Gender Influences \([\text{Ca}^{2+}]_i\) During Metabolic Inhibition in Myocytes Overexpressing the \(\text{Na}^+-\text{Ca}^{2+}\) Exchanger

Kazuro Sugishita, MD; Zhi Su, PhD; Fenghua Li, MD; Kenneth D. Philipson, PhD; William H. Barry, MD

Background—The \(\text{Na}^+-\text{Ca}^{2+}\) exchanger (NCX) may contribute to \(\text{Ca}^{2+}\) overload and injury in ischemic cardiomyocytes. Recently, NCX overexpression was reported to increase ischemia/reperfusion injury in male and oophorectomized female but not in female mice. We therefore measured the effects of gender and estrogen on [\(\text{Ca}^{2+}\)], and [\(\text{Na}^+\)], during metabolic inhibition (MI) in myocytes from wild-type (WT) and transgenic (TG) mice overexpressing NCX.

Methods and Results—Flow cytometry was used with fluo 3 for [\(\text{Ca}^{2+}\)], and sodium green for [\(\text{Na}^+\)], measurements. Male TG mouse myocytes had higher [\(\text{Ca}^{2+}\)], after 30 minutes of MI \((1086\pm160 \text{ mmol/L, } n=8)\) than male WT \((688\pm104 \text{ mmol/L, } n=9, \ P=0.01)\). The increase in [\(\text{Ca}^{2+}\)], during MI induced by NCX overexpression in female myocytes was not significant, however (TG 552\pm62 \text{ mmol/L, } n=9; WT 426\pm44 \text{ mmol/L, } n=7). The magnitude of rise in [\(\text{Ca}^{2+}\)], during MI was greater in male than female myocytes. KB-R7943, an NCX inhibitor, abolished the effect of NCX overexpression but did not totally eliminate the effect of gender on [\(\text{Ca}^{2+}\)], during MI. NCX current density and basal Na\(^+\) pump function were not influenced by gender. The rise in [\(\text{Na}^+\)], during MI was greater in male than in female myocytes. Estrogen attenuated the increase in [\(\text{Ca}^{2+}\)], and [\(\text{Na}^+\)], in male myocytes during MI and abolished the gender difference in [\(\text{Na}^+\)], during MI.

Conclusions—Increased expression of NCX results in a more marked rise in [\(\text{Ca}^{2+}\)], during MI in male than in female mouse myocytes. This gender difference appears to be mediated in part by an inhibitory effect of estrogen on the rise in [\(\text{Na}^+\)], an NCX modifier, during MI. (Circulation. 2001;104:2101-2106.)

Key Words: myocytes ■ ischemia ■ calcium ■ sex ■ sodium

The \(\text{Na}^+-\text{Ca}^{2+}\) exchanger (NCX) is an important \(\text{Ca}^{2+}\) transporter that can cause \(\text{Ca}^{2+}\) overload in certain pathological conditions.\(^{1}\) In ischemic myocytes, intracellular [\(\text{Na}^+\)] increases as a result of activated \(\text{Na}^+-\text{H}^+\) exchange secondary to intracellular acidosis and of impaired \(\text{Na}^+-\text{K}^+\) ATPase function due to decreased ATP.\(^{2}\) This “\(\text{Na}^+\) overload” and membrane depolarization enhance \(\text{Ca}^{2+}\) influx via the reverse-mode function of NCX. The subsequent “\(\text{Ca}^{2+}\) overload” is generally considered to lead to arrhythmias and necrosis. This hypothesis about the role of NCX during ischemia has been supported indirectly by studies using amiloride, an inhibitor of \(\text{Na}^+-\text{H}^+\) exchanger, and by regulation of the extracellular levels of \(\text{Na}^+\) and/or \(\text{Ca}^{2+}\).\(^{3–5}\) In addition, an inhibitor of NCX, KB-R7943, was recently found to reduce \(\text{Ca}^{2+}\) overload in anoxic rat cardiomyocytes.\(^{6}\)

