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The Role of Phosphatidylinositol 3-Kinase (PI3K) in CO_2 Stimulation of the Na^+/HCO_3^- Cotransporter (NBC)

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Abstract. The basolateral Na^+/HCO_3^- cotransporter (NBC) is the major pathway for bicarbonate reabsorption in the renal proximal tubule cells. The cotransporter activity is enhanced by 10% CO_2. Phosphatidylinositol 3-kinase (PI3K) has been shown to regulate the function and trafficking of cellular proteins by promoting their translocation to the plasma membrane. Therefore, we sought to examine the role of PI3K in CO_2-mediated stimulation of NBC activity in OK cells. Our studies showed that wortmannin, a well-characterized PI3K inhibitor, had no effect on baseline NBC activity but prevented the stimulatory effect of 10% CO_2. This effect was concentration-dependent and time-dependent. Another inhibitor of PI3K, LY294002, also prevented the CO_2-mediated increase in NBC activity. CO_2 stimulation of the cotransporter was paralleled by an increase in PI3K enzyme activity and this effect was blocked by wortmannin. Biotinylation studies also showed that 10% CO_2 increased the immunoreactive NBC in the basolateral membranes and this was prevented by wortmannin. We previously showed that 10% CO_2 stimulation of NBC activity involves the Src family kinase pathway. In the current studies, CO_2 stimulation significantly increased Src phosphorylation and this effect was abrogated by wortmannin. In summary, CO_2 stimulation of NBC is mediated at least in part by increased immunoreactive NBC protein in the basolateral membrane, a process which requires the interaction of PI3K with Src family kinase.

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Introduction

Renal proximal acidification is mediated by the apical sodium hydrogen exchanger (NHE3) and by the basolateral sodium bicarbonate cotransporter (NBC). NBC is the main transport system responsible for reabsorption of bicarbonate from the proximal tubule cell into the blood (Alpern, 1985). It may also play an important role in sodium reabsorption and volume regulation (Romero & Boron, 1999). We previously showed that NBC activity is stimulated by respiratory acidosis and this effect is mediated through the Src family kinase and the classic MAPK pathway (Ruiz et al., 1998, 1999).

Phosphatidylinositol 3-kinase (PI3K) is a dual-specificity kinase, which can act as a phosphoinositide kinase or a protein kinase capable of phosphorylating its substrates (Carpenter & Cantley, 1990; Hunter, 1995; Toker & Cantley, 1997). PI3K has also been implicated in mediating various processes such as cell proliferation and migration, vesicle budding, protein transport and cytoskeletal rearrangement (Hansen, Olsson & Casanova, 1995; Wurmser, Gary & Emr, 1999). The enzyme also provides a mechanism for interaction between proteins and cellular motors vital for trafficking (Martin et al., 1996; Rameh & Cantley, 1999; Guilherme et al., 2000). It has also been shown to interact with tyrosine kinase and G protein-coupled signaling pathways (Downes & Carter, 1991; Thelen, Wyman & Lange, 1994). In

3T3-L1 adipocytes, transient expression of PI3K subunit, p110, resulted in GLUT 4 translocation to the plasma membranes and this effect was blocked by wortmannin (Martin et al., 1996). Overexpression of p110 kinase dead subunit of PI3K (p110Δkin) inhibited GLUT 4 activity and its translocation from the intracellular compartments to the plasma membranes. The same observations have been noted in the presence of insulin, providing evidence of the important role of PI3K in the modulation of glucose transport by insulin. This kinase also plays a role in the regulation of NHE1 activity in the Chinese hamster ovary cells through a process of increased endocytosis and inhibition of exocytosis (Kurashima et al., 1998).

Because the activity of NBC is increased by 10% CO_2, we examined the role of PI3K in regulation of NBC in the basolateral membrane. Using specific antibodies against the NBC, we demonstrated that the cotransporter is constitutively localized in the intracellular compartments as well as the basolateral domains (Noboa et al., 2000; Robey et al., 2002). In this regard we examined the effect of PI3K inhibition by wortmannin on NBC protein abundance in the basolateral membrane. In addition, we previously showed that 10% CO_2 stimulation of NBC1 activity involves the Src family kinase pathway (Ruiz et al., 1999). Since PI3K is involved in tyrosine kinase signaling, we hypothesized that CO_2 stimulation of NBC activity may be mediated at least in part by interaction between Src and PI3K.

