Sarcolemmal Na$^+$/H$^+$ Exchanger Activity and Expression in Human Ventricular Myocardium

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OBJECTIVES   To determine sarcolemmal Na$^+$/H$^+$ exchanger (NHE) activity and expression in human ventricular myocardium.

BACKGROUND   Although the sarcolemmal NHE has been implicated in various physiological and pathophysiological phenomena in animal studies, its activity and expression in human myocardium have not been studied.

METHODS   Ventricular myocardium was obtained from unused donor hearts with acute myocardial dysfunction (n = 5) and recipient hearts with chronic end stage heart failure (n = 11) through a transplantation program. Intracellular pH (pH$_i$) was monitored in enzymatically isolated single ventricular myocytes by microepifluorescence. As the index of sarcolemmal NHE activity, the rate of H$^+$ efflux at a pH$_i$ of 6.90 (J$_{H6.9}$) was determined after the induction of intracellular acidosis in bicarbonate-free medium. Na$^+$/H$^+$ exchanger isoform 1 (NHE1) expression in ventricular myocardium was determined by immunoblot analysis.

RESULTS   Human ventricular myocytes exhibited readily detectable sarcolemmal NHE activity after the induction of intracellular acidosis, and this activity was suppressed by the NHE1-selective inhibitor HOE-642 (cariporide) at 1 μmol/L. Sarcolemmal NHE activity of myocytes was significantly greater in recipient hearts (J$_{H6.9}$ = 1.95 ± 0.18 mmol/L/min) than it was in unused donor hearts (J$_{H6.9}$ = 1.06 ± 0.15 mmol/L/min). In contrast, NHE1 protein was expressed in similar abundance in ventricular myocardium from both recipient and unused donor hearts.

CONCLUSIONS   Sarcolemmal NHE activity of human ventricular myocytes arises from the NHE1 isoform and is inhibited by HOE-642. Sarcolemmal NHE activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in unused donor hearts, and this difference is likely to arise from altered posttranslational regulation. (J Am Coll Cardiol 2000; 36:534–40) © 2000 by the American College of Cardiology

The sarcolemmal Na$^+$/H$^+$ exchanger (NHE) of cardiac myocytes is believed to be the ubiquitous NHE isoform 1 (NHE1) of the multigene NHE family (1) and contributes significantly to the integrated control of intracellular pH (pH$_i$) in this cell type (2). Na$^+$/H$^+$ exchanger isoform 1 activity is regulated primarily by pH$_i$ through the interaction of H$^+$ with a "H$^+$-sensor" site on the exchanger's membrane domain in a manner that results in exchanger activation in response to intracellular acidosis (3,4). Consistent with this, the cardiac sarcolemmal NHE is relatively quiescent at physiological pH$_i$, but its activity increases progressively as pH$_i$ declines (2). Sarcolemmal NHE activity is also modulated by a variety of neurohormonal stimuli such as alpha$_1$-adrenergic agonists (5,6), endothelin (7), thrombin (8) and angiotensin II (9) through receptor-mediated mechanisms. These agents appear to increase sarcolemmal NHE activity by increasing the pH$_i$-sensitivity of the exchanger, which is the mechanism known to underlie growth factor-induced stimulation of NHE1 (3,4).

Recently, the sarcolemmal NHE has received attention as a potential mediator of various physiological and pathophysiological phenomena in myocardium such as inotropic responses to a variety of agonists (7,10–13) and muscle stretch (14) and the induction of hypertrophy by mechanical (15) and neurohormonal (16) stimuli. In addition, work with the novel NHE1-selective inhibitors in our laboratory (17–20) and by others (for recent reviews, see Avkiran [21] and Karmazyn et al. [22]) has provided support for the hypothesis that sarcolemmal NHE activity is an important determinant of the severity of arrhythmias, contractile dysfunction and tissue necrosis during myocardial ischemia and reperfusion. Recent evidence suggests that NHE activity may also be involved in the induction of myocyte apoptosis during ischemia and reperfusion (23,24) and metabolic inhibition and recovery (25). These experimental findings have instigated trials with NHE inhibitors in clinical settings of myocardial ischemia and reperfusion, such as the recent GUARDIAN (Guard during Ischemia Against Necrosis) trial in patients with acute coronary syndromes (26).