Transgenic (TG) mice overexpressing NCX in ventricular myocardium have recently been developed that have a 2.5-fold increase in NCX activity but no known secondary adaptations in other ion channels or transporters.\(^{7,8}\) Cross et al\(^{9}\) demonstrated that overexpression of NCX increased ischemia/reperfusion injury in intact hearts of male but not female TG mice. Because bilaterally oophorectomized female TG mice were less tolerant to ischemia/reperfusion than sham-operated female TG mice, they concluded that estrogen might mediate this gender difference, but the mechanisms involved have been not clarified.\(^{9}\) Therefore, we evaluated the effects of gender and estrogen on the rise in [\(\text{Ca}^{2+}\)], and [\(\text{Na}^+\)], in TG and wild-type (WT) myocytes during metabolic inhibition (MI).

Methods

Production of TG Mice
TG mice were developed as described by Philipson et al.\(^{7}\)

Dissociation of Adult Mouse Ventricular Myocytes
As previously described,\(^{8}\) hearts were removed from anesthetized mice and immediately attached to an aortic cannula. \(\text{Ca}^{2+}\)-tolerant left ventricular myocytes were obtained by enzymatic dissociation with collagenase. Cells were used for experiments within 5 hours after dissociation.

Measurement of [\(\text{Ca}^{2+}\)]
[\(\text{Ca}^{2+}\)] measurement was performed by a previously described method.\(^{10}\) Flow cytometry (FACScan, Becton-Dickinson) was used.
with the Ca\textsuperscript{2+}-sensitive fluorescence probe fluo 3-AM (Molecular Probes). For MI, myocytes were exposed to 2 mmol/L sodium cyanide (NaCN) and zero glucose. All experiments were performed at 37°C in solutions containing 25 μmol/L propidium iodide (PI, Molecular Probes) and probenecid 0.5 mmol/L. PI is a marker for nonviability, and probenecid inhibits loss of fluo 3 via the anion transporter.\textsuperscript{21} During flow cytometry, the cells were excited with an argon laser beam (wavelength 488 nm). Side and forward scattering characteristics were observed to separate individual cells from debris. Approximately 2000 myocytes in each sample were analyzed within 2 minutes to calculate average emission fluorescence intensity. Data were collected for emission intensity at wavelengths of 530 nm for fluo 3 and 670 nm for PI and plotted simultaneously. Only those cells with a low PI fluorescence were analyzed.

Current Densities

and WT animals.

voltage clamp at 0 mV, the cell was exposed to 5 mmol/L K\textsuperscript{+}, then calculated for myocytes from that heart with the formula \( \frac{d}{d}[K\textsuperscript{+}] = \frac{K_d(F - F_{min})}{(F_{max} - F)} \), with values of 864 nmol/L for \( K_d \), 1.2 F_{max}, or Ca\textsuperscript{2+}-saturated fluorescence, was estimated as 5X F_{max}, and F_{min}, fluorescence in the absence of Ca\textsuperscript{2+}, as 1.4 F_{max} 10\textsuperscript{-4} F_{min} was obtained from cells exposed to NaCN solution for ≥30 minutes, then to NaCN solution with 10 mmol/L MnCl\textsubscript{2} for 5 minutes. In some experiments, KB-R7943 (10 μmol/L, Kanebo) was added. 17β-Estradiol (Sigma) was prepared as 10 mmol/L stock in ethanol and added in appropriate dilution.

Measurement of [Na\textsuperscript{+}]

Resting [Na\textsuperscript{+}], in single myocytes was measured with a modification of the method of Yao et al.\textsuperscript{16} Myocytes loaded with 10 μmol/L SBFI acetoxy methyl ester (Molecular Probes) were illuminated at 60 Hz by 340- and 380-nm excitation light with an optical switcher (DX-1000, Solamere Technology Group), and the fluorescence at 510 nm was continuously recorded. The ratio (R) of the 340- and 380-nm/fluorescence was used as an indicator for [Na\textsuperscript{+}]. For calibration, the myocytes were sequentially exposed to calibration solutions of 5, 10, and 20 mmol/L (Na\textsuperscript{+}) containing (μmol/L) gramicidin D 2, monensin 40, and streptothricin 100. [Na\textsuperscript{+}], was equilibrated to [Na\textsuperscript{+}], in each solution. For each dissociation, R under resting conditions and in the various [Na\textsuperscript{+}], solutions was recorded from 8 to 10 myocytes, and an average was calculated. [Na\textsuperscript{+}], then calculated for myocytes from that heart with the formula \( [Na\textsuperscript{+}] = K_d(R - R_{min})/(R_{max} - R) \), where \( K_d \) is 17.5 mmol/L. Average values from ≥5 hearts were then averaged to estimate [Na\textsuperscript{+}], in TG and WT animals.

