

Original Paper

Characterization of the $\text{Na}^+/\text{HCO}_3^-$ Cotransport in Human Neutrophils

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Intracellular pH • Ion transport • Blood cells

Abstract

Background: Bicarbonate transport has crucial roles in regulating intracellular pH (pH_i) in a variety of cells. The purpose of this study was to evaluate its participation in the regulation of pH_i in resting and stimulated human neutrophils. **Methods:** Freshly isolated human neutrophils acidified by an ammonium prepulse were used in this study. **Results:** We demonstrated that resting neutrophils have a bicarbonate transport mechanism that prevents acidification when the Na^+/H^+ exchanger is blocked by EIPA. Neutrophils acidified by an ammonium prepulse showed an EIPA-resistant recovery of pH_i that was inhibited by the blocker of the anionic transporters SITS or the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC) selective inhibitor S0859, and abolished when sodium was removed from the extracellular medium. In western blot and RT-PCR analysis the expression of NBCe2 but not NBCe1 or NBCn1 was detected in neutrophils. Acidified neutrophils increased the EIPA-insensitive pH_i recovery rate when its activity was stimulated with fMLF/ cytochalasin B. This increase in the removal of acid equivalents was insensitive to the blockade of the NADPH oxidase with DPI. **Conclusion:** It is concluded that neutrophils have an NBC that regulates basal pH_i and is modulated by chemotactic agents.

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Introduction

Several transporters regulate intracellular pH (pH_i) by removing the excess of H^+ generated during activation of neutrophils. The Na^+/H^+ exchanger (NHE1) was primarily responsible for the elimination of H^+ in resting cells [1] but other mechanisms may also allow the release of acid equivalents during periods of high activity. Surprisingly, information on the presence of bicarbonate transporters in neutrophils is scarce. The Na^+/HCO_3^- cotransport (NBC) has crucial roles in regulating pH_i in a variety of cells, including cardiac, vascular smooth-muscle, glial and renal cells [2-5], and blood cells as lymphocytes [6], monocytes [7] and platelets [8]. However, the presence of NBC in neutrophils has not been reported yet.

All mammalian NHE isoforms are electroneutral. By contrast, electrogenic and electroneutral Na^+/HCO_3^- cotransporters (NBC) have been identified [9]. The presence of electrogenic mechanisms means that when the membrane is depolarized, the entry of HCO_3^- (removal of acid equivalents) is thermodynamically favored.

The stimulation of neutrophils with soluble (fMLF, PMA) or particulate agents (opsonized zymosan) induce the activation of NADPH oxidase, producing efflux of electrons and generation of intracellular H^+ ions [10]. The continuous production of superoxide by this enzyme requires the compensation of these electrical charges and the changes in pH_i . Recently, DeCoursey [11] have proposed that the prominent mechanism for these restitution is the efflux of protons through a voltage-gated channel. However, most experimental evidence has been obtained in the absence of bicarbonate, which is the main buffering system in the organism.

The purpose of this study was to investigate the presence of NBC in neutrophils, characterize its isoform and evaluate its participation in the regulation of pH_i in resting and stimulated neutrophils.

Materials and Methods

The fluorescent indicator 2',7'-bis (carboxyethyl)-5(6) carboxyfluorescein tetra-acetoxymethyl ester (BCECF-AM) was purchased from Invitrogen (Eugene, OR). N-(2-hydroxyethylpiperazine)-N'-2-ethanesulfonic acid (HEPES), 4-acetamido-40-isothiocyanato stilbene-2,20-disulfonic acid (SITS), 5-(N-ethyl-5-isopropyl)amiloride (EIPA) and N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLF) are from Sigma (St. Louis, MO). Cytochalasin B (Cyt B) was purchased from Biomol (Plymouth Meeting, Pa). S0859 was provided by Sanofi-Aventis (Germany). All other chemicals were reagent and analytic grades.

