

# Regression of Isoproterenol-Induced Cardiac Hypertrophy by Na<sup>+</sup>/H<sup>+</sup> Exchanger Inhibition

Irene L. Ennis, Eduardo M. Escudero, Gloria M. Console, Gisela Camihort, César Gomez Dumm, Randolph W. Seidler, María C. Camilión de Hurtado, Horacio E. Cingolani

**Abstract**—Cardiac hypertrophy is often associated with an increased sympathetic drive, and both in vitro and in vivo studies have demonstrated the development of cardiomyocyte hypertrophy in response to either  $\alpha$ - or  $\beta$ -adrenergic stimulation. Because an association between the Na<sup>+</sup>/H<sup>+</sup> exchanger and cellular growth has been proposed, this study aimed to analyze the possible role of the antiporter in isoproterenol-induced cardiac hypertrophy. Isoproterenol alone (5 mg/kg IP once daily) or combined with a selective inhibitor of the Na<sup>+</sup>/H<sup>+</sup> exchanger activity (3 mg · kg<sup>-1</sup> · d<sup>-1</sup> BIIB723) was given to male Wistar rats for 30 days. Sex- and age-matched rats that received 0.9% saline IP daily served as controls. Echocardiographic follow-up showed a 33% increase in left ventricular mass in the isoproterenol-treated group, whereas it did not increase in the isoproterenol+BIIB723-treated group. Heart weight-to-body weight ratio at necropsy was 2.44±0.11 in controls and increased to 3.35±0.10 ( $P<0.05$ ) with isoproterenol, an effect that was markedly attenuated by BIIB723 (2.82±0.07). Intense cardiomyocyte enlargement and severe subendocardial fibrosis were found in isoproterenol-treated rats, and both effects were attenuated by BIIB723. Myocardial Na<sup>+</sup>/H<sup>+</sup> exchanger activity and protein expression significantly increased in isoproterenol-treated rats compared with the control group (1.45±0.11 vs 0.91±0.05 arbitrary units,  $P<0.05$ ). This effect was significantly reduced by BIIB723 (1.17±0.02,  $P<0.05$ ). In conclusion, our results show that Na<sup>+</sup>/H<sup>+</sup> exchanger inhibition prevented the development of isoproterenol-induced hypertrophy and fibrosis, providing strong evidence in favor of a key role played by the antiporter in this model of cardiac hypertrophy. (*Hypertension*. 2003;41:1324-1329.)

**Key Words:** hypertrophy, cardiac ■ signal transduction ■ antiporters ■ fibrosis ■ adrenergic receptor agonists

Increased sympathetic activity is often implicated in the development of cardiac hypertrophy (CH). A correlation between cardiac mass and sympathetic activity was found in young hypertensive humans,<sup>1</sup> and long-term infusion of subpressor doses of norepinephrine leads to CH in dogs and rats.<sup>2,3</sup> This cardiostrophic effect of catecholamines involves both  $\alpha$ - or  $\beta$ -adrenergic receptors.<sup>4</sup> It is well recognized that repeated or continuous injections of the  $\beta$ -adrenoceptor agonist isoproterenol (Iso) causes, within days, clear CH,<sup>5</sup> and therefore it represents a useful experimental model.

Although several mechanisms have been imputed to underlie the cardiostrophic action of Iso,<sup>5-7</sup> the exact nature is still under debate. Because cumulative evidence supports a cause-effect link between the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) and cardiac cell growth (Cingolani and Camilión de Hurtado<sup>8</sup>), we sought to analyze the possible role of NHE activity in Iso-induced CH by taking advantage of the specific, orally active inhibitor against NHE isoform 1 (NHE-1). This study provides evidence indicating a key role

for NHE-1 activity as a mechanism underlying the development of CH and fibrosis induced by Iso.