Changes in [Na\textsuperscript{+}], were measured with sodium green and PI during MI by flow cytometry with excitation and fluorescence detection as described for measurement of [Ca\textsuperscript{2+}], with fluo 3. The cells were exposed to 5 μmol/L of the tetracetate ester of sodium green, a Na\textsuperscript{+}-sensitive fluorescence probe (Molecular Probes), for 60 minutes and then washed for 10 minutes. The resting [Na\textsuperscript{+}], was assigned a value of 12 mmol/L, and changes in [Na\textsuperscript{+}], in viable cells were calculated with the formula \( [Na\textsuperscript{+}] = K_d(F - F_{min})/(F_{max} - F) \), where \( K_d \) is 27.8 mmol/L. Values of F_{max} and F_{min} were estimated by curve fitting with [Na\textsuperscript{+}]-sodium green fluorescence intensity data from the manufacturer.

Measurement of Exchanger and Na\textsuperscript{+} Pump Current Densities

NCX current and peak Na\textsuperscript{+} pump currents were measured by voltage clamp as previously described.\textsuperscript{15-17} Outward exchange current was activated when the myocyte was abruptly exposed to a rapid switcher microstream of solution containing Li\textsuperscript{+} instead of Na\textsuperscript{+}.

To measure peak Na\textsuperscript{+} pump current, the Na\textsuperscript{+} pump was inhibited for 5 minutes by exposure of the cell to zero K\textsuperscript{+}, and then during voltage clamp at 0 mV, the cell was exposed to 5 mmol/L K\textsuperscript{+} with the rapid switcher to abruptly reactivate the Na\textsuperscript{+} pump. The resulting peak currents were normalized to membrane capacitance to calculate peak current densities (pA/pF).

Statistical Analysis

Results were expressed as mean±SEM. n refers to the number of separate flow cytometry experiments, each consisting of an average of data from 200 to 500 viable myocytes obtained from a single animal. Student’s t tests and ANOVA were used for 2-group and multiple comparisons, respectively.

Results

There have been no previous studies of the sensitivity of [Ca\textsuperscript{2+}], to MI in mouse myocytes with flow cytometry. In preliminary experiments, we used an MI protocol (2 mmol/L NaCN, 20 mmol/L 2-deoxyglucose, zero glucose for 45 to 60 minutes) that we had previously used in rabbit ventricular myocytes.\textsuperscript{10} In mouse myocytes, this produced an extreme rise in [Ca\textsuperscript{2+}], and loss of myocyte viability. We therefore used a less intense protocol in mouse myocytes (2 mmol/L NaCN without glucose). Figure 1A shows an example of representative results of flow cytometry, dot-plotted for fluo 3 and PI fluorescence intensities. The PI-negative cells (enclosed by the solid lines) were considered viable cells. Average fluorescence intensity of fluo 3 from viable cells was increased (from 8.28 to 54.83) during the 45 minutes of exposure to NaCN solution. The time course of changes in normalized fluo 3 fluorescence during MI is demonstrated in Figure 1B. MI with NaCN increased the fluorescence in a time-dependent manner. Cell viability did not change after 45 minutes of exposure to MI (control 26.8±2.1%, MI 25.9±2.5%; n=15). Thus, we performed subsequent experiments in mouse myocytes using a 20- to 45-minute exposure to MI.