Experimental media

HEPES-buffered solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 20 HEPES adjusted to pH 7.4 with NaOH. Bicarbonate-buffered saline solution was similar except for the NaCl concentration that was reduced to 115 mM and replaced for 25 mM $NaHCO_3$ which was added instead of HEPES. Sodium-free solutions were made by replacement of $NaHCO_3$ and NaCl for Choline salts. Maintenance of extracellular pH in bicarbonate-containing solutions were performed by equilibration with an appropriate 5% CO_2 -95% O_2 mixture, bubbled in the solutions kept at 37 °C.

Neutrophil isolation

Human neutrophils obtained from fresh peripheral blood drawn by venipuncture of healthy volunteers were isolated by Histopaque double-gradient centrifugation as described by the manufacturer (Sigma-Aldrich, Procedure Nro 1119). Briefly, neutrophils recovered from the interface between two solutions of Histopaque 1119 and 1077 were washed once in 10 ml of the HEPES-buffered solution and resuspended at a density of 2×10^7 cells /ml. After each isolation, the amount of neutrophils was counted in a Neubauer chamber, where it was corroborated that there was marginal contamination with other cell types. In addition, viability was greater than 95 %, as assessed with trypan blue dye exclusion and purity, determined by differential cell counting, was greater than 98%.

Isolation of plasma membranes

Neutrophils (40×10^6 cells/ml) were lysed by sonication on ice for 22 s at power level 1 in a Branson Sonifier 450 sonicator and an equal volume of cold KCl-HEPES relaxation buffer (100 mM KCl, 50 mM HEPES, 5 mM NaCl, 1 mM MgCl₂, 0.5 mM EGTA, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 2.5 mM PMSF, 1 mM DFP (pH 7.2)) was added. Samples were then centrifuged for 7 min at $700 \times g$ for 4°C and 900 µl of supernatants were collected and centrifuged at $100,000 \times g$, 4°C for 45 min. Plasma membranes were collected from a visible disc above the pellet and resuspended in KCl-HEPES relaxation buffer and 2X Laemmli sample buffer.

Determination of pH_i

Neutrophils were incubated with 10 µg/ml BCECF-AM for 30 min at 37°C. Then, the dye-loaded cells were separated by centrifugation ($700 \times g$, 10 min), suspended in HEPES-buffered solution, re-incubated for 15 min in dye-free solution to complete the hydrolysis, washed and suspended at a density of 2×10^7 neutrophils/ml and stored on ice. Aliquots of 50 µl of this suspension were diluted in 2 ml of HEPES-buffered solution for measurement of the pH_i changes in the stirred and thermostated cuvette of a spectrofluorometer Aminco-Bowman series II (Silver Spring, Maryland, USA). The suspension of cells loaded with BCECF was excited at 503 and 440 nm, and the emitted fluorescence was collected at 535 nm. pH_i was calculated in each preparation calibrating with a high potassium-nigericin solution (135 mM KCl replaced the same concentration of NaCl in the HEPES solution, with 10 µM nigericin, titrated with KOH to 7.8). Small volumes of 0.1 M HCl were added to decrease pH step wise to 6.5. The relationship between the ratios of fluorescence 503 nm/440 nm and the pH value obtained in each step was linear.

Acute acid loading of cells by NH₄Cl pulse

The NH₄Cl pulse technique was used to acidify the intracellular environment. Following the dye loading, cells were exposed to 10 mM NH₄Cl for 15 min. Intracellular acidification was then induced by transferring cells to a sodium-free solution. Aliquots of 50 µl of this suspension were diluted in 2 ml of solutions containing 140 mM sodium and the subsequent changes in pH_i measured to calculate the recovery rate.

Blockade of ionic transport with SITS

Concentrated cell suspensions were maintained with 1 mM SITS in the sodium-free media and then re-suspended at 100 µM SITS (final concentration during recovery) to reduce color artifacts in experiments with inhibited bicarbonate transport.

