Inhibition of the cardiac electrogenic sodium bicarbonate cotransporter reduces ischemic injury

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Abstract

Objective: Although it is believed that sodium-driven acid–base transport plays a central role in the development of the reperfusion injury that follows cardiac ischemia, research to date has demonstrated only a role for Na⁺/H⁺ exchange (NHE). However, Na⁺-driven HCO₃⁻ transport, which is quantitatively as important as NHE in cardiac cells, has not been examined. Methods and Results: Here the results show that a neutralizing antibody raised against the human heart electrogenic Na⁺/HCO₃⁻ cotransporter (hhNBC) blocked the recovery of pH after acidic pulse both in HEK-293 cells expressing hhNBC and in rat cardiac myocytes demonstrating the presence of an electrogenic NBC in rat cardiac myocytes similar to hhNBC. Administration of anti-NBC antibody to ischemic-reperfused rat hearts markedly protects systolic and diastolic functions of the heart during reperfusion. Furthermore, using a quantitative real-time RT-PCR (TaqMan) and Western blot analysis we demonstrated that in human cardiomyopathic hearts, mRNA and protein levels of hhNBC increase, whereas mRNA levels of the electroneutral Na⁺/HCO₃⁻ cotransporter (NBCn1) remain unchanged. Conclusion: Our data provide evidence that inhibition of hhNBC, whose role in cardiac pathologies could be amplified by overexpression, represents a novel therapeutic approach for ischemic heart disease.

Keywords: Cardiomyopathy; Gene expression; Ion transport; Ischemia; Reperfusion; Ventricular function

1. Introduction

Ischemic heart disease represents the principal etiology for the development of congestive heart failure. Thus, treatments that reduce post-ischemic injury could be beneficial for heart failure, as exemplified by β-adrenergic antagonists [1], angiotensin-converting enzyme inhibitors [2] or endothelin antagonists [3]. Because disturbances of myocardial pH⁺ have grave functional consequences, cardiac cells possess transporters that maintain pH⁺ within narrow limits [4]. The pH-regulating systems include NHE [5] and two bicarbonate-dependent systems, Na⁺/HCO₃⁻ cotransport [6] (NBC) and Na⁺-independent Cl⁻/HCO₃⁻ exchange [7].

Although the physiological role of NHE in cardiomyocytes has been amply demonstrated [5], that of HCO₃⁻-dependent pH⁺-regulatory mechanisms had long been underestimated because most studies were carried out in HCO₃⁻-free buffers [8,9]. However, the NBC accounts for about 20–30% of total acid extrusion (i.e., sum of H⁺ efflux and HCO₃⁻ efflux) in cardiac Purkinje fibres [6], and for 40–50% in ventricular myocardium [10,11]. Alteration

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of NBC activity could thus play a major role in pathological situations such as myocardial ischemia, where intracellular proton generation is substantially elevated.

Functional and molecular studies indicate the presence of at least two NBCs in the heart. An electroneutral NBC has been described in sheep cardiac Purkinje fibres [6] and in guinea-pig ventricular cells [10], whereas an electrogenic NBC has been characterized in cat papillary muscle and rat cardiac myocytes [11–13]. Following the expression cloning of an electrogenic NBC from amphibian kidney [14], other electrogenic NBCs have been cloned from mammalian kidney [15,16], pancreas [17], heart [18] and brain [19]. More recently, electroneutral NBCs (NBCn1-A–NBCn1-D) cloned from rat smooth muscle have also been shown to be expressed in heart [20].

Here, we show that, in contrast to the electroneutral cotransporter NBCn1-A [20] (also known as NBC3 [21]), the electrogenic hhNBC is overexpressed in hearts from patients with heart failure. We also demonstrate that specific inhibition of hhNBC with a polyclonal antibody raised against an hhNBC-specific peptide is effective at reducing post-ischemic dysfunction in isolated rat hearts. Thus, the specific inhibition of hhNBC could represent a major advance to the treatment of ischemic heart disease, especially in patients with cardiomyopathy.