Next, we exposed the ventricular myocytes of male TG, female TG, male WT, and female WT mice to MI. [Ca\textsuperscript{2+}], increased in a time-dependent manner in all 4 groups (Figure 2). Male TG mouse myocytes had higher [Ca\textsuperscript{2+}], after 30 minutes of MI than male WT (male TG 1086±160 mmol/L, n=8; male WT 688±104 mmol/L, n=9; P=0.01). Female TG (552±62 mmol/L, n=9) had a higher [Ca\textsuperscript{2+}], than female WT (426±44 mmol/L, n=7), but this difference did not reach statistical significance. These results are consistent with the findings of Cross et al.\textsuperscript{16} showing a greater reduction of contractile function and energy metabolites in male (but not female) TG animals compared with WT after ischemia/reperfusion. There was a gender effect on [Ca\textsuperscript{2+}], after MI that was more marked in TG mice (P=0.0009) than in WT mice (P=0.09).

We then examined effects of an inhibitor of NCX, KB-R7943,\textsuperscript{16,17} on Ca\textsuperscript{2+} overload (Figure 3 and Table). This inhibitor has been reported to depress both forward and reverse modes of NCX similarly under conditions of bidirectional exchange,\textsuperscript{18} but to decrease the reverse mode with a high affinity under unidirectional exchange conditions,\textsuperscript{17} such as ischemia. KB-R7943 10 μmol/L inhibits the reverse mode of NCX by 88% in mouse ventricular myocytes (control 1.21±0.15 pA/pF, KB-R7943 0.15±0.06 pA/pF; n=7). There was no significant difference in [Ca\textsuperscript{2+}], during MI in the presence of 10 μmol/L KB-R7943 between male TG (502±72 mmol/L, n=7) and male WT (435±41 mmol/L, n=7) or between female TG (296±45 mmol/L, n=7) and female WT (318±44 mmol/L, n=7). A significant (though less marked) difference was still observed, however, between male TG and female TG in the presence of KB-R7943 (male TG 502±72 mmol/L, n=7; female TG 296±45 mmol/L, n=7, P=0.03). KB-R7943 exposure caused a greater absolute
reduction in $\text{Ca}^{2+}$, during MI in male than female myocytes (Table). Because it did not completely eliminate the gender difference in $\text{Ca}^{2+}$, during MI, however, factors other than the degree of activation in NCX during MI may contribute to gender effects on $\text{Ca}^{2+}$ overload. We detected no difference in NCX current density ($I_{\text{Na/Ca}}$) between male TG (3.1 ± 0.2 pA/pF, n = 11) and female TG (3.3 ± 0.4 pA/pF, n = 9), a finding consistent with the results of Cross et al, who noted no difference in NCX protein levels. We also found no influence of gender on $\text{Na}^+$ pump function as assessed by measuring peak $\text{Na}^+$ pump current in WT animals (male 1.27 ± 0.08 pA/pF, n = 18 myocytes from 4 animals; female 1.30 ± 0.10 pA/pF, n = 27 myocytes from 6 animals).

As mentioned, TG mice show a gender difference in the extent of ischemic myocardial injury. Because oophorectomized female TG mice had greater myocardial injury than female sham-operated TG mice, estrogen was considered to exert cardioprotective effects. Therefore, we examined the acute effects of this hormone on $\text{Ca}^{2+}$, in normal male mouse myocytes (Figure 4). 17β-Estradiol (Est) had an inhibitory effect on the increase in $\text{Ca}^{2+}$, after 30 minutes of exposure to MI at physiological concentrations (MI 1524 ± 241 nmol/L; MI + Est 1065 ± 168 nmol/L, $P = 0.03$; MI + Est 100 nmol/L 689 ± 127 nmol/L, $P = 0.008$; n = 5), but no effect on $\text{Ca}^{2+}$, in non–metabolically inhibited cardiomyocytes.

