Early and Transient Sodium-Hydrogen Exchanger Isoform 1 Inhibition Attenuates Subsequent Cardiac Hypertrophy and Heart Failure Following Coronary Artery Ligation

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ABSTRACT

Na+/H+ exchanger 1 (NHE-1) inhibition attenuates the hypertrophic response and heart failure in various experimental models. As the hypertrophic program is rapidly initiated following insult, we investigated whether early and transient administration of a NHE-1 inhibitor will exert salutary effects on cardiomyocyte hypertrophy or heart failure using both in vitro and in vivo approaches. Neonatal cardiomyocytes were treated with the novel, potent, and highly specific NHE-1 inhibitor BIX (N-[4-(1-acetyl-piperidin-4-yl)-3-trifluoromethyl-benzoyl]-guanidine; 100 nM) for 1 hour in the presence of 10 μM phenylephrine, after which the cells were maintained for a further 23 hours in the absence of NHE-1 inhibition. One-hour treatment with the NHE-1 inhibitor prevented phenylephrine-induced hypertrophy, which was associated with prevention of activation of calcineurin, a key component of the hypertrophic process. Experiments were then performed in rats subjected to coronary artery ligation, in which the NHE-1 inhibitor was administered immediately after infarction for a 1-week period followed by a further 5 weeks of sustained coronary artery occlusion in the absence of drug treatment. This approach significantly attenuated left ventricular hypertrophy and improved both left ventricular systolic and diastolic dysfunction, which was also associated with inhibition of calcineurin activation. Our findings indicate that early and transient administration of an NHE-1 inhibitor bestows subsequent inhibition of cardiomyocyte hypertrophy in culture as well as cardiac hypertrophy and heart failure in vivo, suggesting a critical early NHE-1–dependent initiation of the hypertrophic program. The study also suggests a preconditioning-like phenomenon in preventing hypertrophy and heart failure by early and transient NHE-1 inhibition.

Introduction

The incidence of heart failure has increased markedly over the past several years and continues to rise (Roger et al., 2011). Although various pharmacotherapeutic options are available for the treatment of heart failure, survival rates have not dramatically improved and remain at approximately 50% 5 years after initial diagnosis (Krum and Teerlink, 2011). Identification of novel therapeutic approaches for the treatment of heart failure and the accompanying hypertrophic and remodeling processes represent important initiatives for identifying effective therapeutic strategies. The sodium-hydrogen exchanger (NHE) appears to be a potential key target for limiting the myocardial remodeling process. This antiporter represents an important regulator of intracellular pH homeostasis by virtue of its ability to extrude intracellular protons in exchange for sodium influx via a 1:1 stoichiometric relationship, thus rendering this an electroneutral pathway (Orlowski and Grinstein, 2011). Although 10 NHE isoforms have thus far been identified, NHE isoform 1 (NHE-1) is the primary isoform identified in cardiac tissue (Karmazyn et al., 1999). There is now convincing evidence that NHE-1 activity represents an important mechanism for induction of myocardial hypertrophy, remodeling, and the subsequent evolution to heart failure (Chen et al., 2001, 2004; Engelhardt et al., 2002; Baartscheer et al., 2003; Ennis et al., 2003; Chahine et al., 2005; Kilić et al., 2005). Precisely how NHE-1 activation contributes to these responses is not known with certainty, although with respect to hypertrophy, this likely occurs as a result of the elevation in intracellular sodium concentrations, which then increases intracellular calcium concentrations via sodium-calcium exchange, the latter likely operating in a reverse-mode capacity, thus actively importing calcium ions into the cardiomyocyte (Karmazyn et al., 2008). A particularly important response to an elevation in intracellular calcium concentrations, and the subsequent formation of the calcium-calmodulin complex, is activation of calcineurin, a calcium-activated phosphatase critically important for the hypertrophic program (Frey and Olson, 2003). Indeed, overexpression of cardiac

ABBREVIATIONS: ANP, atrial natriuretic peptide; BIX, N-[4-(1-acetyl-piperidin-4-yl)-3-trifluoromethyl-benzoyl]-guanidine; CAL, coronary artery ligation; MCIP-1, modulatory calcineurin-interacting protein 1; NFAT, nuclear factor of activated T cells; NHE-1, sodium-hydrogen exchanger isoform 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.
NHE-1 in transgenic mice on its own, in the absence of pathologic insult, has been shown to induce calcineurin upregulation and a calcineurin-dependent hypertrophic response (Nakamura et al., 2008; Xue et al., 2010; Mraiche et al., 2011).

