Decreased Expression of Na\(^+\)/H\(^+\) Exchanger Isoform 1 (NHE1) in Non-infarcted Myocardium after Acute Myocardial Infarction

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SUMMARY

Although cardiac NHE1 is activated during myocardial ischemia and reperfusion injury, little is known about changes in expression in non-infarcted myocardium after acute myocardial infarction (AMI). The purpose of this study was to examine left ventricular function and region dependent NHE1 expression after myocardial infarction. Therefore, we produced two AMI models in rats, a small infarction model which was continuously ligated at the branches of the left coronary artery, and an extensive infarction model continuously ligated at the root of the artery. We examined NHE1 mRNA expression using RNase protection assay and protein levels using Western blot analysis in non-infarcted myocardium during the 24 hour period after AMI. The level of NHE1 mRNA and protein expression in the whole heart including the infarcted myocardium did not change after a small infarction. On the other hand, in the case of an extensive infarction, the levels of NHE1 mRNA and protein expression decreased significantly by 21.5% (P<0.05) and by 22.0% (P<0.05), respectively, in non-infarcted myocardium. Left ventricular systolic pressure (LVSP) decreased significantly by 13% and 38% with the branch and root ligation, respectively. However, left ventricular end-diastolic pressure (LVEDP) only increased with the root ligation. These results indicate that NHE1 expression decreased in response to extensive myocardial infarction only in non-infarcted myocardium. The present study may be important in furthering the understanding of NHE1 in myocardial infarction and suggests that decreased expression of NHE1 in non-infarcted myocardium may decrease the extent of cardiac cell injury. (Jpn Heart J 2002; 43: 273-282)

Key words: Na\(^+\)/H\(^+\) exchanger, Non-infarcted myocardium, RNase protection assay, Western blot analysis

HEARTS injured by ischemia and/or reperfusion manifest a variety of functional defects, such as fatal arrhythmia and congestive heart failure. Recent stud-
ies using molecular biological techniques have demonstrated induction of Na⁺/H⁺
exchanger isoform 1 (NHE1) mRNA expression in the ischemic heart¹,²) and a
reduction in ischemia-induced NHE1 mRNA expression levels by adding angio-
tensin-converting enzyme inhibitors and angiotensin II receptor antagonists.³)
Furthermore, inhibition of NHE1 activity by application of cariporide [(HOE-
642), 4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate] re-
duced ischemia-induced Na⁺ overload in rat myocardium prepared by the Lan-
gendorff method,⁴) and rescued rats from ventricular arrhythmia after the coro-
nary artery ligation.⁵) Moreover, in clinical trials, Rupprecht, et al confirmed that
cariporide prevented acute myocardial infarction (AMI) patients from developing
left ventricular dysfunction.⁶) These results suggest that NHE1 is activated in the
ischemic and remodeling myocardium, and may play a key role in the develop-
ment of heart failure after AMI. However, little is known about changes in NHE1
expression in the non-infarcted region after AMI.

In the present study, we examined whether there are changes in the amount
of NHE1 mRNA and protein depending on the degree of the myocardial infarc-
tion. Thus, we produced two AMI models in rats, a small infarction mode land an
extensive infarction model in which either the branch or root of the left coronary
artery, respectively, was continuously ligated for 24 hours. Furthermore, to clar-
ify the possible cause of NHE1 regulation, we measured various hemodynamic
parameters in the infarcted hearts. Our preparation provides new information on
the changes in NHE1 expression in non-infarcted myocardium of infarcted
hearts.

METHODS

The investigation conformed with the Guide for the Care and Use of Labora-
tory Animals published by the US National Institutes of Health (NIH publica-
tion No. 85/23, revised 1996) and was approved by the animal committee of
Kitasato University.

Production of AMI: Male Wistar rats, weighing 280-330 g, were anesthetized
with halothane (1.5-3.0%), and ventilated (10 mL/kg, 60 breaths/min) using a
rodent respirator (5N-480-7; Shimano Manufacturing, Japan). After an incision
was made through the fourth intercostal space, the ribs were retracted and the
pericardium was opened to allow access to the heart. A 6-0 braided polypropy-
lene suture attached to a 9 mm micropoint reverse cutting needle was passed
around either branch or the root of the left coronary artery to produce a small
(n=18) or an extensive (n=29) infarction in the left ventricle, respectively. In
sham-operated animals (n=24), the suture was not passed around the artery. The
incision was closed and the rats were allowed free access to standard rat chow and
water. Electrocardiograms (365; NEC San-ei, Japan) were monitored using standard limb leads to confirm AMI. Myocardial infarct size (small infarction; $n=4$, extensive infarction; $n=5$) was determined by the triphenyl tetrazolium chloride (TTC) technique and the ratio of infarcted area per left ventricular area was calculated using a personal computer (CAMAC-300, NEC, Japan).

