

Figure 9 Mobility of the subdomains of VAP1, ADAM10, and catrocollastatin/VAP2B. (a) Superimposition of the D₂ segments of ADAM10 and VAP1. The D₂/D₁/C_w segments and helix h7 helix of VAP1 and ADAM10 are shown in blue and red, respectively. The C_n segments of VAP1 and ADAM10 are shown in cyan and pink, respectively. The arrow indicates the pivot point between the C_w and C_n segments. Bound calcium ions in VAP1 are shown as black spheres. Superposition of (b) the M domain and; (c) the C_n segments of the six structures of catrocollastatin/VAP2B and VAP1 monomer structure. Two representative catrocollastatin/VAP2B molecules are shown in blue and red, the other four catrocollastatin/VAP2B molecules are shown in gray, and VAP1 is in green. The zinc ion bound to the red molecule is shown as a yellow sphere. The calcium ions bound to the red and the blue catrocollastatin/VAP2B molecules and VAP1 are shown as red, blue, and green spheres, respectively.

domains, primarily due to a different orientation between the C_w and C_n segments (Figure 9(a)). The possibility that different ADAMs have distinct C_w/C_n orientations remains to be determined. While most of the membrane-bound ADAMs have an EGF module between the C domain and the transmembrane segment, the ADAM10 C domain is separated from the cell membrane by only 26 residues, which are likely to be disordered.⁷³

Catrocollastatin/VAP2B

The structures of catrocollastatin/VAP2B determined in three different crystal forms⁷⁷ are the first reported structures of a member of the monomeric class of MDC-domain-containing proteins of the adamalysin/reprolysin/ADAM family. The overall structure of catrocollastatin/VAP2B showed good agreement with each monomer of VAP1, and the structure of each segment was nearly identical between the molecules (Figure 9(b) and (c)). However the relative orientation of the subdomains was quite variable. Comparison of the six catrocollastatin/VAP2B monomer structures and the four VAP1 monomer structures derived from different crystal forms revealed a dynamic, modular architecture of the MDC domain of this family of proteins.⁷⁷ The largest difference was observed when the M domains of the six catrocollastatin/VAP2B molecules were superimposed (Figure 9(b)). The arm portion was rotated by approximately 13° relative to the M domain, bringing about a 15-Å displacement at the distal end of the C_n segment. A bulky hydrophobic residue (Leu, Phe, or Tyr) at position 408 is highly conserved among adamalysin/reprolysin/ADAM family proteins. The side chain of Leu408 in catrocollastatin/VAP2B functions as a universal joint (shoulder

joint) that allows the D₂ segment to adopt various orientations with respect to the M domain.⁷⁷ Intrinsic flexibility may be important for fine-tuning substrate recognition, and adjusting the spatial alignment of the catalytic and adhesion sites during the catalysis.

Structure of RVV-X

The coagulation factor X activator from Russell's viper venom (RVV-X, russelysin) is a unique metalloproteinase of the P-IV class of SVMPs that is composed of an MDC-domain-containing heavy chain and two C-type lectin-like light chains.^{10,79–81} RVV-X specifically activates factor X by cleaving the Arg194–Ile195 bond in factor X, which is also cleaved by physiological convertases.⁹⁰ Cleavage removes the heavily glycosylated amino-terminal 52 residues (active peptide, or AP) of the factor X heavy chain, which results in exposure of the active site. Activated factor X (factor Xa) in turn converts prothrombin to thrombin, which ultimately leads to formation of a hemostatic plug.

The crystal structure of RVV-X resembles a hook-spanner-wrench configuration, in which the MD domains constitute a hook, and the rest of the molecule forms a handle (Figure 10(a)).⁷⁷ The two homologous light chains have a fold similar to the carbohydrate-recognition domain (CRD) of rat mannose binding protein (MBP),⁹¹ but they form an intertwined dimer, in which the central portion of each chain projects toward the adjoining subunit. The RVV-X heavy chain has a unique cysteine residue (Cys389) in the middle of the HVR. Cys389 forms a disulfide bond with the carboxy-terminal cysteine residue of one of the light chains, light chain A (LA). In addition to

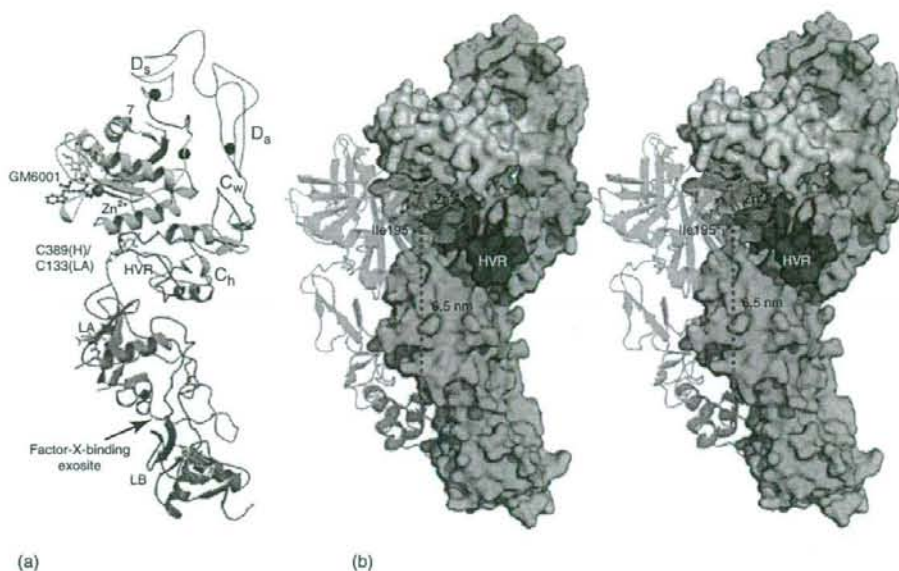


Figure 10 Structure of RVV-X and the factor-Xa docking model. (a) Crystal structure of RVV-X. Each segment of the RVV-X heavy chain is colored as described for VAP1 in Figure 2. Light chain A and B are colored in orange and magenta, respectively. The disulfide bridge between Cys389 in the heavy chain and Cys133 in light chain A, the carbohydrate moieties (in green) linked to asparagine residues, and GM6001 (in magenta) are shown in ball-and-stick representations. Bound calcium and zinc ions are represented as black and red spheres, respectively; (b) factor Xa docking model in stereo. The molecular surface of RVV-X is colored as for (a). Factor Xa is shown in ribbon representation. Ile195 (in stick representation) and the amino-terminal region of the factor Xa heavy chain are shown in magenta. Because the structure of the factor X zymogen is currently unavailable, the model was constructed on the basis of the factor-Xa crystal structures (1XKA and 1IOD).⁷⁸ In the factor Xa structure, the amino terminus of the heavy chain is buried within the protein. However, in the zymogen, the intact scissile peptide bond (Arg194–Ile195) must be situated on the molecular surface, as in the equivalent segments of other serine proteinase zymogen structures.

this interchain disulfide bond, the HVR and surrounding residues are engaged in multiple hydrophobic interactions and hydrogen bonds, which further stabilize the continuous C_h/LA structure. The RVV-X structure represents the first example of HVR-mediated protein–protein interactions in the adamalysin/reprolysin/ADAM family proteins.

The structure of the RVV-X light chains is quite similar to that of the factor X-binding protein (X-bp) from *Deinagkistrodon actus* venom, as determined in complex with the γ -carboxyglutamic acid (Gla) domain of factor X.⁹² This structural similarity and the previous biochemical observations⁸¹ suggest that the concave cleft created between the two light chains in RVV-X may function as an exosite for factor X. A 6.5 nm separation between the catalytic site and a putative Gla-domain-binding exosite suggests the docking model for factor X (Figure 10(b)).⁷⁸ The active-site zinc atom and Ile195 of factor Xa are 16 Å apart because the two molecules are positioned as rigid bodies without any contacts. Intrinsic flexibility between the segments of the RVV-X heavy chain, and conformational changes upon association of RVV-X and factor X zymogen, may allow the catalytic

site of RVV-X to interact directly with the bond between Arg194–Ile195 of factor X in solution. The relatively large separation between the catalytic site and the exosite may explain the high specificity of RVV-X for factor X.

The VAP1 structure suggests a model in which the HVR constitutes an exosite that captures target or associated proteins that are then processed by the catalytic site.⁴⁹ The RVV-X structure⁷⁸ is consistent with this model and provides additional insights into the molecular basis of target recognition and proteolysis by ADAM/adamalysin/reprolysin proteinases. The fold adaptation of the RVV-X structure is also a good example of evolutionary gain of function by multi-subunit proteins for the binding of ligands.

FUNCTIONAL ASPECTS

C-domain-mediated protein–protein interactions

ADAM family proteins are widely distributed and constitute major membrane-bound sheddases that are involved in the proteolysis of cell-surface-protein ectodomains for

cell–cell communications. As such, they have emerged as potential therapeutic targets for various disease conditions. The P-III SVMPs are the most potent hemorrhagins and constitute key toxins in venom-induced pathologies. Thus, they are important targets for antivenom therapeutics. However, the physiological targets of ADAMs and SVMPs and the molecular mechanism of target recognition are poorly understood. The structures of VAP1 and related proteins highlight the potential roles of the C domain in protein–protein interactions.

There is mounting experimental evidence that C domains mediate protein–protein interactions. Peptides encompassing the HVR and the hydrophobic ridge from jararhagin and atrolysin-A (P-III SVMPs from *C. atrox* venom) interfere with platelet interaction and collagen binding.⁹³ Catrocollastatin-C and jararhagin-C, which are proteolytic products (DC-domain fragments) of catrocollastatin/VAP2B and jararhagin, respectively, have been shown to inhibit collagen-induced platelet aggregation.^{94,95} Jararhagin-C also activates early events in the inflammatory response, such as leukocyte rolling and proinflammatory cytokine release.⁹⁶ Recombinant atrolysin-A C domain (A/C) specifically binds collagen type I and von Willebrand factor (vWF), and blocks collagen–vWF interactions.^{97,98} It also binds to the von Willebrand factor A (VWA)-domain-containing extracellular matrix (ECM) proteins collagen XII and XIV, and matrilins 1, 3, and 4.⁹⁹ Binding of vWF in solution to immobilized A/C was inhibited by ristocetin, and preincubation of platelets with A/C abolished ristocetin/vWF-induced platelet aggregation, which indicates that the interaction of A/C with vWF is mediated by the VWA1 domain.¹⁰⁰ Two peptide sequences have been identified in the C domain of jararhagin that bind to vWF and block C-domain binding.¹⁰¹ On the basis of the structures of VAP1 and catrocollastatin/VAP2B, the Jar6 peptide (corresponding to catrocollastatin/VAP2B residues 547PCAPEDVKCG⁵⁵⁶) is located on the surface of the C domain, and thus could play a role in protein–protein interactions. ADAM12 interacts with cell-surface syndecan through its C domain and mediates integrin-dependent cell spreading.³⁷ The DC domain of ADAM13 has been implicated in cell migration events,¹⁰² and binds to the ECM proteins laminin and fibronectin.³⁸ It should be noted, however, that most of these studies do not identify a specific region of the C domain that is involved in these interactions, and so the molecular mechanisms of recognition remain to be elucidated.