NHE-1 is an extensively regulated protein that can be activated by a large number of intracellular messengers (Karmazyn et al., 1999; Orlowski and Grinstein, 2011). Interestingly, the stretching of cardiac tissue can rapidly activate NHE-1, resulting in elevations in intracellular calcium concentrations (Cingolani et al., 1998, 2011), suggesting that myocardial deformation following insult can rapidly activate the exchanger. Consequently, it is possible that early NHE-1 activation represents a critical period during which the antiporter contributes to myocardial hypertrophy, remodeling, and failure. To our knowledge, contribution of early NHE-1 activation to the hypertrophic program has hitherto not been studied. To assess this, we transiently administered a novel and highly specific NHE-1 inhibitor for 1 hour to cardiomyocytes treated for 24 hours with the prohypertrophic factor phenylephrine. Additionally, we subjected rats to experimental myocardial infarction for a 6-week period in which the NHE-1 inhibitor was administered for only the first week following coronary artery ligation, with no treatment during the remaining 5-week follow-up period. Overall, the results suggest that early transient NHE-1 inhibition offers substantial benefit against the development of cardiomyocyte hypertrophy and development of heart failure.

**Materials and Methods**

**Primary Culture of Neonatal Rat Cardiomyocytes.** Neonatal (1–3 days) and adult male (225–250 g) Sprague-Dawley rats were purchased from Charles River (St. Constant, QC, Canada). The investigation protocols followed were in accordance with the University of Western Ontario animal care guidelines and conform to the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada). To inhibit NHE-1, we used the recently developed, highly specific and potent NHE-1 inhibitor BIX (N-[4-(1-acetyl-piperidin-4-yl)-3-trifluoromethylbenzoyl]-guanidine; 100 nM), which exhibits excellent pharmacokinetic and pharmacodynamic properties (Huber et al., 2012). For convenience, the compound is referred to as BIX in this report; its chemical structure is shown in Fig. 1.

Primary cultures of cardiomyocytes were prepared from 1- to 4-day-old Sprague-Dawley neonatal rat hearts as described previously (Gan et al., 2008; Xue et al., 2010; Mraiche et al., 2011). In brief, rats were sacrificed by decapitation, and hearts were perfused in situ via the aorta with a modified Hanks’ balanced salt solution, 20 mM HEPES (pH 7.4), and 1% penicillin/streptomycin. Ventricles were minced and the tissue was subjected to four sequential digestions using a solution containing 0.4% type 2 collagenase in the aforementioned buffer in a water-jacketed Erlenmeyer flask maintained at 37°C. After each extraction, the clear supernatant was removed and the aforementioned buffer in a water-jacketed Erlenmeyer flask maintained at 37°C. After each extraction, the clear supernatant was removed and neutralized with an equal volume of solution containing 1× Hanks’ balanced salt solution, 1% penicillin/streptomycin, and 20% fetal bovine serum to stop collagenase activity. All extractions were pooled, filtered using a 70-μm cell strainer, and centrifuged at 500g for 5 minutes at 4°C. The pellet was resuspended in cell culture medium supplemented with 10% fetal bovine serum and subjected to two 30-minute periods of preincubation to enrich the cardiomyocyte population. Our method of preparation resulted in more than 90% cardiomyocyte yield as demonstrated by myosin staining for noncardiomyocytes.

**Cell Surface Area Measurement.** Cardiomyocytes were cultured for 24 hours in serum-containing medium followed by 24 hours in a serum-free medium. Cells were subjected to treatment with the α1 adrenergic receptor agonist phenylephrine (10 μM) for 24 hours either alone or in the presence of BIX for the first hour of treatment. For the latter treatment group, myocytes were washed with phosphate-buffered saline (PBS), and treatment with phenylephrine was continued for a further 23 hours. Myocytes were photographed using a Leica DM IL (Wetzlar, Germany) inverted microscope equipped with an Infinity1 camera (Lumenera Corporation, Ottawa, ON, Canada) at 100× magnification. At least 60 individual cell surface areas from each sample were measured using SigmaStat Pro 5.0 software (Systat Software, Inc., Chicago, IL).

**RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction.** Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Five micrograms of RNA was used to synthesize first-strand cDNA using the Moloney murine leukemia virus (Life Technologies, Burlington, ON, Canada) according to the manufacturer’s protocol, and was used as a template in the following polymerase chain reactions (PCRs). The expression of atrial natriuretic peptide (ANP), modulatory calcineurin-interacting protein 1 (MCIP-1), and 18S rRNA (as a loading control) genes was determined in 10 μl reaction volumes containing 2× Eva Green quantitative PCR Mastermix-S (Applied Biological Materials, Richmond, BC, Canada), 0.5 μM forward and backward primers, and cDNA. SYBR green fluorescence was measured using an Opticon 2 continuous fluorescence detector and quantified with the Opticon Monitor analysis software from Bio-Rad (Hercules, CA). PCR conditions used to amplify genes were as follows: 30 seconds at 94°C, followed by annealing at 58°C for 20 seconds for ANP and MCIP-1 and 50°C for 20 seconds for 18S RNA, followed by elongation at 72°C for 30 seconds. ANP and MCIP-1 were amplified for 40 cycles, whereas 18S was amplified for 35 cycles. Primer sequences for these genes were as follows: ANP, 5′-CTGCTAGACACCTTGGAAGAGGA-3′ (forward) and 5′-AAAGCTTGTGCAGCCGTCTG-3′ (reverse); MCIP-1, 5′-GGGACTTGGGCAGCAACG-3′ (forward) and 5′-TGATTCTTGTTGGCTGCTC-3′ (reverse); 18S, 5′-CAGGCATTACCAACATG-3′ (forward) and 5′-CCGAGGCACTAGAGAGAC-3′ (reverse).

**Calcineurin Phosphatase Activity Assay.** Calcineurin activity in cardiomyocytes and heart tissue was measured using a Biomol green calcineurin assay kit (Enzo Life Sciences, Ann Arbor, MD) according to the manufacturer’s instructions. Total protein (3 μg) was used to determine the calcineurin phosphatase activity by measuring free phosphate released from the calcineurin-specific RII substrate at 620 nm using a SpectraMax 5 (Molecular Devices, Sunnyvale, CA) plate reader.

**Immunofluorescent Cell Staining.** Cardiomyocytes were grown on collagen- precoated glass coverslips and maintained in serum-containing medium for 48 hours followed by incubation in serum-free media for 24 hours. Following the appropriate treatments, cardiomyocytes were fixed for 10 minutes in ice-cold acetone/methanol (20:80). After fixation, cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 minutes and washed twice again with PBS. Non specific binding sites were blocked with blocking solution (1% bovine serum albumin, 0.1% Triton X-100 in PBS) for 10 minutes and washed twice with PBS. The cardiomyocytes were then incubated with primary antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. Cardiomyocytes were then washed three times with PBS and incubated for 1 hour at room temperature with Alexa Fluor 488–conjugated goat anti-mouse and/or Alexa 594–conjugated goat anti-rabbit secondary antibodies (1:250; Life Technologies). Immunofluorescence was assessed with a Zeiss LSM 510 microscope (Carl Zeiss, Oberkochen, Germany).

**Fig. 1.** Chemical structure of BIX.

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**Measurement of Cellular Calcium and Sodium Concentrations.** To determine intracellular calcium levels, myocytes were loaded with 0.5 μM Fura-2 (Life Technologies) by incubating for 30 minutes at 37°C followed by washing with PBS and incubating for 30 minutes at 37°C in culture medium. Fluorescence was determined at 340-nm excitation and 540-nm emission using a Molecular Devices Spectramax M5 with Softmax Pro plate reader software. For determining sodium concentrations, myocytes were loaded with 1.0 μM CoroNa Red indicator (Life Technologies) using an identical procedure as described for determination of calcium concentrations. Fluorescence was read at 545-nm excitation and 575-nm emission.