Materials and Methods

Reagents

BCECF-AM (2′7′-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein) acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR), amiloride from Research Biochemicals (Natick, MA), wortmannin and LY 294002 from Sigma (St. Louis, MO), anti-PI3K antibody from Upstate Biotechnology (Lake Placid, NY), and (γ^{32}P) ATP from Amersham (Arlington Heights, IL). Buffers and other chemicals of finest quality and highest grade were obtained from Sigma (St. Louis, MO). Affinity-purified rabbit polyclonal antibody was generated against the 18 carboxyl terminal amino-acid residues based on human NBC1 sequence (Genebank accession number NP 003750, Amlal et al., 1998). The affinity-purified antibody (αNBC1c) specifically recognized a protein of the predicted size (130 kDa), as previously described (Noboa et al., 1998; Robey et al., 2002).

Cell Cultures

American opossum kidney cells (OK cells, ATCC CRL 1840) were grown in standard Eagle's MEM medium supplemented with 10% fetal calf serum and maintained in 5% CO_2 at 37°C. To minimize the effects of phenotypic variations, we performed experiments between passages 38 and 43. Prior to each experimental procedure, cells were serum-deprived for 18 hr. Respiratory acidosis was induced by exposure of the cells to 10% CO_2 for the specified times.

PI3K Activity Assay

PI3K activity was measured in OK cells as previously described by Donowitz and his group, with modifications (Khurana et al., 1996). Briefly, confluent cells subjected to each specific treatment were lysed. Cell lysates were centrifuged for 10 minutes and supernatant fractions were incubated with anti-PI3K antibody for 1 hr at 4°C. Protein A agarose suspended in PBS was added for 1 hr at 37°C. The immunoprecipitate was collected and washed with 1% NP-40 three times and with 0.1 M Tris-HCl, (pH 7.4) and 5 mM LiCl and 0.1 mM sodium orthovanadate. Washing with TNE buffer (in mM: 10 Tris-HCl, pH 7.4, 150 NaCl, 5 EDTA, 0.1 sodium orthovanadate) was done twice. Fifty microliters each of TNE, phosphatidylinositol (0.2 mg/ml) and 5 mM MgCl were added sequentially to the complex. Reaction was started with 5 μl each of (γ^{32}P) ATP and 20 mM MgCl and terminated after continued agitation for 10 min at 37°C with 6 N HCl. Radiolabeled lipids were extracted with $CHCl_3$:MeOH [1:1]. Aliquots of organic ($CHCl_3$) phase fraction were spotted on silica TLC plate. Lipids were subjected to chromatography in $CHCl_3$:MeOH:H_2O:NH_4OH [60:47:11:3.2] solvent. Radiolabeled blots were visualized by autoradiography and compared to known standards. Concentrations of aliquoted organic fractions were measured accurately to assure that identical amounts were loaded onto TLC.

Fluorometric Assay of Intracellular pH and NBC Activity

NBC activity expressed as ΔpH unit/min was measured continuously using the pH-sensitive fluorophore BCECF-AM, as previously described (Bernardo et al., 1999). Cells grown to confluence in plastic slides were perfused at 37°C with a chloride-free solution consisting of (in mM) 25 $NaHCO_3$, 110 sodium gluconate, 5 potassium gluconate, 9 HEPES (pH 7.4), 2 $CaSO_4$, 1 KH_2PO_4, 0.5 $MgSO_4$, 10 glucose, and 1 amiloride. Extracellular pH was maintained at 7.4 throughout the experiments. When a stable fluorescence signal was attained, sodium was removed by substitution of equimolar concentration of choline. The absence of sodium resulted in decrease in intracellular pH (pH_i) and pH-sensitive fluorescence intensity. The readdition of sodium caused an immediate increase in pH_i and fluorescence signal. NBC activity was assayed as the initial rate of recovery of pH_i following the readdition of sodium. The rapid pH_i recovery was DIDS-sensitive and in the absence of chloride it was predominantly attributable to NBC activity. Calibration of the pH-sensitive BCECF fluorescence was done routinely in the presence of elevated extracellular potassium and ionophore nigericin to achieve equilibration of the intracellular and extracellular pH. All measurements were performed by dual-wavelength monitoring and ratiometric analysis at pH-sensitive (500 nm) and -insensitive (450 nm) excitation wavelengths (F_{500}/F_{450}).

