Modulation of Na\(^+/\)H\(^+\) exchange isoform 1 mRNA expression in isolated rat hearts

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Gan, Xiaohong Tracey, Subrata Chakrabarti, and Morris Karmazyn. Modulation of Na\(^+/\)H\(^+\) exchange isoform 1 mRNA expression in isolated rat hearts. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H993–H998, 1999.—Na\(^+/\)H\(^+\) exchange (NHE) has been demonstrated to mediate myocardial ischemia and reperfusion injury as well as injury produced by hydrogen peroxide (H\(_2\)O\(_2\)) or lysophosphatidylcholine (LPC). However, changes in gene expression in response to injurious factors have not been extensively studied. We examined Na\(^+/\)H\(^+\) exchange isoform 1 (NHE-1) expression using Southern detection of the RT-PCR product in response to 30 min of global ischemia with or without reperfusion in isolated rat hearts or to 30 min of exposure to either H\(_2\)O\(_2\) (100 µM) or LPC (5 µM). We also determined whether ischemic preconditioning (2× 5-min ischemia) alters basal NHE-1 expression or the subsequent response to insult. Ischemia with or without reperfusion increased NHE-1 expression approximately sevenfold (P < 0.05), whereas either H\(_2\)O\(_2\) or LPC increased expression approximately twofold. Preconditioning reduced NHE-1 message by ~70% (P < 0.05) and significantly attenuated the effects of ischemia, H\(_2\)O\(_2\), or LPC. The internal standard, β-globin was unaffected by any treatment. Our results indicate that NHE-1 expression is rapidly increased in response to ischemia with or without reperfusion as well as in response to H\(_2\)O\(_2\) or LPC. In contrast, preconditioning was associated with downregulation of NHE-1. These results may be important in furthering our understanding of NHE-1 in cardiac disease states and suggest that the antiporter adapts rapidly to cardiac conditions associated with pathology.

NHE, ischemia-reperfusion; preconditioning; hydrogen peroxide; lysophosphatidylcholine

The continuous production of protons in the cardiac cell necessitates the ability of intracellular pH (pHi) regulation by various membrane transporters. In addition to bicarbonate-dependent transport systems Na\(^+/\)H\(^+\) exchange (NHE) represents one of the major mechanisms of pHi regulation and functions to extrude protons concomitantly with Na\(^+\) influx in an electroneutral 1:1 tightly-coupled stoichiometric relationship. Although at least six isoforms of NHE have thus far been identified, the predominant subtype in the heart is the ubiquitous NHE-1 (4, 5). In addition to pHi regulation, there is now extensive and convincing evidence that NHE contributes to various types of cardiac pathologies (reviewed in Refs. 4, 5, 11, 16). One area that has received extensive interest concerns myocardial ischemia and reperfusion. With the use of pharmacological approaches using drugs that inhibit NHE, numerous investigators have reported that inhibition of the antiporter exerts extensive cardioprotective influence against various aspects of cardiac injury associated with both ischemia and reperfusion (1, 4, 5, 11, 16). Oral administration of the NHE-1-specific inhibitor cariporide has profound effects on reducing ventricular arrhythmias and mortality in a rat model of coronary occlusion and reperfusion (9). The contribution of NHE to myocardial ischemic and reperfusion injury is based on the concept that activation of the antiporter during ischemia or reperfusion and the subsequent influx of sodium result in calcium overloading conditions (16). The latter likely reflects an inability to extrude sodium because of inhibition of the sodium-potassium ATPase, and hence calcium increases via sodium-calcium exchange processes. In addition to the beneficial effects of NHE inhibition on the ischemic and reperfused myocardium, there is also evidence that this approach also protects against the deleterious effects of cardiotoxic compounds, particularly those produced by the ischemic myocardium. We have shown that NHE inhibitors can attenuate the cardiotoxic effects of two such factors, H\(_2\)O\(_2\) and LPC (6, 8). In addition, both LPC and H\(_2\)O\(_2\) have been shown to stimulate NHE-1 activity in rat cardiomyocytes (6, 18).

