Overexpression of the Cardiac Na\(^+\)/Ca\(^{2+}\) Exchanger Increases Susceptibility to Ischemia/Reperfusion Injury in Male, but Not Female, Transgenic Mice

Heather R. Cross, Liyan Lu, Charles Steenbergen, Kenneth D. Philipson, Elizabeth Murphy

Abstract—Influx of Ca\(^{2+}\) into myocytes via Na\(^+\)/Ca\(^{2+}\) exchange may be stimulated by the high levels of intracellular Na\(^+\) and the changes in membrane potential known to occur during ischemia/reperfusion. This increased influx could, in turn, lead to Ca\(^{2+}\) overload and injury. Overexpression of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger therefore may increase susceptibility to ischemia/reperfusion injury. To test this hypothesis, the hearts of male and female transgenic mice, overexpressing the Na\(^+\)/Ca\(^{2+}\) exchange protein, and hearts of their wild-type littermates, were perfused with Krebs-Henseleit buffer and subjected to 20 minutes of ischemia and 40 minutes of reperfusion. Preischemic left ventricular developed pressures and +dP/dt\(_{max}\), as well as −dP/dt\(_{max}\), were higher in the male transgenic hearts compared with wild-type, implying a role for Na\(^+\)/Ca\(^{2+}\) exchange in the contraction, as well as the relaxation, phases of the cardiac beat. Postischemic function was lower in male transgenic than in male wild-type hearts (7±2% versus 32±6% of preischemic function), but there was no difference between female transgenic and female wild-type hearts, both at ≈30% of preischemic function. To assess whether this male/female difference was due to female-specific hormones such as estrogen, the hearts of bilaterally ovariectomized and sham-operated transgenic females were subjected to the same protocol. The functional recoveries of ovariectomized female transgenic hearts were lower (17±3% of preischemic function) than those of wild-type and sham-operated transgenic females. The lower posts ischemic functional recovery in the male transgenic and female ovariectomized transgenic hearts correlated with lower recoveries of the energy metabolites, ATP and phosphocreatine, as measured by \(^{31}\)P nuclear magnetic resonance spectroscopy. Alternans were observed during reperfusion in male transgenic and female ovariectomized transgenic hearts only, consistent with intracellular Ca\(^{2+}\) overload. Western analyses showed that alterations in the expression of the Na\(^+\)/Ca\(^{2+}\) exchange or L-type Ca\(^{2+}\) channel proteins were not responsible for the protection observed in the female transgenic hearts. In conclusion, in males, overexpression of the Na\(^+\)/Ca\(^{2+}\) exchanger reduced posts ischemic recovery of both contractile function and energy metabolites, indicating that the Na\(^+\)/Ca\(^{2+}\) exchanger may play a role in ischemia/reperfusion injury. From the studies of females, however, it appears that this exacerbation of ischemia/reperfusion injury by overexpression of the Na\(^+\)/Ca\(^{2+}\) exchanger can be overcome partially by female-specific hormones such as estrogen. (Circ Res. 1998;83:1215-1223.)

Key Words: alternans ■ estrogen ■ ischemia ■ Na\(^+\)/Ca\(^{2+}\) exchange ■ protein expression

The cardiac Na\(^+\)/Ca\(^{2+}\) exchanger mediates the countertransport of 3 Na\(^+\) ions for 1 Ca\(^{2+}\) ion across the sarcolemma (SL) membrane. It is a bidirectional transport process, capable of moving Ca\(^{2+}\) in either direction across the sarcolemma, depending on membrane potential and the transmembrane gradients of Na\(^+\) and Ca\(^{2+}\). A role for the Na\(^+\)/Ca\(^{2+}\) exchanger in mediating Ca\(^{2+}\) influx during myocardial ischemia and reperfusion, leading to Ca\(^{2+}\) overload and cellular injury, has been proposed.\(^1\)-\(^3\) Indirect evidence for this arises from observations that blocking the increase in Na\(^+\) attenuates the subsequent rise in Ca\(^{2+}\), manipulation of ischemic Na\(^+\) levels results in corresponding changes in intracellular Ca\(^{2+}\), and myocardial injury is reduced when hearts are reperfused with Ca\(^{2+}\)-free buffer.\(^1\)-\(^5\) However, as far as we are aware, no study has assessed the role of the Na\(^+\)/Ca\(^{2+}\) exchanger itself, via manipulations of exchanger expression or activity. In addition, it has not been shown that the Na\(^+\)/Ca\(^{2+}\) exchanger actually operates in a Ca\(^{2+}\) influx mode under true ischemic conditions.

A controversial role for the Na\(^+\)/Ca\(^{2+}\) exchanger in contributing to Ca\(^{2+}\) influx during the upstroke, or contraction phase, of the cardiac beat also has been proposed.\(^6\)-\(^7\) Largely conflicting evidence has been presented previously as to whether Na\(^+\)/Ca\(^{2+}\) exchange can operate in the Ca\(^{2+}\) influx...
mode under the conditions of the initial phase of the action potential and whether this Na\(^+\)/Ca\(^{2+}\) exchange-mediated Ca\(^{2+}\) influx could stimulate sarcolemmic reticular Ca\(^{2+}\) release and therefore contribute to contraction.\(^{7-11}\)

By comparing the response to ischemia in hearts from male and female mice overexpressing the Na\(^+\)/Ca\(^{2+}\) exchanger to that of their wild-type littermates, we aimed to provide evidence for the role of Na\(^+\)/Ca\(^{2+}\) exchange in ischemia/reperfusion injury. In addition, by monitoring basal contractility in mouse hearts from transgenic and wild-type animals, we hoped to ascertain whether Na\(^+\)/Ca\(^{2+}\) exchange contributed to the contraction phase of the cardiac cycle in the relatively physiological model of the perfused heart.