NBC Protein Biotinylation, Affinity Precipitation and Immunoblotting of Cell Surface Proteins

OK cells were grown to confluency on 24-mm polycarbonate membranes with a 0.4 μm pore size (Transwell® Corning Costar, Corning, NY). Cells were then placed in serum-free medium for at least 18 hours to establish a quiescent state. Biotinylation procedure was performed as we described previously (Weinman et al., 2001) with some modifications. Briefly, cells were washed three times with PBS buffer containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Sulfo-NHS-SS-biotin (Pierce, Rockford IL) was dissolved

in ice-cold Ca/Mg PBS (0.5 mg/ml), and was added immediately to the basolateral compartment of the Transwell® dish as indicated. The apical compartment was previously quenched with 100 mM glycine. Biotinylation was carried out for 25 min at 4°C with constant agitation. The cells were washed once with serum-free medium to scavenge the unreacted biotin, followed by three washes with Ca/Mg PBS. The polycarbonate membranes were excised from the chambers with a scalpel and placed in 1 ml lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 50 mM EDTA and 1% Triton X-100). The membranes were incubated for 1 hr at 4°C with constant agitation, and then sonicated for 15 sec. The soluble component was transferred to Eppendorf tubes, to which 100 μl of streptavidin agarose (Pierce) was added. The tubes were incubated with constant agitation overnight at 4°C, and then centrifuged (14,000 × *g* for 5 min). The streptavidin agarose beads were washed three times with lysis buffer and one time with low-salt lysis buffer. The biotinylated proteins were precipitated from the cell lysates using streptavidin by elution with buffer (80 mM DTT, 5.6% SDS, 0.008% bromophenol blue, 0.24 M Tris-HCl, pH 8.9 and 16% glycerol). The eluted proteins were electrophoresed in 8% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked for 1 hr with 10% nonfat dry milk in TTBS (Tris-buffered saline with 0.1% Tween 20). The membrane was then incubated in affinity-purified anti-NBC antibody (αNBC1c) for 1 hr. The immunoreactive NBC1 was labeled with anti-rabbit IgG linked with HRP for 1 hr, and detected using NEB developer (New England Biolab, Natick, MA).

Src Phosphorylation Assay

Src phosphorylation was determined through quantitative analysis of whole-cell lysates as previously described (Ruiz et al., 1999; Robey et al., 2002). The total Src abundance and Src tyrosine phosphorylation were measured in parallel, using Src-specific polyclonal antibodies and recombinant monoclonal antiphosphotyrosine antibodies (RC20), respectively, according to manufacturer's recommendation (BD Transduction Laboratories, Lexington, KY).

Data Analysis and Statistics

Data are presented as means ± SE. Statistical analysis was performed using Student's *t*-test for paired data. Analysis of variance was performed as appropriate.