Several studies have indicated that the ADAMs' C domain can influence proteolytic activity. The C domain of ADAM13 was found to be the major determinant of specific developmental events that are mediated by the proteolytic activity of ADAM13.¹⁰³ Ectodomain shedding of interleukin-1 R-II by ADAM17 (TACE) requires the DC domains, whereas tumor necrosis factor (TNF) and p75 tumor necrosis factor receptor (TNFR) shedding by ADAM17 requires only the tethered M domain.¹⁰⁴ The

acidic surface pocket in the ADAM10 C domain serves as a binding site for the ephrin-A5/EphA3 complex in ADAM10-mediated ephrin-A5 proteolysis.²² Jararhagin cleaves vWF at sites adjacent to the VWA1 domain, and digestion is completely inhibited by the A/C and catrocollastatin-C.¹⁰⁰

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Activation of Na⁺/H⁺ Exchanger 1 Is Sufficient to Generate Ca²⁺ Signals That Induce Cardiac Hypertrophy and Heart Failure

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Abstract—Activation of the sarcolemmal Na⁺/H⁺ exchanger (NHE)1 is increasingly documented as a process involved in cardiac hypertrophy and heart failure. However, whether NHE1 activation alone is sufficient to induce such remodeling remains unknown. We generated transgenic mice that overexpress a human NHE1 with high activity in hearts. The hearts of these mice developed cardiac hypertrophy, contractile dysfunction, and heart failure. In isolated transgenic myocytes, intracellular pH was elevated in Hepes buffer but not in physiological bicarbonate buffer, yet intracellular Na⁺ concentrations were higher under both conditions. In addition, both diastolic and systolic Ca²⁺ levels were increased as a consequence of Na⁺-induced Ca²⁺ overload; this was accompanied by enhanced sarcoplasmic reticulum Ca²⁺ loading via Ca²⁺/calmodulin-dependent protein kinase (CaMK)II-dependent phosphorylation of phospholamban. Negative force–frequency dependence was observed with preservation of high Ca²⁺, suggesting a decrease in myofibril Ca²⁺ sensitivity. Furthermore, the Ca²⁺-dependent prohypertrophic molecules calcineurin and CaMKII were highly activated in transgenic hearts. These effects observed in vivo and in vitro were largely prevented by the NHE1 inhibitor cariporide. Interestingly, overexpression of NHE1 in neonatal rat ventricular myocytes induced cariporide-sensitive nuclear translocation of NFAT (nuclear factor of activated T cells) and nuclear export of histone deacetylase 4, suggesting that increased Na⁺/H⁺ exchange activity can alter hypertrophy-associated gene expression. However, in transgenic myocytes, contrary to exclusive translocation of histone deacetylase 4, NFAT only partially translocated to nucleus, possibly because of marked activation of p38, a negative regulator of NFAT signaling. We conclude that activation of NHE1 is sufficient to initiate cardiac hypertrophy and heart failure mainly through activation of CaMKII–histone deacetylase pathway. (*Circ Res.* 2008;103:891–899.)

Key Words: Na⁺/H⁺ exchanger ■ Na⁺ and Ca²⁺ overload ■ cardiac remodeling ■ CaMKII–HDAC pathway
■ calcineurin–NFAT pathway

Intracellular Na⁺ levels are regulated by a network of ion channels and transporters.¹ In the myocardium, Na⁺ homeostasis is closely linked to intracellular Ca²⁺ handling via the Na⁺/Ca²⁺ exchanger (NCX), the principal mechanism for Ca²⁺ efflux from cardiomyocytes. Na⁺ dysfunction alters Ca²⁺ homeostasis, thereby contributing to the pathogenesis of heart failure (HF) in animal models and in humans. The sarcolemmal Na⁺/H⁺ exchanger (NHE)1 is a major Na⁺ influx pathway that also serves as a powerful acid extrusion system. NHE1 couples H⁺ efflux to Na⁺ influx in a 1:1 stoichiometry under the driving force of a Na⁺ gradient formed by the Na⁺ pump. Thus, enhanced NHE activity leads to elevated intracellular Na⁺ concentration ([Na⁺]_i) and cytoplasmic alkalinization. NHE1 activity is controlled by intracellular pH (pH_i) and numerous other factors, such as hormones, catecholamines, and mechanical stimuli, known to be

associated with a failing heart. NHE1 activity has been implicated in myocardial ischemia and reperfusion injury,² and NHE1 inhibition was reported to protect against these injuries in animal models and in patients undergoing coronary interventions.³ However, in other cardiac disease states such as cardiac remodeling process, the role of NHE1 is not fully understood. It has been proposed that enhanced myocardial NHE1 activity is partially responsible for the cardiac remodeling observed in animal models such as guanylyl cyclase-A knockout (GC-A KO) mice,⁴ β₁-adrenergic receptor–overexpressing transgenic (Tg) mice,⁵ and spontaneously hypertensive rats.⁶ However, many signal transduction pathways are activated in these models; therefore, whether activation of NHE1 alone is sufficient to induce hypertrophy remains unknown. We also lack detailed understanding of the molecular and cellular events, including altered intracellular Na⁺

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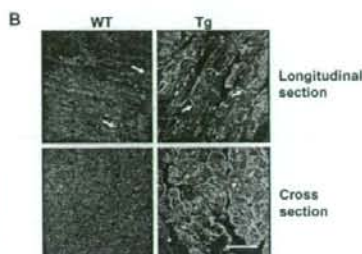
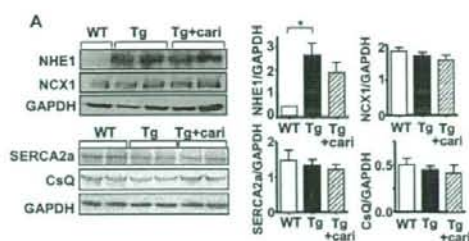


Figure 1. Overexpression of NHE1 protein in Tg hearts. **A**, Immunoblot analysis of NHE1 and other Ca^{2+} regulatory proteins normalized to endogenous GAPDH from WT, untreated, and cariporide-treated Tg hearts ($n=7$ hearts for each group). Two typical examples are shown in the each blot. **B**, Cellular localization of NHE1 pro-

teins. Longitudinal and cross-sections of cardiac muscle from WT and Tg mice were immunostained with anti-NHE1 antibody. Arrows indicate the NHE1 localized to the intercalated disks. Scale bar=100 μm .

and/or Ca^{2+} handling and the activation of signaling pathways that result from NHE1 activation.

To directly address these questions, we generated Tg mice that overexpress an activated form of human NHE1 that lacks the calmodulin-binding inhibitory domain ($\Delta 637$ to 656)⁷ under the control of cardiac α -myosin heavy chain promoter. Previously, we reported that deletion of this domain resulted in constitutive elevation of pH_i sensitivity in quiescent cells.^{7,8} Here, we asked whether overexpressing this mutant form of NHE1 would affect development of cardiac hypertrophy and/or HF in vivo and investigated which signal transduction pathways were involved in NHE1-dependent cardiac pathogenesis. We present evidence that NHE1 serves as an important signal mediator in initiating cardiac remodeling and HF via activation of a Ca^{2+} -dependent hypertrophic pathway.

Materials and Methods

All animal experiments were carried out according to guidelines of the Animal Welfare Committee of the National Cardiovascular Center Research Institute. Tg mice that overexpressed an activated form of NHE1 (human NHE1 with amino acids 637 to 656 deleted) were generated from C57BL/6J mice according to standard procedures; the expression of the transgene was under control of the murine cardiac α -myosin heavy chain promoter. The pH_i , $[\text{Na}^+]_i$, and Ca^{2+} transients in isolated adult myocytes were measured in Hefes-

or bicarbonate-buffered Tyrode solutions by epifluorescent analysis [acetoxymethyl forms of 2',7'-bis (carboxy-ethyl)-5- (and -6)-carboxyfluorescein (BCECF), sodium-binding benzofuran isophthalate (SBFI), and Indo-1, respectively] using an imaging system (AQUACOSMOS, Hamamatsu Photonics). Data are presented as means \pm SD or SEM of at least 3 determinations. We used the paired or unpaired *t* test for statistical analyses. Values of $P < 0.05$ were considered statistically significant (indicated as * versus wild-type [WT] or † versus no-cariporide control, or as otherwise indicated). An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Results

Overexpression of Activated NHE1 Induces Cardiac Hypertrophy and HF In Vivo

We confirmed a marked increase (≈ 5 -fold) in NHE1 protein levels in Tg hearts by immunoblot analysis (Figure 1A). Immunofluorescence analysis of tissue sections revealed that whereas WT hearts expressed low but detectable levels of NHE1 localized to intercalated discs, Tg hearts expressed much higher levels of NHE1 in both intercalated discs and sarcolemma (Figure 1B).

