

Time-dependent activity of Na⁺/H⁺ exchanger isoform 1 and homeostasis of intracellular pH in astrocytes exposed to CoCl₂ treatment

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Abstract. Hypoxia causes injury to the central nervous system during stroke and has significant effects on pH homeostasis. Na⁺/H⁺ exchanger isoform 1 (NHE1) is important in the mechanisms of hypoxia and intracellular pH (pHi) homeostasis. As a well-established hypoxia-mimetic agent, CoCl₂ stabilizes and increases the expression of hypoxia inducible factor-1α (HIF-1α), which regulates several genes involved in pH balance, including NHE1. However, it is not fully understood whether NHE1 is activated in astrocytes under CoCl₂ treatment. In the current study, pHi and NHE activity were analyzed using the pHi-sensitive dye BCECF-AM. Using cariporide (an NHE1-specific inhibitor) and EIPA (an NHE nonspecific inhibitor), the current study demonstrated that it was NHE1, not the other NHE isoforms, that was important in regulating pHi homeostasis in astrocytes during CoCl₂ treatment. Additionally, the present study observed that, during the early period of CoCl₂ treatment (the first 2 h), NHE1 activity and pHi dropped immediately, and NHE1 mRNA expression was reduced compared with control levels, whereas expression levels of the NHE1 protein had not yet changed. In the later period of CoCl₂ treatment, NHE1 activity and pHi significantly increased compared with the control levels, as did the mRNA and protein expression levels of NHE1. Furthermore, the cell viability and injury of astrocytes was not changed during the initial 8 h of CoCl₂ treatment; their deterioration was associated with the higher levels of pHi and NHE1 activity. The current study concluded that NHE1 activity and pHi homeostasis are

regulated by CoCl₂ treatment in a time-dependent manner in astrocytes, and may be responsible for the changes in cell viability and injury observed under hypoxia-mimetic conditions induced by CoCl₂ treatment.

Introduction

Stroke is the most common acute cerebrovascular disease and has high rates of morbidity and mortality (1,2). Despite extensive investigations into the mechanisms of and treatments for stroke, effective therapeutic strategies remain limited. Cerebral ischemia is the major insult caused by stroke, leading to acute neuronal death via a reduction or complete blockade of blood flow to the brain, resulting in oxygen and nutrient deficiency (3). Additionally, cerebral ischemia is associated with changes in several other factors, including vasopressin and endothelin levels, inflammation and intracellular pH (pHi) homeostasis (4). It is understood that pHi is important in modulating neuronal survival following hypoxia injury (5). Under physiologic conditions, the pHi of the brain is maintained at ~7.03. However, mild acidosis during reperfusion injury following transient hypoxia has previously been demonstrated to have a protective effect against ischemic injury (6-8), while mild alkalosis has the opposite effect. Hypoxia significantly affects pH homeostasis via lactate production and the upregulation of glycolysis to maintain ATP production, which facilitates a reduction in pHi (9).

Na⁺/H⁺ exchanger isoform 1 (NHE1) regulates pHi homeostasis and cell volume (10,11) by mediating an electroneutral (1:1) exchange of intracellular H⁺ for extracellular Na⁺ (12). Previous studies have indicated that NHE1 is important during ischemia and reperfusion, and inhibitors of NHE1 have been clinically evaluated for their ability to alleviate ischemia-reperfusion injury (9,13,14). However, it remains unknown whether NHE1 is activated during ischemia, and whether a lower pHi and cellular ATP depletion preclude NHE1 activity (15-17). It has been suggested that the controversies regarding the extent of ATP depletion may be due to differences in the experimental protocols and time courses

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used (15-17). Additionally, certain evidence has suggested that the pH_i and NHE1 activity decline when O₂ tension is reduced to <2% O₂ (18). However, previous studies demonstrated that brain pH_i is not acidic but alkaline (pH_i 7.1-7.4) following stroke (19). Excessive activation of NHE1 was considered to be responsible for this brain intracellular alkalosis, which was demonstrated to correlate with the severity of brain injury (8,20). Furthermore, excess intracellular Na⁺ resulted in increased intracellular Ca²⁺ via the Na⁺/Ca²⁺ exchanger, which may accelerate the Ca²⁺-mediated signaling cascades and lead to secondary ischemic brain injury (21,22).

Hypoxia-inducible factor-1 α (HIF-1 α), a key regulator of the cellular response to oxygen deprivation (18), is a master transcription factor for several genes involved in glucose uptake, angiogenesis, glycolysis, pH balance and metastasis (23). CoCl₂, an established hypoxia-mimetic, can produce a chemical hypoxic-like environment, induce mitochondrial damage and increase the generation of reactive oxygen species (24,25). It is well-established that CoCl₂ exposure activates HIF-1 α under normoxic conditions *in vitro* and *in vivo* (26). In addition, it has been reported reduced pH_i in solutions supplemented with 100 μ M CoCl₂ is associated with solutions pre-equilibrated at 1% O₂ (18). These findings suggest that the model of hypoxic damage induced by CoCl₂ is a suitable tool to investigate the mechanisms of cell injury caused by ischemia (27).

