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Identification of a small Na^+/H^+ exchanger-like message in the rabbit myocardium

Jason R.B. Dyck[a], Gary D. Lopaschuk[a,c] and Larry Fliegel[a,b]

Departments of [a]Pediatrics, [b]Biochemistry and [c]Pharmacology, Faculty of Medicine, University of Alberta, Cardiovascular Disease Research Group, Edmonton, Alberta, Canada

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We examined the Na^+/H^+ exchanger message in isolated perfused rabbit hearts using Northern blot analysis with cDNA encoding for the rabbit cardiac Na^+/H^+ exchanger. A cDNA probe from the coding region of the rabbit myocardial Na^+/H^+ exchanger hybridized to mRNA of 5 kb under high stringency, and to a second 3.8 kb mRNA species under low stringency. When Northern blots were re-probed with a section of the 3'-untranslated region of the cDNA, the 5 kb message was apparent while the smaller 3.8 kb message was not. If isolated working rabbit hearts were subjected to ischemia we observed increases in the 3.8 kb message. Overall, the results show that a 3.8 kb mRNA product, which is homologous to the amiloride sensitive Na^+/H^+ exchanger, exists in the myocardium and increases during ischemia in the myocardium.

Na^+/H^+ exchanger; Myocardium; mRNA; Acidosis; Ischemia

1. INTRODUCTION

The regulation of internal myocardial pH is of special importance to the functioning heart. Resting intracellular pH is 7.0 to 7.3 and can drop dramatically during ischemia. After acidification a rapid recovery of intracellular pH occurs, primarily through the action of the Na^+/H^+ exchanger. The exchanger is responsible for the exchange of one intracellular H^+ for one extracellular Na^+ [1]. The intracellular acidosis that occurs during ischemia may be important during contractile failure associated with ischemia. It has recently been suggested that the Na^+/H^+ exchanger can alter the ability of the heart to recover during reperfusion following ischemia [2,3].

In the kidney and in vascular smooth muscle, the level of Na^+/H^+ exchange activity is increased by external stimuli such as glucocorticoids and metabolic acidosis [4–8]. Studies also demonstrate that incubation of renal proximal tubule cells in acid media will increase the Na^+/H^+ antiporter activity, by a mechanism that is dependent on protein synthesis [9]. In vascular smooth muscle, serum growth factors, platelet-derived growth factors and fibroblast growth factors can increase Na^+/H^+ exchanger mRNA levels up to 25-fold [10]; and Krapf et al. [11] have shown that chronic metabolic acidosis results in increased renal cortical Na^+/H^+ antiporter mRNA levels.

We have recently shown [12] that the amiloride sensitive Na^+/H^+ antiporter [13] is present in the rabbit myocardium. In the present study, we used this rabbit myocardial cDNA clone to examine the regulation of the cardiac Na^+/H^+ exchanger mRNA in response to ischemia and reperfusion in the isolated perfused rabbit heart. We show that there is a related 3.8 kb form of the myocardial Na^+/H^+ antiporter message, in addition to the 5.0 kb form described earlier [13]. Moreover, the relative mRNA levels of this message vary in response to ischemia and reperfusion of the myocardium. This message may be another related form of the Na^+/H^+ antiporter that may be important in clinical situations of ischemia that lead to increased expression of the Na^+/H^+ exchange activity.