Activity of NHE is markedly activated by intracellular acidosis such as that which occurs during ischemia (reviewed in 4, 5, 11, 16) as well as under other pathological conditions such as in the diabetic myocardium (17). Although extensive evidence has now documented the importance of NHE to cardiac pathology on the basis of the cardioprotective effects of NHE inhibitors, little is known about expression of NHE-1, the major cardiac isoform, in the diseased myocardium. Dyck et al. (2) demonstrated increased NHE-1 expression after 3 h of ischemia, a phenomenon that occurred only under moderate conditions of flow reduction. Our study was therefore carried out to further address the issue of NHE-1 expression in the ischemic and reperfused myocardium as well as in hearts exposed to LPC or H\(_2\)O\(_2\). In addition to studying the exchanger under pathological conditions, we also wanted to determine whether myocardial preconditioning, a cardioprotective strategy, also affects NHE-1 expression either on its own or as the subsequent response to the above insults.

METHODS

Animals and heart perfusion. Male Sprague-Dawley rats (250 to 300 g) were purchased from either Charles-River Canada (St. Constant, PQ) or Harlan Sprague Dawley (In-
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diated in the Health Sciences Animal Care facility of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, ON). Rats were killed by decapitation and the hearts were immediately removed and were perfused by way of the aorta at 37°C with oxygenated Krebs-Henseleit buffer consisting of (in mM) 120 NaCl, 4.63 KCl, 1.17 KH2PO4, 1.25 CaCl2, 1.2 MgCl2, 20.1 HCO3- and 8 glucose with pH 7.4 at a flow rate of 10 ml/min. In the first study all hearts were initially equilibrated for 30 min, after which ischemia was induced for 30 min by completely stopping flow with or without subsequent reperfusion for 30 min. A second group in this study consisted of hearts perfused with H2O2 (100 µM) or LPC (5 µM), which was added after 30 min of equilibration. In the second study to determine the effects of ischemic preconditioning, experiments were carried out as above except that hearts were first subjected to two cycles of 5-min global ischemia, each separated by a 10-min reperfusion period. After the second reperfusion period, the hearts were subjected to 30-min ischemia with or without reperfusion or subsequent addition of either H2O2 or LPC. For all studies, control hearts perfused normally for 60 min after the initial equilibration with or without ischemic preconditioning were investigated. This 90-min total period was selected because it represented the maximum perfusion duration of treated hearts.

RNA isolation. At the end of the appropriate treatment period, the atria and large blood vessels were removed, the ventricles were freeze-dried between tongs prechilled in liquid nitrogen, and RNA was isolated using TRIzol reagent (Canadian Life Technologies, Burlington, ON). Tissues were homogenized, and RNA was extracted with chloroform followed by centrifugation to separate the solution into aqueous and organic phases. RNA was recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in DEPC-water. Quantification of RNA was performed by determining the absorbency at 260 and 280 nm.

Reverse transcription-polymerase chain reaction. First strand cDNA synthesis was performed using the Superscript-II system (Canadian Life Technologies). DNA, the quantity of which was determined by prior absorbency readings, was added to oligo(dT) (Canadian Life Technologies), denatured at 65°C for 10 min, and quenched on ice for 10 min. RT was carried out by the addition of 200 µl of Molloney murine leukemia virus-RT, 1 mM dNTPs, and 10 mM DTT at 42°C for 50 min in a total reaction volume of 20 µl. The reaction was terminated by a 15-min incubation at 70°C. The resulting RT products were stored at −20°C. The amplification was carried out using the following cDNA sequences. NHE-1, primer 1 (5’-TACGGTACCCTGCTCTTC-TGCTTCTC-3’) and primer 2 (5’-GATGATGCCGATCTCTTCC-TCTCTTC-3’) with a predicted product size of 649 bp were used (3). PCR reactions were performed in a total volume of 30 µl containing 50 mM HEPES (pH 7.9), 1× PCR buffer, 1.5 mM MgCl2, 250 µM dNTP mix, 1 µl of each amplification primer, 2.5 units Taq polymerase (Canadian Life Technologies) and 4 µl of the RT product. The amplification was carried out as follows: 45 s at 94°C (denaturation), 45 s at 60°C (annealing), and 1 min at 72°C (extension) for 40 cycles. In initial studies of this reaction in our laboratory, we observed that the PCR amplification for 40 cycles is optimal and is still in the linear phase of the reaction. Simultaneously, an internal standard (β-globin) was amplified in a separate set of tubes using the same RT product, 5’-CAACCTTGATCCAGGTCACC-3’ and 5’-GAAGAGCCAAGGACAGGTAC-3’ primer sets with a predicted product size of 270 bp with 1 min at 94°C, 1 min at 58°C, and 45 s at 73°C (extension) for 40 cycles.

