SERCA overexpression reduces hydroxyl radical injury in murine myocardium

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Submitted 14 December 2005; accepted in final form 22 June 2006

Hiranandani, Nitisha, Tepmanas Bupha-Intr, and Paul M. L. Janssen. SERCA overexpression reduces hydroxyl radical injury in murine myocardium. Am J Physiol Heart Circ Physiol 291: H3130–H3135, 2006.—Hydroxyl radicals (·OH) are involved in the pathogenesis of ischemia-reperfusion injury and are observed in clinical situations, including acute heart failure, stroke, and myocardial infarction. Acute transient exposure to ·OH causes an intracellular Ca\(^{2+}\) overload and leads to impaired contractility. We investigated whether upregulation of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase function (SERCA) can attenuate ·OH-induced dysfunction. Small, contracting right ventricular papillary muscles from wild-type (WT) SERCA1α-overexpressing (transgenic, TG) and SERCA2a heterogeneous knockout (HET) mice were directly exposed to ·OH. This brief 2-min exposure led to a transient elevation of diastolic force (F\(_{\text{dia}}\)) and depression of developed force (F\(_{\text{dev}}\)). In WT mice, F\(_{\text{dia}}\) increased to 485 ± 49% and F\(_{\text{dev}}\) decreased to 11 ± 3%. In sharp contrast, in TG mice F\(_{\text{dia}}\) increased only to 241 ± 17%, whereas F\(_{\text{dev}}\) decreased only to 51 ± 5% (P < 0.05 vs. WT). In HET mice, F\(_{\text{dia}}\) rose more than WT (to 597 ± 20%; P < 0.05), whereas F\(_{\text{dev}}\) was reduced in a similar amount. After ~45 min after ·OH exposure, a new steady state was reached: F\(_{\text{dev}}\) returned to 37 ± 6% and 32 ± 6%, whereas F\(_{\text{dia}}\) came back to 238 ± 28% and 292 ± 17% in WT and HET mice, respectively. In contrast, the sustained dysfunction was significantly less in TG mice: F\(_{\text{dia}}\) and F\(_{\text{dev}}\) returned to 14 ± 20% and 67 ± 6%, respectively. Before exposure to ·OH, there is decrease in phospholamban (PLB) phosphorylation at Ser16 (pPLBSer16) and PLB phosphorylation at Thr17 (pPLBThr17) in TG mice and an increase in pPLBSer16 and pPLBThr17 in HET mice. After exposure to ·OH there is decrease in pPLBSer16 in WT, TG, and HET mice but no significant change in the level of pPLBThr17 in any group. The results indicate that SERCA overexpression can reduce the ·OH-induced contractile dysfunction in murine myocardium, whereas a reduced SR Ca\(^{2+}\)-ATPase activity aggravates this injury. Loss of pPLB levels at Ser16 likely amplifies the differences observed in injury response.

Contractile function; calcium pump; ischemia; oxygen radicals

HYDROXYL RADICALS (·OH) are one of the most aggressive species of oxygen free radicals that attack all molecules in the human body (8, 29). These ·OH are involved in the pathogenesis of ischemia-reperfusion injury, which is observed in many clinical situations, including acute heart failure, stroke, and myocardial infarction. When ischemic myocardium is reperfused and oxygen reintroduced, there is a sudden burst of oxygen free radical production. This leads to the formation of damaging reactive species, such as hydroxyl radicals, hydrogen peroxide, and peroxynitrite (2, 28). These reactive oxygen species, especially hydroxyl radicals, interact with lipids, proteins, and nucleic acids and damage cell membranes and impair cellular function. Conditions of ischemia-reperfusion injury are created routinely during heart surgery and transplantation (2).

Oxygen free radicals occurring during ischemia-reperfusion injury have been implicated in the pathogenesis of myocardial stunning and progression of heart failure.

