable to NBC activity (2). pH-sensitive BCECF fluorescence was routinely calibrated in the presence of elevated extracellular K+ and the ionophore nigericin (to equilibrate intracellular and extracellular pHi). All measurements were performed by dual-wavelength monitoring and ratiometric analysis (18) at pH-sensitive and pH-insensitive excitation wavelengths (F500/F430) at the completion of each experiment. Buffer capacity of the cells was determined from the pHi change as described (18).

Lipofection-mediated gene transfer. Transfection with mammalian expression vectors encoding rat (pLXSH/Csk) or chicken (pcDNA1/Csk) Csk was accomplished by lipofection using LipofectAMINE (GIBCO-BRL) according to the manufacturer’s recommendations. Rat Csk was expressed in OK cells under the control of a retroviral long terminal repeat (LTR) promoter, and stable transfectants were selected by hygromycin resistance conferred by the parent pLXSH vector (11). Individual clones were isolated and propagated in complete growth medium supplemented with 100 µg/ml hygromycin. The cells were incubated until they reached confluence before testing. Overexpression of the Csk protein was tested in 10–12 clones by Western blot analysis. Transfection of rabbit proximal tubule cells was achieved in a similar manner but using pcDNA1/Csk, which expresses chicken Csk under the control of a cytomegalovirus promoter (28).

Cell lystate preparation and immunoblotting. Confluent cell monolayers (75 cm2) were rinsed with ice-cold phosphate-buffered saline and harvested in 1 ml of modified RIPA buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with 100 µg/ml phenylmethylsulfonyl fluoride. After incubation on ice for 30 min and centrifugation at 15,000 g for 20 min at 4°C, the resulting supernatant was mixed with SDS gel-loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, and 0.1% bromophenol blue], boiled for 3 min, and separated by 10% SDS-PAGE. Resolved proteins were transferred onto a nitrocellulose membrane, which was then blocked by 2.5% fraction V bovine serum albumin in TBST (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) for 2 h at room temperature. Following incubation with rabbit polyclonal anti-Csk antibodies for 2 h at 4°C, specific bands corresponding to Csk were detected and quantitated by incubation with horseradish peroxidase-labeled secondary antibodies using the ECL detection kit (Amersham).

Detection of specific Src and ERK1/2 phosphorylation. To examine specific phosphorylation, Src immunoprecipitates were analyzed by parallel immunoblot analyses using both rabbit polyclonal anti-Src (Santa Cruz Biotechnologies) and mouse monoclonal anti-phosphotyrosine (RC20) antibodies (Transduction Laboratories). Specific ERK1/2 phosphorylation was similarly examined in ERK1/2 immunoprecipitates using both mouse polyclonal anti-ERK1/2 (Upstate Biotechnology) and mouse monoclonal RC20 antibodies. Detection and quantitation of individual species were uniformly performed using a commercially available chemiluminescent detection system (ECL, Amersham).

Measurement of SFK activity. The SFK activity was evaluated using a commercially available kinase assay kit (Upstate Biotechnology). In brief, we tested the ability of cell lysates to specifically phosphorylate a synthetic peptide substrate of SFKs corresponding to residues 6–20 of cdc2 (9). Samples were incubated in a solution containing 10 mM Tris-HCl (pH 7.2), 12.5 mM MgCl2, 10 mM MnCl2, 0.2 mM EGTA, 25 µM Na3VO4, 0.2 mM dithiothreitol, 50 µM ATP, and 10 µCi [γ-32P]ATP at 30°C for 10 min before stopping the reaction by the addition of trichloroacetic acid. An aliquot was applied to a P81 phosphocellulose paper. Unincorporated 32P was eluted with 7.5% phosphoric acid, and the remaining incorporated radioactivity was determined by liquid scintillation counting.

Measurement of ERK1/2 kinase activity. Total ERK1/2 kinase activity was measured as previously described (30) using a commercially available kit (Upstate Biotechnology). In brief, cell lysates were tested for the ability to specifically phosphorylate myelin basic protein in the presence of inhibitors of PKC, PKA, and calmodulin kinase II. Samples were incubated in 100 µM ATP, 15 mM MgCl2, containing 10 µCi [γ-32P]ATP for 10 min at 30°C before application to P81 phosphocellulose paper. Unincorporated 32P was eluted with 7.5% phosphoric acid, and the remaining incorporated radioactivity was determined by liquid scintillation counting.

Statistical analysis. Results are expressed as means ± SE, and statistical comparisons were performed by t-testing for both paired or unpaired data where appropriate.