**Materials and Methods**

**Animals**

The transgenic mice used in this study were developed by Philipson and colleagues\(^{12-14}\) by microinjection of a transgene construct, containing the canine NCX1 gene under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter, into pronuclei of fertilized C57BL/6xC3H/F1 mouse oocytes. These mice were well-characterized.\(^{13,14}\) Tissue-specific RNA and protein overexpression were confirmed by Northern and Western blot, respectively.\(^{13}\) Increased Na\(^+\)/Ca\(^{2+}\) exchange activity was measured as both an increase in 4\(^{5}\)Ca\(^{2+}\) uptake into SL vesicles and increased Na\(^+\)/Ca\(^{2+}\) exchange current in whole cell patch-clamped myocytes.\(^{13}\) Use of these techniques demonstrated that Na\(^+\)/Ca\(^{2+}\) exchange activity was present at 150% to 300% of that in wild-type mice. In addition, several studies determined that secondary adaptations in Ca\(^{2+}\) homeostasis are minimal in these mice. Specifically, it has been shown that L-type Ca\(^{2+}\) channel density,\(^{13,14}\) sarcolemmic reticulum (SR) Ca\(^{2+}\)-ATPase activity,\(^{14}\) and SR Ca\(^{2+}\) levels\(^{13,14}\) are the same in myocytes from wild-type mice and mice overexpressing the Na\(^+\)/Ca\(^{2+}\) exchanger.

Five male and twenty-four female adult heterozygous transgenic mice weighing 36±2 g were used. Six male and fourteen female of their nontransgenic littermates weighing 38±4 g were used as experimental controls. All female animals were reproducitively competent at the time of experimentation (≈36 weeks old); males were ≈32 weeks old. All animals were treated in accordance with National Institutes of Health (NIH) guidelines.

**Ovariectomized Females**

Excision of both ovaries (bilateral ovariectomy or oophorectomy) was performed in 10 female transgenic mice, and a sham procedure was performed in 8 female transgenic mice. Mice were anesthetized with 2.5% isoflurane and oxygen before surgery. On recovery from anesthesia, 0.05 mg/kg Buprenex (Reckitt-Colman) was infused as an analgesic. Plasma estrogen falls to almost undetectable levels within 1 week of bilateral ovariectomy.\(^{14}\) To ensure that any gene expression effects of estrogen/other female hormones were reversed in the ovariectomized animals, 3 weeks were allowed between surgery and experimentation.

**Heart Perfusion**

The transgenic and wild-type mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body weight). Hearts were excised rapidly and arrested in ice-cold Krebs-Henseleit buffer. Excess tissue was removed and the hearts cannulated, via the ascending aorta, for retrograde perfusion by the Langendorff method using Krebs-Henseleit buffer containing (in mmol/L): NaCl 120, KCl 5.9, MgSO\(_4\)\(_{6}\)\(\times\)H\(_2\)O 1.2, CaCl\(_2\)\(\times\)H\(_2\)O 1.75, NaHCO\(_3\), 25, and glucose 11. The buffer, aerated with 95% O\(_2\)\(\times\)5% CO\(_2\) to give a pH of 7.4 at 37°C, was perfused through the hearts at a constant pressure of 100 cm H\(_2\)O. A water-filled latex balloon, attached via polyethylene tubing to a disposable pressure transducer (Argon), was inserted into the left ventricular cavity via the mitral valve and inflated sufficiently to result in an end diastolic pressure of ≈10 cm H\(_2\)O. Left ventricular pressure, ±dP/dt, and heart rate were recorded using a Maclab/2e System (AD Instruments Inc). Cardiac contractile function was expressed as left ventricular developed pressure (LVDP; peak systolic pressure minus end diastolic pressure).

The perfused mouse hearts were placed in a 10-mm–diameter nuclear magnetic resonance (NMR) sample tube, and coronary effluent was evacuated from an overflow outlet above the heart. Temperature was maintained at a constant 37°C by water-jacketed buffer reservoirs and perfusion lines, and by use of a variable temperature (VT) unit attached to the spectrometer.

**Experimental Protocol**

All hearts were perfused with Krebs-Henseleit buffer for a stabilization period of 30 minutes before the start of no-flow ischemia. Ischemia was initiated by clamping the perfusion line and continued for 20 minutes. Ischemic contracture was observed as a sigmoidal increase in diastolic pressure. The time of onset of contracture and the maximum diastolic pressure reached were recorded. Reperfusion, achieved in all hearts by restoring flow of Krebs-Henseleit buffer at 100 cm H\(_2\)O constant pressure, continued for 40 minutes. The extent of recovery of contractile function was assessed by measurement of LVDP at the end of reperfusion and was expressed as a percentage of preischemic LVDP. At the end of each experiment, hearts were blotted and weighed. Heart weights and heart-weight-to-body weight ratios (HW:BW) did not differ between wild-type and transgenic mice. Male wild-type heart weights were 0.18±0.01 g (HW:BW 4.2±0.4×10\(^{-3}\)), male transgenic hearts were 0.17±0.01 g (HW:BW 4.4±0.3×10\(^{-3}\)). Female wild-type heart weights were 0.13±0.01 g (HW:BW 4.0±0.1×10\(^{-3}\)) and female transgenic hearts were 0.14±0.01 g (HW:BW 4.1±0.2×10\(^{-3}\)).