**In Vivo Model of Postinfarction Remodeling.** Initial studies were carried out to assess the effect of BIX when administered throughout the postsurgery period, as well as to determine the optimal dose by administering the NHE-1 inhibitor in the diet at a concentration of either 45 or 150 parts per million (ppm). In view of the generally superior efficacy seen with the higher BIX dose (see Results), the higher dose was selected for further study to assess benefit with early transient administration. Thus, rats were subsequently assigned to one of four groups: 1) sham group, 2) sham group treated with BIX, 3) coronary artery ligation (CAL) group, and 4) CAL group treated with BIX. Myocardial infarction was induced by ligation of the left main coronary artery as previously described (Chen et al., 2004). In the sham group, the ligature was placed in an identical fashion but not tied. The animals in both groups were placed on a normal diet for an additional 5 weeks after surgery. At the end of the 6-week post-CAL period, rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). An anterior thoracotomy was performed, and the left ventricle was catheterized retrogradely via the right carotid artery using a 3-Fr Millar pressure transducer catheter (Millar Inc., Houston, TX) to obtain left ventricular pressure values.

**In Vitro Studies.** Our initial set of studies was to assess the effect of BIX and determine the optimal dose of the drug when administered to rats throughout the 6-week post-CAL period. These results are summarized in Table 1 and illustrate a substantial attenuation of CAL-induced left ventricular dysfunction by the NHE-1 inhibitor, whereas a more than 3-fold increase in translocation of the latter into nuclei. Previous findings from our laboratory revealed that peak NFAT translocation occurs 3 hours after phenylephrine administration, and therefore, this time point was selected to determine the effects of treatments on NFAT translocation into nuclei. As evident in Fig. 3, C and D, NFAT translocation was completely abrogated by prior treatment with the NHE-1 inhibitor, whereas a more than 3-fold increase in translocation was seen with phenylephrine alone.

Phenylephrine significantly increased intracellular levels of both Ca²⁺ as well as Na⁺, as determined by Fura-2 AM and CoroNa Red fluorescence intensity, respectively, although both effects were significantly attenuated by early treatment with the NHE-1 inhibitor (Fig. 4).

**Statistical Analysis.** All values in the figures and text are presented as the mean ± S.E.M. Multiple comparisons between groups were determined by one-way analysis of variance followed by a post-hoc Tukey test. Differences were considered to be statistically significant at a level of P < 0.05.

**Results**

**In Vitro Studies.** Figure 2 summarizes the treatment protocol for the cultured myocyte studies (Fig. 2A) and demonstrates the hypertrophic response following phenylephrine addition. Phenylephrine produced a significant increase in cell surface area of 47% (P < 0.05) concomitant with a 4-fold elevation in ANP expression after 24 hours of treatment (Fig. 2, B and C). In contrast, early transient treatment with the NHE-1 inhibitor for 1 hour after phenylephrine administration completely prevented the hypertrophic response.

**In Vivo Studies.** Our initial set of studies was to assess the effect of transient NHE-1 inhibition, we determined the degree of calcineurin activation, in view of the importance of this phosphatase to the hypertrophic program. These data are summarized in Fig. 3 and show a general inhibition of calcineurin activation as determined by both phosphatase activity (Fig. 3A) as well as MCIP-1 expression (Fig. 3B), although differences in temporal patterns of inhibition were evident. In particular, NHE-1 inhibition prevented activation of calcineurin 6 and 24 hours after phenylephrine administration, whereas a significant inhibition of MCIP-1 upregulation was seen only after 6 hours of phenylephrine treatment but not after 24 hours. A major function of calcineurin activation is to dephosphorylate the transcriptional factor NFAT, resulting in the translocation of the latter into nuclei. Previous findings from our laboratory revealed that peak NFAT translocation occurs 3 hours after phenylephrine administration, and therefore, this time point was selected to determine the effects of treatments on NFAT translocation into nuclei. As evident in Fig. 3, C and D, NFAT translocation was completely abrogated by prior treatment with the NHE-1 inhibitor, whereas a more than 3-fold increase in translocation was seen with phenylephrine alone.

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**Discussion.** Early transient treatment with the NHE-1 inhibitor in the diet at a concentration of 150 ppm (Research Diet Inc., New Brunswick, NJ) significantly attenuated the effects of phenylephrine administration, whereas a more than 3-fold increase in translocation was seen with phenylephrine alone.