RESULTS

Effects of herbimycin on CO2 stimulation of NBC activity. We have previously shown that 10% CO2-stimulated NBC activity in cultured rabbit proximal tubule cells can be blocked by general tyrosine kinase inhibitors. Pretreatment of OK cells with herbimycin at concentrations known to preferentially inhibit SFKs, <10−6 M (7), had no effect on basal NBC activity, but completely blocked the increase following exposure to 10% CO2 (Fig. 1). These findings are in agreement with our previous observations (20) and are compatible with a postulated role for tyrosine kinases, and SFKs in particular, in regulating NBC activity in proximal tubule cells.
Overexpression of Csk in OK cells and rabbit proximal tubule cells. To better address the role of SFKs on NBC activation, we tested the functional consequences of impaired SFK activation in both OK cells and cultured rabbit proximal tubule cells (see below). To this end, we expressed a Csk transgene and examined the effects on both basal and CO2-stimulated NBC activity. Untransfected cells and cells stably transfected with a β-galactosidase reporter gene were employed as controls. Cultured proximal tubule cells were transiently transfected with the same expression vector. Overexpression of Csk protein was confirmed by Western blot analysis (Fig. 2).

Effect of Csk overexpression on CO2-stimulated NBC activity. Figure 3A shows the effect of 10% CO2 on NBC activity in cultured rabbit proximal tubule cells transfected with pLXSH/Csk or with an empty control vector. In cells transfected with vector alone, 10% CO2 stimulated NBC activity normally from 1.49 ± 0.07 to 2.02 ± 0.19 pH units/min (P < 0.001). In contrast, 10% CO2 failed to stimulate NBC activity in cells expressing Csk [Csk, 1.39 ± 0.09; Csk + 10% CO2, 1.20 ± 0.11 pH units/min; not significant (NS)]. Baseline NBC activity was not different between cells transfected with an empty control vector and pLXSH/Csk. In proximal tubule cells transiently expressing the chicken homolog of Csk, similar results were obtained. The 10% CO2 failed to stimulate NBC (Csk, 1.49 ± 0.10 vs. Csk + 10% CO2, 1.25 ± 0.1 pH units/min; NS, n = 8), suggesting that the effect is not species specific.

Figure 3B shows the results obtained in OK cells stably expressing rat Csk. In cells transfected with control vector alone, 10% CO2 increased NBC activity significantly from 1.0 ± 0.07 to 2.02 ± 0.19 pH units/min (P < 0.01), whereas in cells expressing Csk, 10% CO2 failed to stimulate NBC completely (Csk, 0.7 ± 0.08 vs. Csk + 10% CO2, 0.82 ± 0.08 pH units/min). Baseline NBC activity was not different between control cells and cells stably expressing Csk. Additional experiment showed that Csk-transfected cells had baseline NBC activity not different from controls (1.06 ± 0.05 vs. 1.10 ± 0.07 pH units/min; n = 6, NS).

Effect of CO2 on SFK activity in OK cells. Figure 5A shows the effect of 10% CO2 on SFK activity. CO2 increased SFK activity by over 50% above control levels. Overexpression of Csk in OK cells and rabbit proximal tubule cells. To better address the role of SFKs on NBC activation, we tested the functional consequences of impaired SFK activation in both OK cells and cultured rabbit proximal tubule cells (see below). To this end, we expressed a Csk transgene and examined the effects on both basal and CO2-stimulated NBC activity. Untransfected cells and cells stably transfected with a β-galactosidase reporter gene were employed as controls. Cultured proximal tubule cells were transiently transfected with the same expression vector. Overexpression of Csk protein was confirmed by Western blot analysis (Fig. 2).

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Effect of Csk overexpression on dexamethasone stimulation of NBC activity. Figure 4 shows that stable Csk expression did not prevent the stimulatory effect of 10-8 M dexamethasone on NBC activity [control, 1.30 ± 0.02; dexamethasone, 1.78 ± 0.05 (P < 0.001); Csk, 1.33 ± 0.10; dexamethasone + Csk, 1.80 ± 0.06 pH units/min (P < 0.01)]. The increase in NBC activity by dexamethasone was of the same magnitude in both control and Csk-expressing cells, indicating that the effect of Csk is specific for CO2.

Effect of CO2 on SFK activity in OK cells. Figure 5A shows the effect of 10% CO2 on SFK activity. CO2 increased SFK activity by over 50% above control levels.
within 3 min and then decreased toward baseline levels at 30 min. Activation of NBC and SFK activity by CO\textsubscript{2} was also correlated with increased tyrosine phosphorylation of endogenous Src, which was similarly blocked by herbimycin, suggesting a causal relationship. Figure 5B shows that 10\% CO\textsubscript{2} increased phosphorylation of Src by twofold, and herbimycin decreased phosphorylation to control levels (expressed as percentage of control, 100\%; CO\textsubscript{2}, 212.8 \pm 28.8\%, P < 0.001; CO\textsubscript{2} + herbimycin, 78.3 \pm 13.4\%, n = 4).

Effect of MEK1/2 inhibition on CO\textsubscript{2}-stimulated NBC activity. Because a number of signaling pathways involving Src activation proceed through the classic MAPK pathway (Ras \rightarrow Raf1 \rightarrow MEK1/2 \rightarrow ERK1/2), we utilized the specific MEK1/2 inhibitor PD-98059 to examine the role of this pathway. Figure 6 shows that the inhibitor failed to alter baseline activity of the cotransporter (control, 1.58 \pm 0.17; inhibitor, 1.53 \pm 0.13 pH units/min), but completely abolished the effect of 10\% CO\textsubscript{2} (CO\textsubscript{2}, 2.17 \pm 0.14; CO\textsubscript{2} + inhibitor, 1.57 \pm 0.15 pH units/min; P < 0.02).

