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Differential Effects of Angiotensin AT_1 and AT_2 Receptors on the Expression, Translation and Function of the Na^+-H^+ Exchanger and Na^+-HCO_3^- Symporter in the Rat Heart After Myocardial Infarction

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OBJECTIVES This study investigated the role of angiotensin receptor subtype 1 (AT_1) and angiotensin receptor subtype 2 (AT_2) in the regulation of Na^+-H^+ exchanger (NHE) and Na^+-HCO_3^- symporter (NBC) in the infarcted myocardium.

BACKGROUND The cardiac renin-angiotensin system is activated after myocardial infarction (MI), and both angiotensin AT_1 and AT_2 receptors are upregulated in the myocardium.

METHODS Na^+-H^+ exchanger isoform-1 and NBC-1 gene expression were determined by reverse transcription polymerase chain reaction and Northern blot analysis; protein levels by Western blot analysis; and activity by measurement of H^+ transport in left ventricular (LV) free wall, interventricular septum (IS) and right ventricle (RV) after induction of MI. Rats were treated with placebo, the angiotensin-converting enzyme inhibitor ramipril (1 mg/kg/day), the AT_1 receptor antagonist valsartan (10 mg/kg/day) or the AT_2 receptor antagonist PD 123319 (30 mg/kg/day). Treatment was started seven days before surgery.

RESULTS Na^+-H^+ exchanger isoform-1 and NBC-1 messenger RNA (mRNA) expression and protein levels were increased twofold in the LV free wall after MI, whereas no changes were observed in the IS and RV. Na^+-dependent H^+ flux was increased in the LV free wall. Ramipril inhibited mRNA and protein upregulation of both transporters. Valsartan inhibited the upregulation of NHE-1 mRNA and protein but had no effect on NBC-1 mRNA expression and translation. In contrast, PD 123319 abolished the upregulation of NBC-1 mRNA and protein but had no effect on NHE-1 upregulation. Ramipril and valsartan prevented post-MI increase in NHE-1 activity, whereas ramipril and PD 123319 decreased NBC-1 activity.

CONCLUSIONS Angiotensin II via its AT_1 and AT_2 receptors differentially controls transcriptional and translational regulation as well as the activity of NHE-1 and NBC-1 in the ischemic myocardium and contributes to the control of pH regulation in cardiac tissue. (J Am Coll Cardiol 2001;37:2154–65) © 2001 by the American College of Cardiology

Control of intracellular pH (pH_i) is important in all tissues because the accumulation of metabolic acid and other perturbations of pH_i can have profound effects on cellular homeostasis. A number of pH regulatory proteins exist on the plasma membrane to remove excess acid or its equivalent. Among them are the Na^+-H^+ exchanger (NHE) and the Na^+-HCO_3^- symporter (NBC), which, when activated, induce an intracellular alkalinization (1,2). In the mammalian myocardium, pH_i regulation is of special importance to the functioning heart because acidosis depresses the contractility in cardiac myocytes by affecting virtually every step in the excitation-contraction coupling (3). On the other hand, pH_i regulatory proteins, especially the isoform-1 of NHE, have also been implicated in the pathogenesis of tissue injury, arrhythmias during ischemia, reperfusion and cell necrosis (4,5).

It has been recently recognized that these pH_i-regulating proteins can be acutely activated by angiotensin II (Ang II), endothelin and beta-adrenergic agonists (6–8). Angiotensin II has been shown to increase the activity of the two Na^+-dependent H^+-extruding mechanisms, the NHE-1 (9) and Na^+-HCO_3^- symporter isoform-1 (NBC-1) (6), in cardiac muscle. However, it is not clear whether changes in NHE-1 and NBC-1 expression and activity occur in the clinical setting of ischemic heart disease, particularly after myocardial infarction (MI).

Increasing evidence suggests that the renin-angiotensin system is activated after acute MI. It has been demonstrated that messenger RNA (mRNA) and protein levels for angiotensinogen (10), angiotensin-converting enzyme (ACE) (11) and angiotensin receptors (12,13) are increased in rat hearts after MI, and Ang II is considered to have a profound effect on ventricular remodeling after MI (14).

The angiotensin receptors include at least two different subtypes: the angiotensin receptor subtype 1 (AT_1) is the

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Abbreviations and Acronyms

ACE	= angiotensin-converting enzyme
Ang II	= angiotensin II
AT_1	= angiotensin receptor subtype 1
AT_2	= angiotensin receptor subtype 2
BCECF-AM	= acetoxymethylester of the fluorescence dye 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein
β_i	= cellular buffering power [$\beta_i(mM) = \Delta(NH_4^+)_i/\Delta pH_i$]
cDNA	= complementary DNA
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
IS	= interventricular septum
J_H	= H^+ efflux [$J_H = \beta_i(dpH_i/dt)$]
LV	= left ventricle/ventricular
MI	= myocardial infarction
mRNA	= messenger RNA
NBC-1	= Na^+-HCO_3^- symporter isoform-1
NCE	= Na^+-Ca^{2+} exchanger
NHE-1	= Na^+-H^+ exchanger isoform-1
PCR	= polymerase chain reaction
pH_i	= intracellular pH
PSS	= physiological saline solution
RT-PCR	= reverse transcription polymerase chain reaction
RV	= right ventricle/ventricular

predominant receptor in the adult rat heart and is considered to be the major mediator of the Ang II-induced effects in the cardiovascular system (15). In contrast, angiotensin receptor subtype 2 (AT_2) activation has been reported to inhibit cell proliferation (16); to induce differentiation (17), apoptosis (18) and regeneration (19); and to be involved in the control of voltage-sensitive ion currents (20).

This study was undertaken to investigate the influence of angiotensin AT_1 and AT_2 receptors on pH_i-regulating systems in the rat myocardium at different time points after MI. The NHE-1 complementary DNA (cDNA) (21) and the NBC-1 cDNA (22) have been cloned from rat myocardium, providing us with the possibility of investigating the expression of these genes after MI. The antibodies to cardiac NHE-1 (23) and recently generated antibodies to NBC-1 (24) further enabled us to determine protein levels of both transporters in the heart. To investigate whether transcriptional and translational alterations of NHE-1 and NBC-1 after MI contribute to the control of myocardial pH_i, we separately measured the H^+ efflux through both transporters in tissue samples from infarcted rat hearts. We then examined the effects of chronic pre- and post-MI treatment with an ACE inhibitor, an AT_1 receptor antagonist or an AT_2 receptor antagonist on NHE-1 and NBC-1 mRNA expression, protein levels and transporter activity in the rat myocardium.

Here we demonstrate for the first time an induction of the cardiac NHE-1 and NBC-1 gene and protein expression as well as an increase in transporter activity after MI in vivo, and we also demonstrate a differential role of angiotensin AT_1 and AT_2 receptor subtypes in transcriptional, translational and functional regulation of these cardiac transporters.