Although significant advances have been made, as described above, in understanding of the regulation and roles of the sarcolemmal NHE, these have been achieved exclusively through the use of myocardial tissue and cells from a variety of animal species. As a consequence, the applicability to man of many of the findings is unconfirmed, and little is

Abbreviations and Acronyms

$beta_i$	=	intrinsic buffering power
dpH_i/dt	=	rate of recovery of pH_i
J_H	=	rate of H^+ efflux
$J_{H6.9}$	=	rate of H^+ efflux at pH_i 6.90
NCE	=	Na^+/Ca^{2+} exchanger
NHE	=	Na^+/H^+ exchanger
NHE1	=	Na^+/H^+ exchanger isoform 1
pH_i	=	intracellular pH

known regarding sarcolemmal NHE activity and expression in human myocardium. We have used a microepifluorescence technique to determine, for the first time, sarcolemmal NHE activity in ventricular myocytes isolated from explanted human hearts. In addition, we determined NHE expression in ventricular myocardium of those hearts by immunoblot analysis.

METHODS

Human ventricular myocytes. Ventricular myocardium was obtained from explanted human hearts comprising 11 recipient hearts with chronic end-stage heart failure (eight with ischemic heart disease and three with dilated cardiomyopathy) and five donor hearts that were not used for transplantation due to a low ejection fraction, as described recently (27). Table 1 shows the characteristics of the individuals from whom ventricular tissue was obtained over a period of 15 months and the number and ventricular origin of the cells from each preparation that were used in the microepifluorescence studies. The mean age of patients from whom recipient hearts were obtained was 49.3 ± 3.2 years (n = 11), which was significantly greater (p < 0.05, unpaired t test) than that of the individuals from whom the unused donor hearts originated (33.8 ± 6.2 years, n = 5).

Table 1. Origin of Human Ventricular Myocytes Used in the Study

Heart No.	Status	Age (yr)/ Gender	Cells Studied
1	Recipient (IHD)	55/M	4 (RV)
2	Unused donor	22/F	12 (RV)
3	Unused donor	19/F	16 (RV)
4	Recipient (DCM)	20/M	10 (RV)
5	Recipient (DCM)	50/M	7 (LV)
6	Recipient (IHD)	48/M	11 (LV)
7	Unused donor	42/M	7 (LV)
8	Recipient (IHD)	49/M	4 (RV)
9	Recipient (IHD)	47/M	4 (RV)
10	Recipient (IHD)	55/M	5 (RV)
11	Unused donor	34/F	4 (LV)
12	Unused donor	52/F	4 (LV)
13	Recipient (IHD)	59/M	6 (RV)
14	Recipient (IHD)	56/F	4 (LV)
15	Recipient (IHD)	48/M	10 (RV)
16	Recipient (DCM)	55/M	4 (LV)

DCM = dilated cardiomyopathy; IHD = ischemic heart disease; LV = left ventricular; RV = right ventricular.

Myocytes were isolated by enzymatic digestion of left or right ventricular myocardium, as described in detail previously (28), and only rod shaped cells were used in the microepifluorescence studies. Of the 112 myocytes listed in Table 1, 87 (54 from recipient hearts and 33 from unused donor hearts) were used for determination of sarcolemmal NHE activity. The remainder were used for in situ calibration of the pH-sensitive fluorescent dye carboxyseminaphthorhodafluor-1 (C-SNARF-1), estimation of intrinsic buffering power ($beta_i$) and determination of the NHE-inhibitory efficacy of HOE-642 (cariporide), a potent NHE1-selective inhibitor (29), which we have shown to inhibit sarcolemmal NHE activity in rat ventricular myocytes (19) and which was tested in the GUARDIAN trial (26).

Measurement of pH_i and $beta_i$. The microepifluorescence-based approach that was used to monitor pH_i in single cells has been described in detail previously (8) and used in our earlier studies with rat ventricular myocytes (5,6,8,9,19,30). In brief, cells loaded with C-SNARF-1 were placed on a glass coverslip in a 100 μL chamber and fluorescence recordings made using a dual-emission photometer system (D104C; Photon Technology International Inc.) during continuous superfusion (3.5 mL/min) with bicarbonate-free Tyrode's solution (34°C). Calibration was with nigericin-containing solutions, and $beta_i$ was estimated during stepwise removal of extracellular NH_4Cl, as described (8). The calibration curve was obtained by nonlinear least squares fit of normalized emission ratios; this gave best-fit values for pK and a of 7.08 and -1.46, respectively, which are similar to the values previously obtained in rat ventricular myocytes (8).