Overexpression of NHE1 in vivo resulted in cardiac hypertrophy and dilated cardiomyopathy; the Tg mouse hearts had thinner ventricular walls and greater chamber

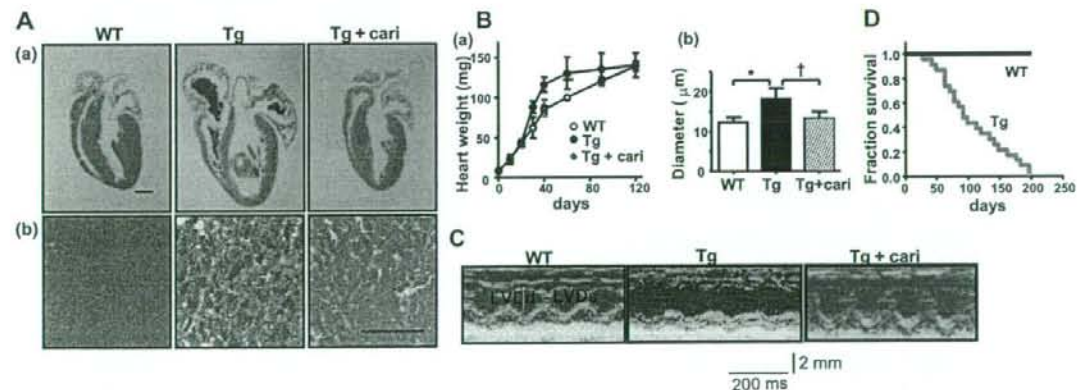


Figure 2. Overexpression of activated NHE1 results in cariporide-preventable cardiac hypertrophy, fibrosis, and HF. **A**, Cardiac sections stained with Masson trichrome. Scale bars: 1 mm (a) and 100 μm (b). **B**, Quantitative analysis of heart weight during development (a) and cardiomyocyte diameters (b) in WT, untreated, and cariporide-treated Tg hearts ($n=5$ separate hearts). **C**, Representative echocardiography obtained from each group. LVdD and LVdS indicate left ventricular dimension at diastole and systole, respectively. **D**, Kaplan-Meier survival analysis of WT and Tg mice. All results were obtained from 40-day-old mice, except in B (a) and D.

Table. Histological and Echocardiographic Analyses of Left Ventricular Dimensions and Function

	WT	Tg	Tg+ Cariporide
Histopathology			
IVSW, mm	1.21±0.03	0.75±0.12*	1.29±0.36†
LVFW, mm	1.18±0.03	0.83±0.08*	1.35±0.32†
LV area, mm ²	1.95±0.30	6.07±0.84*	3.18±0.62†
Echocardiography			
LVDd, mm	2.44±0.15	3.27±0.46*	2.30±0.32†
LVDs, mm	0.56±0.02	1.98±0.04*	0.82±0.02†
% FS	77.8±8.0	33.0±5.3*	64.4±7.2†

IVSW indicates interventricular septal wall; LVFW, left ventricular free wall; LV area, LV surface area measured in short axis at widest section; LVDd, LV dimension at diastole; LVDs, LV dimension at systole; FS, fractional shortening. Values are means±SD; n=5–8 separate hearts for each group. **P*<0.05 vs WT; †*P*<0.05 vs untreated Tg mice.

dilation accompanied by ventricular fibrosis (Figure 2A, supplemental Figure IB and the Table). We detected the onset of progressive hypertrophy in Tg hearts beginning at the age of 20 days (Figure 2B, a), with outstanding enlargement of the atria (supplemental Figure I A). Cardiomyocyte diameters in Tg mice were significantly larger than those in WT mice (Figure 2B, b). These results suggest that each myocyte became bigger at approximately 40 days of age, but they progressively died, as evidenced by chamber dilation. Echocardiography of Tg mice at 40 days of age showed increased diastolic and systolic ventricular diameters and decreased systolic function, which was demonstrated by a decrease in the percentage of fractional shortening (Figure 2C and the Table). Markers of HF, atrial natriuretic peptide, and cardiac troponin I in serum were also increased in Tg mice (supplemental Figure IC and ID), indicating that their hearts had developed HF. Tg mice had a high mortality rate

(Figure 2D) and showed cardiac arrhythmia; this was particularly so for older mice (data not shown). However, all cardiac remodeling events observed in Tg mice were largely prevented by IP administration of cariporide for 20 days (from the age of 20 to 40 days; Figure 2, supplemental Figure I, and the Table) without significant change in NHE1 protein levels (Figure 1A), suggesting that these phenomena resulted from elevated NHE1 activity.

Overexpression of NHE1 did not significantly change the expression levels of other Ca²⁺ regulatory proteins (NCX1, sarcoplasmic reticulum Ca²⁺-pump [SERCA2a], or calsequestrin [CsQ]) with or without cariporide treatment (Figure 1A). In addition, expression of Na⁺/K⁺-ATPase was also unchanged (WT, 0.36±0.04; Tg, 0.35±0.05; and Tg+cariporide, 0.40±0.07; n=3 hearts). As predicted from our previous work,⁹ we detected a marked increase in the expression of calcineurin homologous protein (CHP)1, an obligatory subunit of NHE family members (supplemental Figure IIA),¹⁰ concomitant with the high level of NHE1 expression in Tg mice.

Increased Exchange Activity and [Na⁺]_i in Cardiomyocytes from Tg Mice

We measured pH_i and [Na⁺]_i in freshly isolated ventricular myocytes from WT and Tg hearts. Tg myocytes appeared to be more vulnerable to collagenase treatment and mechanical stress than WT myocytes. Surviving Tg myocytes were larger than WT myocytes (Figure 3A), similar to our observations in whole-heart sections (Figure 2B). In addition, the pH_i recovery of Tg myocytes after acid loading that was cariporide-inhibitable was faster than that of WT myocytes (Figure 3B). Consistent with our previous data using this NHE1 mutant,^{7,8} the pH_i dependence of the H⁺ efflux rate (*J*_H) was alkaline-shifted in Tg myocytes (Figure 3C) after correction for the intracellular H⁺ buffering power, which was similar between

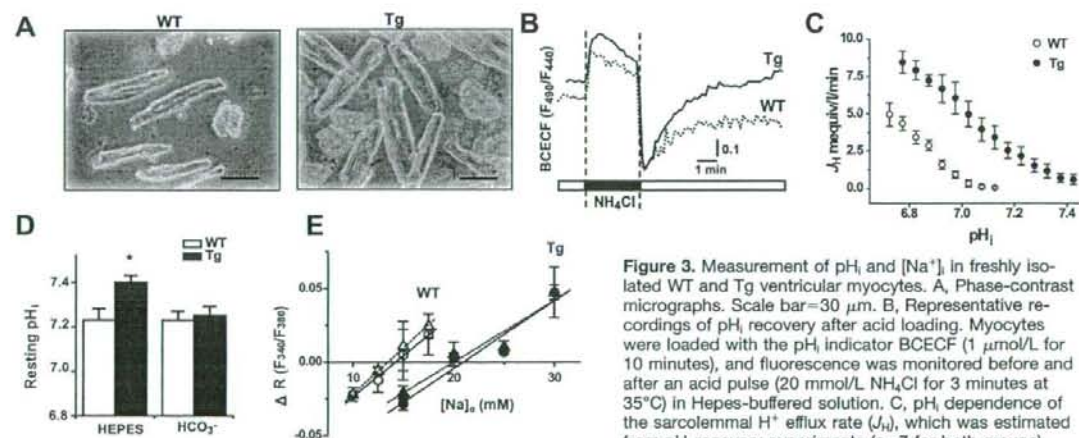


Figure 3. Measurement of pH_i and [Na⁺]_i in freshly isolated WT and Tg ventricular myocytes. **A**, Phase-contrast micrographs. Scale bar=30 μm. **B**, Representative recordings of pH_i recovery after acid loading. Myocytes were loaded with the pH_i indicator BCECF (1 μmol/L for 10 minutes), and fluorescence was monitored before and after an acid pulse (20 mmol/L NH₄Cl for 3 minutes at 35°C) in HEPES-buffered solution. **C**, pH_i dependence of the sarcolemmal H⁺ efflux rate (*J*_H), which was estimated from pH_i recovery experiments (n=7 for both groups).

D, Resting pH_i in HEPES- or bicarbonate-buffered Tyrode solutions was obtained by calibration (n=16 to 19 cells from 6 hearts for each group [see also supplemental Figure IV]). **E**, Evaluation of resting [Na⁺]_i in HEPES-buffered (dotted lines) or bicarbonate-buffered (solid lines) solution in WT (open symbols) and Tg myocytes (closed symbols), using the Na⁺-specific fluorophore SBFI and the null-point approach. Average changes in SBFI ratio (ΔR) vs [Na⁺]_i were fitted, and linear regressions for all points provided the resting [Na⁺]_i (n=25 and 12 cells for each point from 4 Tg and 4 WT hearts, respectively).