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as well as the hypertrophic response with BIX administration in the chow at both 45 and 150 ppm, although the higher dose was generally more effective, particularly in terms of hypertrophy as determined by tissue weights.

We next determined whether treating animals with the NHE-1 inhibitor (at 150 ppm) for only 1 week after CAL, after which treatment was removed and the animals were followed for a further 5-week period, can bestow protection against sustained coronary occlusion (Fig. 5A). At the end of this period, hearts from rats subjected to 6 weeks of CAL exhibited left ventricular hypertrophy as manifested by increased tissue weights as well as a marked elevation in expression of ANP (Fig. 5). These responses to sustained CAL were nearly completely inhibited by early, 1-week treatment with BIX.

Assessment of hemodynamics revealed significantly depressed left ventricular systolic function in terms of developed pressure and rate of pressure development concomitant with diastolic abnormalities as evidenced by a 3-fold elevation in left ventricular end-diastolic pressure as well as attenuation of left ventricular relaxation efficiency (Fig. 6). However, the degree of hemodynamic dysfunction seen 6 weeks after CAL was significantly attenuated by early, 1-week treatment with the NHE-1 inhibitor. No effects were seen on either mean arterial pressures or heart rates in these animals (Fig. 7).
Based on our findings in cultured cardiomyocytes, we further determined whether inhibition of the hypertrophic response in the chronically infarcted myocardium is also associated with diminution of calcineurin activation as assessed both by phosphatase activity and indirectly by determining MCIP-1 expression. Indeed, hearts subjected to 6 weeks of sustained CAL demonstrated significantly increased calcineurin activity, although this was completely suppressed by early and transient NHE-1 inhibition (Fig. 8).

**Discussion**

NHE-1 inhibition has been long recognized to be an effective approach to limiting infarct size in animals subjected to ischemia and reperfusion, although for many reasons, as recently reviewed, this has not translated to a desired efficacy in clinical trials (Karmazyn, 2013). A number of studies using different experimental models have also demonstrated a beneficial effect of NHE-1 inhibition in attenuating hypertrophy and remodeling (Chen et al., 2001, 2004; Engelhardt et al., 2002; Baartscheer et al., 2003; Ennis et al., 2003; Chahine et al., 2005; Kilić et al., 2005). Most experimental studies aiming to reduce heart failure have used approaches in which therapies were administered chronically throughout the monitoring period, a logical approach since myocardial remodeling represents a chronic process reflecting a complex series of intracellular events following initial insult. The contribution of NHE-1 to myocardial hypertrophy and remodeling also likely reflects substantial chronicity since the antiporter is subjected to prolonged stimulation either via pH-dependent mechanisms or through the increased generation of locally generated prohypertrophic autocrine and paracrine factors (Karmazyn et al., 1999, 2008) independent of genomic

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**TABLE 1**

Effect of 6-week treatment with two doses of BIX on heart weights, hemodynamic parameters, and heart rates in rats subjected to 6 weeks of sustained CAL or sham surgery

The sham group represents pooled data for untreated and BIX-treated animals at both doses. Values indicate means ± S.E. with the number of animals in each group shown in parentheses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatments</th>
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<tbody>
<tr>
<td></td>
<td>CAL (8)</td>
</tr>
<tr>
<td>HW/BW (g/kg)</td>
<td>3.4 ± 0.21</td>
</tr>
<tr>
<td>LVW/BW (g/kg)</td>
<td>1.47 ± 0.07</td>
</tr>
<tr>
<td>LVSP (mm Hg)</td>
<td>127 ± 4.3</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>13.2 ± 0.7</td>
</tr>
<tr>
<td>LV+dP/dt(_{\text{max}}) (mm Hg/s)</td>
<td>4983 ± 211</td>
</tr>
<tr>
<td>LV-dP/dt(_{\text{min}}) (mm Hg/s)</td>
<td>5221 ± 234</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>139 ± 11.1</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>428 ± 22</td>
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HR, heart rate; HW/BW, heart weight-to-body weight ratio; LV+dP/dt\(_{\text{max}}\), maximum of the first derivative of left ventricular pressure development over time; LV-dP/dt\(_{\text{min}}\), minimum of the first derivative of left ventricular pressure over time; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular end-systolic pressure; LVW/BW, left-ventricular-weight-to-body-weight ratio; MAP, mean arterial pressure.