Determination of sarcolemmal NHE activity. The rate of acid efflux (J_H) was used as the index of sarcolemmal NHE activity, as in our previous work (5,6,8,9,19,30). After 5 to 10 min of superfusion with Tyrode's solution, myocytes were subjected to intracellular acidosis (in order to activate the sarcolemmal NHE) by transient (5 min) exposure to 30 mmol/L NH_4Cl and its subsequent washout (14 min). Since pH_i was lowered to ≤ 6.90 upon NH_4Cl washout in all cells that were subjected to this protocol, J_H was estimated at a pH_i of 6.90 and termed $J_{H6.9}$.

In experiments in which the NHE inhibitor HOE-642 was used, cells were subjected to two consecutive acid pulses (as described above) separated by 10 min. During the second acid pulse, HOE-642 (1 μmol/L) was included in the superfusate during exposure to NH_4Cl and the first 7 min of NH_4Cl washout; HOE-642 was subsequently removed from the Tyrode's solution to assess the reversibility of drug action. With the same protocol, myocyte contraction was monitored using a video edge-detection system, as described before (31).

Determination of NHE and Na^+/Ca^{2+} exchanger expression. Myocardial expression of NHE1 was determined at protein level by immunoblot analysis. In order to avoid potential problems with differential recoveries of mem-

branes from unused donor and recipient heart samples, immunoblot analysis was conducted using unfractionated tissue homogenates as described recently (32). Na$^+$/Ca^{2+} exchange (NCE) expression was also determined as a positive control for the presence of sarcolemmal protein in the samples. Ventricular tissue samples (approximately 0.2 g) obtained from regions without overt signs of fibrosis or damage were rapidly thawed, weighed and homogenized for 3 to 4 min in lysis buffer (sorbitol [5%], histidine [pH 7.4; 25 mmol/L], Na$_2$EDTA [50 mmol/L], KCl [50 mmol/L], leupeptin [1 μg/μL], PMSF [0.5 mmol/L] and benzamidine [1 mmol/L]). For NHE analysis, 0.5% SDS and 0.1% beta-mercaptoethanol were added to 25 μL of sample containing 100 μg of protein. After boiling for 5 min, 55 μL of lysis buffer and 5 μL of polyoxyethylene-8-lauryl ether (Sigma, Poole, United Kingdom) were added to the sample. After incubation at 37°C for 15 h, 50 μL of 3× SDS-sample buffer was added and the sample boiled for 10 min. For NCE analysis, SDS-sample buffer (×1) was added directly to an aliquot of tissue homogenate to obtain a final protein concentration of 2 μg/μL and the sample boiled for 10 min. After centrifugation, all samples (100 μg protein) were subjected to electrophoresis using a 7.5% SDS-polyacrylamide gel, and the separated proteins were transferred to polyvinylidene difluoride membranes. Immunoblot analysis was performed using mouse monoclonal antibody for NHE1 (1:500 dilution; #MAB3140, Chemicon International Inc., Harrow, United Kingdom) or NCE (1:500 dilution; #C2C12, Cambridge BioScience, United Kingdom) in conjunction with antimouse secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Statistical analysis. For each heart, $J_{H6.9}$ was determined in up to 11 cells of either left or right ventricular origin (Table 1), and an average value was obtained. Data for unused donor and recipient groups are expressed as mean ± SEM, with the n values representing the number of hearts in each group. The unpaired t test was used to compare $J_{H6.9}$ in recipient versus unused donor hearts, and p < 0.05 was considered significant.