2. MATERIALS AND METHODS

2.1. *Heart perfusions*

New Zealand White rabbits of either sex were obtained from a local breeder. Seven-day-old rabbits were separated from the doe on the morning of experimentation, and used within 4 h. All rabbits were anesthetized with an i.p. overdose of Na^+ pentobarbitol (60 mg/kg). When the animals totally lacked sensation, the heart was quickly removed and placed in ice-cold Krebs–Henseleit buffer supplemented with 11 mM glucose, 0.4 mM palmitate, 3% albumin, and 100 μU/ml insulin. Hearts were then cannulated through the aorta and an initial retrograde perfusion at 60 mmHg pressure began within 60 s of initially opening the thoracic cavity. Subsequent cannulation of hearts in the working mode was as described previously [14]. The perfusate was continuously gassed with a 95% O_2/5% CO_2 mixture, resulting in a perfusate pH of 7.4. The hearts were perfused at a 7.5 mmHg left atrial preload and 30 mmHg aortic afterload. Heart rate and pressure

Correspondence address: L. Fliegel, Department of Pediatrics and Biochemistry, Faculty of Medicine, University of Alberta, 408 Heritage Medical Research Center, Edmonton, Alberta, Canada, T6G 2S2. Fax: (1) (403) 492 3383.

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development were measured with a Gould P21 pressure transducer in-line with the aortic outflow lines.

Protocol 1. Non-ischemic heart vs. ischemic heart

To examine the quantity of the Na^+/H^+ exchanger mRNA during extended periods of ischemia we used rabbit hearts subjected to a 60-min period of low-flow ischemia (coronary flow = 0.2 ml/min). In these hearts contractile activity ceased during the ischemic period.

Protocol 2. Ischemic heart vs. ischemic-reperfused heart

We compared the level of mRNA in ischemic hearts and hearts undergoing ischemia and reperfusion in another set of experiments. To do this, we used rabbit hearts subjected to a 40-min period of no-flow ischemia followed by 60 min of aerobic reperfusion. We chose this particular protocol since previous studies demonstrated that the 40-min ischemic period will produce a severe acidosis in these hearts [15]. The second reason we chose this protocol is that it represents a severe, but generally reversible episode of ischemia. As a result of this reversibility, any change in mRNA for the Na^+/H^+ exchanger is not likely a result of a non-specific effect that may occur in hearts that are irreversibly injured and are not capable of recovering a large degree of mechanical function. At the end of all perfusions, hearts were freeze clamped with Wollenberger clamps cooled to the temperature of liquid N_2. Frozen tissue was powdered in a mortar and pestle also cooled to the temperature of liquid N_2.

2.2. *RNA isolation and analysis*

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Bethesda Research Laboratories. The plasmids pTZ18R and 19R were from Pharmacia LKB Biotechnology Inc. [^{32}P]ATP was obtained from New England Nuclear. Immobilon-N filters were from Millipore. All chemicals were of the highest grade available.

Poly(A^+) RNA was prepared from isolated perfused rabbit heart using a modified procedure similar to that described earlier [12]. Ten μg of Poly(A^+) RNA was applied to each lane of the Northern blots. The Northern blot was probed with ^{32}P-labelled random primed cDNA (BRL). The fragments used for labelling were 1–688 and 1264–1892 of the rabbit cardiac cDNA clone [12] corresponding to part of the coding and untranslated regions respectively. To confirm that the RNA samples were of the same quantity and quality, all Northern blots were stripped and reprobed in control experiments using a cDNA fragment containing the mouse ribosomal 28 S subunit [16]. Exposure times were 16 h for Northerns probed with Na^+/H^+ exchanger probe and 4 h when Northerns were reprobed with the mouse ribosomal 28 S subunit. In some experiments Northern blots were reprobed a third time with a cDNA probe encoding for actin. Hybridization and washes were as described earlier, with blots being routinely washed with 1 × SSC at 58°C (medium stringency). Where indicated blots were washed at 0.1 × SSC, 65°C (high stringency, [12]). Scanning densitometry was with a Camag TLC Scanner II and a SP4 290 Camag Integrator.

3. RESULTS

In Protocol 1 we subjected rabbit hearts to a 60-min period of low-flow ischemia (coronary flow = 0.2 ml/min). In these hearts contractile activity ceased during the ischemic period. In a separate series of experiments we determined that if these hearts are reperfused after this time, contractile activity recovers completely to pre-ischemic levels (not shown).