There are two different subcellular defects that may contribute to the development of acute myocardial dysfunction: deranged calcium handling and alteration of myofilament responsiveness. Previous studies have demonstrated that intact contracting cardiac trabeculae from rats and rabbits after acute exposure of ·OH develop a rigor-like contracture marked by an increase in diastolic tension, myofilament proteolysis, and overall decreased cardiac contractility (29). During the acute phase, there are multiple mechanisms that are possibly responsible for calcium overload: sarcoplasmic reticulum (SR) damage, mitochondrial damage, changes in properties/activity of sodium-calcium exchange, and/or changes in L-type channel activity. The relative contributions of these factors to the acute calcium overload are still unknown.

Previous studies demonstrated a decrease in SR Ca\(^{2+}\)-ATPase activity at increasing concentrations of ·OH (7) as well as a clear attenuation of the positive force-frequency relationship after the exposure of ·OH (14, 25, 29). These studies indicate that there may be a direct impairment of SR function due to ·OH exposure. Reverse-mode sodium-calcium exchange (29) has also been reported as a major cause of acute diastolic dysfunction due to ·OH exposure. In addition, calcium channel antagonists (5, 6) have been proposed to (partially) prevent myocardial stunning, showing that L-type calcium channel could be an important route of intracellular calcium overload. Combined from these studies it is clear that calcium overload plays a significant role in acute cardiac dysfunction due to ·OH injury.

The main goal of this study is to test our hypothesis that ·OH injury can be attenuated via (partial) normalization of calcium handling via modulation of SR Ca\(^{2+}\)-ATPase activity. Not only could the total absolute loss of function be attenuated by simply having more calcium pumps available, preserved Ca\(^{2+}\) handling (e.g., preserved SR Ca\(^{2+}\)-ATPase activity) could potentially aid in removing excess Ca\(^{2+}\) that has entered the myocytes as a result of ·OH-induced damages. Our results indeed indicate that modulation of SR Ca\(^{2+}\)-ATPase activity can indeed alter the magnitude of effects of hydroxyl radical injury on the heart; the ·OH-induced injury is substantially less in transgenic mice with higher SR Ca\(^{2+}\)-ATPase activity and aggravated in mice with a reduction in SR Ca\(^{2+}\)-ATPase activity.

MATERIALS AND METHODS

Transgenic mouse models. All mouse models have been published previously. SERCA-overexpressed mice (transgenic, TG) are mice
expressing the (skeletal) SERCA1a isoform (2.5-fold increase in the total amount of SERCA and an approximately twofold increase in SR Ca\(^{2+}\) uptake function) (18). We chose the SERCA1a-overexpressed mice for this proof-of-principle study; SERCA1a possesses faster Ca\(^{2+}\) transport kinetics, but unaltered Ca\(^{2+}\) affinity, pH response, and PLB affinity, and therefore possible positive effects of enhanced SERCA activity (26) would be most pronounced. SERCA2a heterozygous knock-out mice (HET) have a \(~40\%\) reduction in expression of SERCA2a, resulting in a decreased SR calcium reuptake (10). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Muscle preparation and experimental setup. Adult mice (2–3 mo old) were anesthetized with urethane ip (300 mg/1 ml of 0.9% NaCl). With each mouse under deep anesthesia and after bilateral thoracotomy and intraventricular (via the apex) heparin administration (1,000 U), the heart was rapidly removed and perfused retrogradely through the aorta with Krebs-Henseleit (K-H) solution containing (in mmol/l) 120 NaCl, 5 KCl, 2 MgSO4,1.2 NaH2PO4, 20 NaHCO3, 0.25 Ca\(^{2+}\), and 10 glucose (pH 7.4) in equilibrium with 95% O2–5% CO2 at 37°C. The dimensions (length, width, and thickness) of the muscle were measured with an optical micrometer (accuracy \(\pm 1\,\mu\text{m}\)). An aorta was removed from the heart and a continuous flow of oxygenated K-H solution (without the BDM) was switched off, both valve and block ends are cut off on either side of the muscle, and the central part of the muscle is quickly frozen in liquid nitrogen and stored at \(-80\,^\circ\text{C}\). For protein composition analysis, muscles \((\sim 0.1\,\text{mg or less})\) are homogenized in 32.5 μl of homogenization buffer \([25\,\text{mM i}2\text{midazole (pH 7.4), 300 mM sucrose, 1 mM DTT, 20 mM sodium metabisulfite, and protease inhibitor cocktail 10 μl/ml. Samples are applied to 14% PAGE. After electrophoresis and blotting on nitrocellulose membranes, blots are blocked with 5% nonfat dry milk in Tris-buffered saline and Tween 20 overnight and incubated with monoclonal antibodies against Calsequestrin, total PLB, and PLB phosphorylation at Ser16 and Thr17 for 2 h. Calsequestrin concentration was used for normalization. Antigen antibody complexes are visualized by peroxidase-conjugated anti-mouse antibodies using enhanced chemiluminescence (Pierce).