Protein expression

Expression constructs for NBCe1b, the human cardiac splicing variant of NBCe1, [12] and human NBCn1 [13], have been described previously. Expression construct for rat NBCe2 (NBC4c) was a gift from Dr. Jeppe Praetorius (Aarhus University, Denmark). Human Embryonic Kidney (HEK) 293 cells were individually transfected with NBCe1, NBCe2, or NBCn1, as indicated, using the calcium phosphate method [14]. Cells were grown at 37 °C in an air/CO₂ (19:1) environment in DMEM medium supplemented with 10% (v/v).

Immunodetection

Two days post-transfection, cells were washed in PBS buffer (140 mM NaCl, 3 mM KCl, 6.5 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.5) and cell lysates were prepared by addition of 150 µl SDS-PAGE sample buffer to 60 mm Petri dish. Samples of untransfected or transfected HEK293, or samples of neutrophils isolated membranes (50 µg protein), were resolved by SDS-PAGE on 7.5 acrylamide gels. Proteins were transferred to PVDF membranes, and then incubated with rabbit anti-NBCe1 antibody (AB3212 Chemicon; 1:2000 dilutions), rabbit anti-NBCe2 antibody (ab99131 Abcam, Cambridge, MA, USA; 1:2000 dilutions) or rabbit anti-NBCn1 antibody (ab89656 Abcam, Cambridge, MA, USA; 1:2000 dilutions). Membranes were blocked with 5% non-fat milk. Immunoblots were incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase and visualized using the ECL reagent and a Chemidoc Image Station (Biorad).

RT-PCR

Total RNA was isolated from isolated human neutrophils using RNeasy Mini Kits (Qiagen, USA). The RNA was reverse transcribed using 2 U/µl Superscript II Reverse Transcriptase (Invitrogen). PCR (HotStar