Effect of CO\textsubscript{2} on ERK1/2 kinase activity. The finding that PD-98059 inhibits CO\textsubscript{2} stimulation of NBC activity suggests involvement of the classic MAPK pathway. Consistent with this interpretation, 10\% CO\textsubscript{2} increased ERK1/2 activity by over 50\% (Fig. 7A), and this effect was also blocked by PD-98059. Tyrosine phosphorylation of ERK1/2 correlated with activation. Figure 7B and C shows that CO\textsubscript{2} increased phosphorylation of ERK1/2, which was likewise blocked by PD-98059.

Fig. 3. Effect of Csk overexpression on CO\textsubscript{2}-stimulated NBC activity. A: rabbit proximal tubule cells were transiently transfected with an empty control vector or with pLXSH/Csk cDNA, and NBC activity was measured in cells exposed either to 5\% or 10\% CO\textsubscript{2} for 5 min. Each bar represents mean \pm SE; n = 8. B: NBC activity was measured in untransfected (control) and stably transfected OK cells exposed to either 5\% or 10\% CO\textsubscript{2} for 5 min. Each bar represents mean \pm SE; n = 6.

Fig. 4. Effect of Csk overexpression on dexamethasone stimulation of NBC activity. NBC activity was assayed in untransfected (control) and stable pLXSH/Csk-transfected OK cells pretreated with either vehicle or dexamethasone, 10^{-8} M, for 48 h as previously described (24). Each bar represents mean \pm SE; n = 6.
DISCUSSION

The role of SFKs in modulating NBC and NHE3 activities has only recently been examined, but SFKs are clearly involved (10, 32, 34). This closely related family of tyrosine kinases is phylogenetically conserved and in the mouse is composed of at least nine members (Src, Yes, Fyn, Lyn, Lck, Hck, Fgn, Plk, and Yrk) (7, 12, 29). Src, Yes, and Fyn are ubiquitously expressed and have been identified in renal proximal tubule cells (3, 29). Heterologous expression of Csk (COOH-terminal Src kinase) specifically inhibits the activation of all known Src kinase family members (3, 11, 14, 17) and prevents the acidosis-induced increase in NHE3 activity in MCT medullary collecting duct cells (34). A decrease in pHi, both in the presence or absence of extracellular acidosis, increases both Src kinase activity and specific phosphorylation of endogenous 60- to 70-kDa and 120-kDa proteins in these cells (32). We have previously shown that activation of tyrosine kinases by a variety of extracellular stimuli, including insulin, EGF, and carbachol, can increase NBC activity in cultured proximal tubule cells (19). The finding that tyrosine kinase inhibitors prevent the effect of metabolic acidosis on NHE3 and CO2 on NBC activity is consistent with the hypothesis that SFKs may be involved in the regulation of both NHE3 and NBC.

OK cells are derived from proximal tubule cells and retain morphological and functional characteristics of normal proximal tubule, including regulated apical NHE3 and basolateral NBC activities. They are thus ideally suited for the simultaneous study of their regulation (8). Heterologous expression of Csk completely blocked the effects of CO2 on NBC activity in both OK cells and cultured rabbit proximal tubule cells.

CO2 stimulation also increased endogenous SFK activity and phosphorylation. Taken together, these findings suggest that Src plays a role in the stimulation of NBC activity by CO2.

Activation of Src results in signaling through the classic MAPK cascade (Ras → Raf → MEK1/2 → ERK1/2) (1, 3, 5, 6, 8, 13–17, 30, 31). The “classic” MAPK pathway signaling through ERK1/2 is activated by a variety of extracellular stimuli, including growth factors. The upstream activator of ERK 1/2, MEK 1/2, is
specifically inhibited by PD-98059. It is therefore of great interest that PD-98059-inhibitable MEK1/2 and ERK1/2 activation plays a major role in the stimulation of NHE1 by growth factors and arginine vasopressin (AVP) (1,5). The finding that PD-98059 also blocks the effect of CO₂ on NBC activity, coupled with the observation that CO₂ increases both specific ERK1/2 phosphorylation and kinase activity, strongly suggests that the classic MAPK pathway is involved in NBC regulation. The major components of this pathway are ubiquitously expressed along the axial nephron, and both MEK1/2 and ERK1/2 are stimulated by EGF-1 and angiotensin II in the kidney, with the latter acting mainly in the proximal tubule (30). The specific mechanisms whereby ERK1/2 augments a membrane transporter activity have not been elucidated. ERK1/2 kinase activation by AVP in platelets and by growth factors in other tissues can increase NHE1 activity independent of specific Tyr phosphorylation (5,6). In the brain, there is substantial evidence that angiotensin II independently of specific Tyr phosphorylation (5,6). In conclusion, we have demonstrated that CO₂ stimulation of NBC activity involves both SFK and ERK1/2 activation.

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