## Methods

The investigation was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996). Male 16- to 18-week-old Wistar rats with free access to standard rat chow and tap water were randomly assigned to 1 of the following groups: (1) control (Ctrl, n=10), which received a daily injection of 0.9% saline IP; (2) Iso (n=12), which received 5.0 mg/kg ( $\pm$ ) isoproterenol hydrochloride (Sigma) dissolved in 0.3 mL saline IP once daily,<sup>9</sup> and (3) Iso+BIIB (n=8), which received 3.0 mg · kg<sup>-1</sup> · d<sup>-1</sup> BIIB723 (Boehringer-Ingelheim) in the drinking water plus the daily injection of Iso. The solution of BIIB723 was prepared every day according to the daily records of water consumption and body weight (BW), the latter measured every 2 days. Systolic blood pressure, by the indirect tail-cuff method, and heart rate were determined weekly during treatment, which lasted 30 days. At the end of treatment, animals were euthanized under deep ether anesthesia, and the hearts removed and subjected to further analysis. The ventricles were blotted and weighed (HW), and HW normalized by BW (HW/BW ratio, in mg/g) was used as a hypertrophy index. Only 1 rat, from the Iso-treated group, died during the protocol.

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From Centro de Investigaciones Cardiovasculares (I.L.E., E.M.E., M.C.C.de H., H.E.C.), Cátedra de Histología B, Facultad de Ciencias Médicas, UNLP (G.M.C., G.C., C.G.D.); and Boehringer-Ingelheim Pharma KG (R.W.S), Biberach an der Riss, Germany. M.C.C.deH. and H.E.C. are established investigators of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); G.M.C. is an established investigator of Comisión de Investigaciones Científicas Prov. Buenos Aires (CICBA).

Correspondence to Dr Irene L. Ennis, Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, UNLP 60 y 120 (1900), La Plata, Argentina. E-mail iennis@atlas.med.unlp.edu.ar

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## Measurement of $pH_i$

One papillary muscle isolated from the left ventricle (LV) of each heart, isometrically contracting at 0.2 Hz, was used for  $pH_i$  determinations with the epifluorescence approach (BCECF) previously detailed.<sup>10</sup>

## Echocardiographic Examination

Rats were monitored echocardiographically under light anesthesia (35 mg/kg pentobarbital sodium IP) 1 or 2 days before euthanasia. Cardiac geometry and function were evaluated by 2-dimensional M-mode echocardiography with a 7-MHz linear transducer. All measurements, including LV wall thickness and diastolic dimensions, were performed according to the American Society of Echocardiography leading-edge method.<sup>11</sup> LV mass was calculated as previously described.<sup>12</sup>

## Histomorphometric Studies

After isolation of a papillary muscle, the LV was fixed in 10% buffered formaldehyde for 24 hours and embedded in paraffin. Serial sections of 4  $\mu\text{m}$  were stained with hematoxylin-eosin for cell morphology or picrosirius red (Direct Red 80, Aldrich) for collagen quantification. Cardiomyocyte cross-sectional area (CSA), collagen volume fraction (CVF), and the reference area were determined in each group for an average of 10 micrographs. Because the greatest degree of fibrosis in the treated groups was confined to the region below the endocardium, the LV wall was halved into a subendocardial (inner half) and subepicardial (outer half) region, and CVF was separately quantified in both regions.

## Western Blotting

Immunoblot analysis of membrane-enriched fractions was performed with a rabbit polyclonal antibody for NHE-1 (a kind gift from Dr M. Donowitz, Johns Hopkins University, Baltimore, Md). Detection of immunoreactive proteins was performed by chemiluminescence (Amersham), and signals were quantified by densitometric analysis. To confirm the specificity of the primary antibody, blots containing samples of each experimental group were incubated with secondary antibody alone.

## Statistics

Results are expressed as mean  $\pm$  SEM. The Student *t* test or 1-way ANOVA followed by the Student-Newman-Keuls test was used where appropriate. Significance level was set at  $P < 0.05$ .

An expanded Methods section can be found in an online supplement available at <http://www.hypertensionaha.org>.

## Results

BIIB723 is a new acylguanidine member of the BIIB family of NHE inhibitors,<sup>13</sup> with high oral bioavailability. It displays selectivity for NHE-1 over NHE-2 of  $\approx 40$ -fold and an  $IC_{50}$  of  $\approx 30$  nmol/L in cardiomyocytes.<sup>14</sup> The molecule is closely related to sabiporide, whose pharmacological properties were recently reported.<sup>15</sup> In 5 of the Iso+BIIB-treated rats, the serum level of the NHE-1 inhibitor was determined on 3 consecutive days (tenth through the twelfth after the onset of treatment). The plasma concentration of the inhibitor attained an average value of  $410 \pm 84$  nmol/L. No significant difference in BW, systolic blood pressure, or heart rate between the experimental rat groups was observed during the treatment (Table). Echocardiographic examination showed an increase in LV internal end-diastolic diameter, anterior end-diastolic thickness, LV mass, and fractional shortening in the Iso group (Figure 1). All these effects were cancelled when the NHE-1 inhibitor was combined with Iso. Representative echocardiograms of each group are also shown in Figure 1.