**NMR Spectroscopy**

Relative changes in concentrations of phosphorus metabolites were observed during the ischemia/reperfusion protocol by acquiring consecutive 5-minute \(^{31}\)P NMR spectra using a GN-500 MHz spectrometer with an 11.7-T superconducting magnet (Oxford Instruments) at the \(^{31}\)P resonance frequency of 202.47 MHz. A 60° pulse with an interpulse delay of 1.8 s was used, and each spectrum consisted of 120 summed transients. Peak resolution was enhanced by reducing B\(_0\) inhomogeneities; this was achieved by shimming the nuclear magnetic resonance (NMR) sample tube, and coronary effluent was evacuated from an overflow outlet above the heart. Temperature was maintained at a constant 37°C by water-jacketed buffer reservoirs and perfusion lines, and by use of a variable temperature (VT) unit attached to the spectrometer.

**Western Blot Analysis**

Hearts from male wild-type (n=6), male transgenic (n=5), female wild-type (n=14), female transgenic (n=6), female sham-operated transgenic (n=8), and female ovariectomized transgenic mice (n=8) were frozen-clamped and ground in liquid nitrogen before suspension in lysis buffer containing 75 mmol/L NaCl, 20 mmol/L HEPES, 2.5 mmol/L MgCl\(_2\), 0.1 mmol/L EDTA, 20 mmol/L glycerophosphate, 0.05% Triton X-100, 0.5 mmol/L DTT, 0.1 mmol/L Na\(_2\)VO\(_4\), 4 μg/mL leupeptin, and 200 μg/mL PMSF (pH 7.7).\(^{17}\) Protein concentration was determined using a protein assay kit (BioRad). For determination of Na\(^+\)/Ca\(^{2+}\) exchanger overexpression, 50 μg protein was solubilized in sample buffer to yield a final concentration of 2% SDS, 62 mmol/L Tris–HCl, 5% glycerol, 0.01% bromphenol blue, and 25 μL/mL β-mercaptoethanol (pH 6.8). To allow determination of the molecular weights of separated proteins, high molecular weight (Gibco) and rainbow (Amersham Corp) markers were used.
Myocardial Contractile Function During Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart Rate, bpm</th>
<th>LVDP, cm H$_2$O</th>
<th>+dP/dt$_{max}$, cm H$_2$O/ms</th>
<th>−dP/dt$_{min}$, cm H$_2$O/ms</th>
<th>Ischemia, Maximum Contracture, cm H$_2$O</th>
<th>End Reperfusion, Heart Rate, bpm</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>330±13</td>
<td>116±12</td>
<td>4.1±0.5</td>
<td>−3.4±0.7</td>
<td>63±11</td>
<td>330±13</td>
</tr>
<tr>
<td>Transgenic</td>
<td>336±15</td>
<td>165±25*</td>
<td>6.5±1.3*</td>
<td>−5.1±0.9*</td>
<td>82±6*</td>
<td>298±27</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Wild-type</td>
<td>334±16</td>
<td>101±4</td>
<td>3.5±0.2</td>
<td>−2.6±0.2</td>
<td>57±5</td>
<td>311±21</td>
</tr>
<tr>
<td>Transgenic</td>
<td>385±19</td>
<td>128±10</td>
<td>4.6±0.4</td>
<td>−4.0±0.4</td>
<td>73±8</td>
<td>399±41</td>
</tr>
<tr>
<td>Transgenic (SHAM)</td>
<td>308±22</td>
<td>100±4</td>
<td>3.1±0.2</td>
<td>−2.0±0.1</td>
<td>77±6</td>
<td>315±18</td>
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<tr>
<td>Transgenic (OVEX)</td>
<td>336±15</td>
<td>155±9†</td>
<td>5.4±0.4†</td>
<td>−4.4±0.4†</td>
<td>69±6</td>
<td>311±18</td>
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</tbody>
</table>

Data are mean±SEM. SHAM indicates sham-operated; OVEX, ovariectomized. Maximum contracture is defined as the highest diastolic pressure reached during ischemia.

*Males: significant difference from wild-type males (P<0.05).
†Females: significant difference from wild-type females (P<0.001).

Results

Myocardial Contractile Function

Myocardial functional parameters for the 6 groups of mice are shown in the Table. At no time during the protocol were there any significant differences in heart rates between the groups.