NCX modifiers, including $\text{Na}^+$, might contribute to the gender difference and inhibitory effect of estrogen on $\text{Ca}^{2+}$ overload. Therefore, we investigated changes in $\text{Na}^+$, in these experimental conditions. No gender difference in resting $\text{Na}^+$, was measured with SBFI in WT (male 11.9 ± 0.1 mmol/L, n = 5; female 11.1 ± 0.2 mmol/L, n = 5) and TG (male 12.0 ± 0.3 mmol/L, n = 5; female 12.1 ± 0.2 mmol/L,
Next, we measured changes in \([Na^+]_i\) during MI by flow cytometry and sodium green (Figure 5). In preliminary experiments, we found that myocytes loaded with sodium green were more sensitive to injury during MI, with a significant decrease in viability after 30 minutes at 37°C. Therefore, changes in \([Na^+]_i\) were measured after 20 minutes of MI. As expected, \([Na^+]_i\) rose during MI. \([Na^+]_i\) after 20 minutes of MI was higher in males in both WT and TG animals (Figure 5); although \([Na^+]_i\) tended to be slightly higher in TG than WT animals, these differences were not statistically significant. An analysis of the gender effect, combining myocytes from WT and TG animals, showed a significant influence of gender on \([Na^+]_i\) during MI (male 18.8±1.1 mmol/L versus female 15.8±0.7 mmol/L, n=10, P<0.04). Estrogen had a significant inhibitory effect on the rise in \([Na^+]_i\) during MI in males (Figure 5) that was apparent at the 1-nmol/L concentration level. In an analysis combining male WT and TG animals, exposure to 1 nmol/L estrogen reduced the \([Na^+]_i\) level from 18.8±1.1 to 14.8±1.2 mmol/L (n=10, P<0.002). The hormone had no effect on \([Na^+]_i\) in non–metabolically inhibited cardiomyocytes and did not reduce \([Na^+]_i\) during MI in female myocytes (Figure 5).

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<th>Effects of KB-R7943 on ([Ca^{2+}]_i) During 30 Minutes of MI</th>
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Discussion

**Ca^{2+} Overload in Mouse Myocytes During MI**

We previously showed the usefulness of flow cytometry as a method for \([Ca^{2+}]_i\) measurement during MI in rabbit ventricular myocytes.\(^{10}\) In this study, we measured changes in \([Ca^{2+}]_i\), during MI in mouse ventricular myocytes. Time-

**Figure 3.** Effect of KB-R7943 (10 μmol/L) on \([Ca^{2+}]_i\) after 30 minutes’ exposure to MI. There was no significant difference between male TG and male WT.

**Figure 4.** Effect of estrogen on \([Ca^{2+}]_i\) in normal male mouse myocytes. 17β-Estradiol reduced \([Ca^{2+}]_i\) in a concentration-dependent manner in metabolically inhibited cells but had no effect on myocytes during control conditions. Mean±SEM, n=5.

**Figure 5.** Effects of gender and estrogen exposure on \([Na^+]_i\), after 20 minutes’ MI in WT (A) and TG (B) mice. In both groups, \([Na^+]_i\) during MI tended to be higher in males than females. When TG and WT animals were combined, gender difference in \([Na^+]_i\) during MI reached statistical significance (see text). Physiological concentration (1 nmol/L) of 17β-estradiol attenuated rise in \([Na^+]_i\) in males and abolished gender difference. Mean±SEM, n=5.
dependent increases in fluo 3 fluorescence intensity during 45 minutes of exposure to NaCN solutions were observed. In addition, mouse myocytes are more sensitive to MI than rabbit myocytes with respect to [Ca\textsuperscript{2+}], and viability effects. This greater sensitivity to MI might result from a higher intracellular [Na\textsuperscript{+}] and/or higher NCX density\textsuperscript{13} in mouse than rabbit ventricular myocytes, and it supports the idea that Ca\textsuperscript{2+} influx on NCX is a major determinant of injury due to ATP depletion.