Astrocytes have been intensively investigated in the field of ischemic stroke research, as ischemic stroke causes neuronal injury and also damages astrocytes (28-30). Previous studies have demonstrated that, in ischemic infarcts, neurons will not survive if their neighboring astrocytes are not viable (31,32). Greater understanding of NHE regulation and the pattern of pH_i changes in astrocytes in responding to CoCl₂ treatment may aid the understanding of the mechanisms of hypoxia-induced neural injury, and facilitate the design of more effective treatment strategies. Additionally, as numerous physiological pathways, each with a characteristic reaction time, are spontaneously activated following the onset of stroke (33), the aim of the current study was to investigate whether NHE activity and pH_i homeostasis are altered in a time-dependent manner under hypoxia-mimicking conditions induced by CoCl₂ treatment of astrocytes.

Materials and methods

Materials. All cell culture reagents, including Dulbecco's modified Eagle medium (DMEM), antibiotics, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), cariporide, 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Cell culture plastics were purchased from Corning Incorporated (Corning, New York, USA). TRIzol reagent, Super Script III reverse transcriptase and Platinum SYBR Green qPCR Super Mix were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from Beyotime Institute of Biotechnology (Haimen, China).

All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals. BALB/c (n=22; 1-day old; 2 \pm 0.3 g) mice were obtained from the Experimental Animal Centre of Zhengzhou University (Zhengzhou, China), housed in a specific-pathogen-free environment and fed with commercial pellets. All experiments were approved by the Ethics Committee of Life Sciences of Zhengzhou University.

Primary astrocyte culture. Primary astrocytic cultures were prepared as described previously (34). Neonatal mice (1-day-old) were sacrificed by decapitation following anesthesia with Zoletil 100 (30 mg/kg, Virbac Corporation, France). The brains were rapidly dissected, as follows: The cerebellum and olfactory bulbs were removed and the meninges and blood vessels were carefully stripped off using a XYH-4A stereomicroscope (Shanghai Optical Instrument Factory Co., Ltd., Shanghai, China). The cerebral cortices were removed and the tissue was carefully transferred to complete DMEM (supplemented with 10% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin-sulfate) containing 0.0025% trypsin/EDTA to dissociate. Subsequently, the cell suspension was filtered through a 75- μ m pore-size mesh filter (XBSW-200, Shanghai Junsheng Biotechnology Co, Ltd., Shanghai, China). Following centrifugation at 900 x g for 10 min, the cells were seeded (3x10⁴ cells/cm²) in poly-L-lysine-coated tissue culture flasks (Corning BioCoat; Corning Inc., New York, USA) and maintained in complete DMEM at 37°C in a humidified atmosphere with 5% CO₂. The medium was renewed on day *in vitro* 1 (DIV1), DIV5 and DIV7. On DIV9, the microglia were discarded from the astrocytic cultures using the shake-off method (35).

Astrocytes were harvested from the flasks with trypsin and reseeded at a density of 3x10⁴ cells/cm² in complete DMEM/F12 medium containing 10% heat-inactivated FBS, 50 U/ml penicillin and 50 mg/ml streptomycin. The purity of the cultures was monitored by immunocytochemical staining using an antibody against the astrocyte marker glial fibrillary acidic protein (GFAP; 1:1,000; Sigma-Aldrich; cat. no. SAB5500113) and the absence of microglial cells was confirmed using isolectin B4 (1:100; Sigma-Aldrich; cat. no. L5391). The homogeneity of astrocytes was ~90-95% and <5-10% of the cells were isolectin B4 (1:100; Sigma-Aldrich; cat. no. L5391) (36).

Astrocytes were reseeded at a density of 5x10⁴ cells/cm² and incubated at 37°C with 5% CO₂. The culture medium was changed every 3 days and experiments were carried out on DIV19-22 (10-13 days after plating at which point they formed an incomplete monolayer of both stellate- and flat-shaped astrocytes). The cells were then exposed to 100 μ M CoCl₂ and analyzed at distinct time points over 24 h.

Cell viability assay. To determine the effects of hypoxia on the viability of astrocytes, cells were treated with 100 μ M CoCl₂ in serum-free DMEM/F12 medium prior to the measurement of cell viability every 4 h using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, according to manufacturer's instructions.

The assay was performed by adding MTT solution (5 mg/ml) to each well and incubating at 37°C for 4 h. Following removal

of the culture medium, 1 ml dimethyl sulfoxide was added and thoroughly mixed for 10 min. MTT absorbance was measured at 570 and 630 nm by using an SP-Max 2300A2 microplate reader (Shanghai Flash Spectrum Biological Technology Co., Ltd., Shanghai, China).