*P < 0.05 from CAL alone; **P < 0.05 from CAL + 45 ppm BIX.
mechanisms. Indeed, it has been well documented that myocardial stretch rapidly activates NHE-1 as a consequence of agonist-dependent activation of both angiotensin AT₁ and endothelin ET₁ receptors (Cingolani et al., 1998, 2011).

In view of the rapidity by which NHE-1 can be activated, we sought to determine whether early NHE-1 activation represents a critical period during which the antiporter contributes to myocardial hypertrophy, remodeling, and failure. Experiments were therefore carried out on cardiomyocytes exposed to hypertrophic stimuli for 24 hours, but in which the NHE-1 inhibitor was administered for only the first hour of hypertrophic stimulation, or in a rat model of heart failure in which animals were subjected to sustained coronary artery occlusion for a 6-week period and administered the NHE-1 inhibitor for only the first week following coronary artery ligation with no treatment during the remaining 5-week follow-up period.

Although comparisons between experiments using cultured cardiomyocytes and in vivo approaches should be done cautiously, some similarities can, however, be identified particularly with respect to NHE-1. For the cardiomyocyte experiments, we used the α₁-adrenoceptor agonist phenylephrine as the hypertrophic stimulus, primarily because activation of this receptor increases NHE-1 activity in cardiomyocytes, which likely contributes to the hypertrophic response (Wallert and Fröhlich, 1992; Javadov et al., 2006; Kilic et al., 2010). Thus, NHE-1 activation likely plays a critical role in the hypertrophic response to a number of stimulatory factors, both in vitro and in vivo (Karmazyn et al., 2008).

The NHE-1–specific inhibitor used in the present study is a structurally modified form of the benzoguanidine NHE-1 inhibitor sabiporide shown to be an effective cardioprotective agent (Touret et al., 2003), with the major modification of the piperazine moiety to a piperidine, thereby bestowing enhanced efficacy (Huber et al., 2012).

Taken together, our results clearly show that early treatment with the potent NHE-1 inhibitor results in marked attenuation of the hypertrophic response in cultured cardiomyocytes as well as significant attenuation of hypertrophy, remodeling, and left ventricular dysfunction in the 6-week postinfarcted rat heart. The results suggest that early transient NHE-1 inhibition offers substantial benefit against the development of heart failure, likely by preventing an NHE-1–dependent cascade of events during a critical initiation of the hypertrophic program. However, the benefit seen with early and transient BIX administration was substantially less than that seen when an identical dose of the drug was administered throughout the post-CAL period, thus suggesting that sustained drug administration after infarction...
represents the ideal treatment to obtain optimal benefit, further suggesting that NHE-1 activity continues chronically post-infarction and thus contributing to the remodeling process during the period.

The inhibition of the hypertrophic response in both cultured myocytes and intact postinfarcted hearts was associated with sustained diminution of calcineurin activity, suggesting an important role for NHE-1 in initiating calcineurin activation during the early phase of the hypertrophic process. The link between NHE-1 and calcineurin activation is reinforced by a number of previous studies linking the inhibition of NHE-1 activity with prevention of calcineurin activation (Ennis et al., 2007; Kilić et al., 2010; Guo et al., 2011). Moreover, myocardial NHE-1 overexpression in mice in the absence of insult results in hypertrophy and left ventricular dysfunction (Nakamura et al., 2008; Xue et al., 2010). Nakamura and colleagues (2008) have linked this phenomenon to calcineurin activation as a direct consequence of NHE-1–dependent elevation in intracellular Ca\(^{2+}\) concentrations. Moreover, inhibition of calcineurin activity prevented the hypertrophic response to myocardial NHE-1 overexpression (Nakamura et al., 2008). The results in the present study using cultured cardiomyocytes support the concept of a Ca\(^{2+}\)-dependent calcineurin activation resulting in a hypertrophic response based on the fact that early NHE-1 inhibition prevented the increase in intracellular Ca\(^{2+}\) as well as Na\(^{+}\) concentrations following phenylephrine administration. This was associated with significantly reduced translocation of NFAT into nuclei. Additionally, suppression of calcineurin activation was clearly evident in rats subjected to coronary artery occlusion that were treated for the first week with NHE-1 inhibitor. As was seen in studies on cultured myocytes, this was associated with diminution of MCIP-1 expression. The likely primary role of MCIP-1 is to serve as an endogenous suppressor of calcineurin activity, especially at high MCIP-1 concentrations, and in this regard, MCIP-1 overexpression has been shown to be associated with reduced hypertrophy in response to various stimuli, including \(\beta\)-adrenoceptor stimulation, exercise, or overexpression of a constitutively active form of calcineurin (Rothermel et al., 2001). However, MCIP-1 likely exerts diverse influence on cardiac hypertrophy, which reflects the nature of the hypertrophic stimuli: indeed, the hypertrophic response to pressure overload or sustained adrenergic stimulation due to isoproterenol administration is inhibited in MCIP-1–null mice (Vega et al., 2003). In the present study, transient administration of the NHE-1 inhibitor was associated with decreased calcineurin activity as well as MCIP-1 expression, although the exact role of MCIP-1 in mediating changes in calcineurin activity under our experimental conditions is difficult to know at present. In the cultured myocyte study, early NHE-1 inhibition was reflected in reduced NFAT import into nuclei at a relatively early time point of 3 hours. This result may be somewhat analogous to a previous report which suggests that calcineurin inhibition with cyclosporine \(A\) prevents early but not late remodeling in the postinfarcted rat myocardium, although the experimental approach was markedly different from that used in the present report (Youn et al., 2002).