Data analysis and statistics. Data were collected and analyzed on- and off-line using custom-written software in LabView (National Instruments). Data are expressed as means ± SE unless otherwise stated. Data were statistically analyzed using ANOVA or Student’s t-tests (paired or unpaired) where applicable. A two-tailed value of P < 0.05 was considered significant.

RESULTS

Contractile function after \(-\text{OH}^+\) exposure. In accordance with previous studies in other species (7, 8, 29, 30), acute \(-\text{OH}^+\) exposure in murine papillary muscles led to a rapid increase in diastolic force (F\(_{\text{dia}}\)) and a decrease in developed force (F\(_{\text{dev}}\)). Figure 1A shows the raw record of force before, during, and after exposure to \(-\text{OH}^+\) in a murine papillary muscle. As can be clearly seen, direct acute exposure of \(-\text{OH}^+\) for 2 min on
papillary muscles of mice resulted in an increase of $F_{\text{dia}}$ and decrease of $F_{\text{dev}}$. In Fig. 1B, the effect on $F_{\text{dia}}$ and $F_{\text{dev}}$ for $n = 13$ muscles after the exposure of OH is depicted. Contractile parameters were observed until a new steady-state level was reached. This new steady-state level was marked by an elevated $F_{\text{dia}}$ and reduced $F_{\text{dev}}$ compared with preinterventional values.

$F_{\text{dia}}$ at peak of contracture ($\sim$12 min after OH exposure) was increased to $485 \pm 49\% (P < 0.05)$ of its value before OH exposure and after $\sim$45 min returned to a new steady-state level of $238 \pm 28\% (P < 0.05)$. $F_{\text{dev}}$ at peak of contracture was decreased drastically to $11.3 \pm 2.8\% (P < 0.05)$ and returned to a new steady-state level of $37.6 \pm 5.6\% (P < 0.05)$. In another set of control experiments, contractile parameters remained unchanged when muscles were treated similarly but without OH for the equivalent period of time (data not shown), indicating reliability and durability of the preparation, as well as showing that prolonged study of these isolated trabeculae is not complicated by an excessive loss of function over time.

