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Regulation of Renal Na^+/HCO_3^- Cotransporter Stimulation by CO_2: Role of Phosphorylation, Exocytosis and Protein Synthesis

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Abstract. The sodium bicarbonate cotransporter (NBC1) mediates bicarbonate reabsorption in the renal proximal tubule. NBC1 activity is stimulated by 10% CO_2, however, the mechanism is poorly understood. Here, we examined the mechanism of NBC1 regulation by 10% CO_2 using an immortalized human proximal tubule cell line (HK2). In cells exposed to 10% CO_2, the cotransporter activity (measured as ΔpH/min) increased within minutes and this increase was maintained for 6 to 24 h. Early NBC1 stimulation was accompanied by increased NBC1 phosphorylation. Basolateral membrane NBC1 protein increased by 30 min and reached a maximum at 6 h. Increased NBC activity at 6 h was accounted for by increased NBC exocytosis to the basolateral membrane and not by decreased endocytosis. Latruncullin B (an actin cytoskeleton inhibitor) did not prevent CO_2-induced stimulation, while nocodazole (a microtubule-disrupting agent) abrogated the stimulatory effect of 10% CO_2. A significant increase in NBC1 mRNA expression level was observed at 6 h and maintained for 24 h. Total NBC1 protein increased at 12 to 24 h with 10% CO_2 incubation and this effect was blocked by cycloheximide. In summary, the present study demonstrates that early activation of NBC1 activity by 10% CO_2 was mediated by NBC1 phosphorylation. The stimulation of cotransporter activity observed at 6 h was due to exocytosis, while the late effect starting from 12 h was accounted for by increased protein synthesis.

Key words: Na^+/HCO_3^- cotransporter — Acidosis — Exocytosis — Endocytosis — Cytoskeleton — Microtubule

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Introduction

The sodium bicarbonate cotransporters (NBCs) are important for pH regulation in different cell types (Boron & Boulpaep, 1983; Burnham et al., 1997; Romero et al., 1997). NBC1 in the renal proximal tubule is responsible for bicarbonate reabsorption from the cell to the blood. Respiratory acidosis has been shown to enhance HCO_3^- reabsorption by the kidney (Brazeau & Gilman, 1953; Dorman et al., 1954; Adrogue & Madias, 1986; Chen & Boron, 1995) and NBC1 activity (Ruiz, Arruda & Talor, 1989). In rabbit renal proximal tubule cells (primary culture), incubation with 10% CO_2 leads to increased cotransporter activity as early as 5 min, which is maintained until 48 h (Ruiz et al., 1998b). Increased NBC1 activity with 10% CO_2 was also shown in immortalized opossum kidney (OK) cells (Ruiz et al., 1999). The increased NBC1 activity in rabbit proximal tubule and in OK cells is associated with various signaling mechanisms. We have previously reported the role of protein tyrosine kinase C (Ruiz et al., 1998a) and Src family kinases (Ruiz et al., 1999). More recently we have shown that CO_2 shared the same mechanism with non-related stimuli such as carbachol and angiotensin II through Pyk2-Src interaction (Espiritu et al., 2002) in OK cells. These studies suggested a role of NBC1 phosphorylation, however, this possibility has not been studied with 10% CO_2. Moreover, it is interesting that the early effect of 10% CO_2 on NBC1 activity was maintained with prolonged incubation. The late effect of 10% CO_2 stimulation is believed to be due to increased NBC1 synthesis (Ruiz et al., 1998b), but this hypothesis has not been studied directly. In addition, the mechanisms of the intermediate effect between 5 min to 48 h have not been studied.

Incubation of OK cells with acid caused an early increase of Na-H antiporter activity through exocytosis and a late effect through increase in mRNA and protein synthesis (Amemiya et al., 1995; Yang et al., 2000). Based on the well known role of the cytoskeleton in protein trafficking in epithelial cells (Brown & Stow, 1996; Cantiello et al., 1991) and based on the intracellular movement of vesicles and protein targeting (Yang et al., 2000), we examined the possible role of microtubules and actin cytoskeleton on NBC1 regulation by 10% CO_2.

In the present study, we examined the mechanisms of increased NBC1 activity in the presence of 10% CO_2 in human proximal tubule cells (HK2). We aimed at investigating the role of phosphorylation, exocytosis, endocytosis and protein synthesis in response to 10% CO_2 to regulate NBC1 activity.

Materials and Methods

Materials

The pH-sensitive BCECF-AM was purchased from Molecular Probes (Eugene, OR). Protease inhibitor cocktail, phosphatase cocktail inhibitors I and II, amiloride, nigericin and other perfusing reagents were obtained from Sigma (St. Louis, Mo). Polyclonal NBC1 antibodies raised from rabbit were synthesized by ADI (San Antonio, TX) and rabbit anti-phosphorylated proteins (α-Pan) were obtained from Zymed (South San Francisco, CA). Biotin, streptavidin, sulfo-NH2-acetate, iodoacetic acid and mesna (2-mercaptoethanesulfonic acid sodium salt) were purchased from Pierce (Rockford, IL). Immunoblots were analyzed with a chemiluminescence detection system (Phototype) from New England Biolab (Beverly, MA). All other western blotting solutions were obtained from BioRad (Hercules, CA). Cell culture reagents including serum were purchased from Invitrogen (Grand Island, NY). Total RNA was extracted using the ULTRASPEC™-II RNA isolation system obtained from Biotecx (Houston, TX), labeled and detected using the North2South® Direct HRP labeling and detection kit by Pierce Rockfold, II. Nocodazole, latruncullin B and cycloheximide were purchased from Calbiochem (La Jolla, CA).

Cell Culture

Mycoplasma-free human proximal tubule (HK2) cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in 5% fetal bovine serum-supplemented DMEM/F12 (1:1) modified with penicillin, streptomycin and 20 mM HEPES in a 5% CO_2 incubator at 37°C. Cells were serum-free for 18–24 h prior to testing and preincubated with inhibitors for 30 min, where appropriate.

Intracellular pH_i Measurement and NBC1 Activity

Confluent HK2 cells grown in slides were loaded with 6 μM BCECF-AM and pH-dependent changes in fluorescence were measured using a Deltascan dual-excitation fluorometer (PTI) at 37°C, as previously described (Ruiz et al., 1995). This technique has been extensively used (Weinman et al., 2001; Espiritu et al., 2002; Robey et al., 2002; Bernardo et al., 2003). In brief, cells were perfused at 37°C with C1-free physiological solution (in mM): 25 $NaHCO_3^-$, 110 sodium gluconate, 5 potassium gluconate, 2 $CaSO_4$, 0.5 $MgSO_4$, 1 KH_2PO_4, 10 glucose and 9 HEPES, pH 7.40), containing 1 mM amiloride. After establishing a stable baseline signal, Na^+ was removed by equimolar substitution with choline at pH 7.4. Na^+ removal resulted in an immediate decrease in pHi that fully recovered when Na^+ was readded. NBC1 activity was taken as an initial rate of pH recovery after the addition of Na^+ and was calculated from the slope of the line-drawn tangent to the initial deflection for a period of 1 min. All measurements were performed by dual wavelength monitoring and ratiometric analysis at the pH-sensitive (504 nm) and pH-insensitive (440 nm) excitation wavelength, depending on the BCECF-AM batch. Although the buffering capacity was not measured in the present experiments, we have previously shown that buffering capacity was not altered by 10% CO_2. During the measurement of NBC1 activity, cells were perfused at constant extracellular pH of 7.4 and gassed up with 5% CO_2; therefore, the effect of prior exposure to CO_2 was not due to increased HCO_3^- concentration.