Basal Contractility

During the preischemic period, LVDP, +dP/dt$_{max}$, and −dP/dt$_{min}$ were higher (P<0.05) in the male transgenic hearts (165 cm H$_2$O; +6.5 cm H$_2$O/ms and −5.1 cm H$_2$O/ms, respectively) than the male wild-type hearts (116 cm H$_2$O; +4.1 cm H$_2$O/ms and −3.4 cm H$_2$O/ms, respectively). These parameters were also higher in the female transgenic hearts (128 cm H$_2$O; +4.6 cm H$_2$O/ms and −4.0 cm H$_2$O/ms, respectively) than in the female wild-type hearts (101 cm H$_2$O; +3.5 cm H$_2$O/ms and −2.6 cm H$_2$O/ms, respectively), although not reaching statistical significance. Overexpression of Na"/Ca$^{2+}$ exchange in male hearts, therefore, increased basal cardiac contractility. LVDP, +dP/dt$_{max}$, and −dP/dt$_{min}$ were lower in the hearts of wild-type females (P<0.001) and sham-operated transgenic females (100 cm H$_2$O; +3.1 cm H$_2$O/ms and −2.0 cm H$_2$O/ms, respectively; P<0.001) than in the ovariectomized female transgenic hearts (155 cm H$_2$O; +5.4 cm H$_2$O/ms and −4.4 cm H$_2$O/ms, respectively). The lower contractility in female wild-type and female sham-operated transgenic than female ovariectomized transgenic hearts suggests that estrogen, or other female hormones, can result in direct or indirect negative inotropic effects.

Ischemic Contracture and Postischemic Functional Recovery

Hearts were subjected to no-flow ischemia for 20 minutes. Ischemic contracture began at ≈12 minutes and reached a maximum pressure at ≈17 minutes in all hearts (data not shown). The contracture maximum was higher (P<0.05) in male transgenic (82 cm H$_2$O) than male wild-type hearts (63 cm H$_2$O). There were no significant differences in contracture maximum among the female hearts.

By the end of the 40-minute reperfusion period, recovery of contractile function was significantly lower (P<0.001) in the male transgenic hearts (7% of initial LVDP) than in the male wild-type hearts (32% of initial LVDP; Figure 1). In the females, however, the transgenic and wild-type hearts recovered function to the same extent (≈30% of initial LVDP). To determine whether this male/female difference was due to female-specific hormones such as estrogen, a group of bilaterally ovariectomized and sham-operated transgenic females were studied. The postischemic recovery of contractile function was significantly lower (P<0.05) in the ovariectomized females (17% of initial LVDP) than in the wild-type and sham-operated transgenic females (39% of initial LVDP). In summary, in males, overexpression of the Na"/Ca$^{2+}$ exchanger lowered postischemic recovery of contractile function, indicating that the Na"/Ca$^{2+}$ exchanger may play a role in ischemia/reperfusion injury. From the studies of ovariectomized versus sham-operated transgenic females, it appears that this detrimental effect of Na"/Ca$^{2+}$ exchange overexpres-
During reperfusion, the occurrence of mechanical alternans, beats of alternating large and small amplitude at equal intervals (Figure 2), was significantly higher \((P<0.01)\) in the male transgenic hearts \((5/5)\) than in the male wild-type hearts \((0/6)\). The occurrence of mechanical alternans during the reperfusion period was also significantly higher \((P<0.01)\) in the ovariectomized female transgenic hearts \((5/11)\) than in the female wild-type hearts \((0/14)\). There was no significant difference in the occurrence of alternans between female nonovariectomized transgenic \((1/6)\) and female sham-operated transgenic \((1/8)\), compared with female wild-type hearts. Alternans occurred during reperfusion, therefore, only in hearts that also exhibited low recovery of postischemic contractile function.

### Coronary Flow

Preischemic coronary flow was the same in all hearts, at \(1.61\pm0.05\,\text{mL/min}\) in male wild-type, \(1.56\pm0.04\,\text{mL/min}\) in male transgenic, \(1.66\pm0.03\,\text{mL/min}\) in female wild-type, \(1.72\pm0.05\,\text{mL/min}\) in female transgenic, \(1.61\pm0.04\,\text{mL/min}\) in female sham-operated transgenic, and \(1.61\pm0.03\,\text{mL/min}\) in female ovariectomized transgenic hearts. Coronary flow at the end of reperfusion was the same in all hearts, at \(1.57\pm0.04\,\text{mL/min}\) in male wild-type, \(1.48\pm0.05\,\text{mL/min}\) in male transgenic, \(1.56\pm0.03\,\text{mL/min}\) in female wild-type, \(1.63\pm0.07\,\text{mL/min}\) in female transgenic, \(1.54\pm0.03\,\text{mL/min}\) in female sham-operated transgenic, and \(1.49\pm0.03\,\text{mL/min}\) in female ovariectomized transgenic hearts. Coronary flow did not differ significantly in any group between preischemia and end-reperfusion.

### Phosphate Metabolite Levels and Intracellular pH

Phosphate metabolite levels and intracellular pH were measured in all hearts by \(31^P\) NMR spectroscopy to determine whether overexpression of the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger altered myocardial energetics or pH regulation. These measurements also would determine whether the lower postischemic recovery of function in the male transgenic and female ovariectomized transgenic hearts correlated with a lower postischemic recovery of energy metabolites. Representative spectra are shown in Figure 3.