Results

PI3K Inhibitors Prevent CO_2 Stimulation of NBC Activity

To evaluate the role of the PI3K pathway in CO_2 stimulation of NBC activity, we utilized wortmannin and LY294002, which are known specific inhibitors of PI3K (Okada et al., 1994; Vlahos et al., 1994). Wortmannin is a fungal metabolite that inhibits PI3K activity by binding irreversibly to the p110 catalytic subunit of the enzyme. The lower concentration (10 nM) used in our studies was the dose noted to preferentially inhibit PI3K, whereas the non-specific effects of the drug on PKC, PKA, PKG, PI 4-K as well as MAPK and $p70^{s6k}$ were observed at higher concentrations (Ui et al., 1995). Using OK cells, a well-established cell line of renal proximal tubule origin, NBC acitivity was assayed with different concentrations of wortmannin for 30 min prior to CO_2 stimulation. As shown in Fig. 1*A*, wortmannin blocked the stimulatory effect of CO_2 on NBC activity at 0.1 nM and this inhibitory effect was maintained up to 100 nM. Fig. 1*B* shows that the CO_2-induced stimulation of cotransporter activity was inhibited by wortmannin at 1 min and was maximal after 5–30 min. Fig. 1*C* demonstrates that the dose of wortmannin used in subsequent experiments (10 mM) had no effect on baseline NBC activity. To confirm the above findings, we used LY294002, another inhibitor of the PI3K, which binds to the ATP binding site of PI3K. The concentration of LY294002 (50 μM) utilized in our studies has been previously shown to be specific for PI3K (Vlahos et al., 1994). As shown in Fig. 2, LY294002 had no effect on baseline NBC activity, but prevented the stimulatory effect of CO_2.

Wortmannin Prevents CO_2-induced Increase in PI3K Activity

If the stimulatory effects of CO_2 on NBC activity were mediated by PI3K, the increase in NBC activity should be paralleled by corresponding changes in PI3K activity. Therefore, we performed experiments to study if the CO_2-induced stimulation of PI3K activity was blocked by wortmannin. OK cells were exposed to 10% CO_2 in the presence or absence of 10 nM of wortmannin and assayed for PI3K activity. Fig. 3 shows that 10% CO_2 induced a significant increase in PI3K activity as compared to control (expressed as % change from control), and this stimulatory effect of 10% CO_2 was blocked by wortmannin (Control 5% CO_2, 100%; 10% CO_2, 254.3% ± 11.3; Insulin, 219.6% ± 15; 10% CO_2 + wortmannin, 123.2% ± 9.80 where, 10% CO_2 *vs.* 10% CO_2 + wortmannin, $P < 0.05$, $n = 4$). Insulin, which has been shown to increase PI3K activity, was used as positive control, as previously described (Ui et al., 1995; Suga et al., 1997; Sharma et al., 1998; Shepherd et al., 1998; Yang, et.al., 1998).

Wortmannin Prevents the CO_2-induced Increase in Immunoreactive NBC Protein in Basolateral Membrane

To study the possible mechanism whereby PI3K may play a role in stimulation of NBC activity by CO_2, we examined the effects of wortmannin on changes in NBC1 basolateral membrane protein. In these experiments, OK cells grown in Transwell®