Electrophoresis. The amplification products were analyzed on 3% agarose gels stained with ethidium bromide in 1× TAE buffer; 8.0 µl of each PCR product were loaded in each lane and electrophoresed at 110 V for 90 min. At the end of the electrophoresis the gel was visualized under ultraviolet light. Positive amplifications for NHE-1 were demonstrated as single bands at 649 bp. β-Globin amplification was demonstrated as a single 270-bp band.

Southern blotting. The specificity of the amplification was confirmed by Southern blotting after the transfer of the PCR products from gel onto nylon membranes after denaturation and neutralization. Hybridization with biotinylated amplification product-specific oligoprobe was carried out using the NHE-1 oligoprobe 5’-TAGACCTGTGCTGTTAGAA-3’ (3). A similar amplification product-specific oligoprobe (5’-GAGGCGAGGAGCAGGCTGGCA-3’) for β-globin was used. The detection was carried out by incubation of nylon membranes in the dark, at 37°C for 2 h (however, see below for preconditioned hearts) using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system for visualizing the specific blue bands. Semiquantification of the RT-PCR products was carried out densitometry using a Hewlett-Packard 4C scanner and Mocha software (Jandel Scientific). The densitometric values were expressed as arbitrary units per microgram of total RNA. It should be noted, however, that in our initial experiments there was an extremely low signal for NHE-1 message from preconditioned hearts. Therefore, in this set of studies densitometry was carried for all treatment groups after a 4-h incubation period of the PCR product.

Statistical analysis. The data are expressed as means ± SE and were analyzed by ANOVA. P < 0.05 was accepted as indicating significant differences between treatment groups.

RESULTS

Amplification of NHE-1 was demonstrated as a single band at 649 bp on ethidium bromide-stained gels. Similarly β-globin amplification was demonstrated as a single band at 270 bp. As a negative control, when PCR reactions were carried out in the absence of reverse transcription these bands were not visible and there were no other recognizable bands, indicating that these bands originated from mRNA and not from genomic DNA. Southern blots of the gel demonstrated specific binding of oligonucleotides to these bands, confirming the specificity of all amplifications.

Our first set of studies was aimed at determining the effect of global ischemia with or without reperfusion on expression of NHE-1. These results are summarized in Fig. 1, which demonstrates a significant (P < 0.05), approximately sevenfold elevation of NHE-1 expression after 30 min of ischemia. This level of stimulation was maintained in hearts subjected to a further 30 min of reperfusion with values virtually identical to those seen in ischemic, nonreperfused hearts (Fig. 1).

We next determined whether NHE-1 expression is altered in response to two chemical agents, namely, H2O2 or LPC, the deleterious effects of which are attenuated by NHE inhibition producing injurious effects on the myocardium. As summarized in Fig. 2, both insults significantly increased NHE-1 expression after 30 min of treatment with an approximately twofold elevation in response to both factors, which was significantly higher than control (P < 0.05).
Our subsequent set of studies was aimed at determining whether myocardial preconditioning alters NHE-1 expression. We initially speculated that if stressful influences increase NHE-1 expression, the possibility exists that approaches that protect the myocardium from insult may exert opposite effects. Indeed, it should be reemphasized that in our initial studies using identical 2-h incubation duration of the PCR product for Southern detection, as was done for nonpreconditioned hearts, we were unable to detect any NHE-1 message with densitometry. Accordingly, for all experiments with preconditioned hearts the duration of incubation of nylon membranes was increased to 4 h. The results for these studies are summarized in Figs. 3 and 4. The most startling observation was a marked down-regulation of NHE-1 expression after two 5-min ischemic preconditioning cycles that was 66% less than corresponding control values ($P < 0.05$). It should be noted that two complete 5-min cycles were a prerequisite for NHE-1 mRNA downregulation with no effect seen when only one 5-min period was employed. In hearts subjected to two ischemic cycles, 30 min of ischemia with or without reperfusion failed to significantly stimulate NHE-1 expression (Fig. 3) as was observed in nonpreconditioned hearts (Fig. 1).