In Protocol 2 we used a more severe ischemic episode followed by reperfusion. Seven-day-old rabbit hearts were subjected to a 40-min period of no flow ischemia followed by 60 min of aerobic reperfusion. As shown in Fig. 1, heart function recovered to approximately 75% of the pre-ischemic function during the 60-min reperfusion period. As a result, any change in mRNA for the Na^+/H^+ exchanger is not likely a result of a non-specific effect that may occur in hearts which are irreversibly injured and are not capable of recovering a large degree of mechanical function.

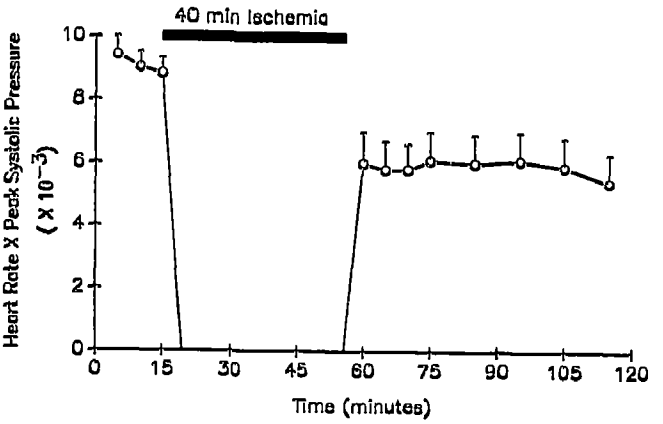

Fig. 1. Effect of a 40-min period of transient no flow ischemia on reperfusion recovery of isolated working hearts from seven-day-old rabbits (Protocol 2). Heart rate × peak systolic pressure development was measured in hearts as described in section 2. Values are the mean ± S.E. of 8 perfused hearts.

Fig. 2a shows RNA blot analysis of poly(A^+) RNA from isolated perfused rabbit hearts probed with a fragment from the coding region. Lane 1 is mRNA from hearts subjected to control aerobic perfusion while lane 2 is mRNA from hearts that subjected to 60 min of low flow ischemia as described in section 2 (Protocol 1). The figure shows that two messages hybridize with the rabbit cardiac Na^+/H^+ exchanger cDNA probe. One larger message is of approximately 5.0 kb in size, and one smaller message is 3.8 kb in size. The smaller hybridizing mRNA species was much more apparent in hearts undergoing the low flow ischemia as compared to the control. This Northern blot was washed at a medium stringency with 1 × SSC at 58°C. To examine the relationship between the 3.8 kb message and the 5.0 kb message, we washed the blot at a higher stringency, 0.1 × SSC at 65°C. The result is shown in Fig. 2b. The 3.8 kb message is no longer apparent while the 5.0 kb message remains. Reprobing the blot with cDNA encoding the 28 S ribosomal subunit confirmed that equal amounts of undegraded RNA had been applied to the gel and that the mRNA was purified to the same degree (Fig. 2c).

To examine the expression of the mRNA for the Na^+/H^+ exchanger during ischemia followed by reperfusion we used Protocol 2. The results are shown in Fig. 3. During reperfusion of previously ischemic myocardium there was a dramatic increase in intensity of the 3.8 kb message in comparison to hearts undergoing ischemia without reperfusion (Fig. 3a). There was also a minor