At necropsy, HW was increased in the Iso-treated rats, and this effect was attenuated by NHE-1 blockade. Figure 2 shows the mean values of HW normalized by BW for each group. The

## General Characteristics of the Experimental Groups

Rat Group	BW, g	SBP, mm Hg	HR, bpm
Control (n=10)			
Before	307.9 $\pm$ 12.5	121.6 $\pm$ 3.1	398 $\pm$ 8
After	335.6 $\pm$ 16.6	122.0 $\pm$ 1.4	381 $\pm$ 9
Iso (n=11)			
Before	314.6 $\pm$ 9.5	119.9 $\pm$ 3.6	395 $\pm$ 8
After	323.3 $\pm$ 10.5	112.1 $\pm$ 3.3	400 $\pm$ 2
Iso+BIIB (n=8)			
Before	328.3 $\pm$ 13.0	107.6 $\pm$ 6.6	401 $\pm$ 9
After	335.6 $\pm$ 14.5	110.6 $\pm$ 3.1	401 $\pm$ 21

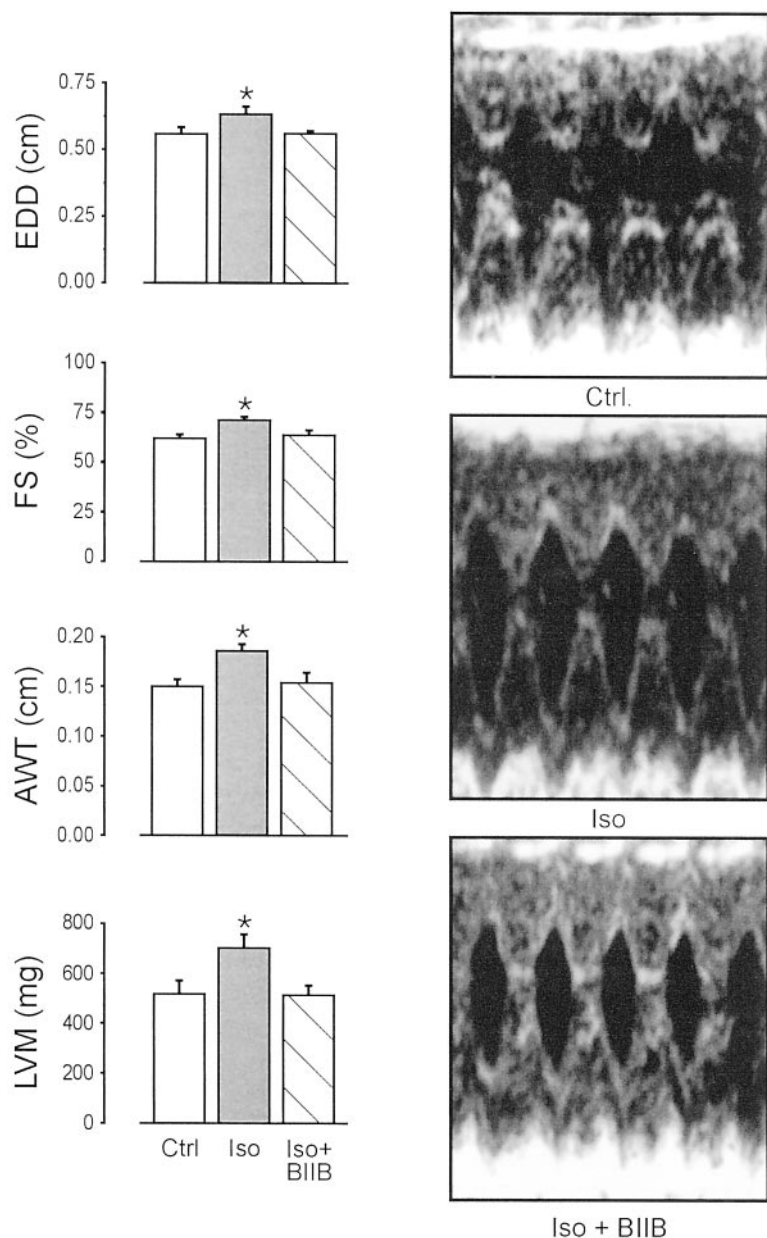
Values are mean  $\pm$  SE; n indicates the number of rats; before and after indicate values at the onset and at the end of treatment, respectively.