NBC1 Immunoblot Analysis

Subsequent immunoblotting of whole-cell lysates for membrane NBC1 was performed, using polyclonal NBC1 antibody directed against the 20 amino acids (DSKPSDRERSPTFLERHTSC) at the carboxyl terminal of human NBC1 (Weinman et al., 2001, Robey et al., 2002). These antibodies recognize NBC1 of human origin and were routinely used to probe for NBC1 in HK-2 cells. These antibodies were shown to be specific for NBC1 using competing peptide (*results not shown*).

NBC1 Phosphorylation and Immunoprecipitation

Quiescent HK2 cells were incubated in 10% CO_2 for 0, 1, 5 and 15 min. Cells were lysed using modified RIPA buffer (50 mM Tris-Cl, pH 7.4, 1% NP 40, 150 mM NaCl and 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails. Whole cell lysates (500 μg) were incubated with anti-NBC antibody for 6 h at 4°C. The resulting immunocomplex was precipitated with protein G/A agarose. Proteins were eluted from the immunocomplex by boiling for 10 min in 2 × SDS sample buffer and were resolved by denaturing PAGE, transferred to nitrocellulose and probed with anti-phosphorylated proteins (α-Pan) antibody. The first antibody was stripped from the same membrane and probed with anti-NBC1.

Biotinylation of Basolateral Membrane NBC1

HK2 cells were grown on 0.45 μm polycarbonate membrane (Transwell®) to confluency and the basolateral membrane was selectively labeled with sulfo-NHS-LC-biotin, as described by Sargiacomo et al. (1989), with some modifications. Cells were washed with PBS and the basolateral membrane was incubated with 0.5 mg/ml biotin in PBS containing Ca/Mg for 25 min at 4°C. The apical membrane was quenched with 100 mM glycine in PBS Ca/Mg. After labeling, cells were lysed using cell lysis buffer (1% Triton X-100, (150 mM NaCl, 5.0 mM EDTA and 50 mM Tris, pH 7.5), supplemented with protease-and phosphatase-inhibitor cocktails. Biotinylated proteins were precipitated with immobilized streptavidin overnight. Biotin-labeled proteins were eluted by boiling the protein-biotin-streptavidin complex with 50 μl elution buffer containing 80 mM DTT, 0.24 mM Tris-Cl, pH 8.9, 0.008% bromophenolblue, 5.6% SDS and 16% glycerol. The proteins were run in SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-NBC1 antibody.

Measurement of NBC1 Insertion into the Basolateral Membrane

Newly inserted membrane NBC1 protein was measured using sulfo-NHS-acetate followed by biotinylation, as previously described (Yang et al., 2000). Sulfo-NHS-acetate irreversibly binds to proteins at the biotin binding site. Pre-exposure with sulfo-NHS-acetate prevents biotin binding, therefore, only newly inserted proteins will be available for biotinylation. In brief, cells grown on polycarbonate membrane were washed with PBS-Ca/Mg and the basolateral membrane proteins were incubated with 1.5 mg/ml sulfo-NHS-acetate in PBS (pH 7.5) for 2 h at room temperature. After the basolateral proteins were blocked, cells were quenched with 100 mM glycine in PBS Ca/Mg, washed with PBS and fresh serum-free medium was added. The cells were reincubated in 10% CO_2 or 5% CO_2 at 37°C. After 6 h, the basolateral proteins were labeled with 0.5 mg/ml sulfo-NHS-LC biotin, as described in Biotinylation and immunoblotted with anti-NBC. The NBC1 detected at 6 h was assumed to be the new NBC1 protein translocated to the basolateral membrane. To make sure that the NBC1 protein detected was, indeed, newly exocytosed NBC1 protein and not due to inefficiency of sulfo-NHS-acetate blockade, basolateral membrane biotinylation was performed immediately after blocking with sulfo-NHS-acetate. The residual NBC1 detected after western blotting was the total unblocked NBC1 that did not reflect exocytosis. The amount of unblocked NBC1 was compared to total NBC1 detected after basolateral membrane biotinylation of cells not exposed to sulfo-NHS-acetate as control. NBC1 exocytosis was calculated as:

$$\%\ NBC1_{exocytosed} = NBC1_{biotinylated} - NBC1_{unblocked}/\text{biotinylated } NBC1_{total} \times 100 \quad (1)$$

where: $NBC1_{unblocked}$ is the biotinylated NBC1 detected after blocking with NHS acetate without CO_2 reincubation, $NBC1_{biotinylated}$ is the biotinylated membrane NBC1 detected after 6 h of reincubation in 5% or 10% CO_2, and biotinylated $NBC1_{total}$ is the biotinylated membrane NBC1 detected without prior NHS-acetate incubation.

Measurement of Endocytosed NBC1

The amount of endocytosed NBC1 was measured using 2-mercaptoethanesulfonic acid sodium salt (mesna) as previously described (Yang et al., 2000). Mesna is a cell-impermeant agent capable of reducing S=S bonds. Reduction of S=S bonds results in the disruption of biotin-protein binding. Biotinylated proteins that are retained in the plasma membrane are cleavable by mesna, while biotinylated proteins that have moved into the cells are not susceptible for cleavage. Resistance to cleavage by mesna is hereby used as a measure of endocytosis. In brief, quiescent cells grown on Transwell® polycarbonate membrane were labeled with water-soluble biotin, 1.5 mg/ml sulfo-NHS-SS-biotin, as described. After biotin labeling, unbound biotin was quenched with 100 mM glycine and the cells were washed with PBS Ca/Mg. Fresh serum free medium was added to the biotin-labeled cells and they were reincubated in 5% CO_2 or 10% CO_2 at 37 °C for 6 h. After 6 h, the cells were washed with cold PBS and the biotin-labeled basolateral membrane proteins were exposed to 1 ml of freshly prepared 10 mM mesna for 30 min. After 30 min, an additional 0.25 ml of 50 mM mesna was added and again in 60 min. After 90 min of mesna incubation at 4°C, mesna was oxidized by addition of 1 ml 500 mM iodoacetic acid for 10 min. Cells were lysed with lysis buffer and biotinylated proteins were precipitated with immobilized streptavidin and immunoblotted with anti NBC1.

Measurement of Increase in NBC1 Protein

Quiescent HK2 cells were incubated in 10% CO_2 for 0, 0.5, 6, 12 and 24 h. Cells were lysed using modified RIPA buffer. Fifty micrograms of lysate were run in SDS-PAGE and immunoblotted with anti-NBC1. The length of CO_2 incubation that gave a significant increase in total NBC1 was identified and was used for the succeeding experiments with cycloheximide. To block protein synthesis, cells were incubated with 100 μM cycloheximide 30 min prior to 10% CO_2 incubation and maintained therein throughout the 12 h 10% CO_2 incubation. After stimulation, cells were lysed and the amount of NBC1 was detected using NBC1 antibody.