2. Methods

2.1. Animals

All animals used in the present study received human care in accordance with the principle of NIH guidelines (publication no. 85-23) for animal care.

2.2. Cell culture

Experiments were performed in HEK-293 cells expressing hhNBC and in isolated rat cardiac myocytes. The complete coding region of hhNBC (GenBank Accession no. AF069510) was cloned into the eukaryotic expression vector pCDNA3.1HisC at the EcoRI site in sense and anti-sense orientation. HEK293 cells stably transfected with sense and anti-sense constructs for hhNBC were grown in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum, 10 mM Hepes and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). When cells reached 80% of confluence, they were transfected with pCDN-hhNBC plasmid, using lipofectamin (Life Technologies, Cergy Pontoise, France).

Stably transfected clones were selected by addition of G418 (1 mg/ml) to the medium. Expression of recombinant hhNBC in HEK-293 cells was tested by RT-PCR, using specific primers for hhNBC (not shown) as well as in Western-blot experiments using anti-hhNBC specific antibodies.

For cardiac myocyte experiments, rats were anesthetized and the heart was rapidly removed. Myocytes were isolated as previously described [22].

2.3. Polyclonal antibodies

The deduced amino acid of hhNBC protein was analysed for highly antigenic regions using the Jameson-Wolf antigenic index. A peptide representing amino acids 629–644 of hhNBC was synthesized (Peptide Synthesizer Model 431A, Applied Biosystems), purified and conjugated to keyhole limpet hemocyanin, using benzidine as the coupling agent. Fourteen-week-old New Zealand rabbits (two animals per peptide) were injected on day 0 with peptide-carrier conjugate (150 μg/injection) in complete Freund’s adjuvant, and every 2 weeks thereafter with peptide-carrier conjugate (50 μg/injection) in incomplete Freund’s adjuvant. Animals were bled 7 days after boosts (days 39, 69 and 95) and sera were tested at various dilutions on ELISA plates coated with unconjugated peptide.

2.4. Expression-pattern studies

Ventricular samples from failing human hearts were obtained from 10 patients with NYHA class VI terminal heart failure undergoing cardiac transplantation (nine men, one woman, mean age 50±3 years). Of these, four patients had history of ischemic cardiomyopathy (ICM) and six had dilated cardiomyopathy (DCM). All patients were treated with diuretics, angiotensin-converting enzyme inhibitors, antiarrhythmics, and anticoagulants until the time for transplantation. Normal RNA and protein microsomes samples were obtained from Clontech and from organ donor candidates (mean age 37.8±8.5 years) whose hearts could not be transplanted for non-cardiac reasons. The use of these samples was approved by the Internal Review Committees and the investigation was performed in accordance with the principles outlined in the Declaration of Helsinki.

The expression level of hhNBC was measured using Northern-blot analysis, real-time quantitative RT-PCR and Western immunoblotting analysis. Total RNA from the ventricles of normal hearts, and hearts from patients with dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) were extracted using the acid guanidinium thiocyanate–phenol–chloroform method [23].

Real-time quantitative RT-PCR were performed on cDNA generated from 100 ng of total RNA using murine Moloney leukemia virus reverse transcriptase (Life technologies) and random hexamers. For the PCR, we used 200 nM of both sense and antisense primers (Genset) as well as 100 nM of TAMRA-labelled primer probe (PE Applied Biosystems) in a final volume of 50 μl, the TaqMan PCR...
cycle threshold (Ct) and to assess PCR efficiency. Results are presented as the PCR cycle number at which exponential PCR-generated fluorescence is first detected; the lower the Ct, the higher the expression. Northern blot analysis was done on 2 μg of poly A RNA using standard methods [24].

In immunoblotting experiments, the polyclonal antibody typically recognized a 137-kDa band, which is believed to be glycosylated hhNBC.