CSA of LV cardiomyocytes was enlarged in rats treated with Iso ( $458 \pm 11$  vs  $294 \pm 4$   $\mu\text{m}^2$ ,  $n=4$  each group;  $P < 0.05$ ). This effect was markedly attenuated by NHE-1 blockade ( $357 \pm 5$   $\mu\text{m}^2$ ,  $n=4$ ;  $P < 0.05$  compared with Ctrl and Iso-treated groups). Representative microphotographs of LV specimens obtained from each experimental group are shown in Figure 3. The images illustrate the Iso-induced enlargement of myocyte CSA and the attenuation of this effect by the NHE-1 inhibitor.

In comparison with Ctrl, myocardial collagen content was increased in rats treated with Iso. The increase in collagen was primarily found within the subendocardial region of the LV wall, in accordance with previous reports (Figure 4).<sup>4,16</sup> The increase in CVF in the subendocardium of rats treated with Iso amounted to  $51.2 \pm 3\%$ , compared with  $2.2 \pm 0.4\%$  in Ctrl ( $n=4$  each group,  $P < 0.05$ ). Simultaneous inhibition of NHE-1 activity significantly reduced the increase in subendocardial fibrosis to  $13 \pm 1\%$  ( $n=4$ ,  $P < 0.05$  vs other groups). Interestingly, Iso did not induce collagen accumulation in areas other than the subendocardial region. In the subepicardial region, CVF was  $1.9 \pm 0.3$  in Ctrl,  $2.9 \pm 0.5$  in Iso, and  $1.9 \pm 0.2\%$  in Iso+BIIB groups ( $P = \text{NS}$ ).

The steady-state value of  $pH_i$  determined in isolated papillary muscles bathed with bicarbonate-free (HEPES)-buffered medium was markedly more alkaline in the Iso than in the Ctrl group (Figure 5). When the NHE-1 inhibitor BIIB723 was simultaneously given, this effect of Iso treatment on myocardial  $pH_i$  was prevented.

Because the  $\beta_i$  values were of similar magnitude in all groups ( $27.62 \pm 4.47$  in Ctrl,  $27.62 \pm 4.47$  in Iso, and  $24.35 \pm 6.11$  mmol/L in Iso+BIIB;  $P = \text{NS}$ ), the aforementioned data strongly suggested that myocardial NHE-1 activity was enhanced by Iso treatment. To further support this contention, the effect of acute addition of a widely used specific inhibitor of NHE-1 activity on myocardial  $pH_i$  was assessed in isolated papillary muscles from Iso-treated and Ctrl rats (Figure 6). When the hypertrophied myocardium of Iso-treated rats was exposed to HOE 642, the  $pH_i$  value in the absence of bicarbonate (HEPES buffer) gradually decreased. Myocardial  $pH_i$  also decreased after HOE 642 application in Ctrl myocardium but to a much lesser extent. The best fit of the HOE 642-induced decrease in  $pH_i$  followed an exponential function that asymptotically approached a similar value in



**Figure 1.** Echocardiographic assessment of Iso-induced CH and its attenuation by the NHE-1 inhibitor BIIB 723. Mean values ( $\pm$ SEM) of end-diastolic diameter (EDD), fractional shortening (FS), anterior wall thickness (AWT), and LV mass (LVM) are shown on the right. Control (n=6); Iso, Iso-treated (n=7); Iso+BIIB, combined Iso plus NHE-1 inhibitor treatment (n=8). Iso induced an increase in all of these parameters, which was prevented by NHE-1 inhibition. \* $P < 0.05$  vs Ctrl and Iso+BIIB. Images on the right show representative 2-dimensional M-mode echocardiograms of each indicated group.