![Fig. 1. Stimulation of Na\(^+\)/H\(^+\) exchange isoform 1 (NHE-1) expression in isolated hearts subjected to 30 min of global ischemia alone (I; A) or with reperfusion (IR; B). RT-PCR products were analyzed by densitometry after Southern hybridization. A: values are means ± SE ($n = 10$ for each group); *$P < 0.05$ from control values. B: representative 3% agarose gel for the various treatments as visualized by a single band of 649 bp; C, control. Inset: representative Southern blot hybridization of the same samples.](image)

![Fig. 2. Stimulation of NHE-1 expression in isolated hearts subjected to 30 min of exposure to either hydrogen peroxide ($H_2O_2$) or lysophosphatidylcholine (LPC). RT-PCR products were analyzed by densitometry after Southern hybridization. Values are means ± SE ($n = 10$ for each group). *$P < 0.05$ from control values.](image)

![Fig. 3. Inhibition of NHE-1 expression in hearts subjected to a preconditioning protocol consisting of 2 × 5-min cycles of global ischemia (PC group) and lack of effect with subsequent 30 min of global ischemia alone (PC + I) or with reperfusion (PC + IR). RT-PCR products were analyzed by densitometry after Southern hybridization. Values are means ± SE ($n = 10$ for each group). *$P < 0.05$ from control values.](image)

![Fig. 4. Lack of stimulation of NHE-1 expression in preconditioned (PC) isolated hearts subjected to 30 min of exposure to either $H_2O_2$ or LPC. The RT-PCR products were analyzed by densitometry after Southern hybridization. Values are means ± SE ($n = 10$ for each group). *$P < 0.05$ from control values.](image)
the pattern of changes was markedly different from that in control hearts in that a tendency toward elevation in the NHE-1 message appeared to occur in the ischemic heart, whereas a reduction in expression seemed apparent after reperfusion (Fig. 3). However, none of these effects was significant compared with the NHE-1 message seen in preconditioned nonischemic hearts. The ability of preconditioning to prevent stress-induced elevation in NHE-1 expression was also observed after nonischemic insult (as shown in Fig. 4) because neither H2O2 nor LPC had any effect in hearts previously subjected to preconditioning.

As the reference gene, β-globin expression was studied in hearts subjected to forms of stress identical to those reported for NHE-1. As can be seen in Fig. 5, neither ischemia nor reperfusion affected β-globin expression, and neither H2O2 nor LPC had any effect. Moreover, myocardial preconditioning on its own failed to alter β-globin expression, and there was no effect on subsequent ischemia or on the addition of either H2O2 or LPC (not shown). Representative Southern blots for the various treatments are shown in Fig. 6.