From a conceptual perspective, the results of the present study bear some degree of similarity to the phenomenon of myocardial preconditioning in which a brief episode or episodes of sublethal myocardial ischemia (ischemic preconditioning) or transient administration of a pharmacologic agent (pharmacologic preconditioning) bestows protection of the ischemic and reperfused myocardium (Ovize et al., 2013), although the duration of treatment in our study was substantially greater than that used for classic preconditioning. This protection can be observed acutely in hearts subjected to ischemia minutes following the preconditioning protocol or in hearts made ischemic 12–24 hours after preconditioning, the so-called second window of protection (Hausenloy and Yellon, 2010). The exact mechanisms underlying preconditioning are not known with certainty, although the production of numerous mediators has been proposed, especially for acute preconditioning, whereas the second window of protection is dependent on de novo synthesis of protective factors (Hausenloy and Yellon, 2010). We believe that the present results suggest a preconditioning-like phenomenon, because intervention with the NHE-1 inhibitor occurred only for a transient initial period following either administration of the hypertrophic stimulus in cultured cardiomyocytes or coronary artery ligation. The latter was particularly both impressive and surprising since sustained coronary artery occlusion occurred for 5 of the 6 weeks in the absence of pharmacologic intervention. How this occurs precisely is still under study, but we believe that early inhibition of NHE-1 following its initial stimulation prevents a cascade of events critical for the subsequent development of hypertrophy and heart failure. As mentioned, this likely reflects attenuation of calcineurin activation, resulting in inhibition of the hypertrophic program by preventing NFAT import into nuclei. However, other potential mechanisms may also be identified with further studies.

In conclusion, our study is the first to show that early transient inhibition of NHE-1 bestows subsequent protection against development of cardiomyocyte hypertrophy and left ventricular dysfunction following coronary artery ligation. This study may therefore have important clinical implications, as it suggests that early events likely related to NHE-1 activation represent an important target for therapeutic intervention to
attenuate subsequent hypertrophy and evolution to heart failure. It is interesting to note that in one clinical study in which the NHE-1 inhibitor eniporide was administered to patients subjected to late (>4 hours following the onset of symptoms) reperfusion, a significant reduction in heart failure was observed despite the absence of infarct size reduction (Zeymer et al., 2001). Although not directly analogous to our experimental model, taken together, these results offer further evidence that NHE-1 inhibition at a relatively critical period following infarction can bestow a reduction in subsequent heart failure in the absence of myocardial tissue salvage.

Authorship Contributions

Participated in research design: Kilić, Madwed, Karmazyn.
Conducted experiments: Kilić, Huang, Rajapurohitam.
Contributed new reagents or analytic tools: Madwed.
Performed data analysis: Kilić, Huang.
Wrote or contributed to the writing of the manuscript: Kilić, Karmazyn.

References


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