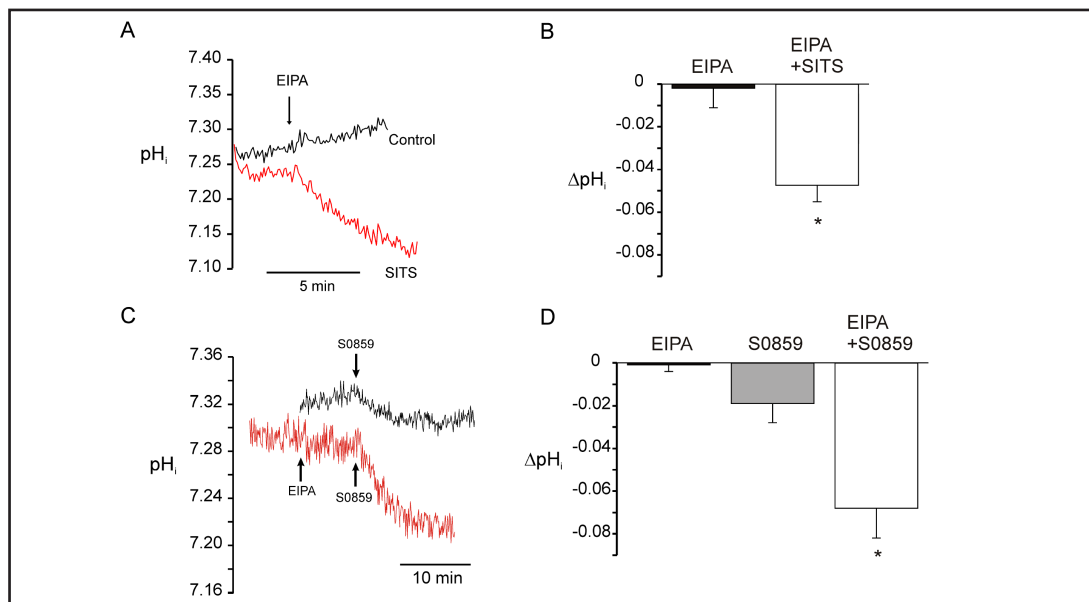


Fig. 1. Regulation of basal pH_i in neutrophils by Na^+/H^+ exchanger and Na^+/HCO_3^- cotransport. Panel A: Representative experiment of pH_i changes after blockade of NHE1 with 2.5 μM EIPA (arrow) in a bicarbonate-buffered solution. Intracellular pH did not decrease unless the transport of bicarbonate was previously blocked by SITS (grey trace). Panel B: mean and SEM of the rate of pH_i change after EIPA without pretreatment (black bar, control, n=4) or pre-incubated with SITS (hollow bar, n=4). Panel C: Representative experiments of pH_i changes after blockade of NBC with 30 μM S0859 in the absence (upper trace) or presence of EIPA (lower trace). pH_i decreased significantly with S0859 (second arrow, lower trace) only when the cells were previously treated with EIPA (first arrow, lower trace). Panel D: mean and SEM of pH_i change after EIPA (black bar, n=5), S0859 without pretreatment of EIPA (grey bar, n=4) or pre-incubated with EIPA (hollow bar, n=5). The asterisks indicate a significant decrease of pH_i ($p < 0.05$).

Taq Master Mix, Qiagen) with 10% cDNA and 1 pmol of each primer (human forward and reverse primers for NBCe1-SLC4A4, NBCe2-SLC4A5 and NBCn1-SLC4A7 transcripts, as previously described) [15], was performed for 30 cycles after 15 min at 95°C, after which denaturation was performed for 30 s at 60°C, annealing for 30 s, and elongation at 72°C for 1 min. PCR for GAPDH (forward: CGAGATCCCTCCAAAATCAA; reverse: TTCACACCCATGACGAACAT) was performed to validate each template. PCR products were separated by 2% agarose gel electrophoresis with GelRed Nucleic Acid Stain (Invitrogen) and photographed under ultraviolet illumination. Human primer pairs has been previously validated by nucleotide sequencing representative PCR products [15].

Statistics

Data were expressed as means \pm S.E.M. and were compared with Student's t test or One-way ANOVA followed by Student-Newman-Keuls post-hoc test. A value of $P < 0.05$ was considered statistically significant (two-tailed test).

Results

Effect of bicarbonate transport blockade on basal pH

In a bicarbonate-buffered solution, after 5 minutes of incubation at 37°C in the absence or presence of the anionic transporters blocker SITS, neutrophils reach similar pH_i values (control: 7.21 ± 0.1 , n=4; SITS: 7.18 ± 0.1 , n=4). Blockade of NHE1 by the addition of 2.5 μM EIPA did not produce a statistically significant change in pH_i in control cells (Fig. 1A-B). However, the application of EIPA on cells pretreated with SITS elicited a significant decrease