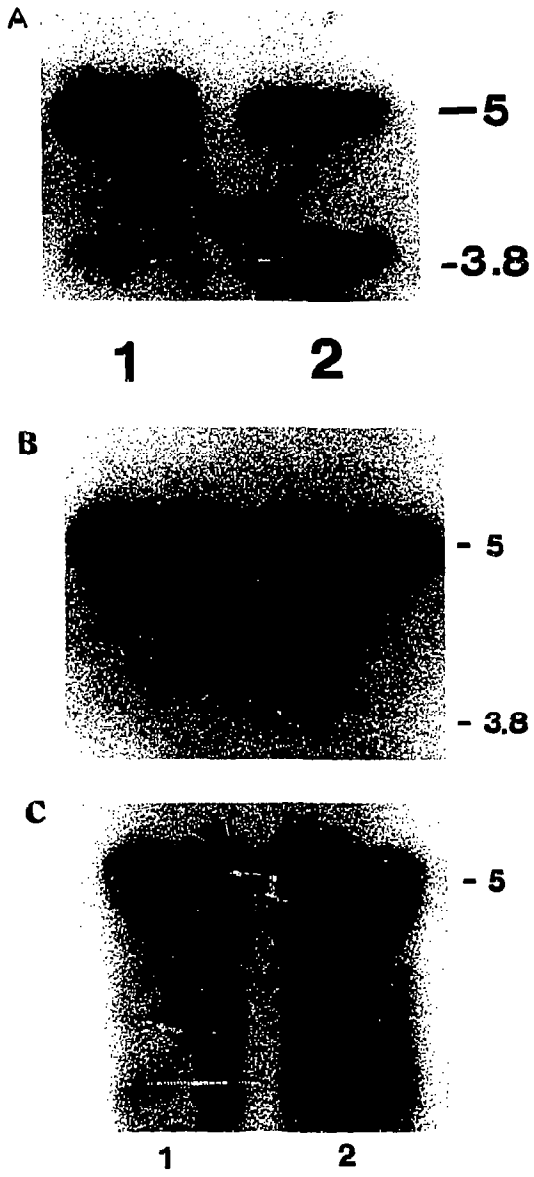

Fig. 2. RNA blot analysis of poly(A⁺) RNA from 7-day-old isolated perfused rabbit hearts. Hearts were perfused as described in section 2 and subjected to either control aerobic perfusion (60 min) (lane 1) or 60 min of no-flow ischemia (lane 2, Protocol 1). Poly(A⁺) RNA was isolated and separated by size on formaldehyde agarose gels. After transfer to nitrocellulose mRNA was probed with residues 1–688 of the rabbit cardiac Na^+/H^+ exchanger [2]. Sizes were estimated based on the positions of the ribosomal subunits run simultaneously on the same gel. The observed (or expected) positions of the 5.0 kb and 3.8 kb isoforms are noted respectively. (A) Exposure of Northern after hybridization and washing at medium stringency as described in section 2. (B) Exposure of Northern after hybridization and washing at high stringency as described in section 2. (C) Exposure of the same Northern after being stripped and reprobed with a fragment of the 28 S ribosomal subunit as described in section 2.

increase in intensity of the 5.0 kb message. The estimated increase in intensity of the two messages from the ischemia (lane 1) to ischemia-reperfusion (lane 2) hearts is 30% for the 5 kb message and over 70% for the 3.8 kb message. We note that with ischemia followed by reperfusion (lane 2, Fig. 3a) the intensity of the 3.8 kb message is now much greater than that of the 5 kb message. Reprobing the blot with cDNA encoding the 28 S ribosomal subunit confirmed that equal amounts of undegraded RNA had been applied to the gel and that the mRNA was purified to the same degree (Fig. 3c).

To examine further the relationship between the 5 kb message and the 3.8 kb message we probed the Northern blot with a cDNA probe from the untranslated region of the rabbit myocardial Na^+/H^+ exchanger. Fig. 3b shows that the 5 kb message is still apparent however the 3.8 kb message is no longer visible.