Changes in myocardial ATP levels during ischemia and reperfusion in the male and female hearts are shown in Figure 4A and 4B, respectively. Throughout ischemia, there were no significant differences in ATP levels in any of the hearts, all falling to \(\approx35\%\) of initial ATP. During reperfusion, ATP levels increased in the male/female wild-type, female transgenic, and female sham-operated transgenic hearts, all reaching \(\approx55\%\) of initial ATP, but remained approximately the same in the male transgenic and female ovariectomized transgenic hearts, at \(\approx30\%\) of initial ATP. Consequently, by the end of reperfusion, ATP levels were significantly lower in the male transgenic hearts than in the male wild-type hearts \((P<0.05)\) and were also significantly lower in the female ovariectomized transgenic hearts than in the female wild-type hearts \((P<0.01)\).

\(\text{PCr}\) decreased rapidly in all hearts at the onset of ischemia, the majority of the ischemic decrease being observed in the first 5 minutes (Figure 5A and 5B). By the end of ischemia, \(\text{PCr}\) levels were the same in all hearts, at \(<10\%\) of initial \(\text{PCr}\). On reperfusion, \(\text{PCr}\) levels increased in all hearts, reaching \(\approx40\%\) of initial \(\text{PCr}\) in the male transgenic and female ovariectomized transgenic hearts and reaching \(\approx60\%\) of initial \(\text{PCr}\) in all other groups. By the end of reperfusion, \(\text{PCr}\) levels were significantly lower in the male transgenic hearts than in the male wild-type hearts \((P<0.05)\) and were also significantly lower in the female ovariectomized transgenic hearts than in the female wild-type hearts \((P<0.05)\).

Intracellular \(\text{Pi}\) increased in all hearts during ischemia and decreased in all hearts during reperfusion. At no time during the protocol were there significant differences in intracellular \(\text{Pi}\) among any of the groups of hearts (data not shown). Phosphomonoester peaks were visible in spectra from all groups of hearts during ischemia and reperfusion, although the broad nature of these multiple peaks precluded reliable quantification.

Intracellular pH decreased during ischemia to \(\approx6.0\) in all hearts and returned to preischemic levels in all hearts during the first 5 minutes of reperfusion (data not shown). There were no significant pH differences among any of the groups during the protocol.
In summary, postischemic recoveries of the energy metabolites, ATP and PCr, were lower in the male transgenic and female ovariectomized transgenic hearts than in the male and female wild-type hearts, therefore correlating with the lower recovery of contractile function found in the male transgenic and female ovariectomized transgenic hearts. The observation that there were no differences in intracellular Pi or intracellular pH during reperfusion between any hearts implies that

**Figure 3.** $^{31}$P NMR spectra from male wild-type and transgenic hearts. Spectra shown were acquired during the preischemic period, at the end of 20 minutes of ischemia, and at the end of 40 minutes of reperfusion. PME indicates phosphomonoester.

**Figure 4.** Effect of ischemia and reperfusion on myocardial intracellular ATP levels in (A) male and (B) female Na$^{+}$/Ca$^{2+}$ exchange–overexpressor and wild-type mice. Points are mean±SEM; n=5 (M WT), n=6 (M Tr), n=14 (F WT), n=6 (F Tr), n=8 (F Tr SHAM), and n=10 (F Tr OVEX). A, At the end of reperfusion, ATP levels were significantly lower in M Tr than M WT (P<0.05). B, At the end of reperfusion, ATP levels were significantly lower in F Tr OVEX than F WT (P<0.01). See Figure 1 legend for expansions to abbreviations.

**Figure 5.** Effect of ischemia and reperfusion on myocardial intracellular PCr levels in (A) male and (B) female Na$^{+}$/Ca$^{2+}$ exchange–overexpressor and wild-type mice. Points are mean±SEM; n=5 (M WT), n=6 (M Tr), n=14 (F WT), n=6 (F Tr), n=8 (F Tr SHAM), and n=10 (F Tr OVEX). A, At the end of reperfusion, PCr levels were significantly lower in M Tr than M WT (P<0.05). B, At the end of reperfusion, PCr levels were significantly lower in F Tr OVEX than F WT (P<0.05). See Figure 1 legend for expansions to abbreviations.
the decreased postischemic contractile function is not due to the direct inhibitory effect of H⁺ and Pi on contractile proteins. There was no apparent effect of Na⁺/Ca²⁺ exchange overexpression on pH regulation in these hearts.

Protein Expression
Because estrogen is known to alter cardiac gene expression, we performed Western analyses on all hearts to determine whether the protection from ischemic injury observed in female Na⁺/Ca²⁺ exchange–overexpressor mouse hearts was due to a male/female difference in expression of Ca²⁺/Ca²⁺ exchange protein.

First, expression of the Na⁺/Ca²⁺ exchange protein was determined. Use of the specific polyclonal antibody directed against the canine NCX1 protein identified the 120-kDa band shown by Philipson et al to correlate with Na⁺/Ca²⁺ exchange activity. Two other dense bands were identified, a 70-kDa band, which is a proteolytic fragment of the 120-kDa protein, and a 160-kDa band corresponding to the nonreduced Na⁺/Ca²⁺ exchange protein. Both bands exhibited the same pattern of density as the 120-kDa band. At the level of protein loading used (50 μg), no 120-kDa bands were visible in male or female wild-type hearts, but strong bands were observed for male transgenic, female transgenic, female transgenic sham-operated, and female ovariectomized transgenic hearts (Figure 6A). Overexpression of the Na⁺/Ca²⁺ exchange protein in the transgenic mice therefore was confirmed.