**Role of SR Ca$^{2+}$-ATPase expression on OH-induced injury.** To determine the effect of SR calcium handling ability on the outcome of acute OH injury, we repeated the above experiments in mice that expressed SERCA1a (higher SR Ca$^{2+}$-ATPase activity than WT) as well as in mice with reduced levels of SERCA2a (HET, with a reduction in SR Ca$^{2+}$-ATPase activity compared with WT). Figure 2A shows the effect of a 2-min OH exposure on the contractile parameters of the papillary muscles in WT, TG, and HET mice. At baseline, average developed force in TG, WT, and HET was $35.9 \pm 2.4$, $26.1 \pm 2.1$, and $22.0 \pm 3.7\, \text{mN/mm}^2$, respectively. Initial diastolic forces were similar in all groups ($\sim 5–8\, \text{mN/mm}^2$). As can be clearly seen, at baseline $F_{\text{dev}}$ is significantly higher in TG mice compared with WT and HET mice ($P < 0.05$). In addition, $F_{\text{dev}}$ is slightly lower in HET mice compared with WT controls ($P = 0.16$). In accordance with the previous experiments, OH exposure of murine papillary muscles led to a rapid decrease in $F_{\text{dev}}$, and increase in $F_{\text{dia}}$. At peak of contracture, $F_{\text{dev}}$ went down from $35.9 \pm 2.4$ to $14.7 \pm 1.3\, \text{mN/mm}^2$, $26.1 \pm 2.1$ to $2.9 \pm 0.7\, \text{mN/mm}^2$, and $22.0 \pm 3.7$ to $2.4 \pm 0.6\, \text{mN/mm}^2$ (P0C = 12 min) in TG, WT, and HET mice, respectively (all $P < 0.05$). At baseline in Fig. 2B, average $F_{\text{dia}}$ was $6.6 \pm 1.7\, \text{mN/mm}^2$. At peak of contracture (occurring at $\sim 12$ min after OH exposure in WT and HET, and earlier in TG mice, $\sim 3$ min) Ca$^{2+}$ overload led to increase in $F_{\text{dia}}$ to $36.7 \pm 3.6$ and $42.1 \pm 3.4\, \text{mN/mm}^2$, respectively, but in TG mice $F_{\text{dia}}$ increased only to $19.2 \pm 1.6\, \text{mN/mm}^2 (P < 0.05$, vs. HET and WT). Contractile parameters were observed until a new steady-state level was reached. This new steady state was marked by a sustained dysfunction, with an elevated $F_{\text{dia}}$ and reduced $F_{\text{dev}}$ in both WT and HET. At this new steady state, $F_{\text{dev}}$ returned to $10 \pm 2$ and $7 \pm 1\, \text{mN/mm}^2$ in WT and HET mice, respectively, whereas $F_{\text{dia}}$ came back to $18 \pm 5$ and $18 \pm 2\, \text{mN/mm}^2$, respectively. In contrast, the sustained dysfunction was significantly less in TG mice: $F_{\text{dia}}$ and $F_{\text{dev}}$ returned to $11 \pm 1$ and $24 \pm 2\, \text{mN/mm}^2$, respectively, compared with the preinterventional values. Figure 3 shows the percentage effect of OH on the contractile parameters of the papillary muscles in WT, TG, and HET mice.

**Phospholamban phosphorylation after OH exposure.** To determine the phospholamban (PLB) phosphorylation levels after OH exposure, small, intact contracting right ventricular papillary muscles from WT, TG, and HET were again directly exposed to OH. At the peak of contractile dysfunction, the muscle is quickly frozen in liquid nitrogen and stored at $-80\, ^\circ\text{C}$. These samples, as well as non-OH-exposed control samples, are analyzed for PLB expression and PLB phosphorylation levels via Western blot (Fig. 4). Our results show that before exposure to OH, TG hearts show a decrease in PLB protein, pPLBSer16, and pPLBThr17, whereas HET mice show a decrease in PLB protein and increase in pPLBSer16 and pPLBThr17 compared with WT. After exposure to OH there is decrease in pPLBSer16 in WT, TG, and HET mice compared with control muscles, but there was no or only little increase in the level of pPLBThr17 in all groups.

**DISCUSSION**

OH-induced injury in isolated murine myocardium. The acute response of murine contracting papillary muscles to OH radicals is very similar to what has been previously described for the rat (8) and rabbit (29). A transient, rigor-like contraction develops several minutes after OH exposure. During the acute phase, this injury is marked by an increase in diastolic tension and a decreased developed force. During the recovery phase,
diastolic force declines but not all the way to pre-\(-\text{OH}\) radical levels, indicating a sustained injury. At this new baseline, diastolic force thus remained elevated, and developed force was decreased.