4. DISCUSSION

We have examined the Na^+/H^+ exchanger message in intact isolated perfused working rabbit hearts. A number of studies have suggested that the gene encoding for the Na^+/H^+ exchanger can be influenced by environmental factors which affect intracellular pH (4–8). The level of Na^+/H^+ exchange activity (V_{max}) is increased by external stimuli such as glucocorticoids and acidosis. In some cases this increase is dependent on protein synthesis. The presence of more Na^+/H^+ exchanger protein has been suggested by an increased number of amiloride analogue inhibitor sites [9,17]. A recent report shows that a high NaCl diet can increase antiporter V_{max} in the proximal tubule. This leaves open the possibility that other environmental stimuli can affect the regulation of the exchanger gene in vivo [18]. The present study examined the Na^+/H^+ exchanger message in the intact myocardium under short term conditions. Though the times involved are relatively short, recent studies on isolated myocytes have shown that a number of different messages can be induced by various stimuli within even shorter time periods [19].

In this study we showed that there is another form of messenger RNA, which is related to the Na^+/H^+ exchanger and cross hybridizes at reduced stringency. This 3.8 kb message is normally present in low amounts in control tissue, relative to the 5.0 kb message. This result confirms our earlier observation on the presence of this message in rabbit skeletal muscle [12]. The results presented here show that the 3.8 kb message can respond to external stimuli such as ischemia followed by reperfusion of the rabbit heart. Under some conditions the 3.8 kb message became more abundant than the 5 kb message (Fig. 3a). The exact mechanism of elevation of these messages by ischemia and reperfusion is not yet known. It is possible that there are either increases in transcription, decreased mRNA degradation or a combination of both. The 5 kb message showed only a slight induction caused by ischemia followed by reperfusion (Fig. 3a). The 5 kb message undoubtedly is the known