METHODS

Animals and study design. Male normotensive Wistar rats (250 g, Charles River Viga, Sulzfeld, Germany) were used in all experiments. Animals were housed individually at a controlled temperature and humidity under a 12-h light/dark cycle. The study was performed in accordance with the German law on animal protection as released in its new version in 1993.

Animals were randomly divided into five groups: 1) sham operation without treatment, 2) MI subjected to placebo treatment (0.9% saline), 3) MI subjected to ramipril treatment (1 mg/kg/day), 4) MI subjected to valsartan treatment (10 mg/kg/day), and 5) MI subjected to PD 123319 treatment (30 mg/kg/day). For surgical procedures, rats were anesthetized by injection of methohexital-Na^+ (10 mg/kg intravenous) and artificially ventilated (70 ventilations/min, 200 mm H_2O, 2.5 ml/ventilation) to perform a left thoracotomy. Myocardial infarction was induced by permanent ligation of the left coronary artery. The AT_1 and AT_2 receptor antagonists valsartan and PD 123319, respectively, were given via osmotic minipumps, and the ACE inhibitor ramipril was given via gastric lavage. Drug dosages for ramipril (25), valsartan (26) and PD 123319 (27) were adjusted to the individual body weight of each rat to ensure effective receptor or enzyme blockade, respectively. Valsartan and PD 123319 were dissolved in 1 N NaOH and buffered with HCl (pH 7.4) before filling into minipumps. Ramipril was dissolved in water. Treatment began one week before induction of MI in all groups and continued until sacrifice. On days 1 and 7 after induction of MI, the rats were decapitated, and the hearts were rapidly excised. The left ventricle (LV) was separated from the atria and the right ventricle (RV) and then divided into the interventricular septum (IS) and the LV free wall by an incision along the line where the RV was cut off from the LV. The LV free wall consisted of scar tissue and area at risk. Tissue samples were rapidly frozen in liquid nitrogen and stored at −80°C until use. The number of animals was six per group.

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. For measurement of gene expression, total RNA was isolated from the fresh-frozen tissue using a single-step isolation method described by Chomczynski and Sacchi (28), and RNA concentration was determined by densitometric measurement of ultraviolet absorption at 260 nm. Poly(A^+)-RNA was isolated from total RNA by using Oligotex mRNA Mini Kit (QIAGEN, Hilden, Germany). Trizol solution, molecular size standards, Super Script Preamplification Systems and Taq polymerase were purchased from Gibco BRL (Eggenstein, Germany). The reverse transcription of total RNA (5 μg) was performed with oligo (dT)

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Table 1. PCR Conditions for Amplification of NHE-1, NBC-1 and GAPDH

Product	PCR Buffer/μl	$MgCl_2$ (1.5 mM/μl)	dNTPs (0.2 mM/μl)	Primers (20 μM/μl)	Taq-Polymerase (2.5 U/μl)	Annealing Temperat. (°C/min)	Number of PCR Cycles
NHE-1	5.0	1.5	2.0	1.0	0.25	57	26
NBC-1	5.0	1.5	2.0	1.0	0.25	56	27
GAPDH	5.0	3.0	1.0	1.0	0.25	60	23

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; NBC-1 = Na^+-HCO_3^- symporter isoform-1; NHE-1 = Na^+-H^+ exchanger isoform-1; PCR = polymerase chain reaction.

primers using Prime-It II Random Primer Kit reagents (Strategene, Heidelberg, Germany). The primer sequences (Pharmacia Biotech, Cambridge, United Kingdom) used in RT-PCR for amplification of cardiac NHE-1 were 5′-AGAAAAAGCAAGAAACAAAGCG-3′ (sense) and 5′-GTGGTAGAAGGAAATGAGCTG-3′ (antisense), resulting in a product size of 209 base pair (bp) and, for amplification of cardiac NBC-1, were 5′-CAAGAAGATGATCAAGCTGGC-3′ (sense) and 5′-AGGACATGAGTGTGATGTCAG-3′ (antisense) (product size 287 bp; from gene bank accession no AF001958) (29). The primer sequences for the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-GAATTCCATTGACCTCAACTACATGG-3′ (sense) and 5′-TTGCTGCAGTCTTACTCCTT-GGAGGCCAT-3′ (antisense) (product size 916 bp). Exact polymerase chain reaction (PCR) protocols for the various amplification reactions are given in Table 1. At the end of amplification reactions, PCR products were incubated at 72°C for 5 min for a final extension. Aliquots of each sample were subjected to electrophoresis on a 1.2% agarose gel and stained with ethidium bromide. The identity of PCR products was verified by sequence analysis (data not shown). To ensure a high reproducibility, each sample was subjected to three independent PCR reactions for NHE-1, NBC-1 and GAPDH.

For Northern blot analysis, 8 μg of Poly(A^+)-RNA from each tissue sample was denatured with formaldehyde, fractionated by electrophoresis through 1.2% agarose gel and transferred and fixated (2 h at 80°C) to a nylon membrane (Hybond-N Membrane, Amersham, Braunschweig, Germany). Hybridizations were carried out in a Rapid-Hyb solution (Amersham, Braunschweig, Germany) using ^{32}P-labelled probes. Membranes were washed using high stringency conditions: once for 30 min at 22°C in 1× SSC/0.1% SDS and once for 60 min at 60°C in 0.1 SSC/0.1% SDS. The blots were then examined by autoradiography. Exposure times were 20 h for Northern blots probed with NHE-1, 24 h for NBC-1 and 4 h for GAPDH. The fragment used for labeling of rat NHE-1 message was 233 to 634 bp of the rat NHE-1 cDNA sequence corresponding with the coding region. The fragment for probing the NBC-1 message was 633 to 822 bp and for GAPDH message was 568 to 989 bp. The cDNA fragments were radiolabeled with [α-^{32}P]dCTP (1×10^9 cpm/μg of DNA, Amersham, Braunschweig, Germany) using the random primer procedure (Strategene).

For the quantification of mRNA, bands were scanned and analyzed using the public domain National Institutes of Health image program. For all RNA samples, the density of the bands were divided by that of the GAPDH mRNA band.

Protein extraction and Western blot analysis. In the second set of the experiments, protein levels of cardiac NHE-1 and NBC-1 were determined in additional sham-operated and placebo-, ramipril-, valsartan- and PD 123319-treated infarcted groups. At day 1 and day 7 after MI, the three tissue samples (RV, IS, LV free wall) of each animal were homogenized in 5 ml of ice cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.5M EDTA, 150 mM NaCl, 0.1% Triton-100, freshly added proteinase inhibitor (100 μg/ml PMSF, 1 mg/ml Trasylol) followed by centrifugation at 14,000 rpm at 4°C for 1 min. The supernatant was collected, and aliquots were mixed with loading buffer (1M Tris-HCl [pH 6.8], 1% SDS, 30% glycerol, 0.8M DTT, 2% bromphenolblue) and solubilized for 10 min at 95°C and then centrifuged 30 min at 14,000 rpm at 4°C. Protein concentration was determined by the method of Bradford (30) using bovine serum albumin as a standard. All preparations were carried out at 4°C.