Measurement of Increase in NBC1 mRNA Expression

Cells were cultured in 100 mM plates to confluency and made quiescent for 18–24 h. Cells were incubated with 10% CO_2 at 0, 0.5, 6, 12 and 24 h and total RNA was extracted using the ULTRASPEC™-II RNA isolation system. Thirty micrograms were run in denaturing agarose gel, transferred to a nylon membrane and hybridized with 200 bases horseradish peroxidase (HRP) NBC1 probe. The NBC1 probe was prepared by amplifying a 200 bp fragment of the amino-terminal coding region of NBC1 using polymerase chain reaction (PCR). The 200 bp product was purified using the Qiagen gel purification kit, denatured, and labeled with HRP according to the manufacturer's instructions (Pierce, Rockford, IL). NBC1 expression was detected using the North2South® Direct HRP detection kit with a stringency of 0.1 ×.

Statistical Analysis

Densitometric analyses of the blots were carried out using NIH Image software. All data are expressed as means ± SEM, and statistical comparison using Student's *t*-test of paired experiments, P values < 0.05 were considered to indicate significant difference.

Results

10% CO_2 Increases NBC1 Activity in Human Proximal Tubule Cells

We have previously shown that 10% CO_2 increased NBC1 activity in rabbit proximal tubule cells as early as 5 min (Ruiz et al., 1998b) and in OK cells (Ruiz et al., 1999; Espiritu et al., 2002). In this study we assessed the effect of 10% CO_2 on NBC1 activity in human proximal tubule (HK-2) cells. 10% CO_2 increased NBC1 activity in HK-2 cells by 42 ± 7% at 30 min. After 6 h of 10% CO_2 stimulation, NBC1 activity in HK-2 cells was maximum and remained unchanged as long as 24 h (Fig. 1). Data obtained as ΔpH/min were converted to % NBC1 activity, using time zero as 100% NBC1 activity.

10% CO_2 Increases NBC1 Phosphorylation after 1 min of Stimulation

The early increase in NBC1 activity has been reported and several pathways have been described that could

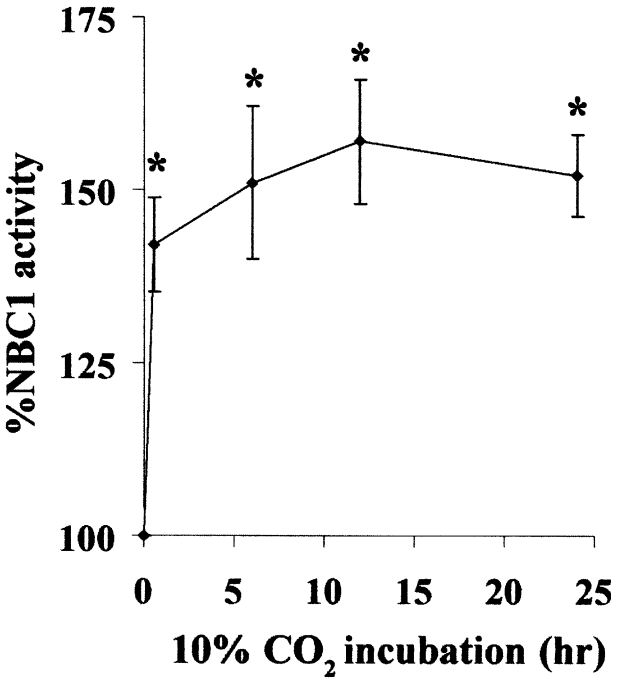

Fig. 1. 10% CO_2 increased NBC1 activity. Quiescent HK2 cells grown on slide were loaded with BCECF-AM. NBC1 activity was measured as ΔpH/min after incubation with 10% CO_2 and compared to a paired 5% CO_2 control. 10% CO_2 significantly increased NBC1 activity as early as 30 min. Increased NBC1 activity was maintained with prolonged incubation. NBC1 activity expressed as ΔpH/min was converted to percent control, using time zero as 100%, and shown as mean ± SEM of 6 paired experiments; $*P < 0.0001$.

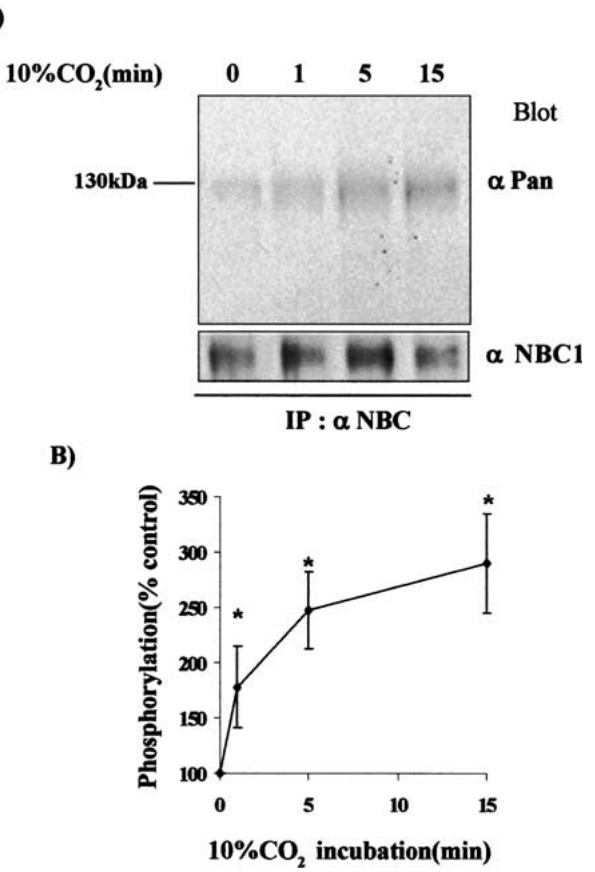

Fig. 2. 10% CO_2 increases NBC1 phosphorylation. (*A*) Quiescent HK2 cells were incubated with 10% CO_2 for 1, 5 and 15 min. Fivehundred micrograms of HK2 cell lysates were immunoprecipitated with anti-NBC1 antibody and blotted with rabbit anti-phosphorylated proteins antibody (α-Pan). Incubation with 10% CO_2 increased the amount of phosphorylated NBC1 at 1 min and maximally at 15 min. The membranes were then stripped and probed with anti-NBC1 to verify protein loading. A representative blot of 4 experiments is depicted. (*B*) Quantitative analysis of NBC1 phosphorylation, using densitometric analysis of 4 independent paired experiments showing significant increase of NBC1 phosphorylation by 10% CO_2; $*P < 0.001$.

potentially lead to NBC1 phosphorylation, but direct NBC1 phosphorylation has not been studied. We assessed NBC1 phosphorylation by the immunoprecipitation method with an antibody that detects all phosphorylated proteins, α-Pan (Edelman, Blumenthal & Krebs 1987; Sengupta et al., 1988). Figure 2*A* shows that 10% CO_2 increased NBC1 phosphorylation after 1 min of stimulation and the level of phosphorylation increased up to 15 min. Figure 2*B* is a quantitative analysis of the blot, showing at least 3-fold increased phosphorylation as compared to control (HK2 cells unexposed to 10% CO_2 were used as control).

10% CO_2 Increases Basolateral Membrane NBC1 Protein

Maximum increase in NBC1 activity was observed at 6 h and this increase was maintained with prolonged 10% CO_2 incubation. We have previously reported NBC1 translocation to the basolateral membrane with AII stimulation (Noboa, 2000; Noboa, 2001; Robey et al., 2002). Figure 3*A* shows that 10% CO_2 incubation increased the amount of NBC1 in the membrane. This representative blot showed a significant increase in membrane NBC1 protein at 6 h that continued to increase with longer incubation. Figure 3*B* is a densitometric analysis of several blots showing that at 24 h the NBC1 protein in the basolateral membrane had increased two-fold compared to cells unexposed to 10% CO_2 (time = 0).