**Hemodynamic parameters:** Hemodynamic parameters were determined in the sham and infarcted rats 24 hours after ligation ($n=4$ for each). Left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were monitored by a needle tip micromanometer (SPR477, Millar, USA) which was directly inserted into the left ventricle through the apex. The dP/dt was calculated with a signal differentiator (G4615-71, Gould, USA). The parameters were recorded at a paper speed of 100 mm/s (RT3200N, NEC San-ei).

**Tissue preparation:** The infarcted and sham-operated rats were decapitated 24 hours after AMI under ethyl ether anesthesia. The hearts were removed immediately and cut transversely to avoid contamination by atrial tissue as follows; 1) Small infarction: included both the left and right ventricles, and 2) Extensive infarction: the heart was divided into the infarcted region (left ventricular free wall) and non-infarcted region (interventricular septum and right ventricle). These pieces of cardiac tissue were then snap frozen in liquid nitrogen and stored at -80°C until use. The cardiac tissues obtained were subjected to RNA or peptide extractions.

**RNase protection assay:** Total RNA was prepared from tissues using the acid guanidinium thiocyanate phenol-chloroform (AGPC) method. The quality and quantity of the RNA samples were monitored by observation of the appropriate 28 S to 18 S ribosomal RNA ratio as determined by ethidium bromide staining of the denaturing gel electrophoresis. The expression of cardiac NHE1 mRNA was evaluated in the small and extensive infarcted hearts of the rats. To obtain the NHE1 cDNA probe, polymerase chain reaction was performed as described previously (corresponding to nucleotide positions 2568-2968 of the rat NHE1 cDNA sequence [GenBank M85299]). The amplified fragments were subcloned into the pGEM-T Easy vector (Promega, USA) and their sequences determined. RNase protection assay was performed using an RPA III kit (Ambion, USA) according to the manufacturer’s instructions. Protected fragments were electrophoresed at 250 V for 60 minutes on 5% polyacrylamide, 8 M urea denaturing gels. Quantification was performed by measuring the photostimulated luminescence (PSL) values using a bioimage analyzer (BAS-2000, Fuji Film, Japan).

**Western blot analysis:** Cardiac tissues were homogenized in 5 mL of ice cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM EDTA, 150 mM NaCl, 0.1% Triton-100, 1 mg/mL pepstatin, 200 mM PMSF, and 1 mg/mL leupeptin. The homogenates were mixed with loading buffer (50 mM Tris-HCl, 100 mM...
dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and solubilized at 95°C for 10 minutes and then centrifuged at 14,000 rpm for 10 minutes. All preparations were carried out at 4°C. For Western blotting, samples containing 100 µg of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to an Immobilon transfer membrane (Millipore Co., USA). After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature, the membrane was incubated with anti-NHE1 antiserum (Alpha Diagnostic International, USA) at a 1:500 dilution overnight at 4°C. After rinsing in TBST, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma, USA) at a 1:3000 dilution for 3 hours at 4°C, and then developed with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium chloride (BCIP/NBT) as chromogenic substrates. Preabsorption was performed by incubating 1 µL of antibodies with 10 µg of antigenic synthetic peptide in 1 mL of TBST overnight at 4°C. The densities of each band in the digitized images were measured using the public domain NIH Image program.

**Statistical analysis:** All quantitative data are described as the mean±SEM. Statistical analyses of hemodynamics, levels of mRNA and protein for NHE1 among the two infarcted groups and the sham group were performed using an unpaired t test. A level of P<0.05 was accepted as statistically significant.

**RESULTS**

**Myocardial infarct size:** As shown in Figure 1, the TTC technique indicated a clear delineation between viable and non-viable tissues. The viable myocardium

![Figure 1](image)

Figure 1. Triphenyl tetrazolium chloride (TTC) staining of the small and extensive infarctions. Rat hearts were excised after 24 hours ligation of either the branch (n=4, left) or root (n=5, right) of the left coronary artery. The infarcted area could not be stained with TTC. The infarction was restricted to the anterior wall of the left ventricle (small infarction), whereas it covered most of the left ventricular free wall and did not extend to the interventricular septum (extensive infarction).
was stained brick red, and was separated from the infarcted tissue. A comparison of planimetry measurements of gross myocardial slices and microscopic giant histologic sections revealed that there was close correspondence between the gross and histologic measurements of the areas of necrosis.\(^7\) In the present study, the infarcted region was small and restricted to the anterior wall of the left ventricle when the left coronary artery was ligated at the branches, whereas it covered most of the left ventricular free wall when the artery was ligated at the root. The mean infarct sizes were 13.4±2.0% (small infarction; \(n=4\)) and 49.3±2.5% (extensive infarction; \(n=5\)) of the left ventricle. The mortality rates were 0% (0 of 18 rats) and 31% (9 of 29 rats), respectively. Nine rats died due to acute pulmonary edema (\(n=4\)) and ventricular fibrillation (\(n=5\)) after root ligation of the coronary artery.