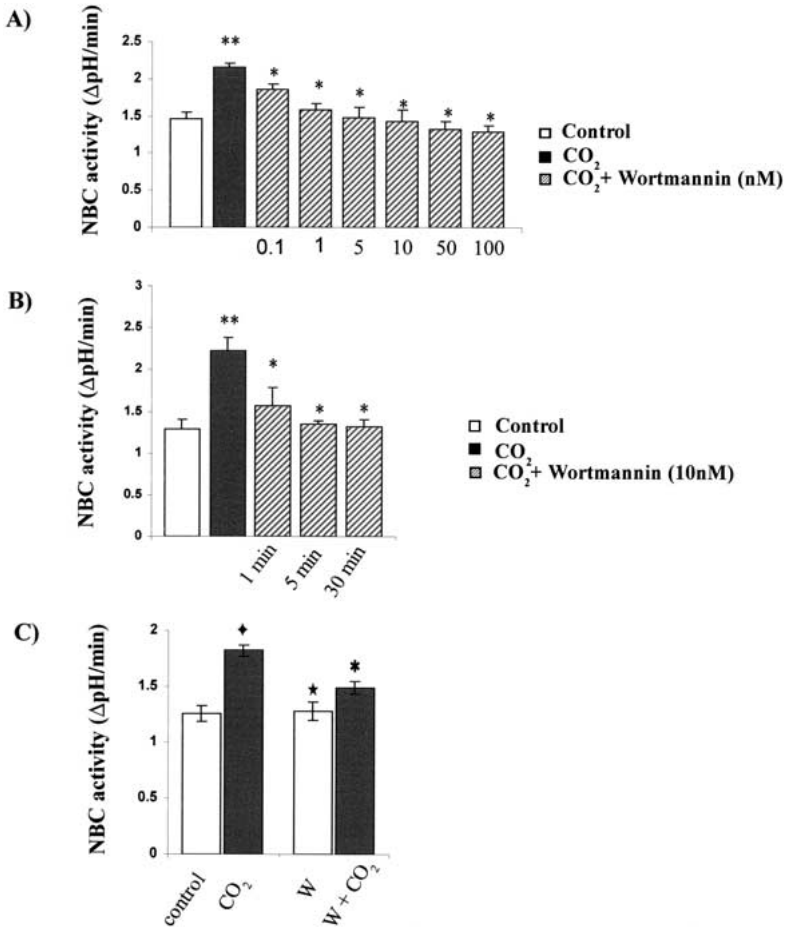

Fig. 1. Dose- and time-dependent effects of wortmannin on CO_2 stimulation of NBC activity. (*A*) OK cells grown to confluence were serum-deprived for at least 18 hr prior to each experiment. Cells were treated with varying concentrations of wortmannin as indicated for 30 minutes and assayed for NBC activity (expressed as ΔpH unit/min) after exposure to 10% CO_2. Data shown are mean ± SE of six experiments (**Control *vs.* CO_2, $P < 0.01$; *CO_2 *vs.* CO_2 + wortmannin, $P < 0.01$). (*B*) Confluent OK cells were serum-deprived for at least 18 hr prior to the planned studies and assayed for NBC activity expressed as ΔpH unit/min. Cells were incubated in 10% CO_2 and treated with 10 nM wortmannin for the indicated time periods. Data shown are mean ± SE of six experiments (**Control *vs.* CO_2, $P < 0.01$; *CO_2 *vs.* CO_2 + wortmannin, $P < 0.01$). (*C*) OK cells grown to confluence were made quiescent for 18 hours. NBC activity (expressed as ΔpH unit/min) was measured at 30 min incubation with wortmannin (10 nM). Data represented are mean ± SE of six experiments. (♦Control *vs.* CO_2, $P < 0.01$; ★Control *vs.* wortmannin, ns; *CO_2 *vs.* CO_2 + wortmannin, $P < 0.01$).

were stimulated with 10% CO_2 in the presence or absence of wortmannin for 30 min and subsequently labeled on the basolateral side with biotin. The labeled surface proteins were precipitated with streptavidin and immunoblotted with affinity-purified NBC antibody (αNBC1c). As shown in Fig. 4, immunoreactive NBC protein is detected in the basolateral membrane. CO_2 significantly increased the amount of immunoreactive biotinylated NBC proteins in the basolateral membranes, and this effect was prevented by wortmannin. This is compatible with the suggestion that the CO_2-induced increase in the detectable immunoreactive NBC in the basolateral membrane is modulated by a PI3K-dependent mechanism.

Effects of Wortmannin on CO_2 Stimulation of NBC Activity when Endocytosis Is Blocked

The increase in NBC activity and detectable immunoreactive NBC protein in the basolateral membrane may be mediated by a PI3K-dependent endocytic mechanism, as described by Grinstein and his group. Therefore, we employed the experimental approach used by Grinstein, where they showed that endocytosis can be virtually abrogated by low temperature