Iso-treated and Ctrl rats, thus canceling the difference in pH<sub>i</sub> between the hypertrophied and normal myocardium (Figure 6B).

In accordance with the functional evidence of enhanced NHE-1 activity, NHE-1 protein expression was found to be elevated in the myocardium of Iso-treated rats. In rats that were simultaneously treated with Iso and the NHE-1 inhibitor, NHE-1 protein expression did not differ from that in the Ctrl group, indicating that NHE-1 inhibition effectively prevented the Iso-induced change in antiporter expression (Figure 7). The picture corresponding to a membrane that was incubated with secondary antibody alone (first 3 lanes of Figure 7A) did not show any band, confirming the specificity of the primary antibody.

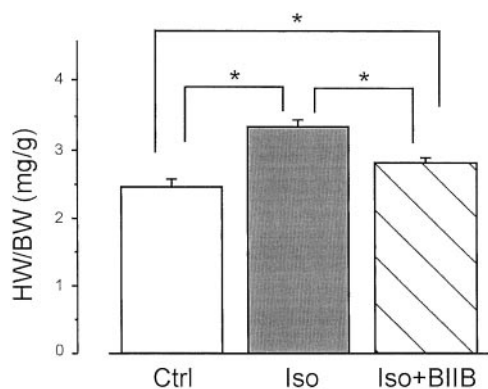
### Discussion

Our results show that Iso-induced myocardial hypertrophy is accompanied by enhanced NHE-1 activity and expression. In this regard, Iso-induced CH seems to differ from other models of

CH in which enhanced NHE-1 activity<sup>17,18</sup> is not correlated with an increase in expression of the antiporter,<sup>17,19</sup> suggesting a role for posttranslational mechanisms. Iso-induced effects are attenuated by chronic inhibition of the exchanger.

Both in vitro and in vivo studies have established that the  $\beta$ -adrenergic receptor agonist Iso induces CH.<sup>2,20</sup> The renin-angiotensin system was proposed to be involved in the development and maintenance of Iso-induced CH.<sup>5,6</sup> More recently, activation of 44- to 42-kDa extracellular signal-regulated protein kinases through a calcineurin-dependent mechanism was shown to participate in the development of Iso-induced CH.<sup>7</sup> Our results show that prevention of CH development by the NHE-1 inhibitor in Iso-treated rats was accompanied by normalization of NHE-1 expression and myocardial pH<sub>i</sub>. The observation that NHE-1 inhibitors exert antihypertrophic effect is not new.<sup>8</sup> NHE-1 inhibition was shown to regress and prevent CH in hypertensive rats<sup>21</sup> and