**Effects of Overexpression of NCX and Gender on Ca\textsuperscript{2+} Overload During MI**

In the present study, NCX-overexpressing TG male mouse myocytes had a significantly higher [Ca\textsuperscript{2+}] during MI than WT male myocytes. The TG mice have been shown to have 2.5-fold increased NCX activity but no secondary adaptations in L-type Ca\textsuperscript{2+} channels, sarcoplasmic reticulum (SR) function, or resting cytosolic Na\textsuperscript{+} and Ca\textsuperscript{2+} concentrations.\textsuperscript{8}

Although Yao et al\textsuperscript{8} found no effect of NCX overexpression on SR Ca\textsuperscript{2+} content, Terracciano et al\textsuperscript{9} reported that resting SR Ca\textsuperscript{2+} content was increased in male NCX TG mice. Taken together, these results directly support the general hypothesis that NCX mediates Ca\textsuperscript{2+} overload in cardiomyocytes subjected to ATP depletion. The inhibitory effect of KB-R7943 on the difference in Ca\textsuperscript{2+} overload during MI (Figure 3) is consistent with this hypothesis, although the selectivity of its action has been questioned.\textsuperscript{17,18}

We detected a striking gender effect on Ca\textsuperscript{2+} overload during MI in TG mice overexpressing NCX. Because an increase in [Ca\textsuperscript{2+}], is thought to be a major contributor to ischemia/reperfusion myocyte injury, this finding is consistent with the report of Cross et al\textsuperscript{9} showing increased myocardial injury induced by ischemia/reperfusion in male but not female TG mice. They concluded that estrogen might be involved, because greater ischemia-induced myocardial damage was demonstrated in oophorectomized female TG mice. This effect of gender is not due to differences in NCX expression, because NCX current density was identical in female and male TG myocytes.

NCX modifiers, including intracellular [Na\textsuperscript{+}] and [H\textsuperscript{+}], might produce the gender difference in the response to ischemia. We have observed that there is a gender difference in the magnitude of [Na\textsuperscript{+}], rise during MI. Because acute exposure to 17\beta-estradiol attenuated the increase in [Na\textsuperscript{+}], during MI at physiological concentrations (1 nmol/L) in male mice and the gender difference in the rise in [Na\textsuperscript{+}], was completely abolished by treatment with the hormone, an estrogen effect appears to contribute to this gender difference. Thus, estrogen may alter the Ca\textsuperscript{2+} influx through reverse NCX during ischemia by reducing the magnitude of the rise in [Na\textsuperscript{+}], during MI.

Acute effects of estrogen may differ from chronic effects, which might be induced via the estrogen receptor and involve altered protein synthesis and other factors that need a longer time period to be manifest. For example, Jovanovic et al\textsuperscript{20} demonstrated that 8-hour pretreatment with 1 to 10 nmol/L estrogen attenuated hypoxia-reoxygenation–induced Ca\textsuperscript{2+} overload in cardiac cells from female guinea pigs, and in vivo estrogen treatment has been reported to increase myocardial glucose oxidation.\textsuperscript{21} A more acute exposure to estrogen, however, can attenuate the oxidative impairment of Na\textsuperscript{+}-K\textsuperscript{+} ATPase function in synaptic cells.\textsuperscript{22} Thus, a beneficial effect of estrogen on Na\textsuperscript{+} pump function during MI could account for some of the gender and acute estrogen effects we have observed. It should be noted that gender differences in [Ca\textsuperscript{2+}], during MI were not completely eliminated by KB-R7943. Further studies will be necessary to determine what factors other than a difference in rise in [Na\textsuperscript{+}], and resulting activation of NCX might also contribute to a gender effect on [Ca\textsuperscript{2+}], during MI.

**Possible Clinical Implications**

Gene expression and protein levels of NCX may be increased in hypertrophied and/or failing myocardium.\textsuperscript{23–26} This may increase the susceptibility of these hearts to ischemic injury, particularly in males, and explain the poor results observed when hypertrophied hearts have been harvested as donor grafts and subjected to a period of ischemia before heart transplantation.\textsuperscript{27} Also, the risk of ischemic heart disease is known to be much lower in premenopausal women. This undoubtedly depends in part on beneficial effects of estrogen on atherosclerosis of coronary arteries.\textsuperscript{28} Gender differences in the extent of Ca\textsuperscript{2+} overload in cardiomyocytes during ischemia, however, might also play a role.

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