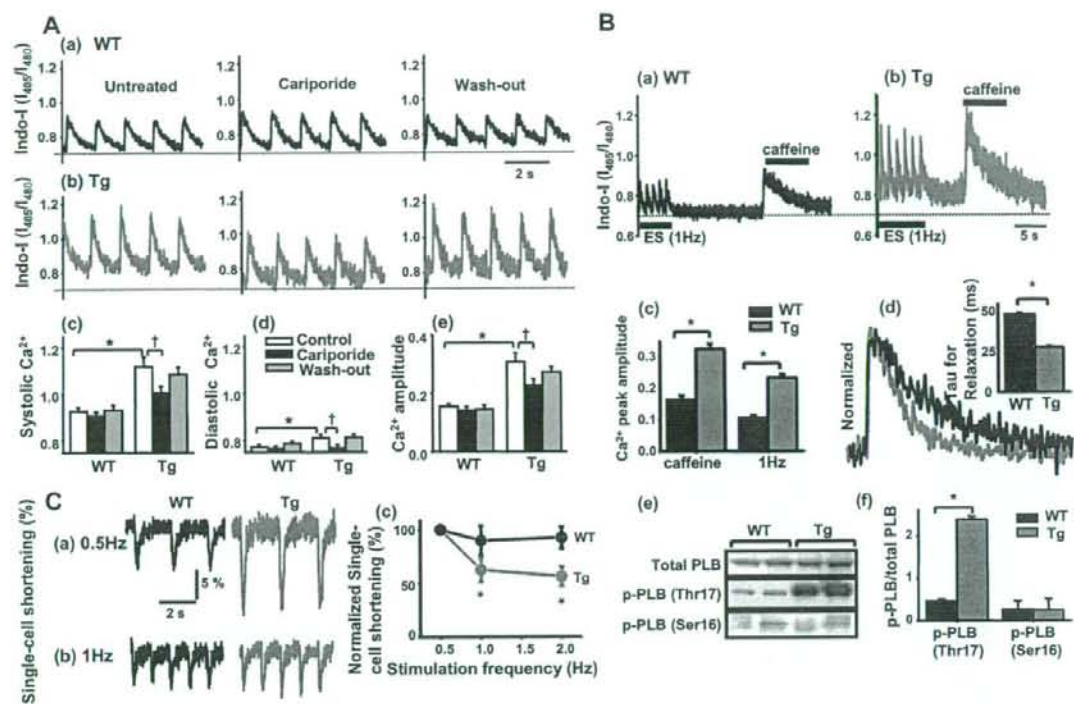


Figure 4. Comparison of the intracellular Ca^{2+} levels, SR Ca^{2+} content, and the amount of single-cell shortening between WT and Tg myocytes. **A**, Intracellular Ca^{2+} transient and acute effects of cariporide. Representative traces of the Indo-1 ratio in single cardiomyocytes stimulated at 1 Hz from WT (a) and Tg mice (b) under baseline conditions (left), 2 minutes after cariporide ($1 \mu\text{mol/L}$) treatment (middle), and 2 minutes after drug washout (right). **c** through **e**, Average data representing the systolic (c), diastolic (d), and peak amplitude (e) of Ca^{2+} transients for each condition ($n=26$ and 18 cells from 3 WT and 3 Tg hearts, respectively). **B**, **A** through **c**, Estimation of SR Ca^{2+} content. Indo-1-loaded, paced (1 Hz) cardiomyocytes were briefly exposed to 10 mmol/L caffeine to monitor SR Ca^{2+} release. Representative traces for WT myocytes (a), Tg myocytes (b), and the averaged data (c) ($n=10$ and 14 cells from 3 WT and 3 Tg hearts, respectively). **d**, Faster Ca^{2+} decline in Tg myocytes. Ca^{2+} transients (0.5 Hz) were normalized and the time constants (τ) were obtained by fitting the decline phase with the first-order exponential decay (inset) ($n=21$ and 52 transients from 3 WT and 3 Tg hearts, respectively). **e** and **f**, PLB in Tg hearts was highly phosphorylated by CaMKII but not by PKA. Representative immunoblots of total and phosphorylated PLBs (e) and normalized phosphorylated levels (f) ($n=4$ independent blots from 2 WT and 2 Tg hearts for all conditions). **C**, Percentage of single-cell shortening elicited by different stimulation frequencies, 0.5 Hz (a) and 1 Hz (b). **c**, Each measurement made at a different stimulation frequency was normalized to the maximal value and then averaged ($n=7$).

WT and Tg myocytes (supplemental Figure III). In addition, the resting pH_i was significantly higher in Tg myocytes under bicarbonate-free conditions (pH_i 7.40 versus pH_i 7.23 in WT; Figure 3D), indicating that overexpressing the active NHE1 mutant led to elevated NHE activity. However, under bicarbonate-buffered conditions, the pH_i was not significantly different between WT and Tg myocytes (Figure 3D, see also supplemental Figure IV), suggesting that pH_i was compensated by bicarbonate-dependent mechanisms. According to the pH dependence of J_{Na} , we can assume that the steady-state exchange activity would be higher in Tg than WT myocytes at the given resting pH_i under physiologically relevant bicarbonate conditions. Indeed, fluorescence measurements using the null-point method¹¹ revealed that $[\text{Na}^+]_i$ was almost 1.5-fold higher in Tg myocytes (17 to 21 mmol/L) than in WT myocytes (12 to 13 mmol/L) under both bicarbonate- and HEPES-buffered conditions (Figure 3E). Treatment with cariporide resulted in a rapid reduction of $[\text{Na}^+]_i$ in Tg myocytes, which reached the value close to the WT myocytes (supple-

mental Figure V). Thus, elevation in $[\text{Na}^+]_i$, rather than pH_i , would be the important event caused by NHE1 activation under physiological conditions.

Tg Myocytes Exhibited Increased Ca^{2+} Levels in the Cytoplasm and Sarcoplasmic Reticulum

As shown in Figure 4A, at 1-Hz stimulation, both diastolic and systolic Ca^{2+} levels were significantly higher in Tg than in WT cardiomyocytes (179 ± 22.7 and 486 ± 18.4 nmol/L in WT and 263 ± 15.8 and 1142 ± 41 nmol/L in Tg myocytes, respectively); Ca^{2+} amplitude (difference between the diastolic and systolic Ca^{2+} levels) was also 2-fold higher in Tg myocytes. Treatment with $1 \mu\text{mol/L}$ cariporide for 2 minutes, which led to 70% to 80% inhibition of NHE1 activity (data not shown), had little effect on Ca^{2+} transients in WT cardiomyocytes, whereas all parameters (diastolic, systolic, and Ca^{2+} amplitude) were at least partly decreased in Tg cardiomyocytes. Two-minute washout reversed the effects of cariporide, suggesting that the increase in intracellular Ca^{2+}

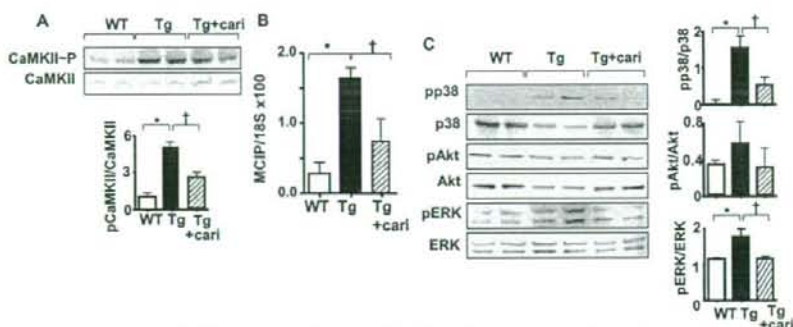


Figure 5. Activation of CaMKII, calcineurin, and p38 signaling pathways related to cardiac hypertrophy in Tg mice. A and C, Relative amounts of phosphorylated CaMKII, p38, Akt, and ERK42/44 were normalized to total protein. B, mRNA level of modulatory calcineurin inhibitory protein-1 (MCIP1), which was used as an indicator of activated calcineurin, was quantified by quantitative RT-PCR (n=7 hearts).

levels in Tg myocytes was attributable to increased NHE1 activity. Furthermore, we also detected marked elevations in diastolic and systolic Ca^{2+} levels at more physiologically relevant stimulation frequencies (up to 3.3 Hz), especially in Tg myocytes; Ca^{2+} amplitude was also significantly higher in Tg myocytes at all stimulation frequencies tested (supplemental Figure VI).

Furthermore, the peak amplitude of the caffeine-induced Ca^{2+} transient, which we used as an index of sarcoplasmic reticulum (SR) Ca^{2+} content, was ≈ 2 -fold higher in Tg versus WT myocytes (Figure 4B, a through c), suggesting that this is one of the mechanisms of increased $[Ca^{2+}]_i$ in Tg myocytes. The rate of decline in the field stimulation-elicited Ca^{2+} transient was significantly faster in Tg than in WT myocytes (Figure 4B, d): the time constant (τ) was reduced to $\approx 60\%$ (inset), indicating accelerated Ca^{2+} removal, mainly by SR Ca^{2+} pumping. In addition, the Ca^{2+} transient upstroke was faster in Tg myocytes than in WT myocytes (supplemental Figure VII), suggesting that the rate of Ca^{2+} release from the SR may also be higher in Tg myocytes. Ca^{2+} /calmodulin-dependent protein kinase (CaMK)II-dependent Thr17 phosphorylation of phospholamban (PLB), a Ca^{2+} pump regulatory protein, was almost 5-fold greater in Tg hearts than in WT hearts, yet no difference was detected at Ser16, a protein kinase (PKA) phosphorylation site (Figure 4B, e and f), strongly suggesting that CaMKII, but not PKA, is activated in Tg hearts and induces PLB phosphorylation, leading to enhanced SR Ca^{2+} loading and subsequent increase in cytoplasmic Ca^{2+} levels.

Effects on Single-Cell Shortening

We measured single-cell shortening in the same myocyte from which the Ca^{2+} transient was obtained, stimulated over a range of frequencies (0.5–2.0 Hz). Interestingly, we observed a clear frequency-dependent decrease in contractility in Tg myocytes after normalizing to the maximal value, although the actual single-cell shortening (percentage) was greater in the Tg than in the WT group at low stimulation frequency (0.5 Hz; Figure 4C). In addition, we observed that Tg myocytes were more susceptible to high frequency stimulation-induced cytotoxicity (supplemental Figure VIII). These results indicate that contractile dysfunction occurs at the cellular level in Tg myocytes.

Activation of Prohypertrophic Molecules in Tg Mice

We assessed the activity of 2 Ca^{2+} -dependent prohypertrophic signaling molecules, CaMKII and calcineurin,¹² in Tg hearts. The level of phosphorylated CaMKII was markedly increased (Figure 5A), consistent with increased CaMKII-dependent phosphorylation of PLB (Figure 4B, e and f). In addition, the amounts of mRNA (Figure 5B) and protein (supplemental Figure IIB) of MCIP1 (modulatory calcineurin inhibitory protein-1), which was used as a sensitive indicator of calcineurin activity, was significantly elevated in Tg hearts. Cariporide largely prevented both effects, suggesting that both CaMKII and calcineurin activation were NHE1-dependent. Furthermore, we detected a marked increase in the phosphorylation of p38, as well as a slight but significant increase in the phosphorylated active forms of extracellular signal-regulated kinase (ERK)42/44 mitogen-activated protein kinases (MAPKs), which were both largely reversed by cariporide treatment. Akt also appeared to be slightly activated in Tg hearts, although this was not statistically significant (Figure 5C).

NHE1-Dependent Translocation of HDAC and NFAT

Calcineurin is known to induce nuclear translocation of the NFAT family of transcription factors during hypertrophy via its dephosphorylation,¹³ whereas CaMKII has been reported to induce nuclear export of histone deacetylase (HDAC)4 by phosphorylating it and thereby promote cardiomyocyte hypertrophy by blocking HDAC-dependent inhibition of target gene transcription.¹⁴ To determine whether these pathways were activated by NHE1 overexpression, we treated primary cultured neonatal rat ventricular myocytes (NRVMs), which are often used as a model system of pathological hypertrophy, with a hypertrophic stimulus from phenylephrine and found that it induced a clear translocation of HDAC4 from the nucleus to the cytoplasm in these cells (supplemental Figure IX). Strikingly, overexpression of NHE1 also triggered translocation of HDAC4–green fluorescent protein (HDAC4-GFP) from the nucleus to the cytoplasm (Figure 6A, a) to an extent similar to that detected in phenylephrine-treated myocytes (Figure 6A, c) and redistributed from the cytoplasm to the nucleus on treatment with cariporide (10 μ mol/L for at least 3 hours; Figure 6A shows the same myocyte before and