Flow cytometry. To confirm the results of the MTT assay, cells were harvested using trypsin/EDTA solution every 4 h following CoCl_2 treatment. The cells were rinsed with binding buffer (0.01 M HEPES; 0.14 M NaCl; and 2.5 mM CaCl_2 , pH 7.4) and collected by centrifugation at $1,500 \times g$ for 5 min. Cell pellets were re-suspended in binding buffer at a concentration of 1×10^6 cells/ml, and incubated with Annexin V-FITC and propidium iodide for 15 min at room temperature in the dark, according to the manufacturer's protocol. Cell injury analysis was performed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA), analyzing 10,000 cells per sample.

pHi measurement. Astrocytes were cultured on glass coverslips until confluent and pHi was determined at distinct time points following CoCl_2 treatment. Cellular NHE activity was determined fluorometrically using the pHi-sensitive dye BCECF-AM, as described previously (37), with modification.

Briefly, cells were exposed to Na^+ solution (138 mM NaCl; 5 mM KCl; 2 mM CaCl_2 ; 1 mM MgSO_4 ; 1 mM NaH_2PO_4 ; 25 mM glucose; and 20 mM HEPES, pH 7.4) with 5 μM BCECF-AM for 1 h at 37°C. During dye loading, cells of the CoCl_2 treatment group were treated with Na^+ solution with or without 100 μM CoCl_2 . Cells were then washed with the Na^+ solution to remove extracellular dye. Filters were mounted in a cuvette, placed in a fluorometer (Photon Technology International, Inc., South Brunswick, NJ, USA), perfused with Na^+ solution and the baseline pHi was measured. Following measurement of the baseline pHi, a standard ammonia pulse technique was used to measure Na^+/H^+ exchange activity (Fig. 1). Cells were briefly exposed to 40 mM NH_4Cl (pH 7.4), which caused alkalization. Then, cells were perfused with Na^+ -free medium (130 mM tetramethyl ammonium chloride; 5 mM KCl; 2 mM CaCl_2 , 1 mM MgSO_4 ; 1 mM NaH_2PO_4 ; 25 mM glucose; and 20 mM HEPES, pH 7.4), resulting in acidification due to the rapid diffusion and washout of NH_3 , leaving behind H^+ ions. Subsequently, the external solution was replaced with the Na^+ solution. Re-addition of extracellular Na^+ allowed activation of Na^+/H^+ exchange and recovery from acidification to basal levels. At the end of each experiment, the fluorescence ratio was calibrated to pHi using nigericin (2 μM ; Sigma-Alrich)/high K^+ solutions of different pH values (38).

The initial rate of Na^+ -dependent recovery from intracellular acidification corresponding to NHE activity was almost linear during the first 1 min of the reaction. NHE activity was measured by calculating the pHi change over the first 1 min using Origin 6.0 (OriginLab, Northampton, MA, USA) and expressed as $\Delta\text{pH}/\text{min}$.

To evaluate which isoforms of the NHE family were responsible for the pHi regulation induced by CoCl_2 treatment, cells were perfused with Na^+ -free medium, Na^+ -free medium with 10 nM cariporide (an NHE1-specific inhibitor) or 25 mM EIPA (a non-specific NHE inhibitor). NHE

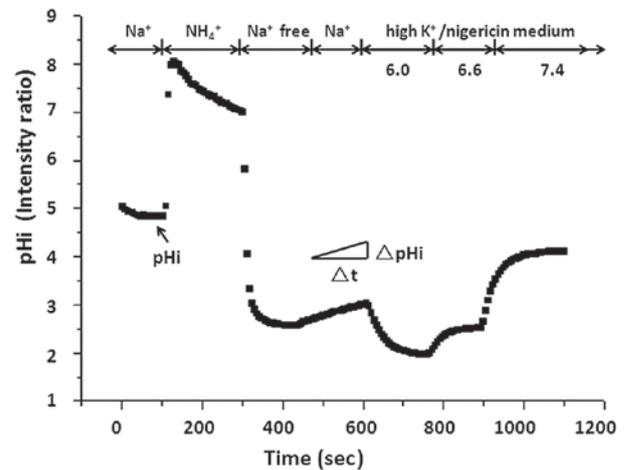


Figure 1. Typical pH recovery experiment for NHE activity and pHi testing. A representative trace of the ammonium pulse technique that was used to monitor baseline pHi and NHE activity in astrocytes. Cells were maintained in Na^+ solutions and measured baseline pHi, then treated with NH_4Cl , which caused alkalization. An $\text{Na}^+/\text{NH}_4\text{Cl}$ -free solution caused cellular acidification due to extrusion of NH_3 while preventing exchanger activity. Re-addition of Na^+ allowed exchanger activity to resume. The initial rates of Na^+ -dependent recovery from intracellular acidification corresponding to NHE activity were measured by calculating the pHi change over the first 1 min recovery period and expressed as $\Delta\text{pH}/\text{min}$. NHE, Na^+/H^+ exchanger; pHi, intracellular pH.

activity was recorded to identify cariporide-sensitive or EIPA-sensitive components of acid extrusion.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To measure the changes in NHE1 gene expression levels induced by CoCl_2 treatment, astrocytes were incubated with 100 μM CoCl_2 over a distinct time course, and then harvested. Total RNA was extracted from astrocytes using TRIzol reagent, according to the manufacturer's protocol, and subjected to DNase treatment. RNA quantity and quality were determined by an EU-2200R Ultraviolet spectrophotometer (Shanghai Onlab Instruments Co., Ltd., Shanghai, China) at 260 and 280 nm. Typically, the equivalent cDNA produced from reverse transcription of 20 ng RNA using a commercial SuperScript III reverse transcriptase kit was used for the RT-qPCR of each sample.