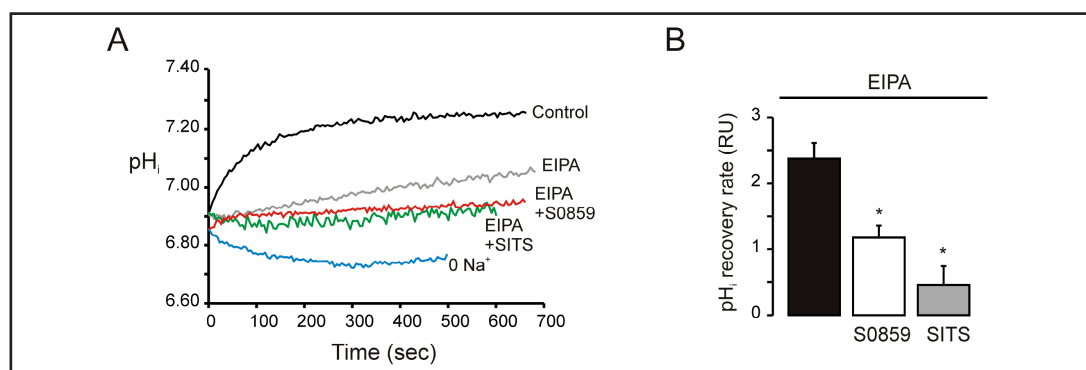


Fig. 2. Recovery of pH_i after an acid load. Panel A: Representative experiment showing the pH_i recovery in bicarbonate-containing media of neutrophils pre-acidified by an ammonium prepulse. Traces from up to down are: without inhibitors, after blockade of NHE1 with $2.5 \mu\text{M}$ EIPA alone, with EIPA and $10 \mu\text{M}$ S0859, with EIPA and SITS and in solution without sodium (choline replaced). Panel B: mean and SEM of the rate of recovery from an acid load in cells treated with EIPA and in the absence (black bar, $n=7$) or presence of blockade with S0859 (hollow bar, $n=4$) or SITS (grey bar, $n=4$). pH_i recovery rate was expressed in rate units (RU), which represent $\text{pH units}\cdot\text{sec}^{-4}$. The asterisk indicates a significant effect of the bicarbonate transport blockers ($p < 0.05$, ANOVA).

in pH_i (Fig. 1A-B), indicating that neutrophils have a bicarbonate transport system that prevents the metabolic acidification when the NHE1 is blocked. In parallel experiments we confirmed that this concentration of EIPA was able to abolish the pH_i recovery in acidified neutrophils in the absence of external bicarbonate (HEPES-buffered solution), experimental condition that allow the study of NHE1 activity in isolation (data not shown).

Figure 1C-D show the effect on basal pH_i of the recently reported selective NBC blocker S0859 [16]. This compound did not significantly affect basal pH_i . However, similarly to SITS, S0859 decreased pH_i in cells pretreated with EIPA, indicating the presence of NBC in neutrophils, which, together with the NHE1, is responsible of maintaining steady basal pH_i .

The recovery from acidosis in bicarbonate media is sodium-dependent and sensitive to SITS and S0859

In order to study the bicarbonate mediated pH_i recovery from acidosis, neutrophils acidified by an ammonium prepulse and maintained in a sodium-free HEPES buffer were transferred to solutions buffered with $\text{HCO}_3^-/\text{CO}_2$ containing 140 mM Na^+ . The NHE1 was blocked with $2.5 \mu\text{M}$ EIPA in order to uncover the involvement of other transporters in the pH_i recovery of acidified neutrophils. This maneuver reduced the initial recovery rate of pH_i to one tenth of the unblocked cells rate (2.38 ± 0.23 vs 23.1 ± 1.46 RU, $n=7$, Fig. 2A). The EIPA-resistant recovery showed a significant reduction in the presence of S0859 or SITS (Fig. 2A-B), suggesting that is mediated by NBC activity.

In a sodium-free (Choline replaced) bicarbonate solution, acidified neutrophils were unable to recover their pH_i (Fig. 2A), showing a decrease during the first minutes (ΔpH_i measured from 1 to 6 min after maximal acidification: -0.139 ± 0.04 pH units, $n=5$), stabilizing after this time period. This experiment indicate that the pH_i recovery is due to a sodium dependent mechanism, ruling out the reverse operation of a $\text{Cl}^-/\text{HCO}_3^-$ exchanger, although this transporter was previously reported to be present in neutrophils [17].

Effect of fMLF/CytB on NBC

fMLF is a chemotactic agent that produces multiple responses in neutrophils including the increase of the activity of the NADPH oxidase accompanied by formation of reactive oxygen species (ROS) and depolarization of the cell. The responses to fMLF are potentiated by cytochalasin B (CytB), a depolymerizer of the actin cytoskeleton [18]. It is well known