10% CO_2 Increases NBC1 Exocytosis

Significant increase in membrane NBC1 protein was observed after 6 h of 10% CO_2 stimulation. This increase in membrane NBC1 protein could be due to either increased exocytosis or to decreased endocytosis of the NBC1 protein. To evaluate the mechanism of the increased amount of NBC in the membrane after 6 h of incubation with 10% CO_2, quiescent HK2 cells grown on Transwell® poly-

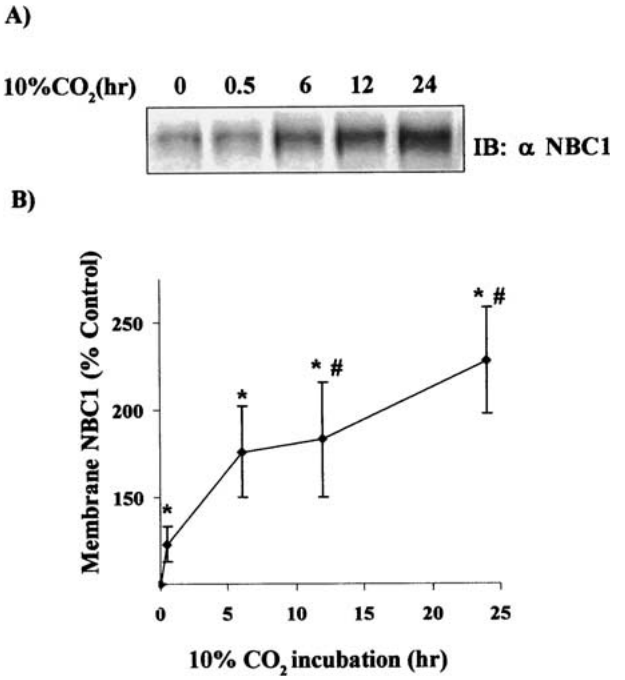

Fig. 3. 10% CO_2 increases basolateral membrane NBC1 protein with prolonged incubation. (*A*) HK2 cells grown on Transwell® polycarbonate membrane were serum-starved for 18–24 h and incubated with 10% CO_2. The basolateral membrane was labeled with biotin and biotinylated proteins were precipitated with immobilized streptavidin. Biotinylated proteins were resolved in SDS PAGE and probed with anti-NBC1. 10% CO_2 increased the amount of NBC1 on the basolateral membrane as early as 30 min. Maximum increase was observed at 6 h. The blot depicted is a representative result of 6 experiments. (*B*) Quantitative analysis of membrane NBC1 using a densitometric method showed significant increased at 6 h, $*P < 0.0001$ *vs.* time 0, # 12 h, 24 h *vs.* 6 h; $P > 0.1$.

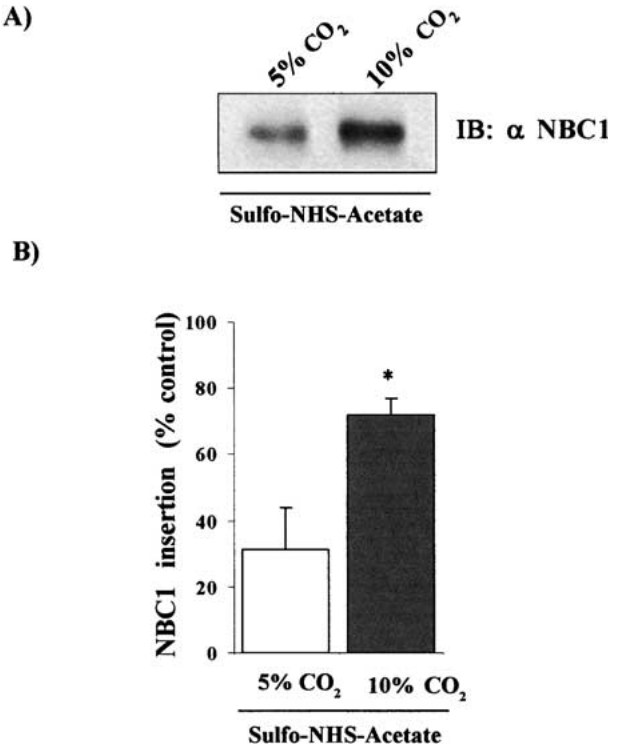

Fig. 4. 10% CO_2 increases NBC exocytosis to the basolateral membrane. New NBC1 inserted into the basolateral membrane was measured by incubating the basolateral membrane with cell-impermeant sulfo-NHS-acetate in order to block biotin protein-binding before 10% CO_2 incubation (*see* Methods). After 2 h blocking, cells were reincubated in 5% or 10% CO_2 for 6 h. After incubation, basolateral membrane proteins were labeled with biotin and precipitated with streptavidin. Biotinylated proteins were resolved in SDS-PAGE and blotted with anti-NBC1 antibody. (*A*) A representative blot of 5 experiments showing increased amount of NBC1 in the basolateral membrane with 10% CO_2 compared with 5% CO_2 incubation. (*B*) Quantitative analysis of 5 blots, showing 3-fold increase of NBC1 exocytosis with 10% CO_2 incubation; $*P < 0.001$.

carbonate membrane were incubated with sulfo-NHS-acetate for 2 h. Sulfo-NHS-acetate binds to all membrane proteins and prevents subsequent biotin binding. After 2 h, cells were washed with PBS and reincubated in 5% or 10% CO_2 for 6 h, as described in methods. Figure 4*A* shows NBC protein in the basolateral membrane with 5% and 10% CO_2 incubation. Figure 4*B* is a quantitative analysis of the blots, showing that 10% CO_2 incubation caused a twofold increase in basolateral NBC1 protein compared to cells incubated in 5% CO_2. Figure 5*A*,*B* shows that prior incubation with sulfo-NHS-acetate blocked biotin binding almost completely and thus the increase of NBC1 protein in the membrane shown in Fig. 4*A*,*B* was due to exocytosis and not due to blocking inefficiency (as explained in Methods).

10% CO_2 Does not Increase NBC1 Endocytosis

The amount of endocytosed NBC1 protein was measured using mesna to cleave exposed biotin-bound proteins, as explained in Methods. The amount of biotinylated NBC1 protein after mesna incubation was assumed to be the endocytosed NBC1 protein. To evaluate the contribution of 10% CO_2 on NBC1 endocytosis, quiescent HK2 cells grown in Transwell® were labeled with biotin. After labeling, cells were reincubated in 5% or 10% CO_2. After 6 h reincubation, basolateral membrane proteins were incubated with mesna and treated with iodoacetic acid. Cells were lysed and immunoblotted with anti-NBC. Figure 6*A* shows that a certain amount of NBC1 protein remained biotinylated after mesna incubation and was assumed to be the total internalized NBC1. Most importantly, 10% CO_2 did not increase NBC1 endocytosis, as with cells incubated with 5% CO_2. Figure 6*B* depicts a quantitative analysis of several blots, showing that 25% of basolateral membrane NBC1 were endocytosed after 6 h reincubation with either 5% or 10% CO_2. The total amount of NBC1 expressed in the basolateral membrane before mesna exposure was used as 100% control.