**Hemodynamic parameters:** The Table presents the body weight and hemodynamic parameters in the sham and infarcted rats 24 hours after the coronary artery ligation. LVSP was significantly decreased by 13% (\(P<0.05\)) in the small infarction group and by 38% (\(P<0.01\)) in the extensive infarction group compared with the sham-operated group. The peak dp/dt was unchanged in the small infarction group, but was significantly decreased (\(P<0.01\)) in the extensive infarction group. As expected, LVEDP was significantly elevated (\(P<0.01\)) only in the extensive infarction group.

**RNase protection assay:** At first, we attempted to analyze NHE1 mRNA expression in heart using Northern blotting, however, we were unable to detect it because of the small quantity of NHE1 mRNA expression. We were able to quantify NHE1 mRNA levels using the RNase protection assay which was more sensitive than Northern blotting and produced fewer artifact signals than the RT-PCR method. The NHE1 message of the ventricles was compared with the sham-operated preparations. In the small infarction group, the NHE1 message from the vent-

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**Table.** Body Weight and Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Small</th>
<th>Extensive</th>
</tr>
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<tbody>
<tr>
<td><strong>BW (g)</strong></td>
<td>311±3</td>
<td>312±12</td>
<td>299±9</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>465±28</td>
<td>436±25</td>
<td>426±26</td>
</tr>
<tr>
<td><strong>LVSP (mmHg)</strong></td>
<td>132±2</td>
<td>115±6*</td>
<td>82±2**</td>
</tr>
<tr>
<td><strong>LVEDP (mmHg)</strong></td>
<td>1.4±0.3</td>
<td>1.5±0.4</td>
<td>5.0±0.4**</td>
</tr>
<tr>
<td><strong>dp/dt (mmHg/s)</strong></td>
<td>6375±375</td>
<td>5688±753</td>
<td>3875±217**</td>
</tr>
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</table>

All hemodynamic parameters were obtained at 24 h after coronary artery ligation. BW=Body weight; HR=heart rate; LVSP=left ventricular systolic pressure; LVEDP=left ventricular end-diastolic pressure; Sham=sham-operated rats. Values are mean±SEM. *\(P<0.05\) vs Sham. **\(P<0.01\) vs Sham.
tricles, including the infarcted region, was not significantly decreased 24 hours after the ligation (Figure 2). On the other hand, in the extensive infarction group, the mRNA of NHE1 from the non-infarcted region significantly decreased ($P<0.05$) by 21.5% (Figure 2). However, it was unchanged in the infarcted region.

**Figure 2.** Quantification of NHE1 mRNA levels in left and right ventricles (A; small infarction), and in the non-infarcted region (B; extensive infarction) 24 hours after AMI. The bands represent the protected fragments of NHE1 (corresponding to nucleotides 2568-2968 of rat NHE1 [GenBank M85299], upper panel) and β-actin (middle panel) mRNA ($n=5$ for each). The 18 S and 28 S ribosomal RNA are shown after staining the agarose gel with ethidium bromide (lower panel). Each lane contains 20 µg of total RNA preparations. The radioactivity of the bands was measured with a bioimage analyzer (BAS-2000; Fuji Film). The mean value of the mRNA levels in the sham-operated rats is 100%. P, probe; Sham, sham-operated rats; S-MI, Small myocardial infarction; E-MI, Extensive myocardial infarction; N, negative control with yeast RNA. *$P<0.05$ vs. Sham.
24 hours after AMI (data not shown). We also examined NHE1 mRNA expression 6 hours after the ligation, but it was unchanged in all cases (data not shown).

**Western blot analysis:** The protein expression of NHE1 was assessed by Western blot analysis (Figure 3). We detected a single band of approximately 110 kDa for cardiac NHE1. The band disappeared by preabsorption of the antibody with NHE1 control peptide. In the small infarction group, the NHE1 protein level was not changed 24 hours after AMI. Moreover, in the extensive infarction group, the protein amount of NHE1 was unchanged in the infarcted region (data not shown).