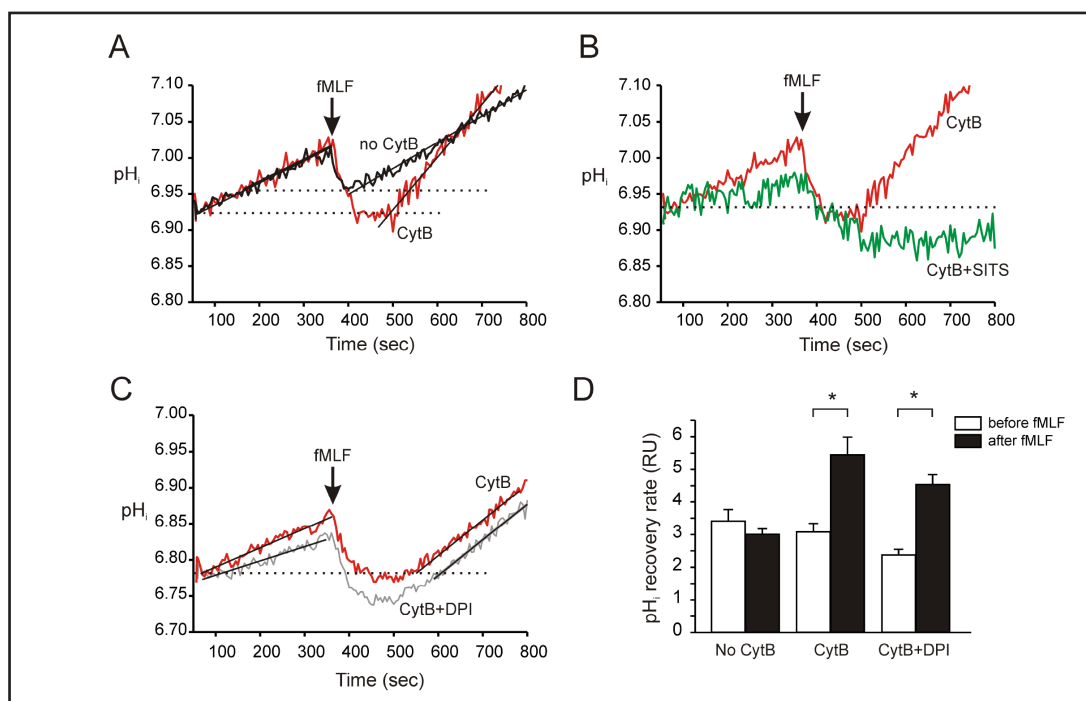


Fig. 3. EIPA-resistant recovery of pH_i from an acid load of stimulated-neutrophils in bicarbonate-buffered solutions. The arrow indicates the addition of fMLF. 2.5 μ M EIPA was applied throughout the experiment. Panel A: comparison of non-primed or CytB-primed neutrophils. Note the change in the slope of the regression line obtained at the same initial pH before and after fMLF (dotted horizontal lines). Panel B: comparison of CytB-primed neutrophils without SITS or blocked with SITS, as explained in materials and methods (displaced to correct color artifact). Panel C: comparison of CytB-primed neutrophils with cells with cells treated with DPI. Panel D: mean and SEM of the rates of recovery of pH_i before (hollow bars) and after fMLF (black bars) measured from the same initial pH_i ($n=5$, 5 and 4 for No CytB, CytB and CytB plus DPI, respectively). pH_i recovery rate was expressed in rate units (RU), which represent pH units. sec^{-4} . Asterisks indicate a potentiation of the EIPA-insensitive pH_i recovery by fMLF ($p<0.05$, paired t test).

that the stimulation of neutrophils with fMLF increases pH_i , mainly by stimulation of NHE1 [19]. In all the experiments shown below, 2.5 μ M EIPA was used to evaluate the NHE1-independent (EIPA-insensitive) rate of pH_i recovery. Independently of the presence of CytB, fMLF produced an initial and possibly artifactual drop in pH_i , which was rapidly reversed by a clear recovery towards alkaline values. The slope of the regression lines starting at the same initial pH_i (horizontal dotted lines in Fig. 3) were measured before and after the addition of fMLF. Non-primed neutrophils have similar rate before and after fMLF. In cells preincubated with CytB, the pH_i recovery was faster after fMLF addition (Fig. 3A and D). This stimulated NBC activity of primed cells was sensitive to SITS (Fig. 3B). The increase in the recovery rate elicited by the chemotactic agent was maintained even when NADPH oxidase was blocked with 2.5 μ M DPI (Fig. 3C and D).