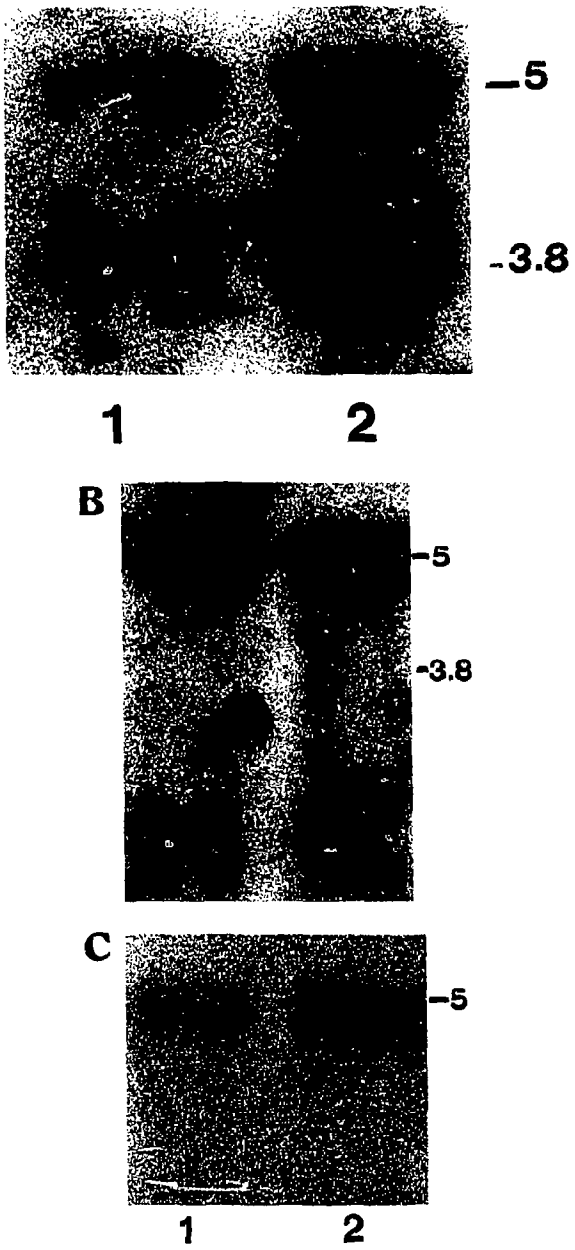

Fig. 3. RNA blot analysis of poly(A$^+$) RNA from 7-day-old isolated perfused rabbit hearts. Hearts were perfused as described in section 2 and subjected to either 40 min of no flow ischemia alone (lane 1) or ischemia followed by 60 min of reperfusion (lane 2, Protocol 2). The blot was prepared and probed as described in Fig. 1A. (A) Exposure of Northern after hybridization and washing at medium stringency with Na^+/H^+ exchanger probe. (B) Exposure of Northern after hybridization and washing at medium stringency with Na^+/H^+ exchanger probe of residues 1264–1892 of the 3'-untranslated region of the rabbit cardiac cDNA clone [2]. (C) Exposure of the same Northern after being stripped and reprobed with a fragment of the 28 S ribosomal subunit as described in section 2.

amiloride sensitive Na^+/H^+ exchanger [13], based on its size and hybridization at high stringency with this clone [12]. The minor increase in the amount of this message is likely not significant. However, it may indicate a trend towards increased expression of this message, which may occur with longer time periods of stimulation as suggested in other tissues [11]. Future studies may examine the effect of long-term ischemia followed by reperfusion of the isolated rabbit heart.

Since the smaller, 3.8 kb mRNA species hybridized to cDNA of the amiloride sensitive Na^+/H^+ exchanger, this leaves open the possibility that this may be another form of the Na^+/H^+ exchanger. When we reprobed the Northern blots with the 3'-untranslated region of the exchanger the 3.8 kb message was not apparent. In addition, washing the blots at increased stringency also eliminated the hybridization to this smaller message. These results suggest that these two messages could originate from different genes. It seems unlikely that the smaller message is a degradation product of the larger one, since it hybridizes at different stringency to our probe and in some instances it was present in larger amounts than the 5 kb message. Also, probing of Northern blots with the 28 S ribosomal subunit showed no differences in quality or purity of the RNA samples. If the smaller transcript did originate from ischemia induced degradation of the larger message, then both bands would consist of amiloride sensitive Na^+/H^+ exchanger mRNA. The increases due to ischemia and reperfusion seen in the two bands would indicate a significant accumulation of amiloride sensitive Na^+/H^+ exchanger transcript. It is also possible that the smaller message codes for a protein that is homologous but has a different activity altogether. However, we believe that this is less likely since the activities of several different forms of the Na^+/H^+ exchanger have been noted earlier in the myocardium [20] and several smaller isoforms of the protein have been identified [21,22]. It is also tempting to suggest that this 'small ischemic form' (SLIF) of the message may represent the amiloride insensitive form of the exchanger which has been reported to be a smaller protein [22]. Recently, several isoforms of the Na^+/H^+ exchanger have been identified [23,24]. Whether this message corresponds to one of these isoforms is not yet known. Early experiments by Sardet et al. [13] used Southern blotting to suggest that only one gene was present which encoded for the amiloride sensitive antiporter. However, these hybridizations were done at high stringency, under conditions that showed no cross hybridization with 3.8 kb message (Fig. 2b). More recently Tse et al. [8] also examined Southern blots of rabbit genomic DNA under both low and high stringency and they noted the presence of another gene which cross-hybridized only under low stringency. They suggested that this represented another Na^+/H^+ exchanger gene or a closely related gene. It is possible that the SLIF message is the product of this gene. Further experiments are necessary to confirm this suggestion.

Overall, our results suggest that in the heart a small

3.8 kb message is present that is related to the amiloride sensitive Na^+/H^+ exchanger. This message increases both with ischemia and during reperfusion following ischemia. The increased message could presumably lead to increased production of the protein, similar to what has been suggested in other tissues for the Na^+/H^+ exchanger [4–7,10,17]. If the SLIF message encodes for a form of the Na^+/H^+ exchanger, it could play a significant role in contributing to reperfusion arrhythmias in the myocardium [2,3]. In addition increased expression of the amiloride sensitive (5 kb message) Na^+/H^+ exchanger could also be significant during long term ischemia and reperfusion of the myocardium. Future studies will explore this possibility.

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