For Western blotting, 100 μg of total protein per lane was separated by a 10% SDS-PAGE gel. Proteins were transferred onto Hybond-C Super transfer membrane (Amersham, Life Science, Germany). The membrane was washed three times for 20 min in TTBS (0.1% Tween 20, 100 mM Tris-HCL, 150 mM NaCl, pH 7.5), blocked for 1 h in 5% nonfat milk/TTBS and incubated with primary rabbit polyclonal antibodies (Alpha Diagnostic, Bio Trend Chemicalien GmbH, Germany) for the cardiac NHE-1 (1:1,000) or NBC-1 (1:300) (31). In control experiments, Western blots were performed with rat kidney homogenates to ensure that the size of the detected bands for cardiac NBC-1 corresponded to those from the kidney. After three washes in TTBS, the membrane was incubated with a 1:1,000 dilution of the anti-rabbit horseradish-peroxidase coupled secondary antibody (Amersham, Braunschweig, Germany) for 30 min at room temperature. After extensive washes in TTBS, NHE-1 and NBC-1 were detected using ECL-reagents (Amersham, Braunschweig, Germany) and exposed to ECL-film according to the manufacturer's instructions. Quantification of Western blots was performed

on a computer using the National Institutes of Health image analysis system (Scion Corporation, Frederick, Maryland).

Preparation of heart tissue samples and pH_i measurement. In a third protocol, sham-operated and infarcted animals treated either with placebo, ramipril, valsartan or PD 123319 were decapitated seven days after MI; the hearts were quickly excised and fixated in a preparation dish containing ice cold physiological saline solution (PSS) (119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.026 mM EDTA, 5 mM HEPES, 5.5 mM D-glucose, pH 7.45 to 7.50 when gassed with 5% CO_2/95% O_2). Under a stereo microscope, the LV was then opened by an incision at the septum to carefully dissect a fresh heart tissue sample from the fringe of the inner endocardial LV free wall, representing a myocardial muscle probe from the ischemic region of the infarcted rat heart. Tissue samples were mounted in a tissue bath placed on the stage of an inverted microscope equipped with a photomultiplier, gassed with 5% CO_2/95% O_2 and heated to 37°C. The preparations were loaded for 60 min with 5 μM of the pH-sensitive membrane-permeable acetoxymethylester of the fluorescence dye 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein (BCECF-AM), as described previously (32). The tissue samples were excited alternately with light at 440 and 495 nm provided by a 75-W xenon lamp, which fed into a monochromator (PTI Deltascan). The emission from the preparation passed through a bandpass filter (515 to 560 nm) and was, via the photomultiplier, fed into the computer where the ratio of emission at the two different excitation wavelengths was calculated after subtraction of background fluorescence. A ratio measurement was obtained every 1 s in these experiments. At the end of the experiments, the ratio was calibrated in terms of pH_i with 4 mg/l nigericin in a 125 mM KCl solution (NaCl substituted with KCl) (33).

In order to determine the cellular buffering power (β_i), the ammonia washout technique was used (34). In brief, an initial alkalinization of the myocardial tissue samples was induced by applying 30 mM NH_4Cl for 8 min in 1-mM amiloride containing Na^+-free PSS (Na^+ substituted with N-methyl-D-glucamine) followed by stepwise (20 mM, 5 mM and 0 mM NH_4Cl) washout of NH_4Cl. The resultant changes in pH_i were used to estimate β_i as follows:

$$\beta_i\ (\text{mM}) = \frac{\Delta[NH_4^+]_i}{\Delta pH_i},$$

where

$$[NH_4^+]_i = \frac{([H^+]_i \times [NH_4^+]_0)}{[H^+]_0} \text{ and}$$

$$[NH_4^+]_0 = [NH_3]_0 \times 10^{(pKa\text{-}pHo)}\ (pKa\ NH_3 = 9{,}27).$$

β_i values were plotted as a function of pH_i and fitted to an exponential.

The H^+ efflux (J_H) was calculated from the equation $J_H = \beta_i(dpH_i/dt)$, where β_i is the buffering power and dpH_i/dt is the rate of pH_i recovery. The data points were plotted as a function of pH_i and fitted to an exponential. The J_H was calculated at pH_i of 7.05 by multiplying β_i with the appropriate dpH_i/dt. To estimate H^+ efflux through the NBC-1, J_H via the NHE-1 measured in HCO_3^--free solution ($NaHCO_3^-$ substituted with NaCl and the solution gassed with 100% O_2) was subtracted from total J_H measured in PSS.

Statistics. Computer-assisted programs based on square fitting methods served to analyze RT-PCR standard curves. Statistical evaluation was determined using one-way analysis of variance with repeated measurements. Means shown to be different between individual groups were compared using the post-hoc unpaired Student *t* test or the Bonferroni test. A p value ≤ 0.05 was considered to be significant. Results are expressed as means ± SEM. Further details of statistical analysis are given in the legends to the figures.

RESULTS

Effects of ACE inhibitor and AT receptor antagonists on cardiac NHE-1 and NBC-1 mRNA abundance after MI. Expression studies of cardiac NHE-1 and NBC-1 mRNA resulted in single bands of the predicted size for NHE-1 of 4.8 kb, for NBC-1 of 7.5 kb and for the house keeping gene GAPDH of 0.9 kb, as previously seen in other organs (21,29). We found identical expression patterns both by RT-PCR and Northern blot analysis. The GAPDH signals used as an internal control did not change in the rat myocardium during the early phase after MI (34). The levels of cardiac NHE-1 and NBC-1 mRNA quantified by densitometric scanning were related to the GAPDH signals and expressed by folds of increase from those of sham-operated rats. In the LV free wall, NHE-1 and NBC-1 mRNA abundance was significantly increased after MI, compared with sham operation. The message of NHE-1 was increased twofold on day 1, was elevated more than twofold seven days after MI and decreased to baseline values 14 days after MI, as shown by RT-PCR and Northern blot analysis (Fig. 1, A and B). Similarly, the message of NBC-1 was elevated on day 1, further increased on day 7 and decreased 14 days after MI (Fig. 2, A and B). Because the RT-PCR reaction was specific for NBC-1, these results demonstrate for the first time an MI-induced upregulation of this transporter in the rat myocardium. In sham-operated rats, NHE-1 and NBC-1 mRNA expression were unchanged on day 1 and day 7 after surgery compared with that of healthy rats (data not shown). In the IS and the RV, NHE-1 and NBC-1 expression were unchanged throughout the seven day post-MI period when compared with that of sham-operated rats (data not shown).