2.5. Intracellular pH measurement

HEK-293 cells were loaded with 1 μM of BCECF-AM [25], the acetoxymethyl ester of the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, Molecular Probes, Eugene, USA) for 30 min at 37°C. pH was measured in a thermostatically controlled holding chamber (37°C) in a Delta Scan dual-excitation spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA). The cells were continuously superfused with Tyrode buffer solution. Fluorescence was detected in individual cells using excitation wavelengths of 440 and 490 nm at an emission wavelength of 535 nm. The pH calibration was generated using the KCl/nigericin technique [26]. The initial rate of pH recovery from an acid load was calculated by fitting a line to the first 2 min of the pH recovery.

2.6. Isolated-heart perfusion

Male Wistar rats (280–320 g) were anesthetized by intraperitoneal injection of sodium thiopental (50 mg kg⁻¹). Hearts were then cannulated via the aorta and perfused using the Langendorff technique with Krebs–Henseleit buffer of the following composition (in mM): NaCl 118; NaHCO₃ 23; KCl 4.7; KH₂PO₄ 1.2; MgCl₂ 1.2; CaCl₂ 1.25; glucose 11; pyruvate 2, gassed continuously with a 95%O₂/5%CO₂ gas mixture and maintained at 37°C. A water-filled latex balloon fixed to a pressure transducer was inserted into the left ventricle for the determination of left ventricular developed pressure (LVEDP) and heart rate as previously described [27]. Positive and negative dP/dt were obtained with a differentiator (Gould Electronics, Cleveland, OH, USA). Balloon volume was adjusted to achieve a left ventricular end-diastolic pressure (LVEDP) of 8–10 mmHg. The volume of the LV balloon was kept constant throughout the experiment, so that changes in LVEDP reflect changes in diastolic chamber distensibility [28]. The exponential time constant of left ventricular pressure decay (τ) was calculated from the linear regression of −dP/dt versus pressure, assuming that the asymptote of the left ventricular relaxation is zero [29]. All measurements were recorded on a thermal multichannel recorder (Gould Electronics) and transferred to computer for analysis. Coronary flow was monitored by collecting coronary sinus effluent. All hearts were initially equilibrated for 30 min after which, zero-flow or low-flow (0.5 ml/min) global ischemia was induced for either 30 or 60 min followed by a reperfusion.

2.7. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) leakage from the myocardium was measured, using an enzymatic assay kit (Boehringer-Mannheim, Germany), in the coronary effluent collected during reperfusion. The activity of LDH was normalized against the wet weight of heart and coronary flow rate as U/min per g wet weight. The absorbance was read at 334 nm using a Beckman DU-640 spectrophotometer.

2.8. Statistics

All data are expressed as the mean±S.E.M. of n preparations. Statistical comparisons were made using either Student’s t-test or one-way analysis of variance.

3. Results

3.1. Functional evidence for hhNBC in cardiac cells

Polyclonal antibodies raised against an extracellular loop of hhNBC were generated and used for both Western-blot experiments and functional studies, either in NBC-transfected HEK-293 or in isolated cardiomyocytes. The anti-NBC antibody detected a single, ~137-kDa band in hhNBC-transfected HEK-293 cells, but nothing in non-transfected cells (Fig. 1a). Similarly, the antibody detected a single band of about 137 kDa in microsomal fractions from rat cardiac tissue (Fig. 1b), indicating that the rat homologue of hhNBC is expressed in myocardium at the protein level and could thus be responsible for the electronegenic transport reported previously [11].