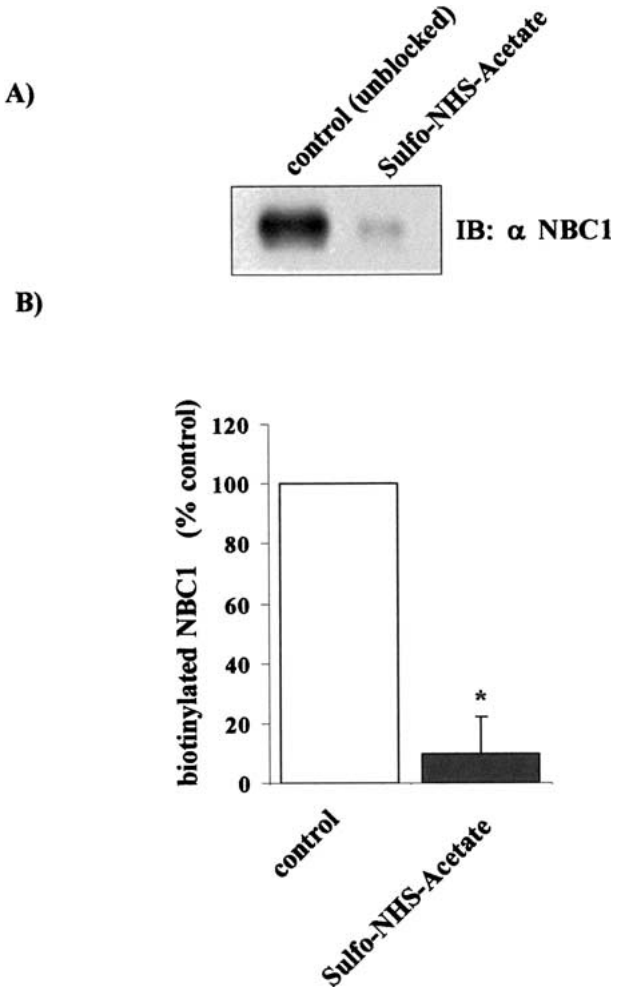

Fig. 5. Sulfo-NHS-acetate efficiently blocks biotin binding. To ensure the efficiency of sulfo-NHS-acetate to block biotin labeling, quiescent cells were incubated with sulfo-NHS-acetate for 2 h and labeled with biotin. The total amount of biotinylated NBC1 protein detected in the membrane without sulfo-NHS-acetate incubation was used as 100% control. The cells were lysed and biotinylated proteins were resolved in SDS-PAGE and immunoblotted with anti-NBC1. (*A*) A representative blot of 5 experiments showing minimal amount of residual membrane NBC1 membrane not blocked with sulfo-NHS-acetate. (*B*) Quantitative analysis showing that sulfo-NHS-acetate is 96% efficient in blocking biotin binding; $*P < 0.0001$.

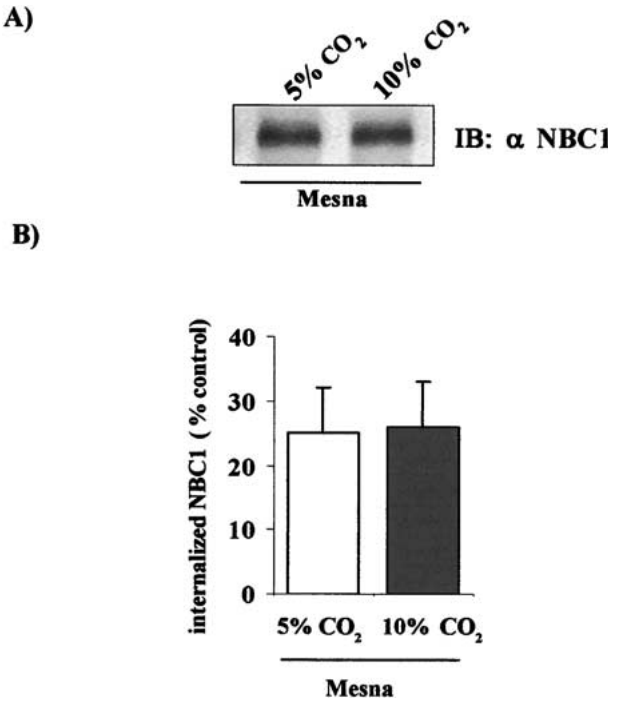

Fig. 6. 10% CO_2 has no effect on NBC1 endocytosis at 6 h. Internalized NBC1 was measured by the amount of biotinylated NBC1 uncleaved by mesna, as explained in Methods. (*A*) A representative blot of 4 experiments showing no difference of endocytosed NBC1 with 10% CO_2 compared to 5% CO_2. (*B*) Quantitative analysis of the blots normalized against biotinylated NBC1 without mesna incubation.

Microtubules but not Actin Cytoskeleton Play a Role in NBC1 Exocytosis

We assessed the role of actin cytoskeleton and microtubules on NBC1 exocytosis. Figure 7*A* shows that 0.1 μM latruncullin B did not block the effect of 10% CO_2 on NBC1 activity. In addition, Figure 7*B*,*C* shows that latruncullin B (0.1 μM) was not able to inhibit the translocation of NBC1 to the basolateral membrane. A higher concentration of latruncullin B was toxic to HK2 cells. Experiments to determine the latruncullin B concentration sufficient to disrupt actin cytoskeleton were performed. Based on the results, 0.1 μM latruncullin B is the highest concentration tolerated by HK2 cells in 6 h (*data not shown*). By contrast, incubation with 50 μM nocodazole completely blocked the effect of 10% CO_2 to increased NBC1 activity (Fig. 8*A*) and prevented the 10% CO_2 -induced NBC1 exocytosis to the basolateral membrane, as shown in Fig. 8*B-C*.

10% CO_2 Increases NBC1 Synthesis at 12 h and Cycloheximide Blocks this Effect

Confluent HK2 cells were incubated in 10% CO_2 for 0, 0.5, 6, 12 and 24 h and lysed as described in methods. Figure 9*A* shows significant increase in total NBC1 protein at 12 h. Figure 9*B* shows that this effect was blocked by 100 μM cycloheximide incubation. Incubation of cycloheximide also prevented the effect of 10% CO_2 on NBC1 activity after 12 h, as shown in Fig. 9*C*.

10% CO_2 Increases NBC1 mRNA Expression at 6 h

Quiescent HK2 cells were incubated in 10% CO_2 for 0, 0.5, 6, 12 and 24 h and total RNA was extracted. Figure 10 showed significant increase of a 7.2 kb NBC1 mRNA at 6 h.