RESULTS

The rate of recovery of pH$_i$ (dpH$_i$/dt) after an intracellular acid load is determined not only by the J_H but also by the beta$_i$ at the relevant pH$_i$ (33). Therefore, to obtain accurate estimates of J_H (as the index of sarcolemmal NHE activity), it was necessary to determine beta$_i$ in both populations of myocytes. Our data revealed no difference in beta$_i$ between myocytes from recipient and unused donor hearts (Fig. 1). Linear least squares regression analysis of all data points gave the equation beta$_i$ = −33.7·pH$_i$ + 260.1, which is very similar to the equation that describes the relationship between pH$_i$ and beta$_i$ in rat ventricular myocytes (8). Basal pH$_i$ values were not significantly different between myocytes from recipient (7.31 ± 0.02) and unused donor (7.29 ±
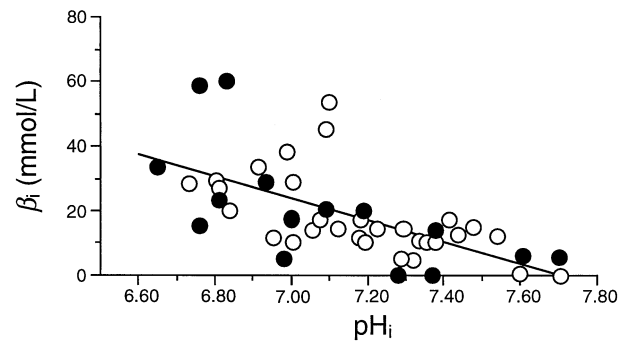

Figure 1. The relationship between pH$_i$ and β_i in human ventricular myocytes obtained from unused donor hearts **(open symbols)** and recipient hearts with end-stage heart failure **(solid symbols)**. Linear least squares regression analysis of all points gave the equation $\beta_i = -33.7 \cdot pH_i + 260.1$. β_i = intrinsic buffering power; pH$_i$ = intracellular pH.

0.03) hearts, and both groups of cells acidified to a similar extent upon washout of NH$_4$Cl, with minimal pH$_i$ values of 6.70 ± 0.03 and 6.71 ± 0.03, respectively. Myocytes from recipient hearts exhibited faster recovery from acidosis, as illustrated by the representative recordings shown in Figure 2A. Quantitative analysis of such data revealed that $J_{H6.9}$ was significantly greater in myocytes from recipient hearts than it was in cells from unused donor hearts (Fig. 2B).
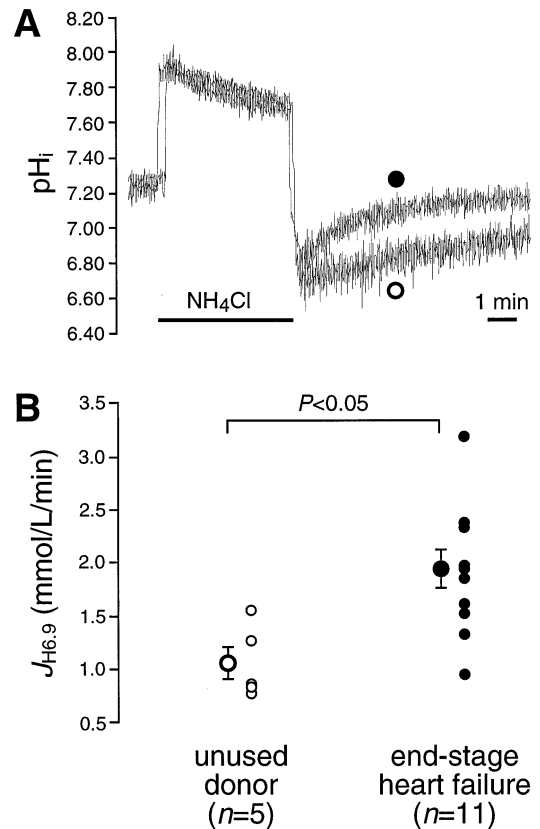