The functional activity of the polyclonal anti-NBC antibody was assessed in NBC transfected HEK-293 cells (Fig. 1c) and in isolated cardiomyocytes (Fig. 1d). In both cases, we acidified the cells with an NH₄⁺ prepulse [30]. Anti-NBC slowed the pH₄ recovery in hhNBC-transfected...
Control 76.2

Table 1
Baseline function

<table>
<thead>
<tr>
<th>Group</th>
<th>LVDP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>$dP/dt_{max}$ (mmHg/s)</th>
<th>$dP/dt_{min}$ (mmHg/s)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.2±2.6</td>
<td>8.7±0.5</td>
<td>2266±79</td>
<td>1701±71</td>
<td>351±9</td>
</tr>
<tr>
<td>Anti-NBC</td>
<td>76.8±2.7</td>
<td>8.8±0.4</td>
<td>2140±76</td>
<td>1568±66</td>
<td>322±11</td>
</tr>
</tbody>
</table>

Data are shown as means±S.E.M. LVDP, left ventricular developed pressure; LVEDP, LV end-diastolic pressure; HR, heart rate.

leading via Na$^+$/Ca$^{2+}$ exchange to a secondary increase in [Ca$^{2+}$], and thus cell damage. Several studies have reported that inhibiting NHE protects against myocardial injury following ischemia–reperfusion [5]. We previously suggested that additional mechanisms, including NBC, could also be involved [31]. To elucidate the role of hhNBC in post-ischemic contractile dysfunction, we investigated the effect of the neutralizing antibody in isolated perfused rat hearts subjected to either (i) 30-min zero-flow ischemia followed by 20-min reperfusion, or (ii) 60-min low-flow ischemia and 30-min reperfusion. NBC polyclonal antibody or pre-immune serum (1:1000 in Krebs buffer solution) was added to the perfusate 15 min before initiating ischemia, and was maintained throughout the experiment, during which serial measurements of hemodynamic parameters were performed. Basal pre-ischemic values of cardiac function were not affected by the presence of anti-NBC (Table 1).

3.2. hhNBC is involved in post-ischemic dysfunction

A well-known consequence of myocardial ischemia is intracellular acidosis. During reperfusion, this low pH$_i$ activates NHE, which may overload the cell with Na$^+$,
within the first 5 min of reperfusion, and coronary flow (Fig. 2d) overshot the pre-ischemic value. Such an increase in coronary flow at the time of reperfusion may be related to the protection of the cardiac function as usually observed with cardioprotective agents.

3.2.2. Low-flow ischemia

During ischemia, LVEDP increased progressively with time (Fig. 3a), while LVDP, \( \frac{dP}{dt_{\text{max}}} \), and \( \frac{dP}{dt_{\text{min}}} \) rapidly fell to zero (Fig. 3b–d). In the presence of anti-NBC antibody, LVEDP increased more slowly during the ischemic period (Fig. 3a), and the complete abolition of cardiac function as determined by LVDP, \( \frac{dP}{dt_{\text{max}}} \) and \( \frac{dP}{dt_{\text{min}}} \), tended to be delayed (Fig. 3b–d).

During reperfusion, treatment with antiserum caused all cardiac function parameters to recover substantially more towards pre-ischemic values than no treatment (Fig. 3a–d). At the end of the reperfusion period, \( \tau \) (an index of isovolumic relaxation time) was \( 99 \pm 26 \) ms in treated hearts, but \( 277 \pm 78 \) ms in controls. Furthermore, the extent of lactate dehydrogenase (LDH) release into the coronary effluent at the time of reperfusion, measured to assess the degree of cell damage following global ischemia and reperfusion, was reduced by anti-NBC treatment (Fig. 4). These data suggest that acute anti-NBC treatment improved contractile recovery and reduced the degree of contracture or irreversible myocardial injury during post-ischemic reperfusion.

3.3. hhNBC expression is increased in human cardiomyopathic hearts

Previous work showed that hhNBC is not measurably expressed in kidney, but is highly expressed in pancreas [17,18] and, to a much lesser extent, in heart [18]. To gain additional insight into the role of the electrogenic NBC in human cardiac pathologies, the expression pattern of hhNBC was measured in normal and in cardiomyopathic hearts. Probes specific for the N-terminus unique to hhNBC were used for Northern experiments illustrated in Fig. 5a. As previously demonstrated, hhNBC mRNA was...
not detected in the kidney, but a very faint band at 7.5 kb was observed in normal hearts. In contrast, easily detectable bands were noted in RNA preparations in either left or right ventricle from patients with end-stage heart failure.