The expression of NHE1, HIF-1 α and β -actin were evaluated by RT-qPCR using Platinum SYBR Green qPCR Super Mix. The cycling conditions used were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. qPCR was performed using an ABI 7500 sequence detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers are presented in Table I. The quantities of gene-specific mRNA expression were determined by the quantification cycle (Cq) method and Cq values for β -actin were used as the internal control. The $2^{-\Delta\Delta\text{Cq}}$ method was used for relative quantitation (39).

Western blot analysis. Following incubation of astrocytes with 100 μM CoCl_2 , cells were washed three times with ice-cold PBS containing 50 mM Tris. Cells were collected and lysed in 500 μl ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.5% Triton X-100). The cell lysate was solubilized for 30 min at

4°C with end-over-end rotation and subsequently homogenized 10 times using a 23-gauge needle (Jintan FiveStar Health & Medical Co., Ltd., Jintan, China). Cellular debris was cleared by centrifugation at 14,000 × g for 15 min. The supernatant was collected, solubilized in loading buffer (5 mM Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; and 1% 2-mercaptoethanol) and boiled for 10 min. Subsequently, samples were loaded onto and size-fractionated by 10% SDS-PAGE, then electrophoretically transferred to a nitrocellulose membrane. Following blocking with 5% non-fat milk in PBS-T (0.05% Tween/PBS) for 1 h at room temperature, the membranes were incubated with rabbit anti-NHE1 (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-28758), mouse anti-HIF-1 α (1:1,000; cat. no. sc-71247) or mouse anti- β -actin (1:2,000; cat. no. sc-47778) antibodies. Membranes were washed with PBS-T three times for 10 min then incubated with horseradish peroxidase-conjugated bovine anti-mouse IgG (1:10,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-2371) and bovine anti-rabbit IgG (1:10,000; cat. no. sc-2370) for 60 min at room temperature in the dark. Blots were detected using an enhanced chemiluminescence western blotting detection system (Beyotime Institute of Biotechnology).

Statistical analysis. Data of multiple experiments are expressed as means \pm standard deviation. Statistical differences among the different time points were conducted using one-way analysis of variance followed by the Student Newman-Keuls test. Statistical analysis was performed with the SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of CoCl₂ treatment on astrocyte cell viability and injury. The effects of CoCl₂ treatment on the cell viability and injury of astrocytes were evaluated every 4 h during a 24-h period by MTT assay and flow cytometry analysis, respectively. Cell viability is presented as the optical density of CoCl₂-treated cells versus the control, and the level of apoptosis is presented as the percentage of cells with FITC-Annexin V binding excluding propidium iodide staining in total cell numbers. Compared with the control group, the levels of cell viability and injury remained at baseline levels in the initial 8 h of CoCl₂ treatment ($P > 0.05$ vs. control; Fig. 2). Subsequently, cell viability decreased significantly following exposure to 100 μ M CoCl₂ for > 8 h, while the level of cell injury continued to increase in each subsequent time interval over the 24-h time course ($P < 0.05$ vs. control).

Role of NHE1 in pH_i regulation in astrocytes. To identify the role of NHE1 in the regulation of pH_i, NHE activity was determined in the presence of a specific NHE1 inhibitor and compared with overall NHE inhibition. The initial NHE activity in astrocytes was 0.205 ± 0.012 Δ pH_i/min (Fig. 3). The recovery rate in the presence of 10 nM cariporide, which specifically inhibits NHE-1 at this concentration, was suppressed to 0.0599 ± 0.004 Δ pH_i/min ($P < 0.05$ vs. control). However, treatment with 25 mM EIPA, which inhibits all NHE isoforms at this concentration, demonstrated no further

suppression of NHE activity, compared with cariporide treatment (0.0492 ± 0.005 Δ pH_i/min; $P > 0.05$ vs. cariporide). These results suggest that NHE1, not other NHE isoforms, is important in regulating the pH_i homeostasis in astrocytes.

Effect of CoCl₂ treatment on pH_i and activity of NHE1. The effects of CoCl₂ treatment on pH_i and the activity of NHE1 in astrocytes were analyzed at multiple time points over 24 h. In the initial 3 h, the pH_i fell from 7.356 ± 0.026 to 6.913 ± 0.038 ($P < 0.05$ vs. control). Subsequently, the pH_i increased steadily and reached 7.441 ± 0.008 at the 24-h time point ($P < 0.05$; Fig. 4A).