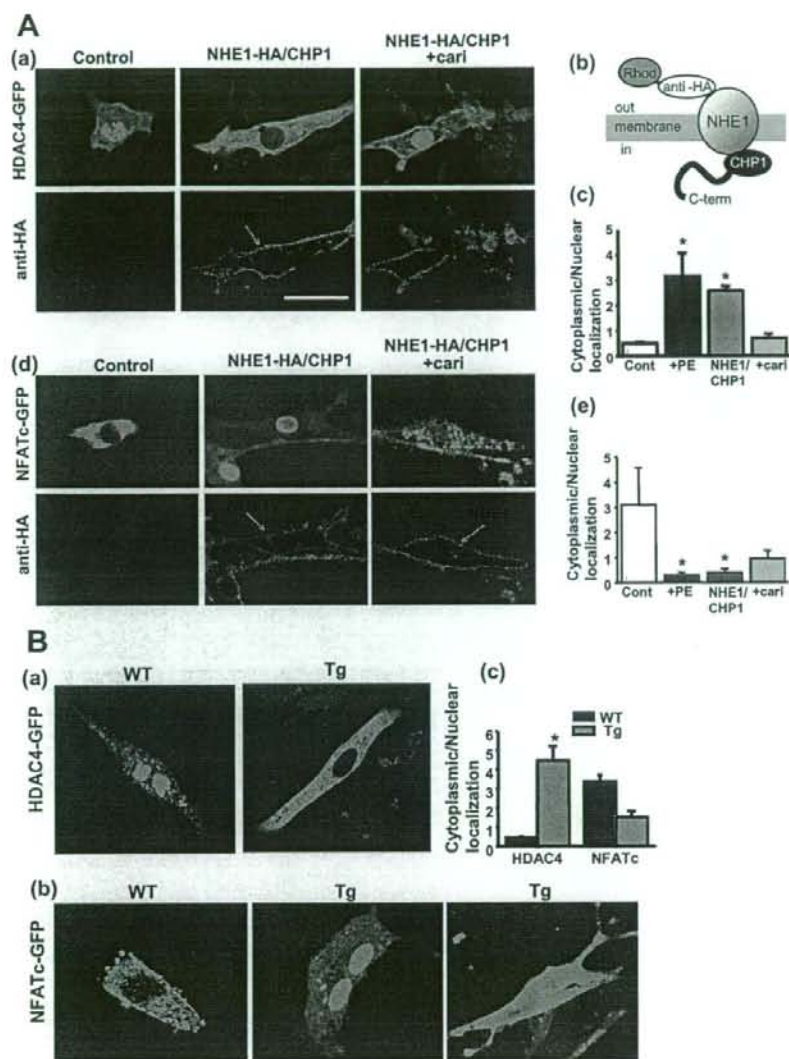


Figure 6. NHE1-dependent subcellular localization of HDAC4 and NFAT. **A**, through **c**, Primary cultured NRVMs were cotransfected with HDAC4-GFP and NHE1-HA (tagged extracellularly) plus its obligatory subunit CHP1 (NHE1-HA/CHP1) or an empty vector. Two days later, NHE1-overexpressing living myocytes were visualized by indirect immunofluorescence (red), and the subcellular localization of HDAC4-GFP was quantified by confocal microscopy (green). Positions of some NHE1-overexpressing cells were marked, and then the same myocyte was chased after cariporide application (10 μ mol/L, \approx 3 hours). **d** and **e**, Similar experiments were performed for NFAT-GFP (* P <0.05 vs control). **B**, Tg myocytes exhibit full activation of the CaMKII-HDAC pathway but only partial activation of the calcineurin-NFAT pathway. Neonatal WT and Tg myocytes cultured for 2 days were transfected with HDAC4-GFP or NFATc-GFP. The subcellular localization of GFP in cardiomyocytes was examined 2 days later ($n \geq 20$ cells from 3 independent preparations). Scale bar = 20 μ m.

after cariporide treatment). Similar NHE1-dependent cytoplasmic accumulation of HDAC4 was observed for endogenous HDAC4 (supplemental Figure IXB). These results strongly suggest that overexpression of NHE1 promotes CaMKII-HDAC signaling. Furthermore, overexpression of NHE1 resulted in nuclear import of NFAT-GFP from the cytoplasm, as observed in the phenylephrine-treated group; this was partially reversed by cariporide treatment (Figure 6A, d and e). These results suggest that overexpression of NHE1 activated the calcineurin-NFAT pathway.

We performed similar experiments in primary cultured mouse ventricular myocytes from WT and Tg hearts. As shown in Figure 6B, HDAC4-GFP accumulated in the cytoplasm of Tg myocytes, whereas it was localized to the nucleus in WT myocytes, as was the case for the NHE1-

overexpressing NRVMs (see also supplemental Figure IXC). On the other hand, NFAT-GFP localized to the cytoplasm in WT cells, but in Tg myocytes, its localization was somewhat intermediate: some localized to the nucleus and some to the cytoplasm (Figure 6B, b and c, for averaged data). These results indicate that the CaMKII-HDAC pathway is fully activated but that the calcineurin-NFAT pathway is only partially activated in Tg myocytes, suggesting that a more complex regulatory mechanism exists in Tg hearts.

Discussion

Despite accumulating evidence of a pathological role for NHE1 in cardiac hypertrophy, remodeling, and HF, it was not known whether direct activation of NHE1 itself could induce

these states. Here, we demonstrated that NHE1 activation is sufficient to initiate these cardiac disease states via activation of Ca^{2+} -dependent prohypertrophic signaling pathways and possibly via acceleration of Ca^{2+} -induced cell death. This conclusion is supported by a number of novel findings from our mouse model: (1) overexpression of an activated form of NHE1 ($\Delta 637$ to 656) resulted in cardiac hypertrophy followed by dilated cardiomyopathy *in vivo*; (2) in isolated Tg myocytes, $[\text{Na}^+]_i$ was increased because of enhanced Na^+/H^+ exchange activity, and both diastolic and systolic Ca^{2+} levels were significantly elevated but with less contractility at higher stimulation frequencies; (3) such remodeling was accompanied by activation of Ca^{2+} -dependent prohypertrophic pathways CaMKII-HDAC and calcineurin-NFAT, as well as p38 and ERK42/44 MAPKs; and (4) these effects observed *in vivo* and *in vitro* were largely prevented by cariporide. Because NHE1 is activated by numerous extracellular stimuli, these findings support the view that NHE1 plays an important role in transducing extracellular stimuli into intracellular Ca^{2+} signals, and therefore has a great impact on cardiac pathogenesis.

Elevated $[\text{Ca}^{2+}]_i$ in Tg Myocytes: Mechanisms and Consequences

The increased resting pH_i and accelerated net H^+ efflux under Hepes-buffered conditions provided direct evidence for enhanced Na^+/H^+ exchange activity in Tg myocytes. Furthermore, we observed that $[\text{Na}^+]_i$ were elevated in Tg myocytes under both bicarbonate-buffered and bicarbonate-free conditions, although no significant changes in pH_i were detected between WT and Tg myocytes under bicarbonate buffer. These findings suggest that the increased $[\text{Na}^+]_i$ rather than pH_i would be an initial signal to trigger the phenotypic changes in myocytes, consistent with previous reports demonstrating the relative importance of increased $[\text{Na}^+]_i$ in cardiac hypertrophy and HF.¹⁵⁻¹⁸ Although the expression level of NCX1 protein was unchanged in our Tg hearts, increased $[\text{Na}^+]_i$ would have influenced the driving force and hence the activity of the NCX by decreasing the forward (Ca^{2+} efflux) and/or increasing the reversed mode (Ca^{2+} influx), resulting in increased $[\text{Ca}^{2+}]_i$, as reported previously.¹⁸ In fact, we observed that both systolic and diastolic Ca^{2+} levels were significantly increased in Tg myocytes and that these levels were further pronounced at higher stimulation frequencies. Previous reports showed that $[\text{Na}^+]_i$ in failing myocardium, which is significantly higher than that in non-failing myocardium, increases more in response to a higher stimulation frequency in both rats and humans.^{19,20} Rapid stimulation-induced membrane depolarization can further activate the reverse mode of NCX1 and promote diastolic Ca^{2+} overload.

Furthermore, we present evidence of altered SR Ca^{2+} handling in Tg hearts, which involved CaMKII-dependent phosphorylation of PLB and subsequent activation of SERCA2a, leading to a higher rate of SR Ca^{2+} pumping. In addition to the higher SR Ca^{2+} content, CaMKII-induced phosphorylation and activation of the ryanodine receptor²¹ might also be involved in the accelerated Ca^{2+} release from the SR in Tg myocytes. Thus, overexpression of NHE1

results in both (1) increased diastolic Ca^{2+} levels and (2) altered SR Ca^{2+} handling. A continuous increase in diastolic Ca^{2+} could activate critical hypertrophic signaling mechanisms and could trigger some death signals. Although reduced SR Ca^{2+} handling is often reported in failing hearts,²² in the case of NHE1 Tg myocytes, the combination of Na^+ -induced diastolic Ca^{2+} overload and enhanced SR Ca^{2+} pumping would ultimately lead SR Ca^{2+} overload, which itself has been reported to activate some Ca^{2+} -dependent protease calpains and induce apoptosis through the mitochondrial death pathway involving BAD, Bid, and caspase12²³; we observed marked degradation of cardiac troponin I in Tg heart (supplemental Figure IIC), which might be a consequence of such an event. Thus, it is plausible that both Na^+ -induced diastolic Ca^{2+} overload and enhanced SR Ca^{2+} pumping promote HF in Tg hearts.

As has been observed in human failing hearts,¹ we observed negative force-frequency dependence in Tg myocytes (see Figure 4C) without any change in the diastolic cell length (data not shown). Preservation of the high Ca^{2+} amplitude relative to diminished contractility observed in Tg mice at high stimulation frequency within the same myocyte suggests that myofibril Ca^{2+} sensitivity might be decreased. Possible mechanisms of this phenomenon are presently unknown. However, a recent report demonstrated that a reduction in myofilament Ca^{2+} sensitivity was introduced by PKD-dependent cardiac troponin I phosphorylation,²⁴ which may occur in cardiac disease states.²⁵ We also detected a slight but significant increase in the phosphorylation of troponin I in Tg hearts (supplemental Figure VIII B).

Overall, the results of the single-cell experiments suggest the cellular mechanisms of hypertrophy/HF observed *in vivo*. However, we must consider that *in vivo* hearts consist of a heterogeneous population of cardiomyocytes that includes dead cells that are not available for *in vitro* experiments after cell isolation.