Pretreatment with the ACE inhibitor ramipril prevented the increase in cardiac NHE-1 mRNA expression on day 1

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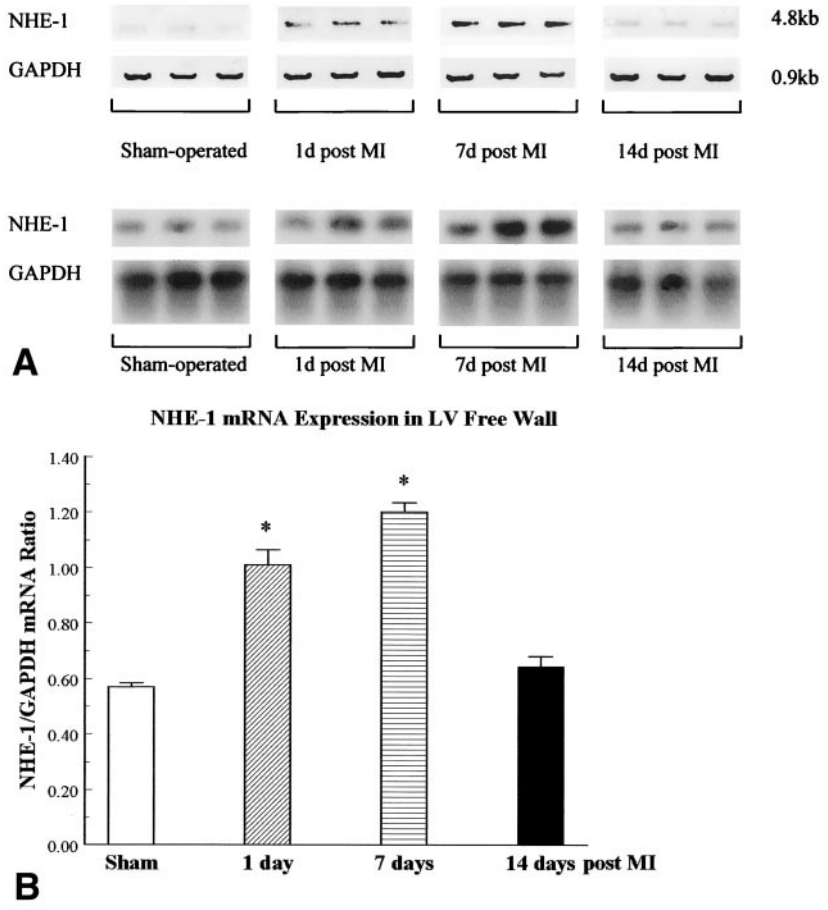

Figure 1. (A) Representative experiments showing upregulation of Na^+-H^+ exchanger isoform-1 (NHE-1) messenger RNA (mRNA) in the myocardium of sham-operated and placebo-treated animals with myocardial infarction (MI) one day and seven days after MI. Total RNA obtained from the left ventricular (LV) free wall was amplified by reverse transcription polymerase chain reaction **(upper line)** and analyzed by Northern blot **(lower line)**. **(B)** Relative changes in mRNA expression were determined by densitometric analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals. *$p < 0.05$ compared with sham operation. Data represent mean ± SEM, n = 6.

and markedly attenuated the increase in NHE-1 expression on day 7 (Fig. 3A) and prevented the upregulation of NBC-1 mRNA expression on days 1 and 7 (Fig. 3B) in the LV free wall of the infarcted rat myocardium. These results indicate that Ang II is involved in the upregulation of NHE-1 and NBC-1 in the ischemic region of the infarcted rat heart. To determine which angiotensin receptor subtype mediated the upregulation of NHE-1 and NBC-1 mRNA expression after MI, animals were pretreated one week before and up to sacrifice after MI with the selective AT_1 and AT_2 receptor antagonists valsartan and PD 123319, respectively. The results illustrated in Figure 3A show that the increase in mRNA expression of NHE-1 was completely prevented on day 1 and markedly inhibited on day 7 by the AT_1 receptor antagonist valsartan but not by the AT_2 receptor antagonist PD 123319. In contrast, PD 123319 totally prevented the NBC-1 mRNA upregulation at both time points, whereas valsartan did not affect the NBC-1 mRNA expression (Fig. 3B). These findings demonstrate that the increase in NHE-1 expression after MI is mediated to a large extent by the AT_1 receptor, whereas the AT_2 receptor controls NBC-1 upregulation.

Effects of ACE inhibitor and AT receptor antagonists on NHE-1 and NBC-1 protein levels. Protein levels for cardiac NHE-1 and NBC-1 were determined in the RV, IS and LV free wall of sham-operated and infarcted animals with placebo, ramipril, valsartan or PD 123319 treatment one day and seven days after MI using Western blot analysis. The density of the digitized signals was quantified as a percentage of the signal density of sham-operated rats, which was set to 100%, respectively. In the rat myocardium, we detected a single band of approximately 110 kDa for cardiac NHE-1. For the NBC-1, we saw a strong band at approximately 115 to 120 kDa and a weaker band at approximately 135 kDa in the rat myocardium. With the antibody used, we had previously seen in rat kidney a band at approximately 135 kDa, which was reduced to approximately 116 kDa after deglycosylation (31). Therefore, it is likely that the two bands represent the glycosylated and the nonglycosylated NBC-1. As demonstrated for NHE-1 mRNA expression, the protein levels of this transporter were increased in the LV free wall of placebo-treated infarcted animals one day after MI and reached maximum values seven days after MI compared with those of sham-