\textit{Attenuation of \(-\text{OH}\)-induced injury by increase SR Ca}^{2+}-ATPase activity.\ The main goal of this study was to test our hypothesis that improved SR Ca}^{2+}-ATPase function can attenuate the acute \(-\text{OH}\) injury. The present study is the first to directly investigate the influence of altered SR Ca}^{2+}-ATPase activity on the contractile response of the cardiac muscle to \(-\text{OH}\)-induced injury. We observed that enhanced SR function in TG mice expressing SERCA1a (16, 18) significantly attenuated the \(-\text{OH}\)-induced injury. Both diastolic and systolic performance of the myocardium were much less affected compared with the response in WT littermates. The protective effect of enhanced SR Ca}^{2+}-ATPase activity is potentially twofold. First, an increase in the number of SR calcium pumps will increase the overall capacity to transport Ca}^{2+} back into the SR during diastole (16). If a number of pumps or even a percentage of this elevated capacity is affected, increased basal activity will still ensure a larger capacity of SR Ca}^{2+} transport after injury compared with mice with normal basal SR calcium pump levels. This (albeit reduced compared with preinjury baseline) capacity could potentially be sufficient to handle “normal” diastolic Ca}^{2+} levels. Second, Ca}^{2+} overload can be dealt with more efficiently as well. Increased capacity may partially alleviate the high Ca}^{2+} levels as they exist during acute injury. The functional capacity of the SR Ca}^{2+} pumps after \(-\text{OH}\) injury appears not large enough to completely prevent the contractile dysfunction, but a significant attenuation of acute dysfunction was observed. On the other hand, it may be that the capacity of the SR is simply insufficient to deal with the Ca}^{2+} overload. It is likely that the total amount of calcium that has entered the cell during the injury exceeds the SR storage capacity, and thus SR Ca}^{2+} pump level may not be the limiting factor anymore. In WT mice, a limited SR capacity is clearly not the case because increased SR Ca}^{2+}-ATPase activity in the TG animals does clearly result in a significant reduction in injury. For proof-of-principle that increased expression of SERCA is indeed solely responsible for the reduction in \(-\text{OH}\)-induced injury, we repeated the experiments with

\begin{figure}[h]
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\caption{Percent effect of \(-\text{OH}\) on the contractile parameters of the papillary muscles in WT, TG, and HET mice (\(n = 8\) each group). At peak of contracture (occurring at \(-12\) min after \(-\text{OH}\) exposure in WT and HET) Ca}^{2+} overload led to significant decrease in developed force (A) and significant increase in diastolic force (B) in WT and HET mice compared with TG mice. The new steady state was also marked by significant elevated diastolic force and reduced developed force in both WT and HET but again came back near baseline in TG compared with preinterventional values. Stimulation frequency was \(4\) Hz and temperature was \(37^\circ\)C throughout the experiment. *Preservation of function vs. WT, \(P < 0.05\). **Loss of function vs. WT, \(P < 0.05\).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Western blot analysis of total PLB and PLB phosphorylation at Ser16 and Thr17 before and after exposure to \(-\text{OH}. A: before exposure to \(-\text{OH}, TG\) hearts show a decrease in phospholamban (PLB) protein and pPLBSer16 and pPLBThr17, whereas HET mice show a decrease in PLB protein and increase in pPLBSer16 and pPLBThr17 compared with WT. B: after \(-\text{OH}\) exposure in all groups, WT exposed (WTe), TG exposed (TGe), and HET exposed (HETe) displayed a marked decrease in level of PLB phosphorylation at Ser16, but there was no significant change in PLB phosphorylation at Thr17 compared with unexposed controls. Calsequestrin (CSQ) concentration was used as an internal control for normalization.}
\end{figure}
mice (HET) in which SERCA2a expression and overall function was significantly reduced (22). With the use of the identical protocol, in HET mice the -OH-induced injury was found to be aggravated; diastolic force rose significantly higher than in WT mice during the acute injury phase, whereas also several other contractile parameters likewise were worse or, at best, equal when compared with WT mice. Thus from the HET experiments we concluded that a reduced expression of SERCA2a aggravates the -OH-induced injury. A reduced SR function is one of the hallmarks of end-stage cardiac failure (1, 19). Reduced SR Ca\(^{2+}\)-ATPase activity contributes to both diastolic and systolic failure and is one of the main changes compared with normal myocardium that is responsible for the alteration in force-frequency behavior (14, 25) and \(\beta\)-adrenergic response (7). Thus a similar -OH-induced injury would likely result in a greater amount of injury in myocardium with low SR Ca\(^{2+}\)-ATPase activity.