Consistent with the time-dependent manner of pH_i changes, NHE1 activity behaved in a similar manner (Fig. 4B). NHE1 activity decreased by $\sim 50\%$ after 3 h of exposure to 100 μ M CoCl₂ ($P < 0.05$ vs. control; Fig. 4B), then increased significantly throughout the remaining recording period ($P < 0.05$).

Effect of CoCl₂ treatment on NHE1 and HIF-1 α mRNA expression. The effects of CoCl₂ treatment on the mRNA expression levels of NHE1 and HIF-1 α were analyzed by RT-qPCR assay. The expression levels of NHE1 mRNA were decreased by $\sim 20\%$ at the 2 h time point following CoCl₂ treatment ($P < 0.05$ vs. control). Subsequently, NHE1 mRNA expression levels increased significantly between 2 and 12 h, compared with the control levels ($P < 0.05$) and plateaued between 12 and 24 h at ~ 1.5 -fold expression (Fig. 5A).

Following CoCl₂ treatment, HIF-1 α mRNA expression levels were also determined. When astrocytes were exposed to 100 μ M CoCl₂, expression levels of HIF-1 α mRNA significantly increased immediately and reached an ~ 2.0 -fold increase at the 12 h time point. The increase in HIF-1 α expression levels remained at ~ 2.6 -fold throughout the remaining recording period ($P < 0.05$ vs. control; Fig. 5B).

Effect of CoCl₂ treatment on protein expression levels of NHE1 and HIF-1 α . The protein expression levels of NHE1 and HIF-1 α were determined by western blot assay (Fig. 6A). CoCl₂ treatment demonstrated minor effects on NHE1 protein expression in the initial 4 h of treatment ($P > 0.05$ vs. control; Fig. 6B). Subsequently, NHE1 protein expression levels increased significantly over the remaining treatment time course and reached an ~ 2.1 -fold increase at the 24-h time point ($P < 0.05$ vs. control).

HIF-1 α protein expression levels also remained at baseline levels in the initial 4 h of CoCl₂ treatment ($P > 0.05$ vs. control). Subsequently, the expression levels began to increase and reached ~ 4 -fold at the 24-h time point ($P < 0.05$ vs. control).

Discussion

The present study investigated the changes in cell viability and injury at distinct time points following exposure of astrocytes to 100 μ M CoCl₂. Cell viability and injury remained unchanged in the initial 8 h of treatment followed by a decrease in cell viability and an increase in cell injury during the remaining recording period. Additionally, the current study demonstrated that CoCl₂ treatment significantly affects pH_i homeostasis and NHE1 expression in a time-dependent manner. NHE1

Table I. Specific primers for the expression of Na⁺/H⁺ exchanger 1, hypoxia inducible factor-1 α and β -actin.

Gene name	Primer sequence (5'-3')	Product size, bp	Amplicon, bp
Na ⁺ /H ⁺ exchanger 1	F: CACCCTTTGAGATCTCCCTCT	21	68
	R: GGGGATCACATGGAAACCTA	20	
Hypoxia inducible factor-1 α	F: AACAGAATGGAACGGAGCAA	20	119
	R: TTCACAATCGTAACTGGTCAGC	22	
β -actin	F: CTAAGGCCAACCCTGAAAAG	20	104
	R: ACCAGAGGCATACAGGGACA	20	

F, forward; R, reverse.

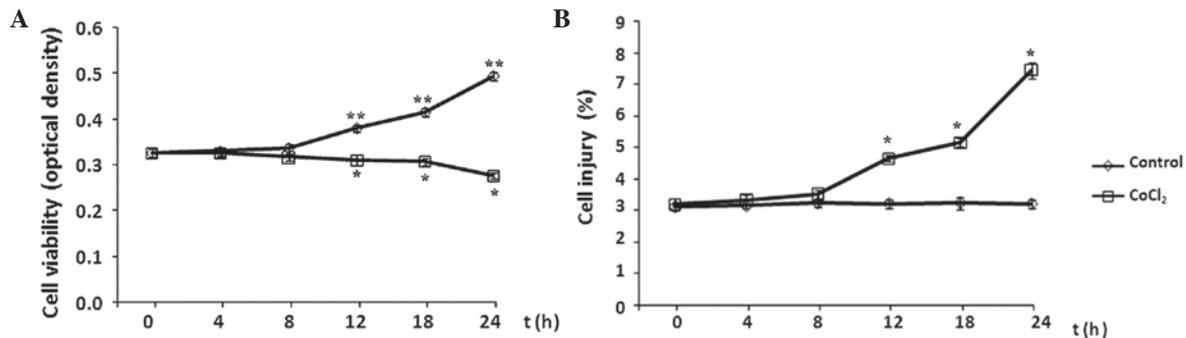


Figure 2. Effect of CoCl₂ treatment on cell viability and injury. (A) Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay following astrocyte exposure to 100 μ M CoCl₂ over a distinct time course of 24 h. Cell viability was presented as optical density. (B) Cell injury was detected by flow cytometry analysis when astrocytes were exposed to 100 μ M CoCl₂ for a distinct time course of 24 h. Cell injury was presented as the percentage of cells with fluorescein isothiocyanate-Annexin V binding excluding propidium iodide staining in total cell numbers. Results are presented as the mean \pm standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Statistical differences among the different time points were determined using one-way analysis of variance followed by the Student Newman-Keuls test. *P<0.05 vs. control in the control group and **P<0.05 vs. control in the CoCl₂ group and