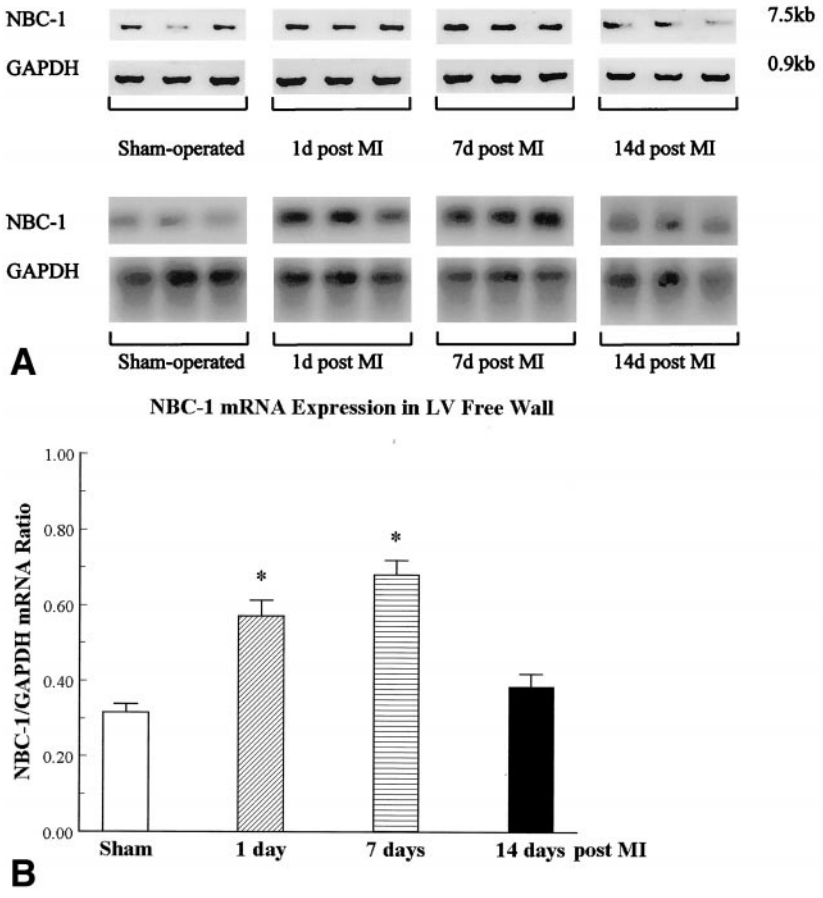

Figure 2. (A) Representative experiments showing upregulation of Na^+-HCO_3^- symporter isoform-1 (NBC-1) messenger RNA (mRNA) in the myocardium of sham-operated and placebo-treated animals with myocardial infarction (MI) one day and seven days after MI. Total RNA obtained from the left ventricular (LV) free wall was amplified by reverse transcription polymerase chain reaction **(upper line)** and analyzed by Northern blot **(lower line)**. **(B)** Relative changes in mRNA expression were determined by densitometric analysis and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals. *$p < 0.05$ compared with sham operation. Data represent mean ± SEM, n = 6.

operated animals (Fig. 4A). Fourteen days after MI, protein levels of NHE-1 showed lower values compared with seven days after MI values (data not shown). The protein amount of NHE-1 was not significantly different in the RV and IS of infarcted and sham-operated rat hearts at both time points measured (data not shown). Chronic treatment of infarcted animals with the ACE inhibitor ramipril and the AT_1 receptor antagonist valsartan completely prevented (one day after MI) and significantly reduced (seven days after MI) NHE-1 protein upregulation in the LV free wall compared with that of placebo-treated MI animals. In contrast, the AT_2 receptor antagonist PD 123319 had no effect on protein upregulation of cardiac NHE-1 at both time points (Fig. 4A). No significant differences in NHE-1 protein values in the RV and IS were observed between the four treated MI groups at any time point measured (data not shown).

One day after MI, protein levels for NBC-1 were increased in the LV free wall of placebo-treated infarcted animals and reached maximum levels on day 7 after MI (twofold) compared with those of sham-operated animals (Fig. 4B). Protein levels of this transporter were reduced 14 days after MI compared with seven days after MI (data not shown). Similarly to mRNA expression, the MI-induced increase in NBC-1 protein levels in the LV free wall was totally prevented by chronic treatment with the ACE inhibitor ramipril and the AT_2 receptor antagonist PD 123319 but not influenced by the AT_1 receptor antagonist valsartan compared with placebo-treated infarcted animals (Fig. 4B). The NBC-1 protein concentrations in the RV and IS of sham-operated animals did not differ significantly from those of infarcted animals at both time points measured and was unaffected by chronic treatment with ramipril, valsartan or PD 123319 (data not shown).

Effects of ACE inhibitor and AT receptor antagonists on Na^+-dependent H^+ transport after infarct. To investigate the contribution of the membrane transporters NHE-1 and NBC-1 to the recovery from an intracellular acid load in the ischemic myocardium, the activity of these transporters was assessed by measuring Na^+-dependent H^+ efflux (J_H) in the presence and absence of bicarbonate. The Na^+-dependent J_H in the absence of bicarbonate (NHE activity) was increased in the LV free wall of placebo-treated infarcted animals (threefold) on day 7 after MI compared

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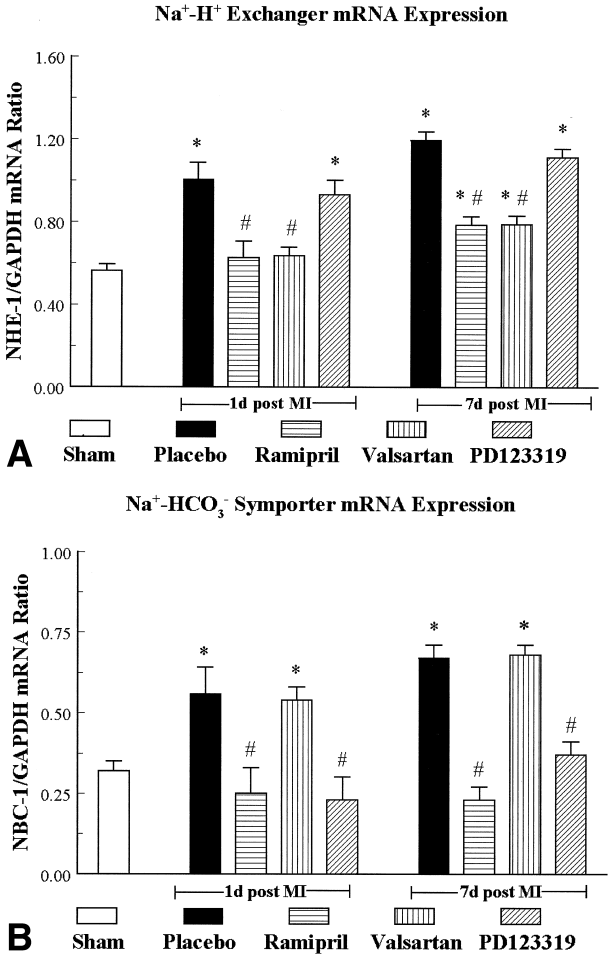

Figure 3. Effects of chronic treatment with the angiotensin-converting enzyme inhibitor ramipril (1 mg/kg/day), the AT_1 receptor antagonist valsartan (10 mg/kg/day) and the AT_2 receptor antagonist PD 123319 (30 mg/kg/day) on messenger RNA (mRNA) expression of Na^+-H^+ exchanger isoform-1 (NHE-1) **(A)** and Na^+-HCO_3^- symporter isoform-1 (NBC-1) **(B)** in the left ventricular free wall one day and seven days after myocardial infarction (MI) compared with sham-operated and placebo-treated animals with MI. $^*p < 0.05$ compared with sham operation; $\#p < 0.05$ compared with placebo MI. Data represent mean ± SEM, n = 6.