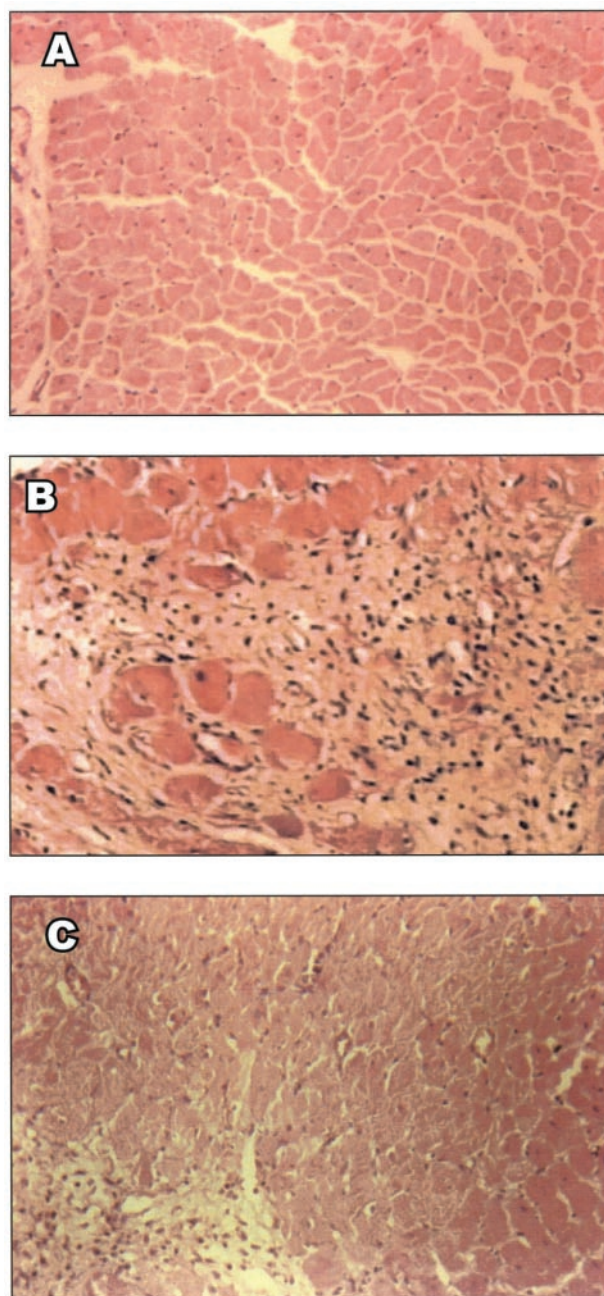




**Figure 2.** Mean values ( $\pm$ SEM) of HW normalized by BW (HW/BW) at necropsy. Groups are labeled as in Figure 1. Iso-induced CH was reduced by NHE-1 blockade. \* $P < 0.05$ .

mice overexpressing  $\beta_1$ -adrenergic receptors,<sup>22</sup> as well as to attenuate the development of cardiomyocyte hypertrophy after myocardial infarction<sup>23</sup> and mechanical stretch.<sup>24</sup> To our knowledge, this is the first demonstration that NHE-1 mediates Iso-induced CH and its prevention by an orally active NHE-1 inhibitor. The prevention of increased NHE-1 expression in rats that received Iso combined with BIIB723 seems contradictory with our recent observation that chronic administration of cariporide to normal rats induced upregulation of the antiporter.<sup>25</sup> However, it is in accordance with previous studies reporting the normalization by cariporide of the increased NHE-1 expression observed in hypertrophied hearts from mice overexpressing the  $\beta_1$ -adrenoreceptors<sup>22</sup> and after myocardial infarction.<sup>23</sup> Whether the difference is due to dissimilar responses of normal hearts and of those exposed to a trophic stimulus is not evident at present.

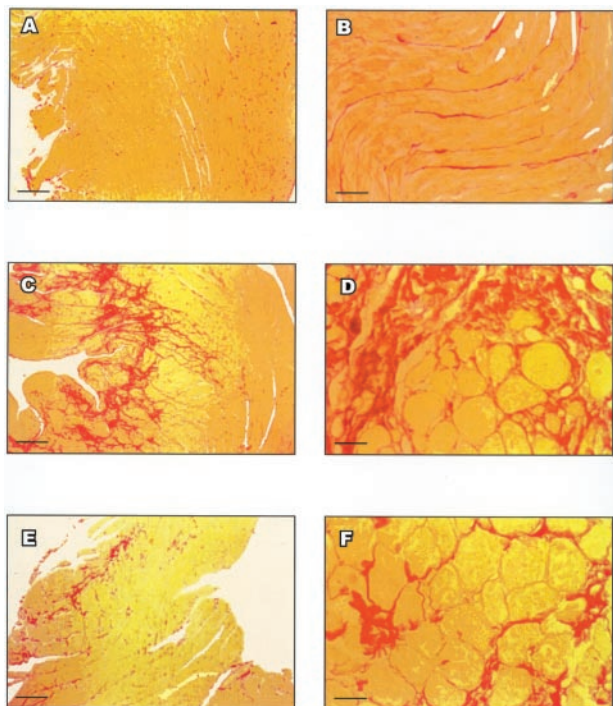
The mechanism through which  $\beta$ -receptor activation causes increased expression of functional NHE-1 protein units is not yet apparent. Acute Iso stimulation was shown to inhibit NHE-1 and activate the  $\text{Na}^+$ -independent- $\text{Cl}^-/\text{HCO}_3^-$  exchanger activities in cardiac tissue.<sup>26,27</sup> Both effects tend to cause intracellular acidification, and one might speculate about a compensatory feedback mechanism increasing the expression of NHE-1 protein. It was shown that chronic intracellular acidosis stimulates NHE-1 expression in renal tissue,<sup>28</sup> and isolated cardiomyocytes increased NHE-1 activity in response to chronic external low pH.<sup>29</sup> The elevated NHE-1 activity will increase  $\text{Na}^+$  influx, increasing its cytosolic concentration, which in turn will lead to a secondary increase in  $\text{Ca}^{2+}_i$  through  $\text{Na}^+/\text{Ca}^{2+}$  exchange.<sup>30,31</sup> The notion that the increase in  $\text{Ca}^{2+}_i$  is a primary signal for CH is supported by numerous studies.<sup>32–34</sup> The rise in  $\text{Ca}^{2+}_i$  activates several intracellular signaling pathways like protein kinase C<sup>35</sup> and calcium-calmodulin-dependent phosphatase (calcineurin) and kinases involved in the hypertrophic response. In this regard, a calcineurin-dependent mechanism was shown to participate in the development of Iso-induced CH.<sup>7</sup> On the other hand, the link between  $\text{Na}^+$  influx, activation of protein kinase C- $\delta$  and  $\epsilon$ , and hypertrophy has been previously suggested.<sup>36</sup> Alternatively, it can be argued that increased NHE-1 expression is an epiphenomenon of the trophic effect of  $\beta$ -adrenergic stimulation. However, the fact that NHE-1 inhibition prevented CH seems to