Downstream Signaling Pathways Leading to Cardiac Hypertrophy and HF

Numerous signaling pathways are known to coordinate pathological hypertrophy and HF, including calcineurin and CaMKII, PKC, and MAPKs (ERK42/44, ERK5, p38, and JNK).¹² We found that the Ca^{2+} -dependent signaling molecules CaMKII and calcineurin are both highly activated in Tg hearts and that this activation was reversed by cariporide treatment. These findings suggest that NHE1, coupled with NCX1, can supply a Ca^{2+} source for activating both the CaMKII and calcineurin pathways. Indeed, recent reports have suggested that both NCX¹⁸ and calcineurin²⁶ are involved in endothelin-1-induced hypertrophy in rat myocytes, secondary to NHE1 activation. Furthermore, overexpression of NHE1 triggered nuclear export of HDAC and nuclear import of NFAT in a cariporide-sensitive manner, providing direct evidence that alterations in the sarcolemmal Na^+/H^+ exchange activity can modulate hypertrophy-associated gene expression in cardiomyocytes.

In contrast to the exclusive NHE1-dependent translocation patterns of NFAT and HDAC in NRVMs, NFAT was incompletely nuclear translocated in Tg myocytes, suggesting

that activation of NFAT was partially blocked in Tg myocytes despite calcineurin activation. In addition to Ca^{2+} -dependent pathways, we found that p38 and ERK42/44 MAPKs were significantly activated in Tg hearts. Because p38 (as well as GSK3 β , the downstream molecule of Akt) is known to negatively regulate calcineurin-NFAT signaling via phosphorylation of NFAT,¹² we assume that p38 activation would be one of the mechanisms for partial activation of the NFAT signal in Tg myocytes. This is consistent with the recent finding that CaMKII rather than calcineurin might serve as a major NHE1-dependent hypertrophy molecule in GC-A KO mice.⁴ Indeed, overexpression of activated CaMKII itself results in pronounced hypertrophy and dilated cardiomyopathy.²⁷ Because p38 activation could have been caused by stress and/or receptor activation secondary to enhanced mechanical load, we do not exclude the possibility that the calcineurin pathway predominates in the initial period of hypertrophy, as previously reported.²⁶

Pathological Implications of NHE1 in Cardiac Hypertrophy and HF

NHE1 activity is upregulated in several in vivo and in vitro models of cardiac hypertrophy/HF,^{4,5,28} and NHE1 inhibitors prevent the detrimental effects,² suggesting that NHE1 contributes to cardiac remodeling. Consistent with these reports, we directly demonstrated that NHE1 activation is sufficient to induce hypertrophy and HF; however, there are also some differences between our model and those used in other studies. For example, hearts of GC-A KO mice exhibit hypertrophy but do not develop HF.⁴ This may be a matter of the degree of NHE1 activation, because the expression level of NHE1 protein was unchanged in GC-A KO mice. In addition, there was a significant increase in p38 phosphorylation in our Tg hearts. Because it has been shown that p38 activation in MKK3 or MKK6 Tg mice induced dilated cardiomyopathy and HF without causing hypertrophy,²⁹ it is possible that there are independent mechanisms for inducing hypertrophy and HF, both of which might be activated in our mouse model (supplemental Figure X).

Our data suggest that NHE1-induced Na^+ overload and altered Ca^{2+} handling are sufficient to introduce cardiac hypertrophy mainly through activation of the CaMKII-HDAC4 pathway. Whether or not this is "the necessary event" remains to be determined. However, our results do demonstrate that NHE1 activation can be an initiation signal to promote cardiac remodeling.

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Disclosures

None.

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Down-regulation of P-glycoprotein expression by sustained intracellular acidification in K562/DOX cells

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ABSTRACT

We have investigated the involvement of intracellular pH (pH_i) in the regulation of P-glycoprotein (P-gp) in K562/DOX cells. The selective Na⁺/H⁺ exchanger1 (NHE1) inhibitor cariporide and the "high K⁺" buffer were used to induce the sustained intracellular acidification of the K562/DOX cells that exhibited more alkaline pH_i than the K562 cells. The acidification resulted in the decreased P-gp activity with increased Rhodamine 123 (Rh123) accumulation in K562/DOX cells, which could be blocked by the P-gp inhibitor verapamil. Moreover, the acidification decreased MDR1 mRNA and P-gp expression, and promoted the accumulation and distribution of doxorubicin into the cell nucleus. Interestingly, these processes were all pH_i and time-dependent. Furthermore, the change of the P-gp expression was reversible with the pH_i recovery. These data indicate that the tumor multidrug resistance (MDR) mediated by P-gp could be reversed by sustained intracellular acidification through down-regulating the P-gp expression and activity, and there is a regulative link between the pH_i and P-gp in K562/DOX cells.

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As the major obstacle of chemotherapy, MDR is mediated by P-gp, a 170 kDa membrane protein encoded by MDR1 gene, which leads to the chemoresistance and the decreased survival in leukemia's [1]. With the development of P-gp modulators to the third generation, the regulation of P-gp is still unsatisfactory [2]. Despite more than 30 years of research, the mechanisms underlying MDR reversal have not been fully clarified. Consequently, doing the future investigations of tumor MDR on a rational basis, and developing new strategies to overcome MDR upon this basis are especially necessary.

In recent years, it has been reported that cancer cells show a strong tendency towards an alkaline deviation of the entire homeostasis when they are resistant to therapeutic intervention [3–5], and malignant cells can live and multiply at pH_i levels from 7.46 to 7.6 and even higher [4,6]. This anomalous "malignant alkalosis" has already been considered to represent one of the most specific characters of the cancerous state by Reshkin and others [6,7]. Furthermore, a large variety of MDR modifiers known to be able to reverse resistance to chemotherapeutic drugs have been shown to

exert their cellular effects through a pH_i-acidifying process; also, a decrease in pH_i has been shown to sensitize cancer cells of diverse origins to apoptosis and hyperthermia [8]. In all these cited cases, a cellular acid-base change is considered to be essential to break through drug resistance.

In order to clarify the internal correlation between pH_i and P-gp in MDR leukemia cells, we experimentally induced sustained intracellular acidification in K562 and K562/DOX cell lines with selective NHE1 inhibitor cariporide and the "high K⁺" buffer method. Cariporide, a powerful and specific inhibitor of NHE1, could inhibit NHE1 and block the efflux of H⁺ to achieve intracellular acidification [9]. The "high K⁺" buffer method is widely used to induce intracellular acidification directly. It is suitable to acidify the cytoplasm for certain duration with little toxicity, and could also regulate the pH_i to the requested value [10,11].

We tested a potential role for sustained intracellular acidification in reversing of MDR through P-gp regulation, and assessed the possibility of a regulative link between pH_i and P-gp expression and activity. Our findings are the first results showing the reverse effect of MDR by sustained intracellular acidification in K562/DOX cells through P-gp regulation, and points out a regulative link between pH_i and P-gp expression. These findings suggest a novel role for pH_i in the regulation of MDR and provide important data for the further tumor MDR research.

Abbreviations: MDR, multidrug resistance P-gp, P-glycoprotein NHE1, Na⁺/H⁺ exchanger1 pH_i, intracellular pH Rh123, rhodamine 123 BCECF-AM, 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl

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Materials and methods

Materials

RPMI 1640 media were purchased from Gibco-BRL Life Technologies, Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Human monoclonal antibody against P-gp, rabbit polyclonal antibody against GAPDH and horseradish peroxidase-conjugated anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced Chemiluminescence Reagent Plus (ECL) reagents were from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine 123 (Rh123), 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl (BCECF-AM), verapamil, doxorubicin and cariporide were purchased from Sigma (Shanghai, China).

Cell culture and experimental conditions

The MDR cell line K562/DOX was derived from K562 cells by selection in increased concentrations of doxorubicin. The derivative cell line was shown to have MDR phenotype by its elevated expression of P-gp, wide cross-resistance and defect in intracellular drug accumulation. K562 cells were cultured in RPMI 1640 with 10% (v/v) FBS, penicillin (50 U/ml), streptomycin (50 µg/ml) and L-glutamine (2 mmol/l) at 37 °C in humidified air with 5% CO₂. Medium for K562/DOX cells were further supplemented with doxorubicin 2 µmol/l. Prior to use in experiments, K562/DOX cells were cultured in drug-free medium for two weeks.

Measurement of pHi and induction of intracellular acidification

Measurement of pHi: pHi of cells was assessed by flow cytometry using the pH-sensitive fluorescent probe BCECF-AM [12]. We did not observe any reduction of intracellular BCECF fluorescence intensities in K562 and K562/DOX cell lines, nor was loss of BCECF during the time course of the experiment in the two cell lines (data not shown). Cell suspensions in serum-free RPMI 1640 were washed and labeled with BCECF-AM. The labeled cells were analyzed with an excitation wavelength of 488 nm, and the ratio of the fluorescence at 530 and 640 nm was plotted vs. pHi. In order to obtain the calibration curve, a linear regression within the pHi range 6.2–7.4 was obtained.

Intracellular acidification: Cell suspensions in serum-free RPMI 1640 medium were washed and resuspended (1×10^6 cells/ml) with cariporide 3 mg/l in serum-free RPMI 1640 or the "high K⁺" buffer containing H⁺/K⁺ ionophore nigericin 5 µmol/l to induce the pHi to 7.2, 7.0 and 6.8 for 1 h and 3 h as described previously [10,11].

Real-time RT-PCR

Total RNAs were isolated from K562/DOX cells treated with cariporide 3 mg/l or "high K⁺" buffer using TRIzol reagent (Invitrogen, San Diego, CA). Real-time PCR was done with 1 µl reverse transcription product in a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA) by using SYBR Green PCR Supermix (Bio-Rad). Human MDR1 primers were 5'-TGC TCA GAC AGG ATG TGA GTT G-3' (forward) and 5'-TTA CAG CAA GCC TGG AAC CTA T-3' (reverse; 120 bp); Human GAPDH primers used as an internal control were

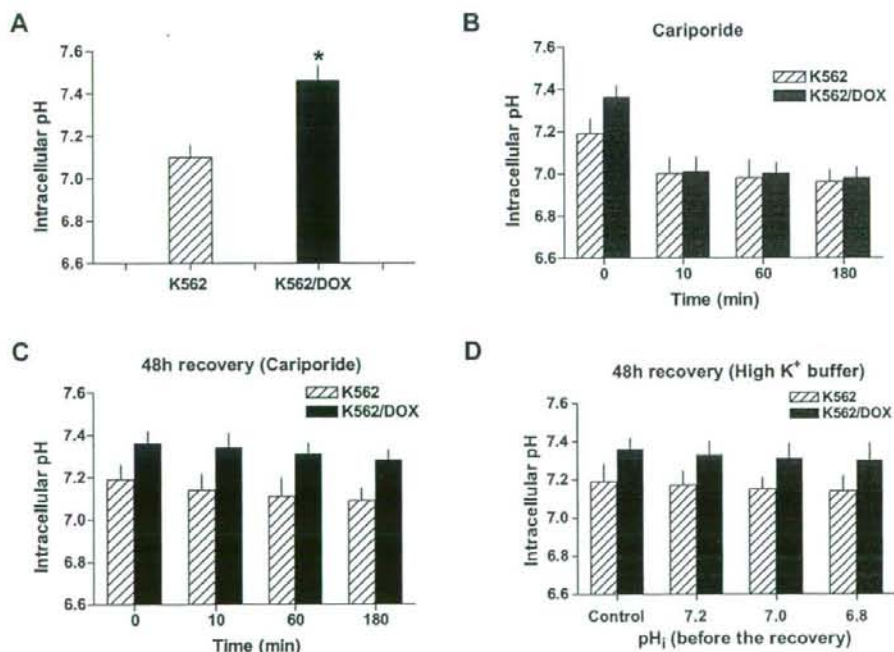


Fig. 1. Determination of pHi in K562 and K562/DOX cells. (A) The pHi of K562 and K562/DOX cells. (B) The pHi of K562 and K562/DOX cells after treated with cariporide in 180 minutes. (C) The pHi of K562 and K562/DOX cells after 48 h recovery from cariporide treatment for 3 h. (D) The pHi of K562 and K562/DOX cells after 48 h recovery from "high K⁺" buffer treatment for 3 h. Data represent means±SD of results obtained from three different experiments performed in triplicate. Asterisks indicate statistical significance (*p* value <0.05, Student's *t*-test).