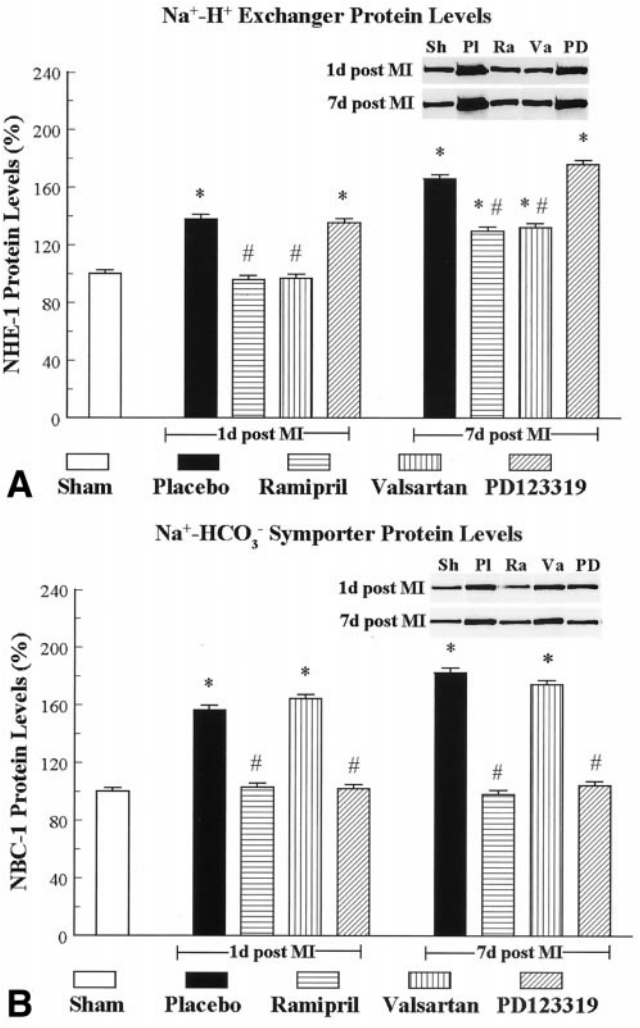

Figure 4. Effects of chronic treatment with the angiotensin-converter enzyme inhibitor ramipril (1 mg/kg/day), the AT_1 receptor antagonist valsartan (10 mg/kg/day) and the AT_2 receptor antagonist PD 123319 (30 mg/kg/day) on protein levels of Na^+-H^+ exchanger isoform-1 (NHE-1) **(A)** and Na^+-HCO_3^- symporter isoform-1 (NBC-1) **(B)** in the left ventricular free wall one day and seven days after myocardial infarction (MI) compared with sham-operated and placebo-treated animals with MI. $^*p < 0.05$ compared with sham operation; $\#p < 0.05$ compared with placebo MI. Data represent mean ± SEM, n = 6. PD = PD 123319; Pl = placebo; Ra = ramipril; Sh = sham-operated; Va = valsartan.

with sham-operated animals. Chronic treatment with the ACE inhibitor ramipril and the AT_1 receptor antagonist valsartan prevented the MI-induced increase in the activity of this transport, whereas the AT_2 receptor antagonist PD 123319 did not significantly affect the H^+ efflux through NHE-1 (Fig. 5 and 6A). At the same time point, the Na^+- and bicarbonate-dependent J_H (NBC activity) was also increased in placebo-treated infarcted animals (fourfold) when compared with sham-operated animals. Treatment with ramipril and PD 123319 prevented the increase in the activity of NBC-1 in the LV free wall. In contrast, valsartan had no effect on the activity of NBC-1 in the LV free wall of the infarcted rat heart (Fig. 5 and 6B).

DISCUSSION

In this study, we demonstrate for the first time an increase in steady-state NHE-1 and NBC-1 mRNA expression and protein levels as well as a rise in the Na^+-dependent J_H consistent with an increase of NHE-1 and NBC-1 activity in the ischemic region of the infarcted rat heart. Furthermore, our data point to a differential role of angiotensin AT_1 and AT_2 receptors in the regulation of NHE-1 and NBC-1 gene expression, protein translation and transport activity in the ischemic myocardium.

Contribution of NHE-1 and NBC-1 in pH_i regulation of the ischemic myocardium. Our results show that MI induces an upregulation of NHE-1 and NBC-1 mRNA and protein in the LV free wall but not in the IS and RV during the early phase after MI. The exact mechanism underlying these events has yet to be established. It is possible that there is an increase in transcriptional and translational regulation, a decrease in mRNA and protein degradation or

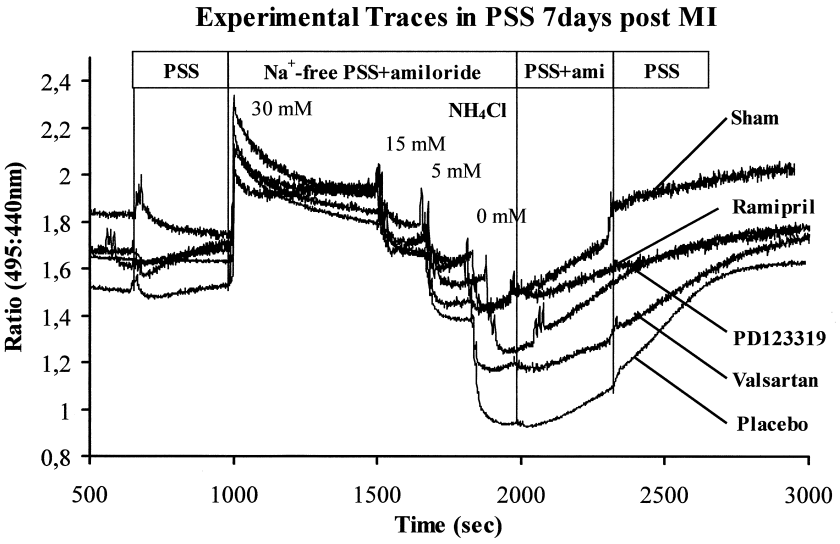

Figure 5. Representative original traces showing recovery from intracellular acidosis after stepwise washout of 30 mM NH_4Cl of endocardial tissue preparations from infarcted rat hearts. Effects of chronic treatment with the angiotensin-converting enzyme inhibitor ramipril, the AT_1 receptor antagonist valsartan and the AT_2 receptor antagonist PD 123319 on puffering power (β_i) and proton efflux (J_H) seven days after myocardial infarction (MI) compared with sham-operated and placebo-treated animals with MI. ami = amiloride; PSS = physiological saline solution.

a combination of both. In these experiments, permanent ligation of the left coronary artery results in an ischemia of the LV free wall known to produce an intracellular acidosis within this region, whereas the IS and the RV are not ischemic (35). Acidosis can act as a specific mechanism to increase gene expression and protein translation or to decrease mRNA and protein degradation of both NHE-1 and NBC-1 (36,37). Additionally, translational and transcriptional upregulation of the cardiac membrane transporters was accompanied by an increase in the activity of Na^+-dependent H^+ flux consistent with a functional importance of the transporter upregulation.