Second, expression of the L-type Ca²⁺ channel (DHP receptor) protein was determined. Use of the specific monoclonal anti-mouse DHP receptor α-2 subunit antibody identified the 140-kDa band described by Morton and Froehner. The 140-kDa band from the male wild-type hearts appeared to be denser than the 140-kDa bands from all other hearts (Figure 6B), but densitometric analysis showed no significant differences between groups. The observation that L-type Ca²⁺ channel expression was not higher in male transgenic and female ovariectomized transgenic hearts is consistent with the role of increased Na⁺/Ca²⁺ exchange activity in mediating the increased ischemic injury observed in these hearts, rather than a secondary increase in other mechanisms of Ca²⁺ influx such as L-type Ca²⁺ channels.

Discussion
The Na⁺/Ca²⁺ Exchanger
The Na⁺/Ca²⁺ exchanger mediates the countertransport of 3 Na⁺ ions for 1 Ca²⁺ ion across the SL membrane. It is a bidirectional transport process, capable of moving Ca²⁺ in either direction across the sarcolemma, depending on the membrane potential and the transmembrane gradients of Na⁺ and Ca²⁺ as described by the following equation:

\[
\text{Equilibrium potential} = -\frac{RT}{F} \ln \left( \frac{C_{a0}}{C_{ai}} \right) \left( \frac{N_{a0}}{N_{ai}} \right)^3
\]

where the equilibrium potential (or reversal potential) is the membrane potential at which the Na⁺/Ca²⁺ exchanger is at equilibrium.

Using the intracellular concentrations of Na⁺ and Ca²⁺ found in murine myocytes (Naₐ=15 mmol/L; Caₐ=0.13 μmol/L) and the extracellular concentrations of Na⁺ and Ca²⁺ used in our buffer solution (Naₒ=145 mmol/L; Caₒ=1.75 mmol/L), the equilibrium potential was calculated to be −73 mV. At membrane potentials more negative than the equilibrium potential, the Na⁺/Ca²⁺ exchanger will operate in the direction of Ca²⁺ influx. Conversely, at membrane potentials more positive than the equilibrium potential, the Na⁺/Ca²⁺ exchanger will operate in the direction of Ca²⁺ efflux. As the resting potential of the cell is −80 mV, the Na⁺/Ca²⁺ exchanger will operate normally in the Ca²⁺ efflux mode, and it has been demonstrated that Na⁺/Ca²⁺ exchange is the major Ca²⁺ efflux mechanism in the heart.

The Contribution of Na⁺/Ca²⁺ Exchange to Basal Contractility
The cardiac excitation-contraction coupling process (reviewed by Callewaert and Bers) begins with the propagation of an action potential along the SL T-tubules and activation of the voltage-dependent SL L-type Ca²⁺ channels (DHP receptors). Ca²⁺ influx via these channels then stimulates release of Ca²⁺ from the SR via SR Ca²⁺ release channels (ryanodine receptors). The resultant large increase in cytosolic Ca²⁺ activates the myofilaments, causing contraction, and also activates the SL Na⁺/Ca²⁺ exchanger and the SR Ca²⁺-ATPase (SERCA). Activation of these 2 transporters decreases the level of intracellular Ca²⁺ and facilitates the downstroke, or relaxation phase, of the cardiac contractile cycle.

In the myocardium, changes in intracellular Ca²⁺ are reflected directly by changes in contractile pressure, provided intracellular pH and Pi remain constant, such as during the preischemic period in this study. Under these conditions, therefore, an increase or decrease in the rate of change of intracellular Ca²⁺ will be manifested as an increase in the rate of contraction (+dP/dtₑₘₙ) or relaxation (−dP/dtₑₘₙ), respectively, and a higher total intracellular Ca²⁺ will be manifested as a higher maximum contraction (LVDP).
In this study, we observed a faster rate of relaxation (−dP/dt_{min}) in the male transgenic hearts compared with wild-type. These data are consistent with the role of Na+/Ca2+ exchange in the relaxation phase of the cardiac cycle. However, we cannot distinguish whether this is due to a direct or indirect role of Na+/Ca2+ exchange. Another possible explanation for the faster rate of relaxation observed in the transgenic hearts is an increase in SERCA activity. Yao et al.\textsuperscript{14} however, showed that SERCA activity during relaxation was the same in control and transgenic myocytes.

A role for Na+/Ca2+ exchange in mediating Ca2+ influx during the contractile phase of the cardiac cycle is controversial.\textsuperscript{6,8–10,26} During the cardiac action potential, the SL membrane depolarizes; therefore, on the basis of the equation, it is predicted that the Na+/Ca2+ exchanger could operate in a Ca2+ influx mode. We found that the hearts of male transgenic Na+/Ca2+ exchange–overexpressor mice had both an increased +dP/dt_{max} and LVDP compared with the hearts of their wild-type littermates. This finding is consistent with a role for Na+/Ca2+ exchange in contributing to the contractile phase of the cardiac cycle, although this role could be direct or indirect. The higher LVDP and +dP/dt_{max} in the transgenic hearts could also be due to higher SR Ca2+ levels. Adachi-Akahane et al.\textsuperscript{13} and Yao et al.\textsuperscript{14} however, showed that SR Ca2+ levels were the same in myocytes from transgenic Na+/Ca2+ exchange–overexpressor mice as in wild-type mice. Adachi-Akahane et al.\textsuperscript{13} and Yao et al.\textsuperscript{14} also showed that there was no increase in L-type Ca2+ channel current density in the myocytes from the transgenic mice, indicating that an increase in L-type Ca2+ channels is not responsible for increased Ca2+ influx leading to the higher LVDP and +dP/dt_{max}.