Expression of NBC isoforms

Immunoblots of membranes isolated from human neutrophils were probed using specific commercial antibodies against the NBC isoforms NBCE1, NBCE2 or NBCn1. The anti-NBCE2 antibody (Fig. 4A) demonstrated a band with a molecular mass of \sim 130 kDa, corresponding to NBCE2 [12]. A band with a similar molecular mass was detected in lysates of HEK293 cells transfected with NBCE2 used as controls (Fig. 4B). The presence of this NBC isoform in human neutrophils was corroborated by the detection of its mRNA in RT-PCR experiments (Fig. 4C).

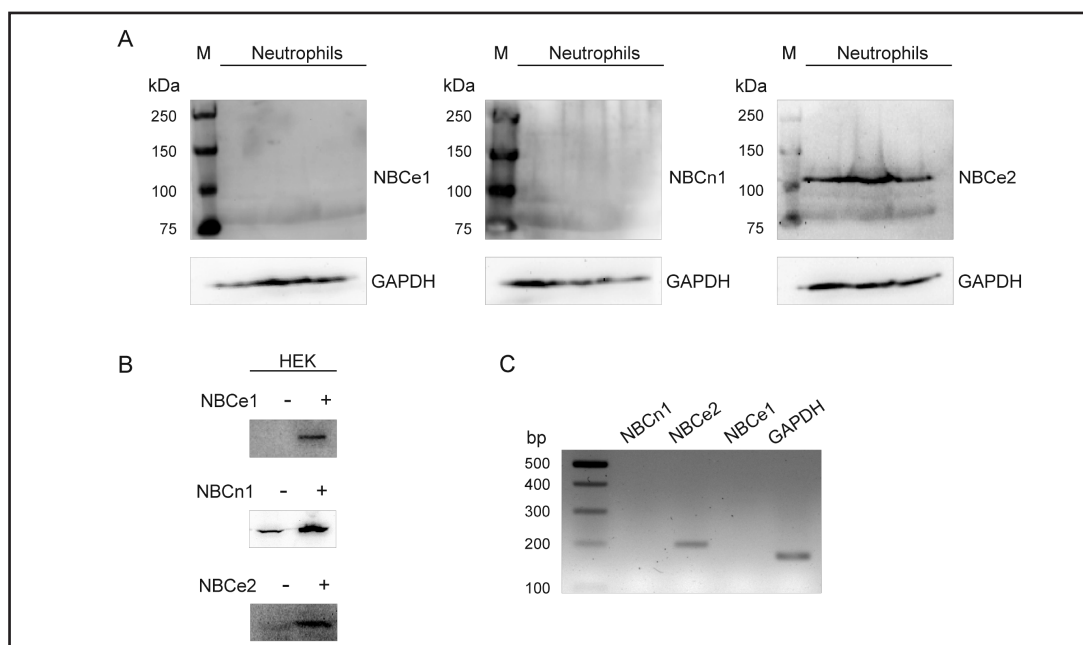


Fig. 4. Identification of the Na⁺/HCO₃⁻ cotransport isoforms in human neutrophils. Panel A: Human neutrophils membranes samples were resolved by SDS-PAGE on 7.5% polyacrylamide gels, transferred to PVDF membrane and probed with commercial antibodies against NBCe1, NBCn1, and NBCe2. Membranes were obtained under the same experimental conditions from neutrophils isolated from three different human beings. Panel B: Sample of lysates corresponding to untransfected (-) human embryonic kidney (HEK293) cells, or HEK293 cells transfected (+) with NBCe1, NBCn1 and NBCe2, as controls for the antibodies used in panel A. Panel C: RT-PCR analysis of NBCe1-SLC4A4, NBCe2-SLC4A5 and NBCn1-SLC4A7 transcripts. Total RNA from isolated human neutrophils was reverse transcribed and first strand cDNA was produced using Superscript II Reverse Transcriptase. Amplicons were analysed on a 2% agarose gel containing GelRed Nucleic Acid Stain. 100-bp ladder was used.

The presence of NBCe1 and NBCn1 was not detected in the human neutrophils, neither at the protein (Figure 4A) nor at the mRNA level (Fig. 4C). Figure 4B shows positive controls performed in HEK293 cells transfected with NBCe1 and NBCe2.