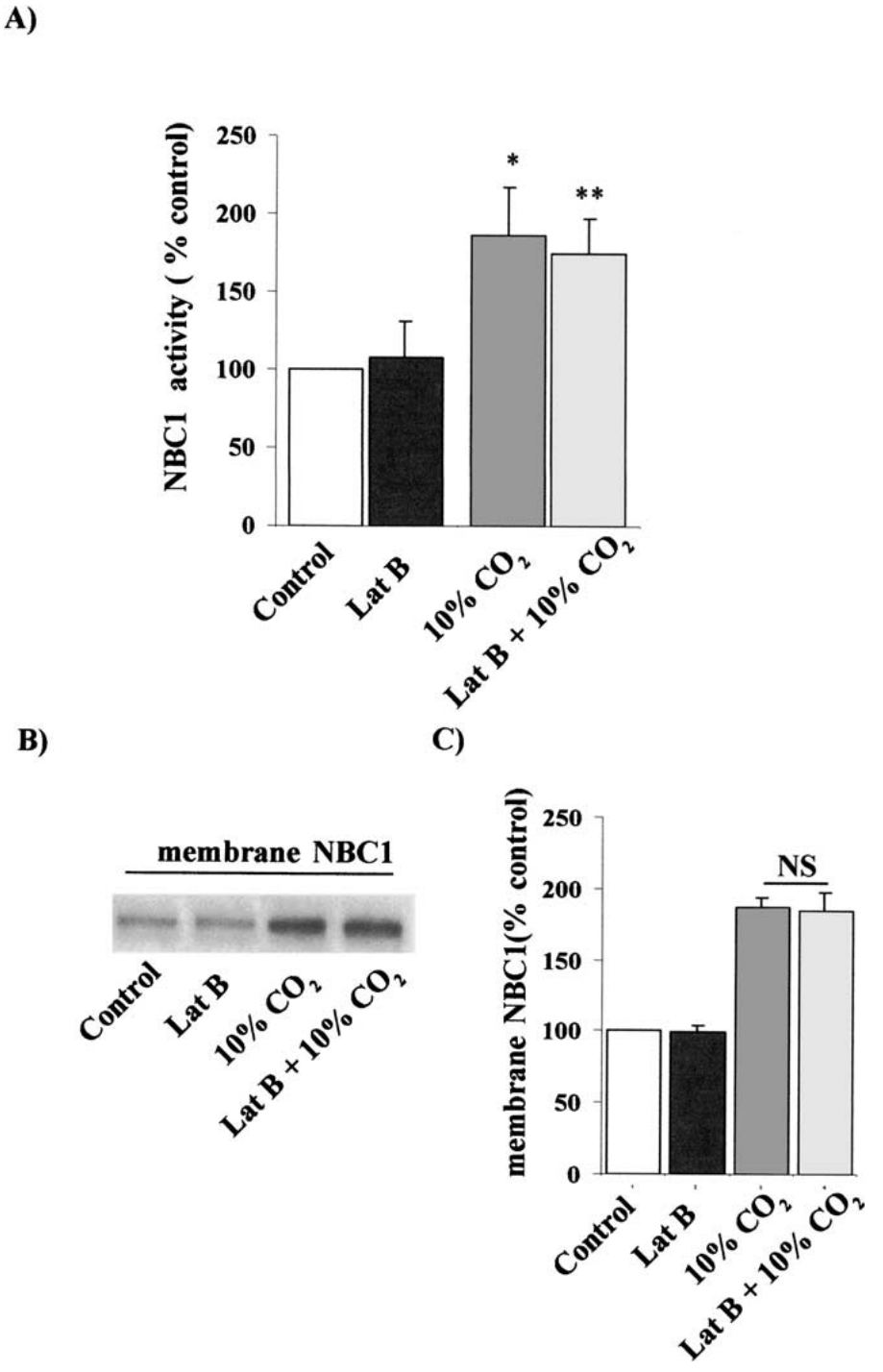

Fig. 7. CO_2 -induced membrane insertion and stimulation of NBC1 did not involve actin cytoskeleton. The role of actin cytoskeleton in CO_2 -induced membrane insertion and stimulation of NBC1 was evaluated using latruncullin B. Quiescent cells were preincubated with 0.1 μM latruncullin B for 30 min and maintained during 10% CO_2 incubation. (*A*) 10% CO_2 stimulates NBC1 activity and latruncullin B was not able to prevent this effect. Data shown are mean ± SEM of 10 paired experiments (*control *vs.* CO_2, $P < 0.001$; **CO_2 *vs.* Lat B + CO_2, NS). (*B*) A representative blot of 4 experiments showing increased expression of NBC1 in the basolateral membrane with 10% CO_2 stimulation. Incubation of latruncullin B did not block this effect. (*C*) A quantitative analysis of 4 blots, (*control *vs* CO_2, $P < 0.0001$; **CO_2 *vs.* Lat B + CO_2, $P < 0.6$).

Discussion

We have previously shown that 10% CO_2 stimulates NBC1 activity in the opossum kidney (OK) cells and in primary cultures of rabbit proximal tubule (Ruiz et al., 1998b). The early effect of 10% CO_2 was shown to be dependent on PKC, PI3-K and tyrosine kinase (Ruiz et al., 1998b; Ruiz et al., 1999; Espiritu et al., 2002; Bernardo AA, 2003), while the late effect was blocked by protein synthesis inhibitor. The present studies were aimed at further understanding the cellular mechanisms involved in the early and late stimulatory effect of NBC1 activity by 10% CO_2. Studies of NHE3 regulation by metabolic acidosis in OK cells have shown that the early effect of metabolic acidosis is mediated by exocytosis of NHE3 protein, which is regulated by microtubules and microfilaments (Yang et al., 2000). The late effect was associated with increased expression of the protein, suggesting enhanced protein synthesis.