**Figure 3.** Representative microphotographs showing LV cardiomyocytes with central nucleus in Ctrl (A), Iso-treated (B), and Iso+BIIB (C) groups. In Iso-treated rats, enlarged cardiomyocytes with isolated mononuclear infiltrate were observed. Instead, those rats treated simultaneously with the NHE-1 inhibitor presented cardiomyocytes of smaller size and stromal reaction with capillaries and mononuclear infiltrate. Hematoxylin-eosin stain; bar=100  $\mu\text{m}$ ; magnification  $\times 160$ .

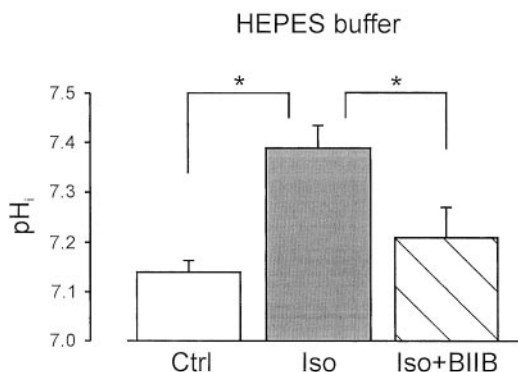
indicate a cause-effect relation between increased NHE-1 activity and the induction of CH.

Significant subendocardial fibrosis was noted in the hypertrophied hearts of Iso-treated rats, which was attenuated by combined treatment with the NHE-1 inhibitor. There are conflicting reports about the presence of fibrosis in Iso-induced CH. Although some authors found no evidence or even a decrease in collagen content, an increase in the amount

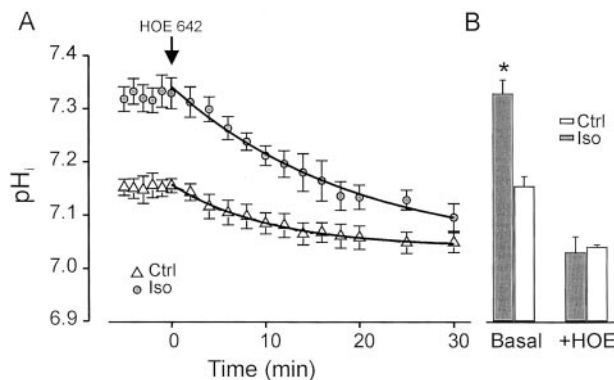


**Figure 4.** Microphotographs of picosirius red-stained histological LV sections. Few isolated collagen fibers are seen in the LV from Ctrl rats (A, B), whereas there is increased collagen deposition primarily confined within the subendocardial region of the ventricular wall in Iso-treated rats (C, D). The Iso+BIIB group presented a marked reduction in the level of fibrosis (E, F). Picosirius red stain; In A–C, bar=400  $\mu$ m; magnification  $\times$ 25; in D–F, bar=25  $\mu$ m; magnification  $\times$ 400.