5'-GAA GGT GAA GGT CGG AGT-3' (forward) and 5'-GAA GAT GGT GAT GGG ATT TC-3' (reverse; 226bp).

Western blotting

Proteins isolated from cell lines treated with cariporide 3 mg/l or the "high K⁺" buffer were resolved by 6% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h and then incubated first with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies for 2 and 1 h, respectively. Specific proteins were visualized with enhanced chemiluminescence detection reagent and determined by densitometric analysis with a Lynx video densitometer (Biological Vision).

Flow cytometry and confocal laser microscopy

Flow cytometry was used to measure intracellular the fluorescent of P-gp substrate Rh123, which was transported by P-gp. After treated with cariporide 3 mg/l or the "high K⁺" buffer for 3 hours, K562 and K562/DOX cells, with or without the pretreatment of verapamil 50 μmol/l for 30 min, were incubated for 1.5 h in serum-free RPMI 1640 containing Rh123 5 μmol/l. After incubation, the cells of each group were collected and resuspended in ice-cold PBS containing 1% fetal bovine serum and kept on ice until analysis by flow cytometry. In another group, K562 and K562/DOX cells treated with cariporide 3 mg/l or the "high K⁺" buffer for 3 h were washed twice by HEPES buffer, and then treated with doxorubicin 10 μmol/l in serum-free RPMI 1640 for 1 h and washed twice by HEPES buffer before imaging on Bio-Rad 1024 confocal laser microscope.

Data analysis

Statistical analyses were made with Student's paired *t*-test using GraphPad Prism (San Diego, USA). Significance was assumed for *P* values less than 0.05.

Results

Intracellular pH of K562 and K562/DOX cells

The pHi of K562/DOX cells was 7.46 while that of K562 cells was only 7.1 (Fig. 1A). After 3 h treatment with cariporide 3 mg/l, the pHi of K562 and K562/DOX cells were both decreased to about 7.0 in 10 min, and the value changed little in the following 170 min. After 48 h recovery in normal culture condition, the pHi of K562 and K562/DOX cells treated with cariporide returned to the similar value of the primary levels (Fig. 1B). The pHi of K562 and K562/DOX cells treated with "high K⁺" buffer of different K⁺ concentration for 1 and 3 h were induced to 7.2, 7.0 and 6.8 in a few minutes and changed little in the following time. After 48 h recovery in normal culture condition, the pHi of K562 and K562/DOX cells treated with the "high K⁺" buffer also returned to the similar value of the primary levels (Fig. 1C and D). These data suggested that the pHi of the resistant K562/DOX cells is more alkaline than the sensitive K562 cells. The two kinds of cells could be acidified with cariporide and the "high K⁺" buffer method, and the process was reversible.

Decreased pHi down-regulated the expression of MDR1 mRNA in K562/DOX cells

In K562/DOX cells, cariporide down-regulated MDR1 mRNA expression in a time-dependent manner and the expression were

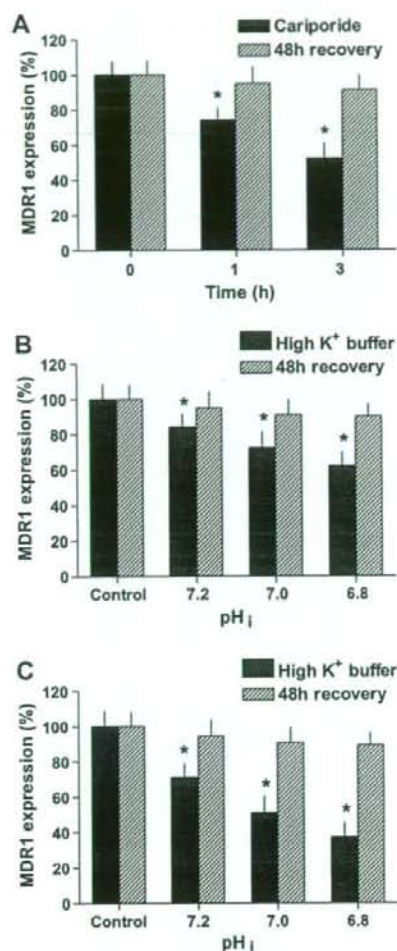


Fig. 2. Determination of MDR1 mRNA expression in K562/DOX cells. (A) The MDR1 mRNA expression of K562/DOX cells after treated with cariporide for 1 and 3 h, and after 48 h recovery from cariporide treatment. (B) The MDR1 mRNA expression of K562/DOX cells after treated with "high K⁺" buffer for 1 h, and after 48 h recovery from "high K⁺" buffer treatment. (C) The MDR1 mRNA expression of K562/DOX cells after treated with "high K⁺" buffer for 3 h, and after 48 h recovery from "high K⁺" buffer treatment. Data represent mean \pm SD of results obtained from three different experiments performed in triplicate. Asterisks indicate statistical significance (*p* value < 0.05, Student's *t*-test).

decreased to 74% and 52% of the control after 1 and 3 h treatment. After 48 h recovery in normal culture condition, the MDR1 mRNA expression of K562/DOX cells returned to the similar value of the primary levels (Fig. 2A). The pHi-effect on MDR1 mRNA was obvious at pHi 7.2, 7.0 and 6.8 induced by the "high K⁺" buffer. The expression was decreased to 84%, 72% and 62% of the control after 1 h treatment and to 71%, 51% and 37% after 3 h treatment. These data indicated that the decrease of MDR1 mRNA expression was also time-dependent. After 48 h recovery in normal culture condition, the MDR1 mRNA expression of K562/DOX cells returned to the similar value of the primary levels (Fig. 2B and C). Nevertheless, these data indicated that the sustained intracellular acidification down-regulated P-gp at mRNA level and the process was reversible.

Decreased pH_i down-regulated P-gp expression in K562/DOX cells

In K562/DOX cells, cariporide down-regulated P-gp expression in a time-dependent manner and the expression was decreased to 78% and 60% of the control after 1 and 3 h treatment as determined by densitometry analysis (Fig. 3A1). After 48 h recovery in normal culture condition, the P-gp expression of K562/DOX cells returned to the similar value of the primary levels (Fig. 3A2). The pH_i -effect on P-gp expression was remarkable at pH_i 7.2, 7.0 and 6.8 induced by the "high K^+ " buffer, and the P-gp expression was decreased to 87%, 76% and 64% of the control after 1 h treatment (Fig. 3B1), and to 78%, 58% and 42% after 3 h treatment (Fig. 3C1). After 48 h recovery in normal culture condition, the P-gp expression of K562/DOX cells returned to the similar value of the primary levels (Fig. 3B2 and C2). These data suggested that the sustained intracellular acidification down-regulated P-gp expression also in a pH_i and time-dependent manner, which was in accordance with the results of MDR mRNA expression, and this process was also reversible in K562/DOX cells.

Intracellular acidification enhanced the Rh123 and doxorubicin accumulation in K562/DOX cells

The Rh123 effectively accumulated in K562 cells and was not affected by cariporide and the "high K^+ " buffer treatment. The Rh123 accumulation was increased by 3.5-fold compared to the control after cariporide treatment, and by 2.2-, 3.2- and 4.0-fold at pH_i 7.2, 7.0 and 6.8 after the "high K^+ " buffer treatment

in K562/DOX cells. When K562/DOX cells were pretreated with verapamil, the inhibitor of P-gp, no obvious change of Rh123 accumulation was observed by cariporide and the "high K^+ " buffer treatment (Fig. 4A). The accumulation of doxorubicin was much less in the K562/DOX cells compared with the K562 cells. The accumulation was not influenced by cariporide or the "high K^+ " buffer in K562 cells, but in K562/DOX cells, it was obviously increased in a pH_i -dependent manner when the cells were pretreated with cariporide and the "high K^+ " buffer. The increased doxorubicin distribution into K562/DOX cells nucleus was also observed after the treatment, which might enhance the therapy effect of this drug, as doxorubicin was applied by binding to tumor cells DNA and inhibiting the synthesis of nucleic acid (Fig. 4B). These results suggested that the transport activity of P-gp was down-regulated and the sensitivity of the tumor cells to the chemotherapeutic drug was increased by the sustained intracellular acidification in K562/DOX cells.