protein directly interacts with various apoptotic-related and other proteins involved in cell growth and proliferation (12), however, it is controversial whether NHE1 is one of the key factors responsible for the cell death induced by CoCl₂ treatment (40,41). Thus, the current study investigated whether the time-dependent changes to cell viability and injury were associated with the regulation of pHi and NHE activity following CoCl₂ treatment.

The NHE gene family includes 9 different isoforms (NHE1-9). NHE1 is expressed in the majority of cells and tissues, and is by far the most abundant NHE isoform in the plasma membrane of rat brains (42,43). However, there is currently little information available on the functional expression of different isoforms in astrocytes (44). To evaluate which isoforms of the NHE family are responsible for pHi regulation induced by CoCl₂ treatment, the inhibitors cariporide and EIPA were used. Cariporide, a potent and highly selective NHE1 inhibitor (IC₅₀: NHE1, 0.01 μ M; NHE2, 1.6 μ M; NHE3, 1,000 μ M) (45), specifically inhibits NHE1, not the other NHE isoforms, at a concentration of 10 nM (43). In the present study, it was observed that 10 nM cariporide successfully suppressed NHE1 activity, but 25 mM EIPA, which was previously demonstrated to block all NHE isoforms at this concentration (46), had no further effect on the recovery rate. This suggests that it is NHE1, rather than other NHE isoforms, that is important in regulating pHi

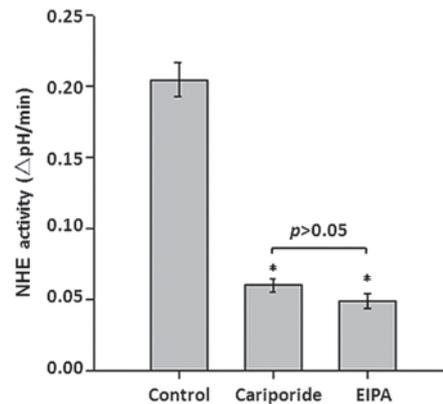


Figure 3. Role of NHE isoforms in intracellular pH regulation in astrocytes. NHE activity was measured in the presence of 10 nM cariporide or 25 mM EIPA compared with Na⁺-free medium as the control using the ammonium pulse technique and expressed as Δ pH/min. Results are presented as the mean \pm standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Statistical analysis was performed with one-way analysis of variance followed by Student-Newman-Keuls test. *P<0.05 vs. control. NHE, Na⁺/H⁺ exchanger; EIPA, ethyl-isopropyl amiloride.

homeostasis in astrocytes. Additionally, the recovery of pHi from acid loading still occurred but at a slower pace in the presence of 25 mM EIPA, indicating that other H⁺ extrusion systems may exist in cultured astrocytes.

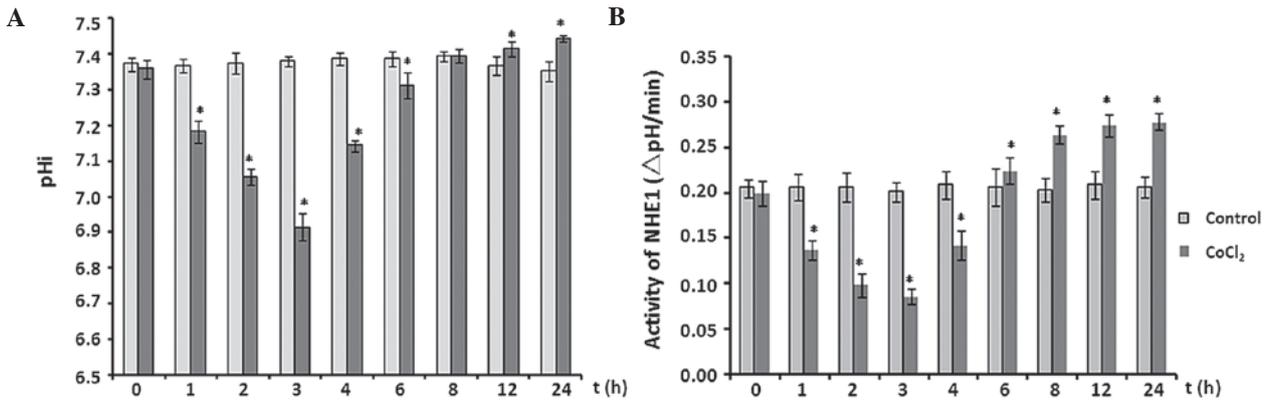


Figure 4. Effect of CoCl₂ treatment on pHi in astrocytes. (A) The effect of CoCl₂ treatment on pHi was tested following astrocyte exposure to 100 μM CoCl₂ over a 24-h time course. Astrocytes were maintained in Na⁺ solution and initial pHi was measured. (B) NHE1 activity was measured using the ammonium pulse technique and expressed as ΔpH/min. Results are presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Statistical differences among the different time points were determined using one-way analysis of variance followed by the Student Newman-Keuls test. *P<0.05 vs. control. NHE1, Na⁺/H⁺ exchanger isoform 1; pHi, intracellular pH.