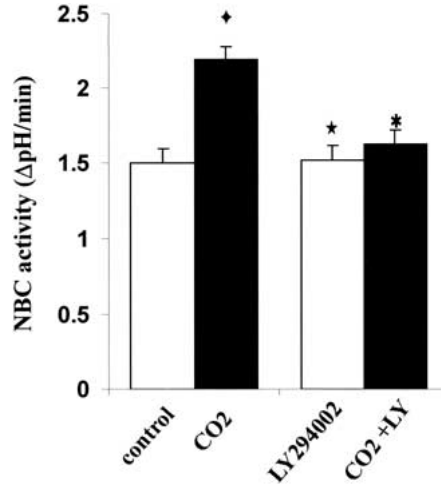

Fig. 2. Effects of LY294002 on CO_2-mediated stimulation of NBC activity. OK cells were made confluent and serum-deprived for at least 18 hr and exposed to 10% CO_2 as indicated, with or without prior incubation with 50 mM LY294002 for 30 minutes. NBC activity (expressed as ΔpH unit/min) was measured fluorometrically as described above (*see* Methods). In other experiments, OK cells were treated only with LY294002. Each data point represents 6 experiments expressed as mean ± SE of six experiments (♦Control *vs.* CO_2, $P < 0.05$; ★Control *vs.* LY294002, ns; *CO_2 *vs.* CO_2 + LY294002, $P < 0.05$).

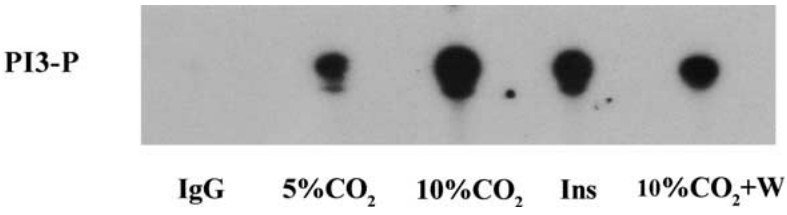

Fig. 3. Effects of wortmannin on NBC activity are paralleled by changes in PI3K activity. OK cells were grown to confluence and incubated in 10% CO_2. The cells were lysed and determination of PI3K activity was performed as described (*see* Methods). Different treatments were done as labeled. Insulin, a known stimulus of PI3K activity, was used as positive control. IgG was used as negative control. The above data are representative of four different experiments.

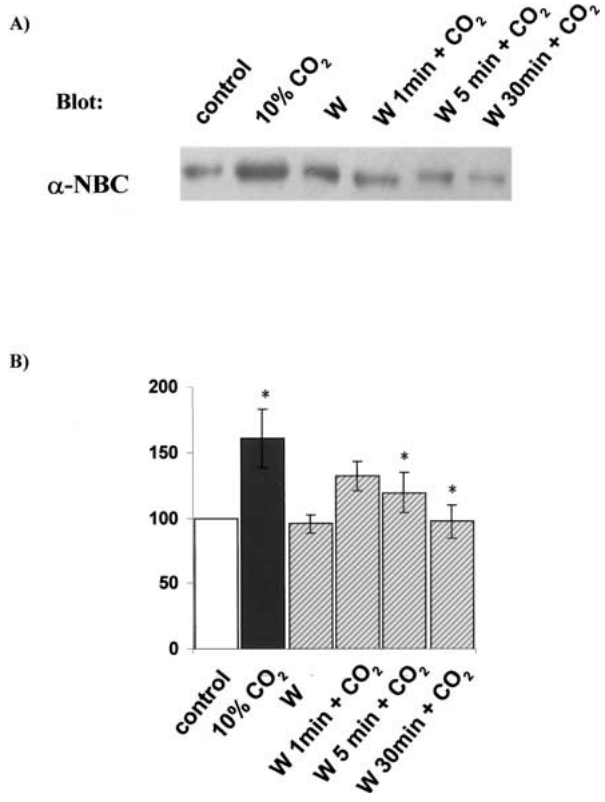

Fig. 4. Wortmannin prevents CO_2-induced increase in immunoreactive NBC abundance in the basolateral membrane. Confluent OK cells were treated with wortmannin, 10 nM for indicated times. Biotinylation was performed as described in Methods. Biotin-labeled proteins were precipitated by streptavidin and immunoblotted with specific anti-NBC antibody (αNBC1c). (*A*) Representative immunoblot analysis of four different experiments. (*B*) Quantitation of four independent experiments (mean ± SE). Data were analyzed by one-tailed *t*-test (Control *vs.* CO_2, $n = 4$, $P < 0.03$; 10% CO_2 vs. *W 5 min + CO_2, $n = 4$, $P < 0.03$; 10% CO_2 *vs.* *W 30 min + CO_2, $n = 4$, $P < 0.03$).