The primary function of cardiac NHE-1 is to regulate intracellular pH (pH_i) by extruding H^+ in exchange for Na^+ in a one-to-one fashion. Thus, NHE-1 also participates in the regulation of intracellular Na^+ (Na^+_i), as demonstrated by Frelin et al. (38) using rat and chicken heart cells. In that study, amiloride and its analogues prevented Na^+ influx after H^+ gradient-induced activation of NHE-1. Activation of NHE-1 was shown to account for as much as 50% of the basal permeability of the membrane to Na^+ (38). In cardiac cells, which have been shown to possess both Na^+-H^+ and Na^+-Ca^{2+} exchanger (NCE) systems, changes in pH_i result in an alteration of the intracellular Ca^{2+}-concentration (Ca^{2+}_i) because of the close linkage of these two exchanger proteins via cytosolic Na^+ (38,39). As a result of cardiac NHE-1 activation after intracellular acidosis, accumulated Na^+ can be exchanged for Ca^{2+} via cardiac NCE at a 3:1 ratio leading to an increase in Ca^{2+}_i (Fig. 7). The idea that stimulation of NHE-1 induces an intracellular Ca^{2+}-overload via NCE has been addressed in a number of investigations (40,41). In our experiments, it is possible that the activation of NHE-1 in the rat myocardium after MI, although important for the restoration of normal pH_i, might result in a paradoxical aggravation of tissue injury because the increase in Na^+_i due to activation of NHE-1 would lead to an enhanced Ca^{2+}_i via NCE, which has been shown to be associated with cell death and arrhythmias (4,5). An enhanced expression of cardiac NHE-1 after MI could thus lead to an increased incidence of tissue injury, cell necrosis and arrhythmias. Conversely, inhibitors of NHE-1 have been found to effectively protect the myocardium from damage after MI (42).

The NBC activity has been shown to have a stoechiometry of 3 HCO_3^- to 1 Na^+ in the kidney. In the heart, an electroneutral NBC activity has been described (43) in cardiac myocytes from sheep but an HCO_3^-:Na^+ ratio of 1.63 has been suggested based on experiments in rat cardiac myocytes (44). In this study, we specifically assessed NBC-1, which is an electrogenic NBC with a stoechiometry different from 1:1. Our functional assays did not address the question of which NBC isoform was involved. However, the good correlation between NBC-1 regulatory and functional data is consistent with the interpretation that the HCO_3^-- and Na^+-dependent net H^+ influx to a large extent reflects the activity of NBC-1 (3:1 ratio). This transporter has been shown to be colocalized directly with the Na^+-K^+ pump within the plasma membrane (45), suggesting that the NBC-induced Na^+ accumulation could be removed immediately from the cytosol via the coupled Na^+-K^+ pump in exchange to K^+ (3:2 ratio). The Na^+-K^+ pump mediated Na^+ efflux causes a hyperpolarization of the cell membrane that might protect the myocardium against arrhythmias (Fig. 7). Thus, an enhanced expression, translation and activity of the electrogenic NBC in the ischemic myocardium could play a cardioprotective role.

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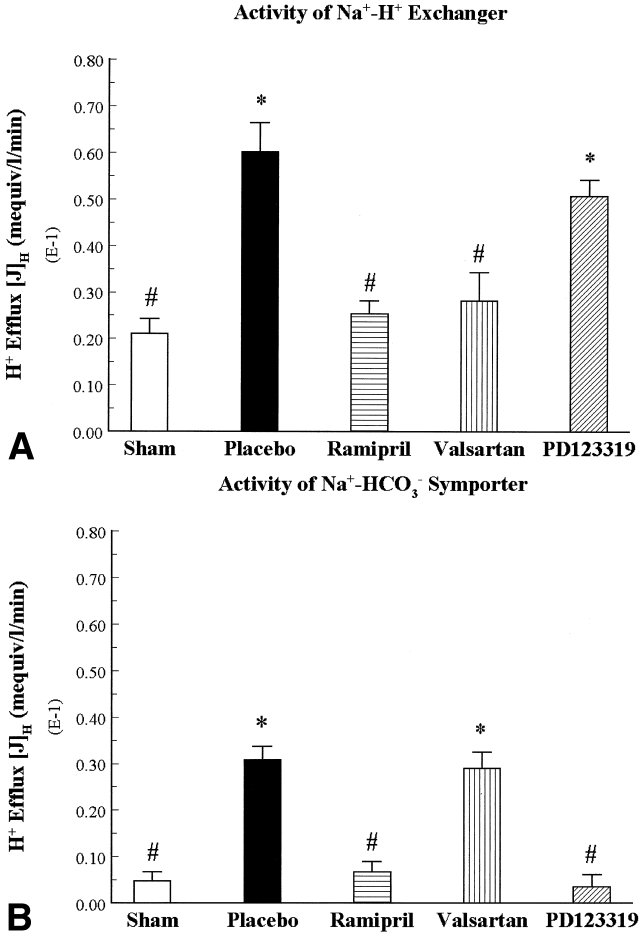

Figure 6. Effects of chronic treatment with the angiotensin-converting enzyme inhibitor ramipril (1 mg/kg/day), the AT_1 receptor antagonist valsartan (10 mg/kg/day) and the AT_2 receptor antagonist PD 123319 (30 mg/kg/day) on Na^+-dependent HCO_3^--independent net H^+ influx (NHE activity; **A**) and Na^+ and HCO_3^--dependent net H^+ influx (NBC activity; **B**) in endocardial tissue preparations seven days after myocardial infarction compared with sham-operated and placebo-treated animals with myocardial infarction. *$p < 0.05$ compared with sham operation; #$p < 0.05$ compared with placebo-treated myocardial infarction. Data represent mean ± SEM, n = 10 to 12.

Effects of ACE inhibitor and AT receptor antagonists on pH_i regulation in the ischemic myocardium. A central aim of our study was to investigate the differential role of angiotensin AT_1 and AT_2 receptor subtypes in the regulation of NHE-1 and NBC-1 in the rat myocardium after MI. The localization of angiotensinogen and renin within cardiac myocytes and fibroblasts has been shown using in situ hybridization and immunofluorescence techniques (46). Additionally, an activation of the tissue-specific cardiac ACE has been demonstrated in experimental heart failure (11), supporting the view of the existence of an intracardiac renin-angiotensin system. Angiotensin-converting enzyme inhibition has been shown to have an influence on cardiac remodeling and to prolong survival in patients with LV dysfunction after MI (47,48). Ennis et al. (49) demonstrated that the ACE inhibitor enalapril induced normalization of pH_i regulatory mechanisms in the hypertrophic myocardium of spontaneously hypertensive rats, which exhibit increased NHE-1 activity. This is in accordance with our observation that the ACE inhibitor ramipril inhibited the mRNA and protein upregulation of NHE-1 and NBC-1 as well as the activation of the Na^+-dependent J_H.