Discussion

Acid-loaded neutrophils were found to regain near-normal pH_i by means of a Na⁺-dependent process. NHE1 activation was observed under chemotactic factors treatment [1, 20]. Studies of the pH_i-dependence of the H⁺ extrusion rate indicate that chemotactic factors increase the [H⁺]_i sensitivity of NHE1 [21]. As far as we know, the involvement of bicarbonate on the recovery of acid-loads and its regulation by chemotactic factors has not been studied yet. A former work performed in neutrophils [22] suggested that a DIDS-sensitive process facilitates HCO₃⁻ entry into the cell, possibly mediated by the presence of a hitherto-undescribed NBC. The experiments presented herein reveal the existence of a bicarbonate and sodium-dependent mechanism, sensitive to SITS and S0859 that could participate in the recovery from acid loads and in the maintenance of steady basal pH_i. Since SITS is a highly nonspecific inhibitor, S0859 (a putative NBC inhibitor) [16] has also been used to block bicarbonate transport. S0859 has no effect on acid-extruding NHE1 in the ventricular myocyte at doses that completely inhibit generic NBC [16]. These results demonstrate for the first time the presence of NBC in human neutrophils.

Distinct NBC isoforms co-exist in the mammalian cells, the electrogenic isoforms, NBCe1 (or NBC1) [23, 24] and NBCe2 (or NBC4) [25, 26], with a stoichiometry of 1 Na⁺: 2 or 3 HCO₃⁻, and the electroneutral isoform NBCn1 (or NBC3) [27], with a stoichiometry of 1 Na⁺:1 HCO₃⁻. The electrogenic NBCe1 and NBCe2 are encoded by the *SLC4A4* gene [24], and the *SLC4A5* gene [25, 28], respectively, while NBCn1 is encoded by the *SLC4A7* gene [27]. According to the immunoblot and RT-PCR experiments performed in the present manuscript, we can report for the first time the presence of NBCe2 in human neutrophils.

The present study shows that in cells primed by CytB, a potent enhancer of oxidative burst [29], the pH_i recovery from the acid load was similar to that of the non-primed cells, but the further response to fMLF induced a faster recovery (Fig. 3). These results reveal the participation of a regulatory mechanism that facilitates pH_i regulation in circumstances of high activity. The increase in the rate of recovery seems to be independent of the activity of the NADPH oxidase because persists after blockade of the enzyme with DPI. Then, we can propose that NBCe2 is regulated by fMLF by a direct pathway upstream NADPH oxidase activation. Nevertheless, it is important to mention that NADPH oxidase functions optimally at neutral pH and we performed our study after acidification by an NH₄⁺ prepulse. Thus, the possible activity of NBCe2 during the respiratory burst exceeds the scope of the present manuscript and might deserve a deeper investigation in a future study.

In summary, herein we are describing for the first time the presence in neutrophils of a bicarbonate and sodium-dependent pH_i regulator mechanism that participates in the maintenance of basal pH_i and in the recovery from acid loads. The stimulation of the cell with a strong chemotactic stimulus activates the recovery from the cell acidification. These functional results indicate the existence of a bicarbonate transporter that may allow the release of acid equivalents during periods of low and high activity. The molecular characterization suggested that this bicarbonate transporter is the electrogenic NBC isoform denominated NBCe2.

Abbreviations

NHE1(Na⁺/H⁺ exchanger); NBC (Na⁺/HCO₃⁻ cotransport); pH_i (Intracellular pH); SITS (4-acetamido-40-isothiocyanatostilbene-2,20-disulfonic acid); EIPA (5-(N-ethyl-5-isopropyl)amiloride); fMLF (N-formyl-L-methionyl-L-leucyl-phenylalanine); CytB (Cytochalasin B); NBCe1 (Electrogenic Na⁺/HCO₃⁻ cotransport isoform 1); NBCe2 (Electrogenic Na⁺/HCO₃⁻ cotransport isoform 2); NBCn1 (Electroneutral Na⁺/HCO₃⁻ cotransport isoform 1).

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