**DISCUSSION**

Despite extensive evidence demonstrating a role for NHE in cardiac pathology, particularly that associated with ischemic heart disease, there are few studies documenting the influence of disease states or other factors on NHE-1 expression in the myocardium. The evidence that myocardial NHE-1 may be altered under pathological conditions was first demonstrated by Dyck and co-workers (2), who showed that a 70% reduction in coronary flow of isolated rat hearts for 3 h resulted in increased NHE-1 expression. Indeed, these investigators failed to observe any changes in NHE-1 expression in hearts subjected to more severe degrees of ischemia, including both 90 and 100% reductions in coronary flow (2). Therefore, our results differ from that previous report in that we show that zero-flow ischemia can produce an upregulation of NHE-1 after only 30 min of treatment with or without subsequent reperfu-
sion. Although the reasons for these differences are not known, when taken together the studies strongly support the concept that myocardial NHE-1 expression is markedly and relatively rapidly increased in the acutely ischemic myocardium. To further assess NHE-1 expression in response to pathology, we also determined the effects of H$_2$O$_2$ and LPC. These chemical stressors were selected first, because their production is known to be increased in the ischemic and reperfused myocardium and because they have been implicated as important contributors to tissue damage produced by ischemia and reperfusion (6, 7, 10, 15, 19–21). The emerging importance of H$_2$O$_2$ has recently been reinforced in a study showing that mouse hearts overexpressing catalase, a major enzyme that breaks down H$_2$O$_2$, demonstrate resistance to ischemia and reperfusion injury (13). In addition, we further considered the investigation of these compounds of particular relevance because the toxic effects of both agents can be attenuated by NHE inhibitors (7, 8) and, moreover, both factors stimulate NHE activity in cardiac cells (6, 18). The potent ability of both H$_2$O$_2$ and LPC to increase NHE-1 expression is of particular interest because it suggests that stimulation of NHE-1 expression may represent a general adaptive response to tissue stress, not selectively in response to ischemic conditions per se.

In contrast to increased NHE-1 expression in response to stress, our results also demonstrate a rapid downregulation of NHE-1 after two 5-min cycles of ischemia. Moreover, after this procedure the ability of ischemia, H$_2$O$_2$, or LPC to further increase NHE-1 expression was significantly inhibited or totally prevented.

Two questions that are reasonable to propose include: 1) Are the potential mechanisms underlying the rapid changes in NHE-1 expression dependent on the nature of the insult? and 2) Are these effects important in understanding the role of NHE in cardiovascular disease? The first question is difficult to address because the regulation of NHE-1 expression in mammalian myocardium has not been elucidated. Although acidosis may be a factor in stimulating NHE-1 expression, the ability of diverse and dissimilar stimuli to increase NHE-1 expression argues against this as a potential mechanism. Moreover, Fliegel’s group has demonstrated a lack of effect of acidosis on either NHE-1 expression or the NHE-1 promoter, although a relatively mild acidosis (pH 6.9) was employed in that study (23). A potentially interesting and important corollary to our findings is the rapid stimulation of various regulatory genes in early myocardial stress, including the protooncogenes c-fos and c-jun as well as a family of stress-stimulated MAP kinases that have been suggested to play important roles in the myocardial response to injury (12, 24). Although the nature of the involvement of these factors in myocardial injury is not clearly understood, many of the protooncogenes are involved in transcriptional regulation of various other genes. Interestingly, both AP-1 and AP-2 regulatory sites have been identified on the NHE-1 gene (4, 14), which suggests that altered expression of the antiporter may reflect transcriptional regulation via intracellular messengers. To our knowledge, the question of whether changes in protooncogene expression occur in the preconditioned myocardium has not been studied. Although extrapolation from noncardiac tissue must be done cautiously, it is interesting to note that protection in the preconditioned ischemic gerbil brain was found to be associated with increased c-jun expression, suggesting the possibility of altered transcriptional regulation of various genes (22). Our present study using an acute insult in isolated intact tissue does not lend itself to explore these mechanisms, although the use of cultured myocytes exposed to different stimuli would likely be an effective approach to study the important question of regulation of NHE-1 cardiac pathology.

In conclusion, the major findings of the present study are that relatively rapid regulation of NHE-1 expression can occur in response to a variety of factors in the acutely ischemic myocardium, the nature of which is dependent on the type of stimulus. The mechanisms underlying changes in NHE-1 expression need to be investigated. Moreover, although our studies provide strong evidence that this gene can be regulated reasonably quickly, the long-term implications of this finding need to be addressed particularly by utilizing chronic models of heart disease. Whether altered gene expression of NHE-1 translates into changes in levels of the NHE-1 protein is also important to determine in such chronic models of disease, particularly to demonstrate whether changes in gene expression and any subsequent alterations in NHE-1 protein production are important adaptive responses to cardiac disease states.

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