Angiotensin II binds to at least two different types of receptors, referred to as AT_1 and AT_2 (50), which both have been shown to be expressed in the rat myocardium (51). It has been demonstrated that AT_1 and AT_2 receptors are significantly increased in the rat heart during the early phase after MI (13,52). Our present data show that the Ang II-induced stimulation of NHE-1 is mediated, at least in part, via activation of the AT_1 receptor, whereas AT_2 receptor activation induced the stimulation of NBC-1. These findings extend previous observations showing that Ang II has an acute activating effect on NHE activity (9,53,54) by demonstrating long-term upregulation of NHE in vivo.

Angiotensin II has also been shown to acutely activate NBC in neonatal myocytes, an effect that was ascribed to the AT_2 receptor (6). In this study, the upregulation of NBC-1 at mRNA and protein level was abolished at both time points after MI by the AT_2 receptor antagonist PD 123319, indicating that this receptor is indeed crucial for the enhanced expression and translation of cardiac NBC-1 under ischemic conditions. On the other hand, the fact that the upregulation of NHE-1 was abolished by the AT_1 receptor antagonist valsartan on day 1 after MI, whereas, on day 7 after MI, this inhibition was not complete, suggests that Ang II-independent mechanisms also contribute to the regulation of NHE-1 at later time points after MI. For instance, in rat ventricular myocytes, it has been demonstrated that α_1-adrenergic stimulation acutely causes an alkalinization of pH_i and an enhanced rate of NHE-mediated recovery from an acid load (55). Endothelin (56) and thrombin (57) have also been shown to activate the cardiac NHE-1 of rat myocytes.

One of the important findings of this study is the discrimination between the mechanism involved in the expression of NHE-1 and NBC-1 by using an in vivo animal model of MI. The possible clinical consequences of this intriguing observation deserve comments. Many reports suggest that the AT_1 receptor subtype plays a major role in cardiac pathophysiology, and the administration of AT_1 receptor antagonists has been shown to reduce infarct size (58) and LV loading (59) and to improve hemodynamics and coronary angiogenesis (14) after MI. Consistent with these and our present findings, Harada et al. (60) have recently reported on reduced reperfusion arrhythmias in AT_{1a} receptor knockout mice. The role of cardiac AT_2 receptors in the control of heart function remains to be clarified. In isolated ischemic rat hearts and after MI, the cardioprotective effect of AT_1 receptor blockade may be mediated, in part, by endogenously released Ang II via AT_2 receptor stimulation (61). Since ACE inhibitors, in addition to reducing NHE-1 expression, also reduce NBC-1 expres-

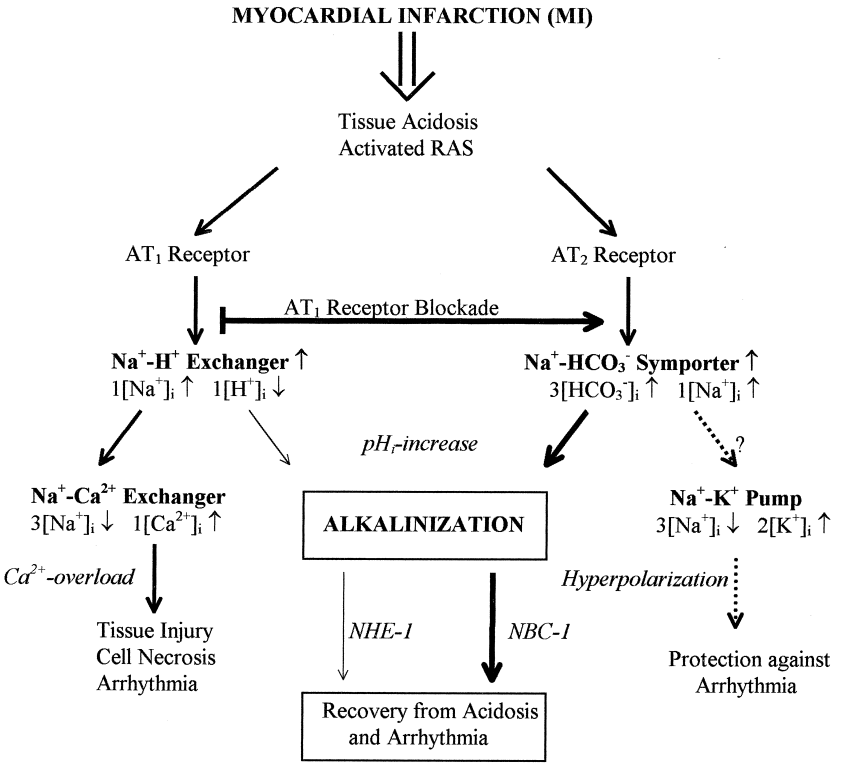

Figure 7. Possible contribution of angiotensin receptor subtype 1 (AT_1) and angiotensin receptor subtype 2 (AT_2) in the regulation of intracellular pH after myocardial infarction (MI). NBC-1 = Na^+-HCO_3^- symporter isoform-1; NHE-1 = Na^+-H^+ exchanger isoform-1.

sion, they prevent the selective AT_2 receptor-mediated effect of NBC-1 upregulation during ischemia. On the other hand, since AT_1 receptor antagonists block NHE-1 upregulation but stimulate NBC-1 activity via the AT_2 receptor, they may have a potential clinical advantage in the early phase therapy of MI (Fig. 7).

Conclusions. In summary, the results of this study demonstrate that NHE-1 and NBC-1 mRNA and protein levels are increased in the LV free wall but not in the IS and the RV of rat hearts in the first week after MI. Additionally, MI induces an increase in the Na^+-dependent J_H consistent with an elevated activity of both transporters playing a role for the recovery from intracellular acidosis. Chronic treatment with an ACE inhibitor effectively reduced mRNA upregulation, protein upregulation and transport activity of cardiac NHE-1 and NBC-1. Treatment with an AT_1 receptor antagonist markedly reduced mRNA−, protein upregulation and transport activity through NHE-1 but had no effect on NBC-1 expression, whereas treatment with an AT_2 receptor antagonist significantly reduced the upregulation of the NBC-1 mRNA−, protein and transport activity but had no effect on NHE-1 upregulation. The results suggest that the myocardial cell damage induced by NHE-1 upregulation after MI can be prevented either by ACE inhibition or AT_1 receptor blockade. Additionally, our findings suggest that AT_1 receptor antagonists may shift the recovery from ischemia-induced myocardial acidosis from an NHE-1-mediated to a more NBC-1-mediated mechanism of alkalinization by engaging the angiotensin AT_2 receptor.

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