Figure 2. **(A)** Representative single-cell pH$_i$ recordings during acid pulses and **(B)** individual and mean $J_{H6.9}$ values in ventricular myocytes obtained from unused donor hearts **(open symbols)** and recipient hearts with end-stage heart failure **(solid symbols)**. In **(B)**, n indicates the number of hearts in each group. pH$_i$ = intracellular pH; $J_{H6.9}$ = rate of H$^+$ efflux at pH$_i$ 6.90.
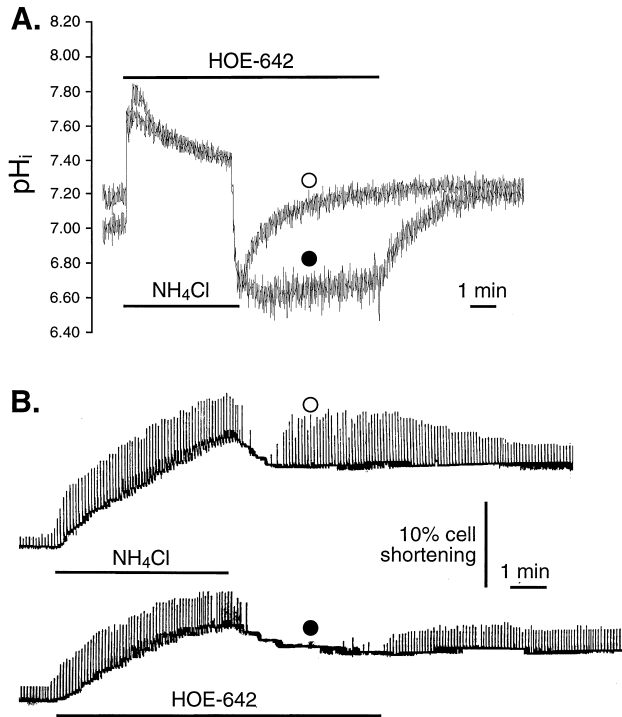
Figure 3. Representative recordings of **(A)** pH$_i$ and **(B)** cell contraction in human ventricular myocytes from recipient hearts with end-stage heart failure during two consecutive acid pulses. The first acid pulses **(open symbols)** were under control conditions whereas during the second acid pulses **(solid symbols)** HOE-642 (1 μmol/L) was present during exposure to NH$_4$Cl and the first 7 min of NH$_4$Cl washout, as indicated by the **horizontal bars.** The baseline changes in **(B)** reflect changes in resting cell length. pH$_i$ = intracellular pH.

To confirm that, under the conditions used, recovery of pH$_i$ from acidosis in human ventricular myocytes occurred predominantly by H$^+$ efflux through the sarcolemmal NHE, we exposed myocytes to intracellular acidosis in the presence of HOE-642. As illustrated by the representative recordings shown in Figure 3A, although there was rapid recovery from acidosis under control conditions, such recovery was markedly suppressed in the presence of HOE-642. The effect of HOE-642 was rapidly reversible, such that when the inhibitor was removed from the superfusion solution, pH$_i$ recovered from acidosis at a rate comparable with that seen under control conditions (Fig. 3A). Figure 3B illustrates that the changes in pH$_i$ were paralleled by changes in the amplitude of cell contraction. Thus, intracellular alkalosis during NH$_4$Cl exposure was associated with an increase in contraction amplitude, while intracellular acidosis after NH$_4$Cl washout was accompanied by a reduction in contraction amplitude. Furthermore, HOE-642 depressed the recovery of cell contraction after NH$_4$Cl washout, in parallel with its inhibitory effect on pH$_i$ recovery from acidosis.

Immunoblot analysis of a random selection of ventricular myocardium from unused donor and recipient hearts revealed that the 110 kDa NHE1 protein was expressed in similar abundance in all samples (Fig. 4A). Na/Ca^{2+} exchanger expression was also readily detected in all samples as

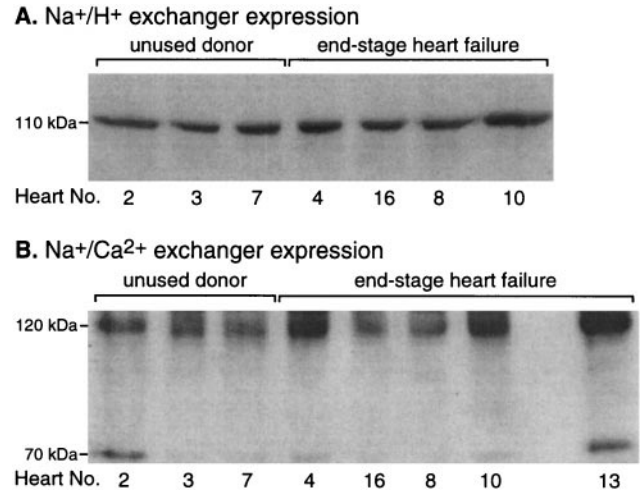**Figure 4.** Autoradiograms illustrating protein expression of **(A)** the Na$^+$/H$^+$ exchanger (NHE1 isoform) and **(B)** the Na$^+$/Ca^{2+} exchanger in ventricular samples from unused donor hearts and recipient hearts with end-stage heart failure. Heart numbers relate to Table 1; in **(B)** the lane between heart numbers 10 and 13 contained size markers.