By densitometry, the signal intensity increased from an average of 1.0±0.3 arbitrary units in control preparations to 6.9±1.0 in heart failure (P<0.05).

In an additional set of experiments, we used real-time quantitative RT-PCR to investigate expression of hhNBC and NBCn1 in hearts from patients with ischemic and dilated cardiomyopathies. This approach, which is more sensitive than Northern analysis, easily detected hhNBC in normal heart (Fig. 5b). Moreover, in cardiomyopathic hearts, the cycle threshold for detecting hhNBC was significantly reduced. Therefore, confirming the results obtained by Northern analysis, both dilated and ischemic cardiomyopathy increase the expression of hhNBC. The number of copies per ng of total RNA increased from 1154±183 in normal to 1877±200 in ischemic cardiomyopathy and to 2262±156 in dilated cardiomyopathy. In contrast, cardiomyopathy did not change the cycle threshold for detecting NBCn1 (Fig. 5c).

Finally, the immunoblot data shown in Fig. 5d confirm that, in human heart failure, the higher hhNBC expression observed at the RNA level was correlated with an increase at the protein level.
4. Discussion

The regulation of $\mathrm{pH}_1$ in cardiac myocytes primarily depends on the activity of at least three membrane transporters. While activation of the $\mathrm{Cl}^-\mathrm{HCO}_3^-$ exchanger acidifies the cell, activation of the NHE and the NBC alkalinises it. Furthermore, Vandenbergh and colleagues suggested that during myocardial ischemia metabolic washout, i.e., lactate and $\mathrm{CO}_2$, may represent a significant contributor to net $\mathrm{pH}_1$ recovery [32]. Although
the role of NHE in cardiac pathophysiological states has been widely investigated [5], the role of NBC had been undefined. Characterization of NBC isoforms is still in its early stage, and both electroneutral [6,10] and electrogenic [11–13] Na\(^+\)/HCO\(_3\)\(^-\) cotransporters have been identified in heart. At the mRNA level, both an electroneutral NBC [20,21] (NBCn1) and an electrogenic NBC [18] (hhNBC) have been identified in human heart. Here, we have demonstrated, at the protein level, that the electrogenic NBC is expressed in both rat heart (Fig. 1b) and human heart (Fig. 5d), and that the specific inhibition of this electrogenic transporter using a neutralizing antibody protects the heart against ischemic injury.

Antibodies to synthetic peptides based on amino acid sequences of a protein have proven useful for understanding the structure, analyzing the molecular forms and studying functional aspects of anion exchangers. Specific anti-Cl \(^-\)/HCO\(_3\)\(^-\) (AE1 isoform) raised against loops of the transporter significantly decreased the exchange function of the AE1 protein [33]. We developed polyclonal antibodies raised against an immunogenic region of hhNBC. Because the synthetic peptide had the same sequence as hhNBC between amino acids 629 and 644, the antibodies are likely to recognize an extracellular loop of the protein. Consistent with our knowledge that the amino acid sequence of this loop in hhNBC [17,18] is extremely similar to the homologous region in the electrogenic rat NBCs [15,19], the anti-NBC antibody recognised a single band in both HEK-293 cells expressing hhNBC and in rat cardiac tissue. Because there is little similarity between the hhNBC peptide used to produce the antibody and the homologous region of the electroneutral NBCs [20] (12.5% identity), and because our antibody did not recognize a second band in Western blots, it is unlikely that the antibody inhibits the electroneutral Na\(^+\)/HCO\(_3\)\(^-\) cotransporter. Furthermore, the anti-NBC antibody neutralized NBC activity in both hhNBC-transfected HEK-293 and isolated rat cardiac myocytes. Taken together, these results suggest that an electrogenic NBC is expressed and functional in rat cardiac cells, as previously suggested from electrophysiological approaches [12]. We further demonstrated that, in contrast to what we observed for the electroneutral NBC, the expression level of hhNBC, while rather low in normal human heart, is increased in cardiomyopathic hearts. It could be speculated that the increased expression of the electrogenic NBC would alter the electrophysiological and contractile parameters of the cardiac cells.