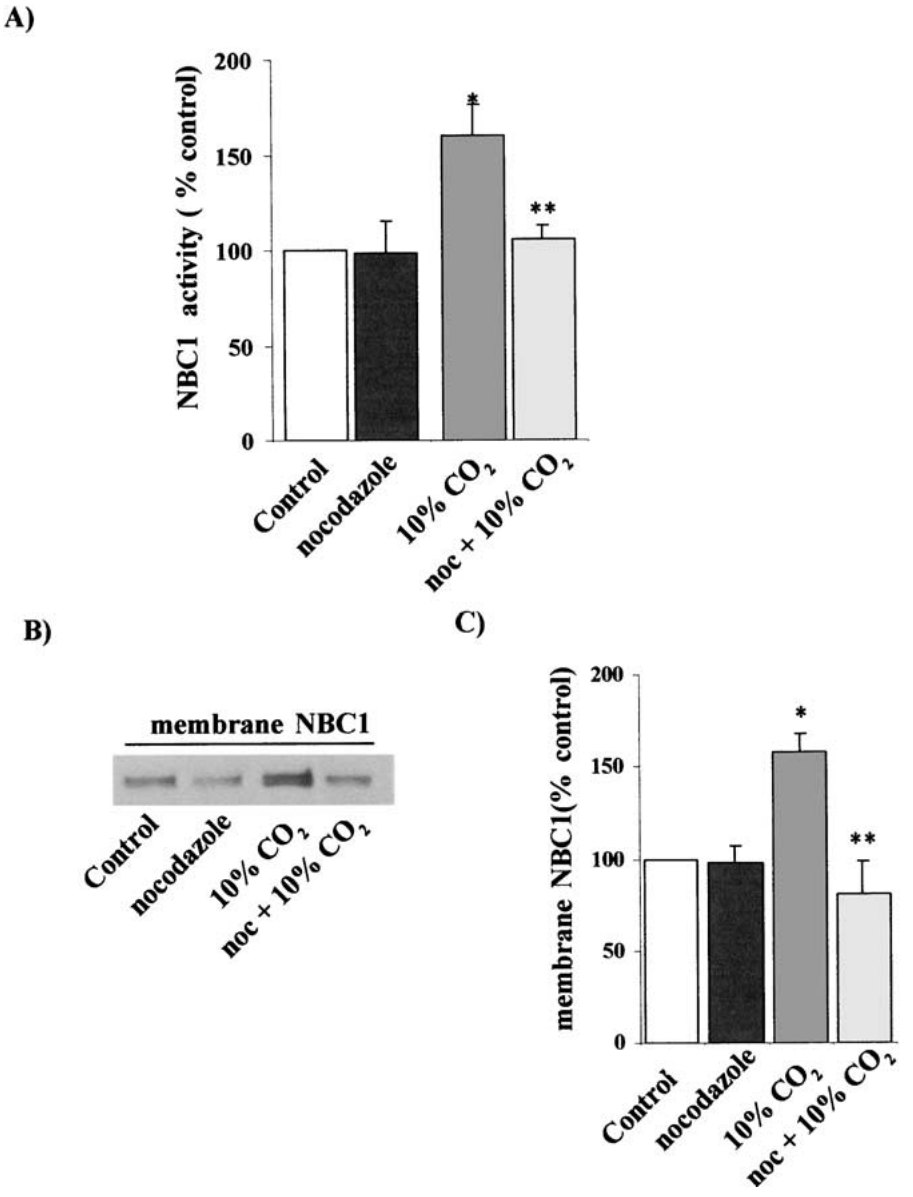

Fig. 8. Microtubule depolymerization prevents both NBC1 exocytosis and increase NBC1 activity with CO_2 stimulation. Quiescent cells were preincubated with 50 μM nocodazole for 30 min and maintained during CO_2 incubation. (*A*) Nocodazole abrogated the CO_2 -induced increase of NBC1 activity. Data shown are the mean ± SEM of 6 paired experiments (*control *vs.* CO_2, $P < 0.001$; **CO_2 *vs.* nocodazole, $P < 0.0001$). (*B*) A blot showing that nocodazole prevented the NBC1 membrane insertion in the presence of CO_2. The experiment was repeated 4 times, showing the same results. (*C*) A quantitative analysis of the blots, presented as the mean ± SEM of 4 experiments, *control *vs.* CO_2, $P < 0.01$ and **CO_2 *vs.* nocodazole, $P < 0.001$).

Within minutes of 10% CO_2 stimulation, NBC1 activity increased and this was associated with enhanced phosphorylation of a protein with a molecular weight of approximately 130 kDa. Our results strongly suggest that this is phosphorylated NBC1 because we immunoprecipated the lysate with an antibody specific against NBC1 and probed with an antibody that recognizes all phosphorylated proteins. Further evidence that this band was phosphorylated NBC1 protein is shown by the fact that a band of the same size was detected when the membrane was stripped from the antibody that recognized phosphorylated proteins and was then probed with NBC antibody (Fig. 2). These results show that 10% CO_2 causes early phosphorylation of NBC1 protein, which is in agreement with previous results from our laboratory, showing that several kinase inhibitors prevent the stimulation of NBC1 activity (Ruiz et al., 1998, 1999, Robey et al., 2002). Further studies using phosphopeptide mapping, mutation of those residues and ^{32}P incorporation are needed to closely define which specific amino-acid residues are phosphorylated by CO_2.

Increased NBC1 activity could be due to increased exocytosis and/or decreased endocytosis of NBC1 protein. Our results clearly show that early CO_2 stimulation was associated with increased amount of NBC1 protein in the basolateral membrane without an increase in total NBC protein content. This suggests increased exocytosis of NBC1 to the basolateral membrane. This increased exocytosis was blocked by a microtubule inhibitor, but not by the actin filament inhibitor. These results are similar to those described with metabolic

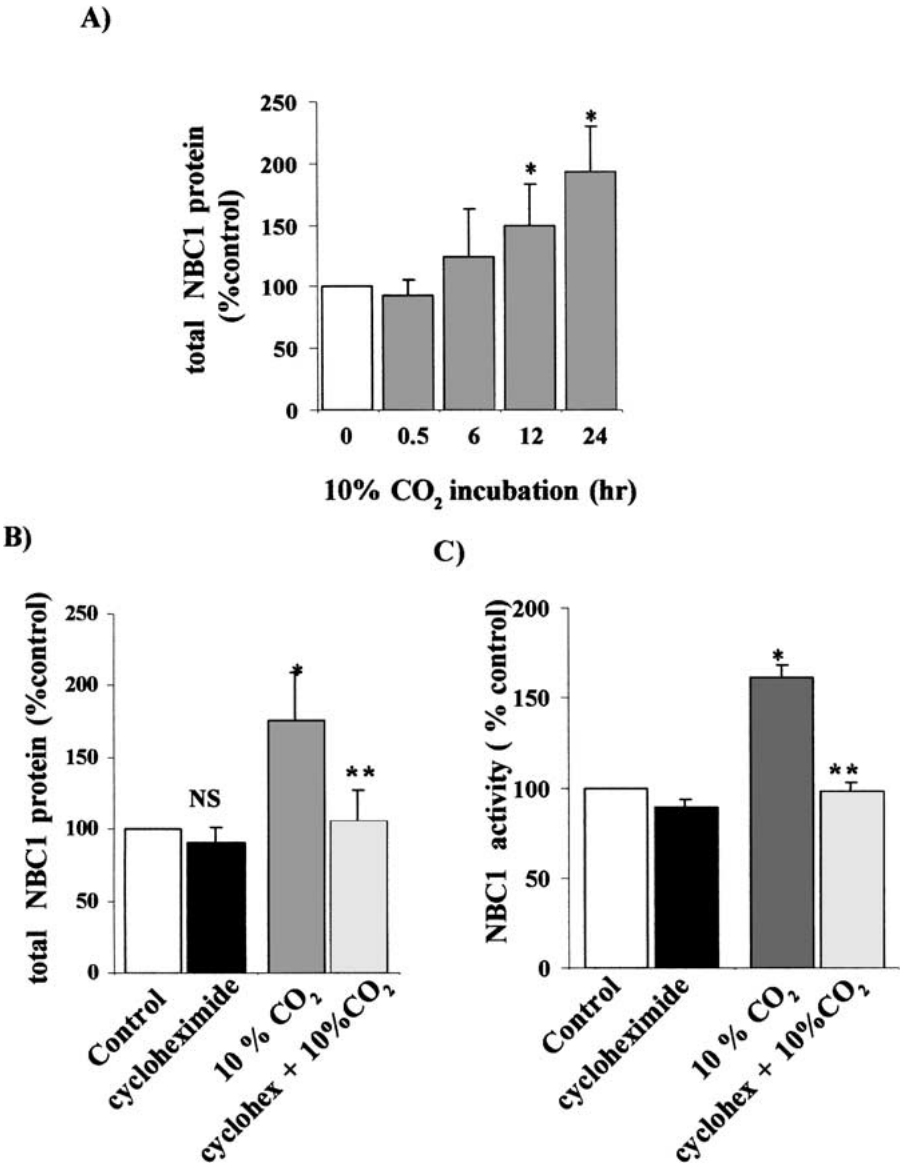

Fig. 9. 10% CO_2 increases total NBC1 protein and cycloheximide blocks both NBC1 activity and synthesis at 12 h. Cells were starved and treated with 10% CO_2 and lysed after different incubation times. (*A*) 10% CO_2 significantly increased total NBC1 protein at 12 h, $*P < 0.001$, $n = 5$. (*B*) 100 μM cycloheximide prevented the effect of 10% CO_2 to increase total NBC1 protein. Quantitative result from 5 paired experiments showing that cycloheximide prevented NBC1 synthesis induced by 10% CO_2. (*C*) Incubation of cycloheximide prevented increased NBC1 activity due to 10% CO_2 stimulation after 12 h, *control *vs.* CO_2, $P < 0.009$; **CO_2 *vs.* CO_2 + cycloheximide, $P < 0.01$.