(Kurashima et al., 1998). Using their protocol with some modifications, OK cells were treated with 10 nM wortmannin for 30 min at 37°C. This allowed wortmannin to react with PI3K and to bind irreversibly with the p110 catalytic subunit PI3K (Okada et al., 1994; Vlahos et al., 1994; Ui et al., 1995). The cells were extensively washed to remove excess wortmannin. Incubation was subsequently carried out for 60 min at 4°C (4°C temperature inhibits endocytosis) or at 37°C (37°C temperature allows endocytosis to occur). The cells were prepared thereafter for measurement of NBC activity (*see* Materials and Methods) at 37°C in 5% or 10% CO_2. In OK cells with prior exposure to 4°C and wortmannin, NBC activity measured at 37°C was inhibited by 10% CO_2 to the same degree as cells exposed to wortmannin at 37°C (Control, 1.5 ± 0.21; CO_2, 2.17 ± 0.08; CO_2 + wortmannin, 37°C, 1.48 ± 0.13; CO_2 + wortmannin, 4°C, 1.25 ± 0.15 (Control *vs.* CO_2, $n = 6$, $P < 0.01$; Control *vs.* CO_2 + wortmannin, 37°C, $n = 6$, ns; Control *vs.* CO_2 + wortmannin, 4°C, $n = 6$, ns). Therefore, prevention of endocytosis by prior incubation of cells at 4°C does not prevent the effect of wortmannin to block CO_2 stimulation of NBC activity.

CO_2-induced Src Phosphorylation Is Blocked by Wortmannin

To examine the relationship between Src and PI3K, we studied the effects of PI3K inhibition by wortmannin on Src-family kinase tyrosine phosphorylation. As shown in Fig. 5, incubation of OK cells in 10% CO_2 resulted in an increase in tyrosine phosphorylation of Src family kinase. Wortmannin prevented tyrosine phosphorylation, suggesting an interaction between Src-family kinase and PI3K in CO_2 stimulation of NBC activity.

Discussion

The mammalian PI3K is a heterodimeric complex made up of two subunits (p85 and p110). The p85 kDa regulatory subunit contains two carboxyl terminal Src homology 2 (SH2) domains and an amino-terminal SH3 domain. Extracellular signals promote the interaction between the Src-family kinases with the SH3 domains of p85 subunit of PI3K (Cantley et al., 1991; Carpenter et al., 1993; Liscivitch & Cantley, 1994). Additionally, the SH2 domain of Src and p85 subunit of PI3K can bind competitively against tyrosine-containing proteins (Fukui & Hanafusa, 1991; Grey

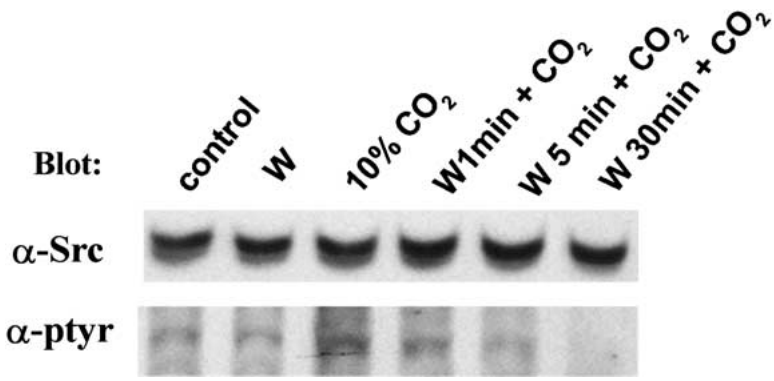

Fig. 5. Effects of wortmannin on Src phosphorylation. OK cells were grown to confluence and incubated with 10 nM wortmannin for the specified time. The cells were then exposed to 10% CO_2 at 37°C for 30 min. Specific tyrosine phosphorylation of Src family kinase was determined using specific anti-phosphotyrosine antibody (*see* Methods). Equal amount of immunoprecipitated Src protein was loaded onto each well and confirmed by western blotting with anti-Src antibody. The above data are representative of three experiments showing Src phosphorylation.