Although this remains to be further investigated, NBC could contribute to the control of [Na\(^+\)]\(_i\), [6,20]. Therefore, the overexpression might lead to an increase in intracellular Na\(^+\) concentration which via activation of the Na\(^+\)/Ca\(^{2+}\) exchange may trigger arrhythmias. Obviously, we cannot exclude an increase in the activity of the electroneutral NBC similar to what has been shown for the NHE [34] but we could hypothesize that the electrogenic NBC might contribute to the higher incidence of ischemia-related arrhythmias in the failing heart [35].

The protection afforded by inhibiting hhNBC was observed on systolic contractility and also on diastolic function in both zero- and low-flow global ischemia. Although the molecular mechanism responsible for such a pronounced protection is not defined yet, we suggest that a reduction of intracellular calcium cycling may be involved, as evidenced by the reduced contracture during both ischemia and reperfusion. During ischemia and reperfusion, intracellular acidosis leads to an activation of NBC associated to an increase Na\(^+\) influx. In this respect, recent data clearly indicated that during no-flow ischemia, myocardial [Na\(^+\)] markedly increased in perfused rat hearts [36]. Increasing [Na\(^+\)] will increase reverse Na\(^+\)/Ca\(^{2+}\) exchange and raise [Ca\(^{2+}\)]\(_i\) in cardiac cells thereby causing cardiac function, especially diastolic function, to deteriorate during ischemia and reperfusion. We showed that anti-NBC exerts beneficial effects during ischemia but more profoundly during early postischemic reperfusion, especially, the heart rate returned to normal immediately at the onset of reperfusion. In this respect, it has been reported that certain interventions at the time of reperfusion or before ischemia may be expected to reduce reperfusion injury or stunning. The reperfusion in isolated ferret hearts undergoing zero-flow ischemia, with acidic perfusate prevent subsequent contractile dysfunction [37]. Moreover, using isolated perfused hearts it was reported that reperfusion with acidic buffer (pH 6.4 or 6.6) attenuated the gain in tissue Na\(^+\) and Ca\(^{2+}\) contents [38]. Finally, reoxygation injury produced in rat neonatal cardiac myocytes has been shown to be attenuated when restoration of oxygen was carried out with acidic medium [39].

Although further studies on [Na\(^+\)], and [Ca\(^{2+}\)], changes during ischemia–reperfusion remain necessary, our results suggest that anti-NBC-induced protection seems at least in part to be mediated by reduction of the ischemia–reperfusion-induced Ca\(^{2+}\) overload since the time of onset and amplitude of ischemia- and reperfusion-induced contracture were significantly reduced compared to the non-treated heart. Because hhNBC is overexpressed in cardiomyopathic hearts we could hypothesize that this transporter may play a more pronounced role in patients with heart failure. In particular, upregulation of hhNBC expression could contribute to both mechanical dysfunction and arrhythmogenesis in the failing heart. Obviously this needs to be further investigated with the aim of determining whether this overexpression is cause or consequence of the disease. In conclusion, the results of the present study suggest that the electrogenic NBC, hhNBC, like the Na\(^+\)/Ca\(^{2+}\) exchange [40], is overexpressed in heart failure, and that its inhibition could reduce the deleterious effect of intracellular Ca\(^{2+}\) overload, and therefore could be useful...
additional therapeutic target for a variety of human cardiac diseases, such as myocardial infarction and heart failure.

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