The Role of Na+/Ca2+ Exchange in Ischemia/Reperfusion Injury

During ischemia, anaerobic glycolysis and ATP degradation produce H+ that activate the Na+/H+ exchanger,\textsuperscript{29,30} causing Na+ influx. Na+ efflux is attenuated because the Na+/K+-ATPase is inhibited during ischemia.\textsuperscript{31} Therefore, Na+/H+ exchange activity leads to increased intracellular Na+.\textsuperscript{4,32,33} The increase in intracellular Na+ during ischemia is accompanied by an increase in intracellular Ca2+, and it is this high Ca2+ that is thought to cause myocardial injury.\textsuperscript{1,2,4,5,34}

Confirmation of the role of Na+/Ca2+ exchange in mediating Ca2+ influx and ischemia/reperfusion injury is complicated by the difficulty of studying the Na+/Ca2+ exchanger during ischemia. No specific inhibitor of Na+/Ca2+ exchange exists, except possibly for the XIP peptide,\textsuperscript{35} which only functions if present intracellularly and therefore is difficult to use in a true ischemia model. Theoretically, the Na+/Ca2+ exchanger could operate in the Ca2+ influx mode during ischemia, thereby mediating Ca2+ overload and injury. As described above, membrane depolarization could switch Na+/Ca2+ exchange from the normal Ca2+ efflux mode to a Ca2+ influx mode. Membrane depolarization occurs during ischemia,\textsuperscript{30} and the high ischemic intracellular Na+ concentration would also favor Ca2+ influx by Na+/Ca2+ exchange. To investigate the role of Na+/Ca2+ exchange in ischemia/reperfusion injury, we studied the effect of overexpression of Na+/Ca2+ exchange during ischemia and reperfusion in the intact heart.

There are 2 possibilities for the mode of operation of the Na+/Ca2+ exchanger during ischemia/reperfusion. First, Na+/Ca2+ exchange may continue in the normal, Ca2+ efflux mode during ischemia. In this case, overexpression of the Na+/Ca2+ exchanger would be expected to increase Ca2+ efflux during ischemia/reperfusion, resulting in decreased injury. Second, Na+/Ca2+ exchange could operate in the Ca2+ influx mode during ischemia, as previously assumed, leading to increased intracellular Ca2+. According to this second model, overexpression of the Na+/Ca2+ exchanger would be expected to increase Ca2+ influx during ischemia/reperfusion, resulting in increased injury.

We found that, in males, overexpression of the Na+/Ca2+ exchanger reduces posts ischemic recovery of both contractile function and energy metabolites. This suggests that the Na+/Ca2+ exchanger may play a role in ischemia/reperfusion injury and is consistent with the hypothesis that the Na+/Ca2+ exchanger operates in the Ca2+ influx mode during ischemia/reperfusion. Further support for Na+/Ca2+ exchange operating in the Ca2+ influx mode and thereby leading to increased intracellular Ca2+ is provided by the observation of higher maximum contracture in the male Na+/Ca2+ exchange–overexpressor mice, the extent of contracture being related to intracellular Ca2+ levels,\textsuperscript{37} provided ATP levels are comparable.\textsuperscript{38} In addition, alternans were observed in the male transgenic hearts during reperfusion but were absent in the male wild-type hearts. As alternans are thought to be caused by Ca2+ overload leading to spontaneous SR Ca2+ release,\textsuperscript{39,40} this further supports the existence of elevated calcium in the male transgenic, compared with male wild-type hearts. The observation that decreased posts ischemic contractile function could not be explained by lower intracellular pH or higher Pi during reperfusion in the Na+/Ca2+ exchange–overexpressor mice supports the conclusion that there is greater injury, either reversible (stunning) or irreversible, rather than simply decreased contractility due to inhibition of contractile proteins.

Secondary adaptations in Ca2+ transport mechanisms in this transgenic mouse model also could give rise to increased intracellular Ca2+ and ischemia/reperfusion injury. However, studies have demonstrated that the major Ca2+ transport mechanisms are unaltered in the Na+/Ca2+ exchange–overexpressor mice.\textsuperscript{13,14} Secondary adaptations in other ion transport mechanisms cannot, however, be excluded.

A Protective Effect of Estrogen?

One intriguing observation from this study was the male/female difference in response to myocardial ischemia in the Na+/Ca2+ exchange–overexpressor mice. As discussed in the previous section, hearts from the male transgenic mice showed a lower posts ischemic recovery of contractile function and energy metabolites when compared with hearts of their wild-type littermates. In contrast, the female Na+/Ca2+ exchange overexpressor mouse hearts recovered function and energy metabolites to the same extent as hearts from wild-type mice. One explanation for the higher recovery in the female transgenic mouse hearts would be a protective effect...
of female-specific hormones such as estrogen with regard to ischemia/reperfusion injury.