two proteins of 120 and 70 kDa; these have been shown previously to represent the intact NCE and a proteolytic fragment, respectively, in human myocardium (32). In contrast to NHE1, however, in three of the five recipient hearts that were studied (heart numbers, 4, 10 and 13 in Table 1), NCE protein was present in markedly greater abundance (Fig. 4B). This difference in NCE abundance, which is consistent with earlier reports (27,34), did not arise from differential protein loading since Coomassie blue staining (not shown) revealed comparable loading of samples.

DISCUSSION

Sarcolemmal NHE activity in human ventricular myocytes. This study is the first to measure sarcolemmal NHE activity in ventricular myocytes from human hearts. Our data show that, after the induction of intracellular acidosis, sarcolemmal NHE activity is readily detectable in human myocytes, as has been shown to be the case in ventricular myocytes from other species (2,8,11). Interestingly, sarcolemmal NHE activity in ventricular myocytes from unused donor hearts appeared to be lower than that in ventricular myocytes from normal rat hearts, measured using the same equipment and methodology. Thus, mean $J_{H6.9}$ was 1.06 ± 0.15 mmol/L/min in five unused donor hearts (33 cells) in this study, but 2.76 ± 0.26 mmol/L/min in 37 rat cells randomly selected from those studied during an overlapping period (5). This suggests the existence of species-specific differences in the expression or regulation of the sarcolemmal NHE.

Our finding that pH$_i$ recovery from acidosis in human ventricular myocytes was inhibited by 1 μmol/L HOE-642 confirms that such recovery arose from H$^+$ efflux through the sarcolemmal NHE. Furthermore, this finding indicates that the sarcolemmal NHE of human ventricular myocytes

is indeed NHE1 since at 1 μmol/L HOE-642 is a selective inhibitor of this isoform (29). This is consistent with work by Fliegel and colleagues (35) who cloned NHE1 from a human cardiac cDNA expression library. We also found that changes in pH$_i$, induced by NH$_4$Cl pulses in the absence or presence of HOE-642, produced parallel changes in the amplitude of myocyte shortening. This is in keeping with the established importance of pH$_i$ in regulating myocardial contractility (36) and consistent with the recent observations of Ito et al. (37) on the effects of NH$_4$Cl pulses on cell shortening in rat ventricular myocytes.

Unused donor versus recipient myocardium. An interesting and potentially important finding of this study is the significantly greater sarcolemmal NHE activity of myocytes in recipient hearts with chronic end-stage heart failure relative to unused donor hearts with acute myocardial dysfunction. That the abundance of NHE1 protein was similar in ventricular tissue from unused donor hearts and recipient hearts with end-stage heart failure suggests that posttranslational mechanisms are likely to be responsible for this difference in sarcolemmal NHE activity. Although these mechanisms cannot be identified on the basis of the present findings, it is interesting to note recent evidence that the activities of protein kinase C (38) and Ca^{2+}/calmodulin dependent kinase (39) are increased in human myocardium with end-stage heart failure since both kinases have been proposed as stimulatory regulators of sarcolemmal NHE activity in rat ventricular myocytes (6–9,40).

In addition to a potential role for the kinase-mediated signaling pathways outlined above, it may be argued that an altered intracellular Na$^+$ concentration, arising from an increased NCE activity (see below), could also contribute to the greater sarcolemmal NHE activity in recipient hearts with end-stage heart failure. This is unlikely, however, since recent work in sheep Purkinje fibers (41) has shown that variation in the intracellular Na$^+$ concentration is not a physiologically important regulator of NHE activity in the heart.