Discussion

Chemotherapy as the most potent tools in contemporary medicine has been endangered by the resistance of tumor cells to chemotherapeutic agents. The alkaline pH_i of many and possibly all kinds of malignant cells is known to decrease the retention of anticancer drugs [13–16]. A failure to induce cytosolic acidification has been proposed to be the main factor underlying drug resistance in both the highly alkaline cancer cells and in malignant cells with slightly elevated pH_i [13,17]. It was reported that the pH_i

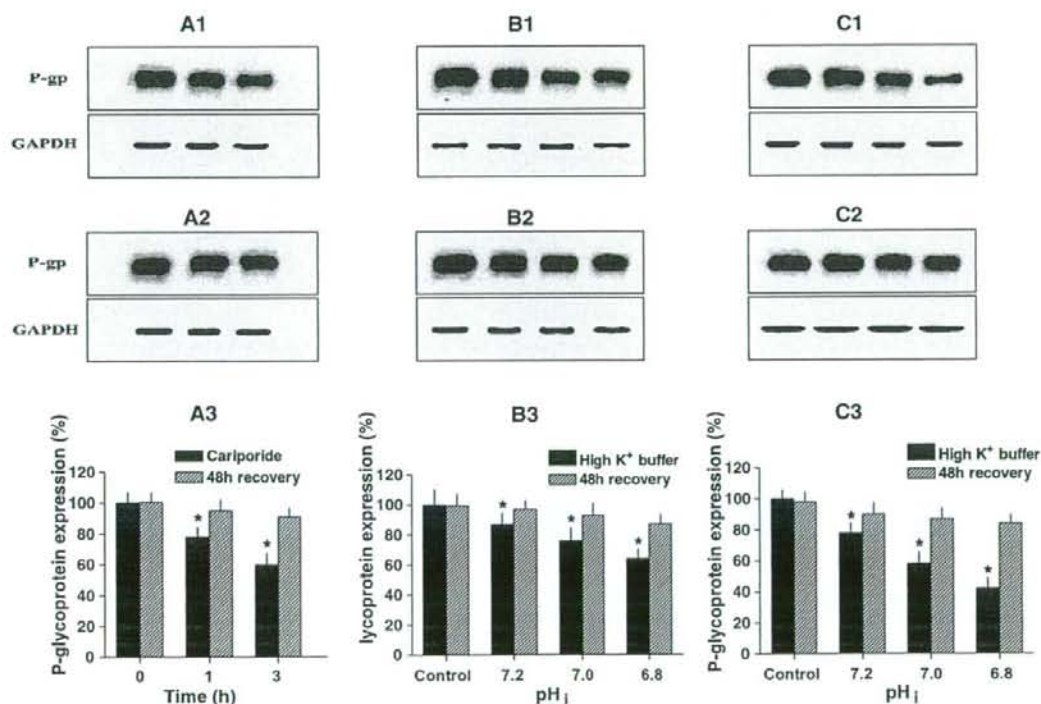


Fig. 3. Determination of P-gp expression in K562/DOX cells. The P-gp expression of K562/DOX cells treated with cariporide for 1 and 3 h (A1) and with "high K^+ " buffer for 1 h (B1) and 3 h (C1). The P-gp expression of K562/DOX cells after 48 h recovery from cariporide treatment for 1 and 3 h (A2), and from "high K^+ " buffer treatment for 1 h (B2) and 3 h (C2). The intensity of P-gp expression of K562/DOX cells treated with cariporide for 1 and 3 h (A3) and with "high K^+ " buffer for 1 h (B3) and 3 h (C3). The intensity of P-gp was quantified by densitometric analysis of the Western blot bands. Data represent means \pm SD of results obtained from three different experiments performed in triplicate. Asterisks indicate statistical significance (p value < 0.05 , Student's t -test).

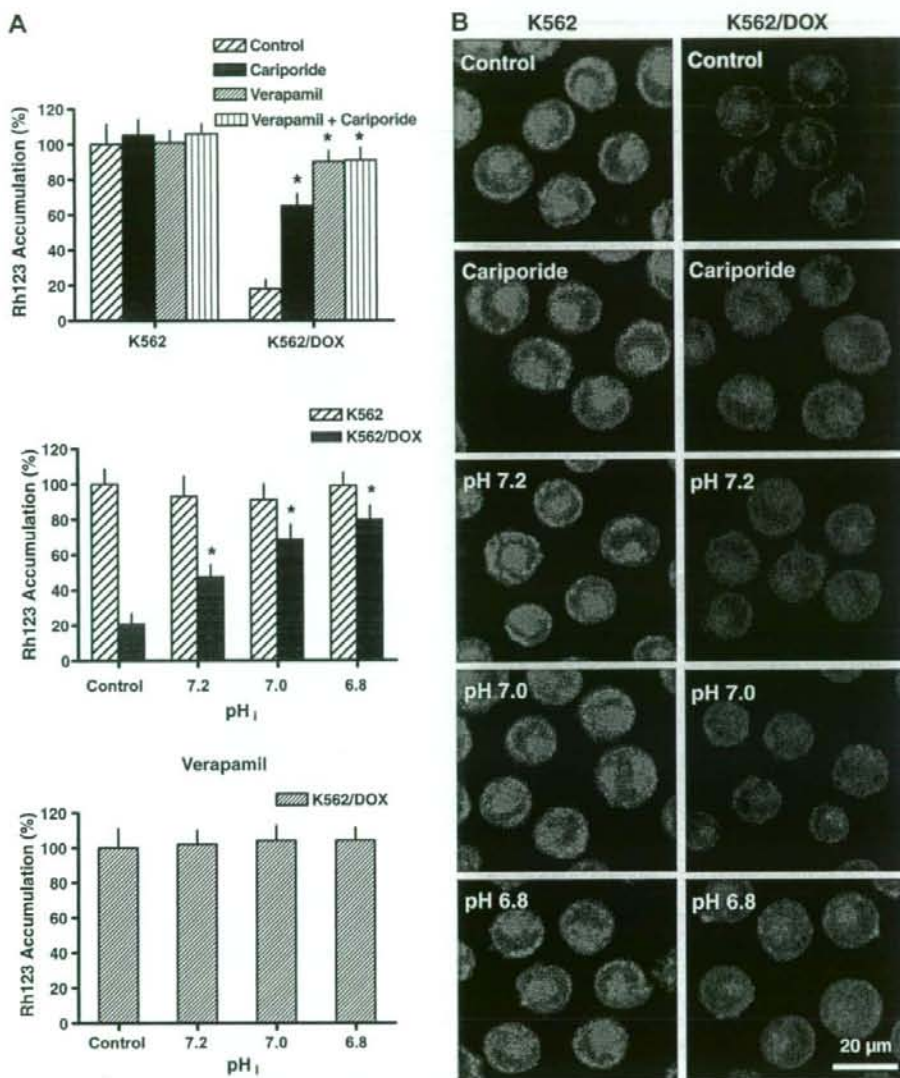


Fig. 4. Effect of sustained intracellular acidification on Rh123 and doxorubicin accumulation in K562 and K562/DOX cells. (A) Rh123 accumulation in K562 and K562/DOX cells treated with cariporide for 3 h, and pretreated with verapamil for 30 min before the cariporide treatment (top). Rh123 accumulation in K562 and K562/DOX cells treated with "high K⁺" buffer for 3 h (middle). Rh123 accumulation in K562/DOX cells pretreated with verapamil 50 μ mol/l before the "high K⁺" buffer treatment (bottom). (B) After the K562 and K562/DOX were treated with cariporide, and the pH_i were induced to 7.2, 7.0, 6.8 with the "high K⁺" buffer treatment for 3 h, respectively, the doxorubicin was added to the medium 1 h prior to viewing with confocal laser microscope (500 \times) as described under Materials and Methods. Data represent means \pm SD of results obtained from three different experiments performed in triplicate. Asterisks indicate statistical significance (*p* value < 0.05, Student's *t*-test).

was significantly higher in doxorubicin-resistant human colon carcinoma cells HT29-dx than sensitive HT29 cells, and HT29-dx cells accumulated less doxorubicin than HT29 cells [18]. Similar results have been reached by other groups: with doxorubicin and daunomycin in myeloma [19], adriamycin in the more alkaline MCF-7 human breast cancer cells [20].

The data showed that K562/DOX cells had more alkaline pH_i than K562 cells. The pH_i 7.46 of K562/DOX cells was obviously higher than pH_i 7.1 of K562 cells. The pH_i of the two kinds of cells were induced to about 7.0 by cariporide, and 7.2, 7.0, and 6.8 by "high K⁺" buffer. Sustained intracellular acidification increased the accumu-

lation of Rh123 in a pH_i-dependent manner in the K562/DOX cells, but had no effect on the K562 cells. In contrast to these results, intracellular acidification had no apparent effect on Rh123 accumulation in K562/DOX cells pretreated with P-gp inhibitor verapamil, which implied the intracellular acidification might influence the Rh123 kinetics through regulating P-gp activity. The sustained intracellular acidification also increased the doxorubicin accumulation in K562/DOX cells together with the change of doxorubicin distribution, but had no effect on K562 cells. The Fig. 4B showed the increased doxorubicin distribution into the cell nucleus, which might enhance the therapy effect of this drug. The K562/DOX cells

are known to over-express P-gp, but the K562 cells are not. The ability of sustained intracellular acidification to affect Rh123 and doxorubicin kinetics supports the conclusion that the sustained intracellular acidification acts by interfering with a process associated with the P-gp activity. It might occur primarily at the MDR1 mRNA and P-gp expression levels. The data showed that sustained intracellular acidification decreased the MDR1 mRNA expression in a time and pH_i -dependent manner by real-time RT-PCR analysis. Furthermore, over-expression of P-gp in K562/DOX cells was effectively inhibited by the intracellular acidification. The densitometry analysis showed that the expression of P-gp was decreased also in a pH_i and time-dependent manner. The sustained intracellular acidification might affect the expression of MDR1 mRNA and then result in the decreased expression of P-gp, and finally reduce the activity of this membrane protein. After 48 h recovery with the cells pH_i returning to the primary value, the expression of MDR1 mRNA and P-gp were also reverted to the primary levels. The results indicated that the regulative effect of the sustained intracellular acidification on P-gp was reversible, which proved the direct correlation between pH_i and P-gp.

Although the molecular pathways of the regulation of P-gp by pH_i have not been elucidated yet, it was reported that inhibition of the p38-MAPK pathway down-regulated P-gp expression level and diminished the cellular multidrug resistance [21]. SB203580, a specific inhibitor of p38-MAPK pathway, significantly reduced the degree of the vincristine resistance in L1210/VCR cells [22]. Interestingly, it was also reported that NHE1 played a central role in the regulation of MAPK activity [23]. A specific NHE1 inhibitor BIIB722 prevented p38-MAPK phosphorylation, and strongly attenuated apoptosis in cardiomyocytes [24]. And the mechanism of intracellular acidification-induced down-regulation of P-gp activity might be through p38-MAPK pathway, although alternative possible mechanisms may exist.

The available data indicate that any mechanism that leads to an elevation of pH_i might contribute to the onset and maintenance of tumor MDR. Treating malignant diseases along with MDR in the unmodified conditions may continue to limit the therapy success, if efforts are not made to induce intracellular acidification. Therefore, future studies are needed to investigate the specific molecular mechanisms of the regulation of P-gp by pH_i , and the cellular pH_i deserves to be analyzed as a possible important influence factor on the MDR status of tumor cells to the chemotherapy.

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