of collagen deposition was found in other studies.<sup>4,16,37,38</sup> Benjamin et al<sup>39</sup> proposed that increased fibrosis after Iso treatment was predominantly related to myocyte necrosis. Increased cell necrosis confined to the subendocardial region induced by Iso treatment has been attributed to the increased susceptibility of subendocardial cells to ischemia.<sup>40</sup> In this regard, the protective effect of NHE-1 inhibitors to ischemia/reperfusion injury might have a role in the attenuation of the fibrotic response in hearts of Iso-treated rats.<sup>23</sup> Alternatively, the trophic effect of  $\beta$ -receptor



**Figure 5.** Myocardial steady pH<sub>i</sub> value in papillary muscles bathed with bicarbonate-free (HEPES)-buffered medium. Group labels are the same as in Figure 1. In Iso-treated rats, myocardial pH<sub>i</sub> was more alkaline, suggesting increased acid extrusion. This effect was not observed in rats that were simultaneously treated with Iso plus the NHE-1 inhibitor BIIB723. \**P*<0.05

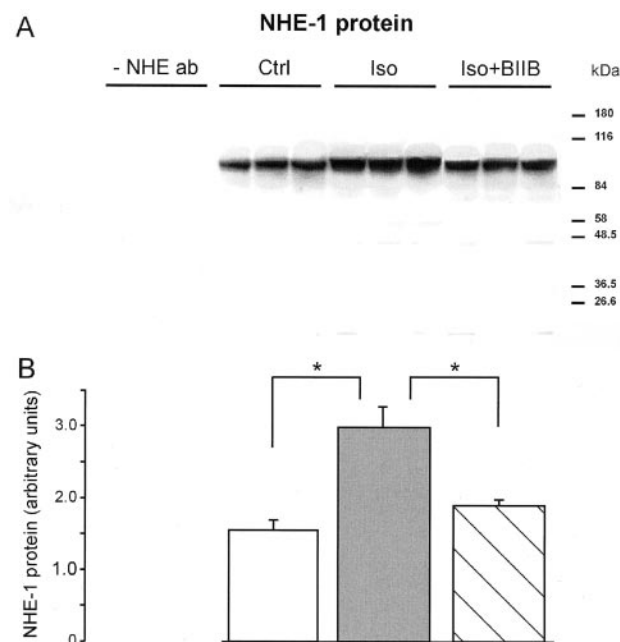


**Figure 6.** Effect of acute inhibition of NHE-1 activity on the resting pH<sub>i</sub> value of hypertrophied and normal myocardium. A, Time course of the decrease in pH<sub>i</sub> induced by application of 10  $\mu$ mol/L HOE 642 (n=4 each). Inhibition of NHE-1 activity induced a larger decrease in pH<sub>i</sub> in the hypertrophied myocardium, thus cancelling the difference with Ctrl myocardium. B, Bars show average resting pH<sub>i</sub> values before HOE 642 (basal) and the steady pH<sub>i</sub> value predicted from the exponential fit of the data shown in A (+HOE). \**P*<0.05.

stimulation might stimulate fibroblast proliferation and collagen synthesis through an NHE-1-dependent pathway. A similar antifibrotic action of cariporide was reported in the hypertrophied hearts of mice overexpressing  $\beta_1$ -adrenoceptors.<sup>22</sup>

### Perspective

CH has been established as 1 of the most powerful predictors of cardiovascular morbidity and mortality. In the past few



**Figure 7.** Increased NHE-1 protein expression in myocardial tissue from Iso-treated rats and its normalization by treatment with an NHE-1 inhibitor. A, Western blots from the indicated rat cardiac membranes. The first 3 lanes correspond to the picture obtained when the membrane was incubated with the secondary antibody alone. B, Mean  $\pm$  SEM NHE-1 protein expression (arbitrary units) (n=3 each); group labels have the same meaning as in Figure 1. \**P*<0.05.



years, there has been impressive progress in understanding of the mechanism(s) of myocardial hypertrophy, and sound evidence has emerged about the main role played by NHE-1 activity in many, if not all, types of CH. The inhibition of NHE-1 activity appears to constitute a potential, novel therapeutic strategy to interfere with the hypertrophic process independently of load mechanisms.

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