Decrease in PLB phosphorylation after -OH exposure. In close accordance to previous work, we show that before being exposed to to -OH, TG hearts show a decrease in PLB protein and PLB phosphorylation, whereas HET mice show a decrease in PLB protein and increase in PLB phosphorylation when compared with WT. The latter finding indicates a specific role for the phosphorylation of PLB on basal contractile function and represents a compensatory mechanism via which the calcium handling is normalized in the presence of altered SERCA activity induced by the transgene or knockdown. Our results indicate that after -OH exposure, all groups displayed a marked decrease in the level of PLB phosphorylation at Ser16. The sustained cardiac dysfunction (rise in diastolic force and loss of developed force) was significantly less in TG mice compared with WT and HET mice. We conclude that under control conditions, PLB phosphorylation partially compensates for the decreased SERCA activity/expression in HET mice. After -OH exposure, dephosphorylation of PLB at Ser16 contributes to the contractile dysfunction in WT mice, whereas TG mice are less susceptible to the -OH-induced dysfunction, because they already have low PLB phosphorylation levels before -OH exposure and thus maintain their normal SR calcium uptake capacity. In sharp contrast, in HET mice the high levels of PLB phosphorylation at Ser16 that were present at baseline are now lost after -OH exposure, thereby unmasking the reduced SR calcium uptake capacity, and thereby aggravating the injury response to -OH. Overall, our results indicate PLB dephosphorylation at Ser16 contributes to the dysfunction observed after -OH exposure. Experiments in which we investigated phosphorylation of the Thr17 site reveal that -OH exposure did not decrease the phosphorylation at Thr17.

Limitations of study. We used two different lines of mice in our studies. To show that a reduced SR Ca\(^{2+}\)-ATPase activity aggravated the -OH-induced injury, we used the SERCA2A HET mouse, in which one allele is mutated resulting in reduced SERCA2a expression, which results in a decreased SR Ca\(^{2+}\) uptake activity when compared with WT mice. To show the other end of the spectrum, we used the SERCA1a transgenic mouse line. This mouse expressed the SERCA1a isoform (~80% of total SERCA) in conjunction with the native cardiac isoform SERCA2a (~20%). The objective of our studies was to test whether enhanced calcium handling would partially prevent -OH-induced injury, which according to our data was clearly the case. Although SERCA1a and SERCA2a are different isoforms, when expressed in the heart, apart from the higher activity, they have been reported to behave virtually identical. Ji and coworkers (15) showed that only the maximal velocity was changed and that the apparent affinity for Ca\(^{2+}\) ATP affinity, Hill coefficient, pH dependence of Ca\(^{2+}\) uptake, and turnover rate were similar to SERCA2a. In addition, Lalli and coworkers (16) found that SERCA1a can substitute both structurally and functionally for SERCA2a in the heart and that SERCA1a overexpression can be used to enhance SR Ca\(^{2+}\) transport and cardiac contractility. Thus, although technically the SERCA1a mouse has a reduced SERCA2a expression, it has an enhanced SR Ca\(^{2+}\)-ATPase activity (because of SERCA1a expression) under the conditions studied, evident by the enhanced contractions at baseline. This model thus does not allow us to conclude that the great reduction in the injury response is due to SERCA2a specifically, but it does allow to prove our main hypothesis in that SR Ca\(^{2+}\)-ATPase activity modulated -OH-induced injury.

In conclusion, the acute injury that occurs after -OH exposure is dependent on the level of SERCA activity and PLB dephosphorylation specifically at Ser16. Increased SR Ca\(^{2+}\)-ATPase activity can partially rescue the heart from -OH-induced injury, whereas a reduction in SR Ca\(^{2+}\)-ATPase activity can aggravate the -OH-induced contractile dysfunction. Thus interventions primarily aimed at restoration of depressed contractility in various cardiomyopathies via improvement of SR Ca\(^{2+}\)-pumping [regulation of SR Ca\(^{2+}\)-ATPase activity (9) and/or downregulation/blocking of phospholamban] (4) may also be beneficial during reperfusion injury situations by providing a means to more effectively deal with the calcium overload that results from the oxidative stress/reperfusion injury.

ACKNOWLEDGMENTS

We thank Dr. M. Periasamy for making the TG and HET mice available, and we thank A. Kalyanasundaram for phenotyping of the mice.

GRANTS

This study was supported by National Institutes of Health Grants MMPC DK-59630, RO1 HL-73816, and KO2HL-083957 to P. M. L. Janeesen.

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