A possible protective role of estrogen has been investigated by several groups in an attempt to explain recent clinical findings indicating that females are protected from a variety of cardiac events. For example, some studies have shown that premenopausal women possess a lower risk for ischemic heart disease than age-matched males and that this protection is lost after menopause unless estrogen replacement therapy is implemented. Experiments have shown that delivery of estrogen to males for days or weeks before experimentation caused a receptor-mediated reduction in infarct size in an in vivo model of rabbit myocardial ischemia. Increased recovery from global ischemia in perfused rat hearts, and increased resistance to anoxia in isolated ventricular strips.

To assess whether the protection from ischemic injury observed in the female transgenic hearts was an estrogen-mediated effect, a group of hearts from bilaterally ovariectomized and sham-operated female transgenic mice was subjected to the ischemia/reperfusion protocol. Plasma estrogen falls to almost undetectable levels within 1 week of a bilateral ovariectomy. In this study, experiments were performed 3 weeks after ovariectomy. Postischemic functional recovery in the ovariectomized female transgenic hearts was lower than that of the sham-operated female transgenic hearts and almost as low as in the male transgenic hearts. Therefore, the protection from ischemic injury observed in the female transgenic hearts was attenuated by ovariectomy, indicating that a female-specific hormone such as estrogen may be responsible for the protection.

Mechanisms Underlying the Protective Effect of Estrogen

Gene Expression Effects

Because of the known effect of estrogen on gene expression in the heart, we performed Western analyses on all hearts to determine whether the protection from ischemic injury observed in the female Na+/Ca2+ exchange--overexpressor mouse hearts was due to a male/female difference in expression of Ca2+ transport proteins. First, we measured levels of the Na+/Ca2+ exchange protein. We aimed to determine whether the Na+/Ca2+ exchange protein actually was overexpressed in the female transgenic mice or whether the observation that ischemic injury was not exacerbated in female transgenic hearts was due to a down-regulation of Na+/Ca2+ exchange expression. The level of Na+/Ca2+ exchange protein expression was the same in all transgenic hearts; therefore, the protection from ischemic injury observed in the female transgenic mouse hearts was not due to a down-regulation of Na+/Ca2+ exchange expression.

Second, a recent report by Johnson et al demonstrated that DHP binding was increased in estrogen-receptor knockout mice. As DHP binds to the DHP receptor, otherwise known as the L-type Ca2+ channel, this observation implies that estrogen normally down-regulates L-type Ca2+ channel expression. As Ca2+ also theoretically could enter via the L-type Ca2+ channel during ischemia, we determined the levels of L-type Ca2+ channel expression in these groups of hearts. Despite the increased susceptibility to ischemic injury observed in the male transgenic and female transgenic ovariectomized mouse hearts, L-type Ca2+ channel expression did not differ significantly between any of the groups. Therefore, the protection from ischemic injury observed in the female transgenic mouse hearts could not be explained by an estrogen-mediated decrease in L-type Ca2+ channel expression.

Lack of Protection in Female Wild-Type Hearts

Any proposed mechanism for the protective effect of estrogen must also explain the lack of a male/female difference in response to myocardial ischemia in the wild-type mice. If estrogen were to reduce Ca2+ influx via Na+/Ca2+ exchange, estrogen would be more protective in mice that overexpress the Na+/Ca2+ exchanger. In this study, we showed that expression of Na+/Ca2+ exchange was unaltered in both transgenic and wild-type females compared with males, but estrogen may affect Na+/Ca2+ exchange activity. Alternatively, estrogen may counteract a mechanism that is only activated at the high levels of intracellular Ca2+ reached during ischemia/reperfusion in the transgenic mice. Also, Na+/Ca2+ exchange overexpressor mice may be more responsive to receptor-mediated effects of estrogen via Ca2+ activation of estrogen receptors. Another question arising from the observations of this study is why the protection in the female transgenic mice was not reversed fully by ovariectomy. One possibility is that some of the hormone-mediated protective effects in females could be developmental and therefore be irreversible.

In summary, by comparing basal contractility in mice overexpressing the Na+/Ca2+ exchanger to that of their wild-type littermates, we provided evidence for Na+/Ca2+ exchange contributing not only to the relaxation phase of the cardiac contractile cycle but also to the contraction phase. In addition, the differences in peak contracture during ischemia, in postischemic recovery of myocardial energetics and contractile function, and in the occurrence of reperfusion alternans in male transgenic and male wild-type hearts suggest that the Na+/Ca2+ exchanger indeed can play a role in ischemia/reperfusion injury. We also have shown that exacerbation of ischemia/reperfusion injury by overexpression of the Na+/Ca2+ exchanger is not observed in females, unless ovariectomized, implying a protective effect of female-specific hormones such as estrogen. Although the mechanism underlying this protective effect is as yet unclear, we have demonstrated that an alteration in the expression of the Na+/Ca2+ exchange or L-type Ca2+ channel proteins is not involved.

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Heather R. Cross, Liyan Lu, Charles Steenbergen, Kenneth D. Philipson and Elizabeth Murphy

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