![Western blot analysis of the NHE1 protein levels in left and right ventricles (A; small infarction), and in the non-infarcted region (B; extensive infarction) 24 hours after AMI.](image)

**Figure 3.** Western blot analysis of the NHE1 protein levels in left and right ventricles (A; small infarction), and in the non-infarcted region (B; extensive infarction) 24 hours after AMI. The NHE1 protein was detected as the 110 kDa band in sham rats and infarcted rats (n=5 for each). The band disappeared with preabsorption of the antibody with NHE1 control peptide. Positions of molecular mass markers are shown on the left. The densities of the bands in the digitized images were measured using the public domain NIH Image program. The mean value of the protein levels in the sham-operated rats is 100%. Sham, sham-operated rats; S-MI, Small myocardial infarction; E-MI, Extensive myocardial infarction

*P*<0.05 vs Sham.
However, the protein level of NHE1 decreased significantly ($P<0.05$) by 22.0% in the non-infarcted region 24 hours after AMI.

**DISCUSSION**

In this study, we have shown that (1) LVSP decreased in the small and extensive infarction groups, (2) LVEDP only increased in the extensive infarction group after AMI, and (3) In the small infarction group, the level of NHE1 mRNA and protein expression in whole heart, including the infarcted myocardium, did not change 24 hours after AMI. On the other hand, in the extensive infarction group, the level of NHE1 mRNA and protein expression decreased significantly in non-infarcted myocardium 24 hours after AMI.

These results suggest that NHE1 mRNA and protein expression decrease spontaneously and this may be associated with the congestive heart failure due to extensive myocardial infarction. Because NHE1 is an important regulator not only for extracellular H+ to regulate cell pH but also for intracellular Na+ to maintain cell volume, it is possible that the spontaneous reduction of NHE1 expression prevents Na+ overload *in vivo* in the rat heart, and results in a cardioprotective effect as well as NHE1 inhibition. Numerous investigators have demonstrated that NHE1 inhibitors reduce the activation of NHE1 and Na+-entry in ischemia and protect the myocardium in experimental studies and clinical trials, so these studies are consistent with the present results.

Dyck, *et al* using rats prepared by the Langendorff method, demonstrated that NHE1 mRNA expression levels did not change under either severe low flow ischemia or global ischemia for 3 hours, although it increased in response to mild low flow ischemia for 3 hours. Further, Gan, *et al* confirmed that short term global ischemia (30 minutes) also produced upregulation of NHE1 mRNA expression. Moreover, in an *in vivo* infarction model, ischemia-induced tissue acidosis elevated local renin activity in rat hearts and NHE1 mRNA expression levels were decreased in the presence of a specific inhibitor of angiotensin II receptors. These results suggest that ischemic tissue may upregulate NHE1 mRNA and protein expression, most likely via angiotensin II receptors, because structural cardiac remodeling by fibrous tissue following AMI is established in tissue repair of the infarcted heart. However, under our experimental conditions, upregulation of NHE1 mRNA and protein was never observed in infarcted myocardium 24 hours after AMI. Our finding is inconsistent with that of Sandmann (2001), but agrees with those of Dyck, *et al* (1995) and Gan, *et al* (1999) using Langendorff methods.

This study is the first to report that the NHE1 message decreased significantly in non-infarcted myocardium (Figure 2). Moreover, this change is indeed
accompanied by changes in protein levels (Figure 3). NHE1 mRNA and protein levels change in non-infarcted myocardium 24 hours after AMI, suggesting that the regulation of NHE1 expression is not the direct result of myocardial ischemia, but rather some factors related to myocardial ischemia. There are several lines of evidence which indicate that neurohumoral factors, such as the renin-angiotensin system and catecholamines, are activated during AMI in humans and play an important role in cardiac remodeling and/or the prognosis after AMI. In addition, angiotensin II and kappa-opioid receptor stimulation regulate myocardial NHE1 in the in vivo rat heart. Thus, the regulation of NHE1 in non-infarcted myocardium may be partially mediated by neurohumoral factors, such as the renin-angiotensin system. On the other hand, NHE1 is regulated in response to a variety of extracellular stimuli, not only agonists such as growth factors and hormones, but also mechanical stimuli such as osmotic stress and cell spreading. It is possible that mechanical stimulation or lack of it, for example the loss of regular beating of cardiac contraction and relaxation, may decrease myocardial NHE1 in non-infarcted myocardium.

In conclusion, decreased levels of NHE1 mRNA and protein expression in non-infarcted myocardium may be associated with cardiac dysfunction due to extensive AMI. Spontaneous down-regulation of NHE1, by pharmacological treatment for example, may contribute to cardioprotection.

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