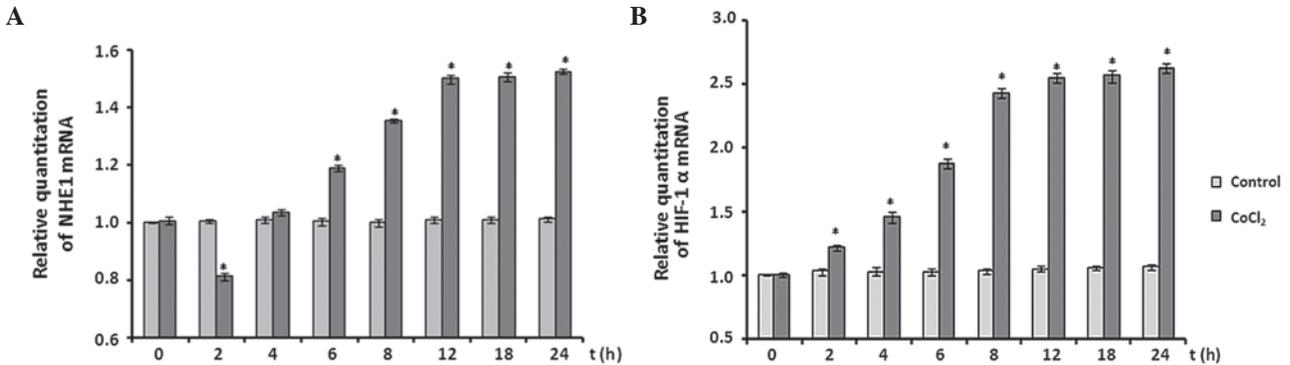


Figure 5. Effect of CoCl₂ treatment on NHE1 and HIF-1α mRNA. Reverse transcription-quantitative polymerase chain reaction assay was used to clarify the expression levels of (A) NHE1 and (B) HIF-1α mRNA were measured every 2 h during the 24-h time course. Quantification cycle (Cq) values were used to determine the quantities of gene specific mRNA expression and the 2^{-ΔΔCq} method was used for relative quantification. β-actin was used as an internal reference gene. Results are presented as the mean ± standard deviation of three independent experiments and samples were analyzed in triplicate (n=9). Values are presented as ratios over control (0 h time point). Statistical analysis at all time points between two groups was performed using the t-test. *P<0.05 vs. control. NHE1, Na⁺/H⁺ exchanger isoform 1; HIF-1α, hypoxia inducible factor-1α.

It has been previously reported that the effects of CoCl₂ treatment are very rapid, with pH and NHE activity significantly decreased in parallel within 10 min when O₂ tension was reduced to <2% O₂ (18,47-49). The time-course experiment within the current study revealed that the steady state pHi in astrocytes began to fall within the initial 3 h of CoCl₂ treatment. Additionally, NHE1 activity was downregulated over the same time period. These findings are in agreement with previous studies (18,47-49).

The present study also observed that the pHi and NHE1 activity increased between 4 and 8 h, and remained at high values throughout the remaining time course. These results are supported by other previous studies (9,18). Considering that CoCl₂ treatment significantly affects pH homeostasis and that the upregulation of glycolysis to maintain ATP production facilitates a decrease in pHi (9,18), it is likely that NHE-1 may be activated by intracellular acidosis to exchange intracellular H⁺ for extracellular Na⁺. The current study evaluated the expression levels of NHE1 mRNA and protein, and used HIF-1α as a marker of hypoxic stress and a key regulator of hypoxia. It was observed that HIF-1α protein increased gradually during

the initial 12 h of CoCl₂ treatment and remained at high levels over the remaining time course. These findings are supported by previous studies (2,12). However, the NHE1 protein expression did not change in the first 4 h, whereas NHE1 mRNA expression was decreased by 20% in the initial 2 h of CoCl₂ treatment. Subsequently, the protein and mRNA levels of NHE1 increased significantly throughout the time course.