Ventricular myocytes from human myocardium with end-stage heart failure exhibit varying degrees of hypertrophy (42). Therefore, the possibility that the observed difference in $J_{H6.9}$ between recipient and unused donor hearts may simply reflect a difference in the myocyte membrane surface area to volume ratio needs to be considered. In this context, a recent paper (43) has reported the first direct measurements of membrane surface area (measured by cell capacitance) and cell volume (measured by confocal microscopy) in control versus hypertrophied ventricular myocytes. The findings of that study (43), which used rat ventricular myocytes, have revealed that the membrane surface area to volume ratio remains constant over a threefold increase in cell volume, with no significant difference in this ratio between control and hypertrophied cells. Therefore, the difference in $J_{H6.9}$ observed in this study between unused donor versus recipient hearts is unlikely to be an artefact that arises from myocyte hypertrophy in the latter; instead, it is

likely to reflect a true difference in sarcolemmal NHE activity.

It is notable that, although sarcolemmal NHE activity of myocytes was significantly greater in recipient hearts with end-stage heart failure under conditions of intracellular acidosis, basal pH$_i$ was not altered. This may indicate that, at physiological values of pH$_i$ ($>$7.10), the sarcolemmal NHE of myocytes was quiescent in both recipient and unused donor hearts, as is the case in ventricular myocytes from a variety of animal species (2,8,11). Maximum NHE activity could not be determined in this study because it was not possible to lower pH$_i$ below approximately 6.70 without compromising myocyte viability.

Potential clinical relevance of findings. The NHE phenotype of healthy human myocardium is unknown and may differ from that of the unused donor hearts used in this study. However, if it is assumed that our novel data reflect increased sarcolemmal NHE activity in end-stage heart failure, then this change could have important (patho)physiological consequences. In particular, the greater sarcolemmal NHE activity of failing myocardium may increase its susceptibility to injury and dysfunction during ischemia and reperfusion, in view of the proposed role of the exchanger in this setting (see introduction). Indeed, experimental studies have suggested that failing myocardium is more susceptible to contractile dysfunction (44) and ventricular fibrillation (45) during ischemia and reperfusion. In this context, it is important to note that: (1) the mechanisms that underlie the detrimental effects of increased NHE activity during myocardial ischemia and reperfusion are thought to involve Ca^{2+} influx through NCE, operating in reverse mode (46), and (2) expression of NCE protein is increased in failing human myocardium (as shown in previous studies [27,34] and confirmed here), and this is accompanied by greater NCE activity (47). In the light of our findings, the question of whether increased NHE activity contributes to the development of heart failure also needs to be addressed, particularly in view of the in vitro data that pharmacological NHE inhibition attenuates the development of hypertrophy in response to mechanical and neurohormonal stimuli in neonatal (15) and adult (16) rat ventricular myocytes.

Our data may also have wider clinical relevance because they represent the first direct evidence that human ventricular myocytes express a functional NHE1 protein whose activity is inhibited by HOE-642 in a readily reversible manner. This NHE1-selective inhibitor was used in the recent GUARDIAN trial, whose primary objective was to determine whether NHE inhibition decreases the combined incidence of mortality and myocardial infarction (both Q-wave and non-Q-wave) in patients with acute coronary syndromes (26). The preliminary results of this trial, as presented at the 48th Scientific Sessions of the American College of Cardiology (48), have shown no significant reduction in the composite incidence of death and myocardial infarction in response to drug treatment in the overall study population. Nevertheless, with the highest dose of

HOE-642 (120 mg intravenously three times a day), there were significant reductions in the composite incidence of death and myocardial infarction in patients undergoing surgical revascularization and in the incidence of Q-wave myocardial infarction in the other patient populations (48). Although many factors may have contributed to these findings, including the presence or absence of timely reperfusion (without which NHE inhibition would not be expected to provide significant benefit [21]), it would be important to determine whether an NHE-inhibitory concentration (≥ 1 μmol/L) of HOE-642 was maintained in the circulation during the period of risk in the various study groups.

Concluding comments. Our present findings have shown that ventricular myocytes from explanted human hearts exhibit sarcolemmal NHE activity, which arises from the NHE1 isoform and is inhibited by HOE-642 in a reversible manner. Such activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in unused donor hearts with acute myocardial dysfunction. This difference in sarcolemmal NHE activity occurs in the absence of a difference in NHE1 protein expression in recipient versus donor myocardium, which suggests the involvement of posttranslational regulatory mechanisms. Identification of the relevant molecular mechanisms and determination of the functional significance of the observed difference in sarcolemmal NHE activity require further investigation.

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