acidosis in OK cells except for the finding that in OK cells microfilaments played a role in exocytosis. In both the OK cells (Yang et al., 2000) and in our studies, decreased endocytosis did not appear to play a role in the stimulation of NHE3 or NBC1 activity. After 24 h of CO_2 stimulation (late effect), increased NBC1 activity was associated with both increased NBC1 mRNA expression (Fig. 10) and total NBC1 protein content (Fig. 11), suggesting that de novo NBC1 protein synthesis was involved in this process. In support of this contention, we found that the protein synthesis inhibitor cycloheximide prevented both the increased NBC1 activity and total NBC1 content. We do not have an explanation for the stable NBC1 activity after 6–24 h, despite the fact that NBC1 mRNA and protein expression continued to increase. It is possible that although protein synthesis was increased, exocytosis at 24 h failed to increase in parallel (Fig. 3), thus explaining the lack of continuous increase in NBC1 activity (Fig. 11). Again, these findings are in complete agreement with results observed in OK cells exposed to metabolic acidosis and provide further support for the notion that NHE3 and NBC1 are regulated in parallel.

In conclusion, in HK2 cells, 10% CO_2 induced an early increase in NBC1 activity, which is mediated by phosphorylation and exocytosis, and the late effect is due to increased NBC1 mRNA expression and increased NBC1 protein content.

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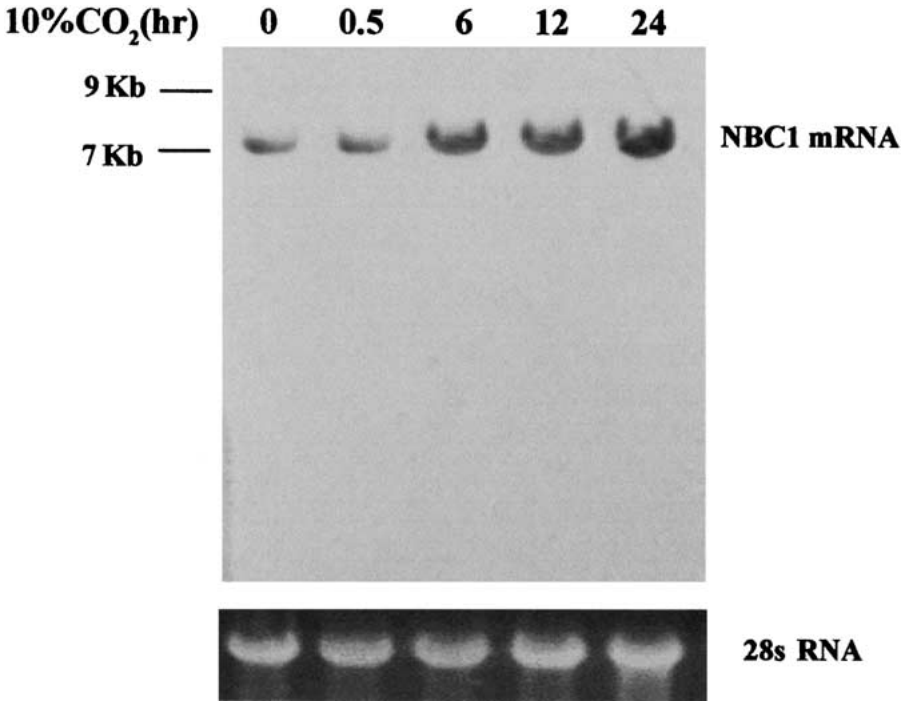

Fig. 10. 10% CO_2 increases NBC1 mRNA expression at 6 h. Thirty microgram of total RNA were run in denaturing agarose gel. Lower panel is 28s RNA detected using ethidium bromide stain to check the consistency of total RNA loading. The same RNA was transferred to a nylon membrane and probed with single-stranded labeled NBC1 DNA fragment (*see* Methods). Upper panel shows that 10% CO_2 significantly increased NBC1 mRNA level at 6 h and this was maintained until 24 h.

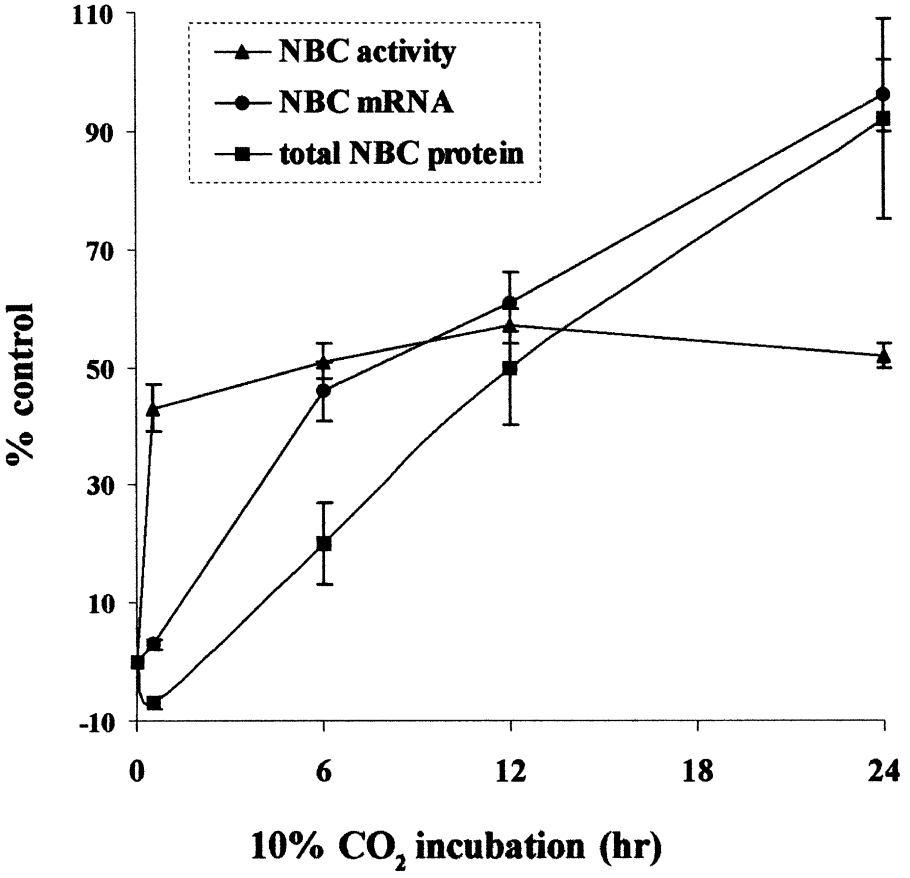

Fig. 11. 10% CO_2 increases NBC1 activity, NBC1 mRNA and total NBC1 protein in a time-dependent manner. NBC1 activity, NBC mRNA and total cellular NBC are plotted as percent control *vs.* time. Control is baseline NBC1 activity at time = 0, NBC1 mRNA or total cellular NBC1 without 10% CO_2 incubation.

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