et al., 2000). Overexpression of the p110 subunit of PI3K enhanced the Src-family kinase-mediated tyrosine phosphorylation of Btk (Bruton's tyrosine kinase) and this effect was blocked by the PI3K inhibitor, wortmannin. PI3K has also been shown to play an important role in cellular trafficking of proteins (Chasserot-Golazs et al., 1998). The role of PI3K in membrane trafficking was first noted because of the sequence homology of the mammalian PI3K catalytic subunit and the yeast protein (Vps34p), which plays an important role in protein trafficking in yeast (Carpenter & Cantley, 1990; Wurmser, et al., 1999). In eukaryotic cells, PI3K has been implicated in various cellular processes, such as receptor internalization, membrane budding and protein sorting (Downes & Carter, 1991). Vesicular sorting of glucose transporter GLUT4, ENaC and NHE1, have been shown to be mediated by PI3K-dependent pathways (Martin et al., 1996; Kurashima et al., 1998; Blazer-Yost et al., 1999; Paunescu et al., 2000).

We previously showed that the increase in NBC activity in response to CO_2 is mediated by tyrosine phosphorylation of Src-family kinases (Ruiz et al., 1999). Stimulation of NBC activity by CO_2 is blocked by herbimycin at a concentration known to preferentially inhibit Src-family kinases. Overexpression of Csk (COOH-terminal Src kinase), which is a negative regulator of SFKs, abrogated the stimulatory effects of CO_2 on NBC activity. CO_2 stimulation also increased SFK activity and Src phosphorylation. While phosphorylation may provide a mechanism that mediates the stimulatory effect of CO_2 on NBC activity, it is likely that other processes, such as increase in protein abundance in the basolateral membrane, may also be important to this response. Therefore, we studied the role of PI3K in regulation of NBC activity and protein abundance in the basolateral membrane.

To study the role of PI3K in the regulation of NBC activity by CO_2, we used the inhibitors wortmannin and LY294002. Our data showed that wortmannin and LY294002 had no effect on basal NBC activity, but prevented the CO_2-induced stimulation of the NBC activity. The time course of the inhibitory effect of wortmannin on NBC activity was noted to be maximal at 5 min and sustained inhibition was noted up to 30 min. The CO_2 stimulation of cotransporter activity was associated with increased PI3K activity, which was blocked by wortmannin, consistent with a role for a PI3K-dependent mechanism.

Using specific antibodies generated against the 18 carboxyl-terminal residues of the human NBC1, we studied the distribution of the NBC in HK2 cells by biotinylation studies. In the basal unstimulated condition, we demonstrated that immunoreactive NBC1 was localized in the intracellular compartment as well as in the basolateral membrane (Noboa et al., 2000; Robey et al., 2002). Since PI3K is involved in protein trafficking, we examined the role of an increase in immunoreactive NBC protein in the basolateral membrane in response to CO_2. In the present studies, we showed that CO_2 increased the amount of immunoreactive biotinylated NBC proteins in the basolateral membrane. This effect was blocked by inhibition of PI3K with wortmannin.

It must be noted that the increase in NBC activity may be due to decreased endocytosis or increased exocytosis or summation of the two processes. Hence, using the method described by Grinstein and his group (Kurashima et al., 1998), we showed that wortmannin inhibited CO_2-stimulated NBC activity even when endocytosis was prevented by low temperature.

The specific mechanism of interaction between tyrosine kinase signaling and the effect of PI3K on NBC activity has not been examined. We previously showed that the increase in NBC activity in response to CO_2 is mediated by Src-family kinases (Ruiz et al., 1999). CO_2 stimulation increased SFK activity and tyrosine phosphorylation of Src family kinases. Our current studies showed that wortmannin blocked the tyrosine phosphorylation of Src family kinases. This suggests that PI3K activity is important for tyrosine phosphorylation of Src family kinases. In summary, the current paper provides the first evidence for regulation of NBC activity and trafficking through in-

teraction between the signaling pathways involving Src-family kinases and PI3K.

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