Although the protein expression demonstrated no detectable change, the function of NHE1 was inhibited and NHE1 mRNA levels were decreased during the early period of CoCl₂ treatment. Taken together, these findings may explain the drop in pHi and NHE1 activity during the early period of CoCl₂ treatment. Notably, despite reduced pHi and NHE1 activity, there were no significant changes in cell viability and injury in the early period of CoCl₂ treatment. These findings are consistent with previous reports (18,50). It was suggested that hypoxia-induced acidification appears to be beneficial for matching ATP production and consumption by suppressing metabolic activity. By contrast, during the later period, the mRNA and protein expression levels of NHE1 were increased and remained at elevated levels. The activation of NHE1

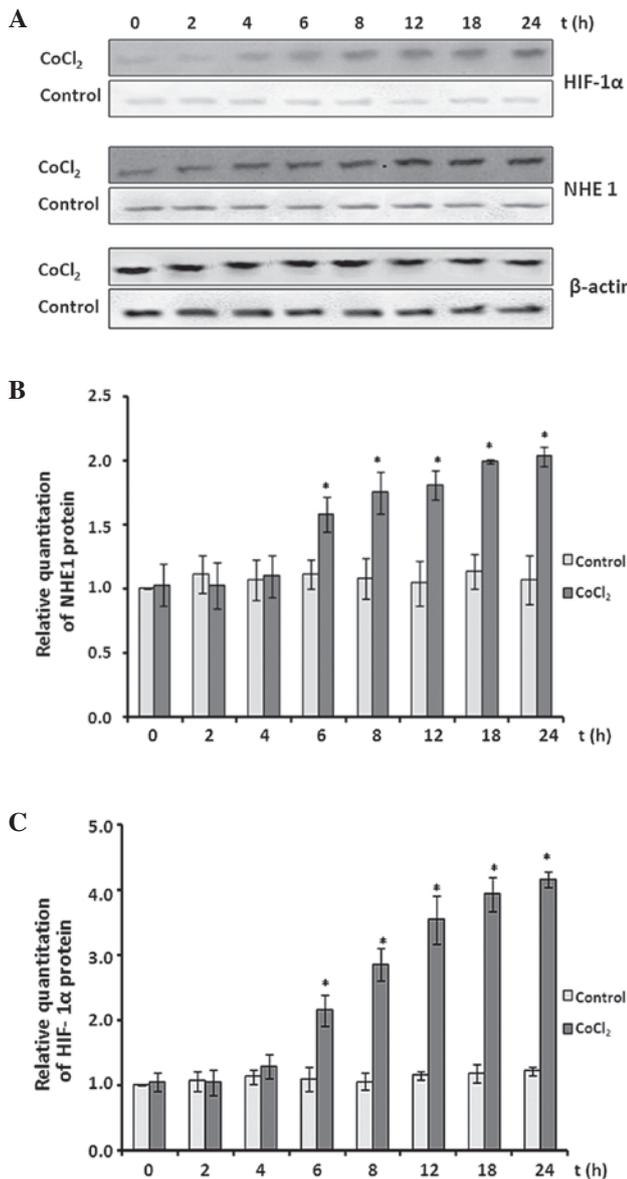


Figure 6. Effects of CoCl₂ treatment on the protein expression levels of NHE1 and HIF-1α. Astrocytes were exposed to 100 μM CoCl₂ for a distinct time course, then (A) western blot analysis was performed to determine the protein expression levels of NHE1 and HIF-1α, using β-actin as the loading control. Densitometry was performed, and (B) NHE1 and (C) HIF-1α values normalized to β-actin and presented as ratios over the control (0 h time point). Results are presented as the mean ± standard deviation of three independent experiments (n=3). Statistical analysis at all time points between two groups was performed using the t-test. *P<0.05 vs. control. NHE1, Na⁺/H⁺ exchanger isoform 1; HIF-1α, hypoxia inducible factor-1α.

may result in the acceleration of the Ca²⁺-mediated signaling cascade to initiate deleterious events (22). The current study observed decreased cell viability and increased injury after 8 h of CoCl₂ treatment, and this appeared to be associated with enhanced pHi and NHE1 activity.

In summary, NHE1, rather than other NHEs isoforms, appeared to be dominant during the regulation of pHi homeostasis in astrocytes under CoCl₂ treatment. NHE1 activity and pHi homeostasis changed over time under CoCl₂ treatment, and CoCl₂-induced NHE-1 activity occurred independently of HIF-1α activity. We propose, for the first time, that CoCl₂ treatment exerts early effects (in the initial 2-3 h of CoCl₂

treatment) on pHi homeostasis, resulting in cellular acidification associated with the inhibition of NHE1, and later effects (after 2-3 h of CoCl₂ treatment) on pHi homeostasis, resulting in cellular alkalosis caused by the stimulation of NHE1 activity. Additionally, it is likely that the time-dependent changes in pHi and NHE activity induced by CoCl₂ treatment were responsible for the changes in cell viability and injury. The findings of the current study emphasize the relevance of CoCl₂ treatment on the functional regulation of NHE1. These results may be beneficial for the elucidation of the mechanisms of human stroke injury, the development of neuroprotective treatment strategies and the determination of an optimal time lapse for the initiation of therapy following stroke.

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