Hydroxyl Radical–Induced Acute Diastolic Dysfunction Is Due to Calcium Overload via Reverse-Mode Na⁺-Ca²⁺ Exchange

Oliver Zeitz, A. Eveline Maass, Phuc Van Nguyen, Geerd Hensmann, Harald Kögler, Karsten Möller, Gerd Hasenfuss, Paul M.L. Janssen

Abstract—Hydroxyl radicals (OH) are involved in the development of reperfusion injury and myocardial failure. In the acute phase of the OH-mediated diastolic dysfunction, increased intracellular Ca²⁺ levels and alterations of myofilaments may play a role, but the relative contribution of these systems to myocardial dysfunction is unknown. Intact contracting cardiac trabeculae from rabbits were exposed to OH, resulting in an increase in diastolic force (F\text{di}) by 540%. Skinned fiber experiments revealed that OH-exposed preparations were sensitized for Ca²⁺ (EC\text{50}: 3.27±0.24×10⁻⁶ versus 2.69±0.15×10⁻⁶ mol/L; P<0.05), whereas maximal force development was unaltered. Western blots showed a proteolytic degradation of troponin T (TnT) with intact troponin I (TnI). Blocking of calpain I by MDL-28.170 inhibited both TnT-proteolysis and Ca²⁺ sensitization, but failed to prevent the acute diastolic dysfunction in the intact preparation. The OH-induced diastolic dysfunction was similar in preparations with intact (540±93%) and pharmacologically blocked sarcoplasmic reticulum (539±77%), and was also similar in presence of the L-type Ca²⁺-channel antagonist verapamil. In sharp contrast, inhibition of the reverse-mode sodium-calcium exchange by KB-R7943 preserved diastolic function completely. Additional experiments were performed in rat myocardium; the rise in diastolic force was comparable to rabbit myocardium, but Ca²⁺ sensitivity was unchanged and maximal force development was reduced. This was associated with a degradation of TnI, but not TnT. Electron microscopic analysis revealed that OH did not cause irreversible membrane damage. We conclude that OH-induced acute diastolic dysfunction is caused by Ca²⁺ influx via reverse mode of the sodium-calcium exchanger. Degradation of troponins appears to be species-dependent but does not contribute to the acute diastolic dysfunction. (Circ Res. 2002;90:988-995.)

Key Words: diastolic dysfunction ■ oxidant stress ■ calcium handling ■ myofilament ■ contractility

Hydroxyl radicals (OH) are one of the most aggressive species of oxygen free radicals.1,2 OH are known to play an important role in several pathophysiological processes. Oxygen free radicals occurring during reperfusion after ischemia are thought to be involved in the development of “stunned myocardium” and in the development and progression of heart failure are associated with increased oxidative stress,3-6 Free radical–induced myocardial dysfunction is characterized on different biological levels: oxygen free radical–exposed myocardial tissue and single cells show a severe diastolic dysfunction,1,2 and contracting intact cardiac trabeculae from rabbit7 and human8 develop a rigor-like contracture marked by a severe and in part reversible increase in diastolic force and a loss of developed force. However, the mechanisms whereby oxidative stress acts remain speculative.3

The cause of the acute phase of myocardial diastolic dysfunction after free radical exposure may likely have 2 components: both increased intracellular calcium levels and alterations of the myofilaments are hypothesized to be involved. It has been shown that OH exposure causes a severe intracellular calcium overload and multiple mechanisms including sarcoplasmic reticulum (SR) damage, mitochondrial damage, L-type calcium current, and sodium-calcium exchange may contribute to this calcium overload.1,3,7,9,10 Furthermore, altered myofilament responsiveness may be involved in OH-induced dysfunction.11 The troponin complex may be a potential target, whereby radicals may interact directly with the molecules or initiate specific, possibly Ca²⁺-dependent, proteolytic pathways.3,12,13

The relative contribution of these different subcellular defects to the development of acute myocardial dysfunction...
after OH exposure remains unresolved. Accordingly, we investigated the mechanisms underlying the early unresolved diastolic function after application of OH. The results indicate that Ca\(^{2+}\) influx via the sodium-calium exchanger is responsible for the increase of diastolic force. Furthermore, although degradations of the troponin complex are observed, these degradations are ruled out to significantly contribute to the acute contractile dysfunction after OH exposure.

**Materials and Methods**

**Animals and Preparation**

After anesthesia, hearts from Chinchilla bastard rabbits (Harlan, Borchen, Germany) (either sex, 1.5 to 2.5 kg; fed ad lib) were prepared as described previously.\(^{7}\) Experiments were conducted according to institutional and national guidelines. Small, right ventricular trabeculae (average dimensions \(\approx 200 \times 150 \times 3000 \ \mu m\)) were dissected and mounted in the setup as published previously.\(^{7}\)

**Generation of Hydroxyl Radicals**

Hydroxyl radicals (OH) were generated from H\(_2\)O\(_2\) via the Fenton reaction. For catalysis, a complex of Fe\(^{3+}\) and nitrotriaceitate (Fe-NTA) were used. This method has been shown to generate reproducible amounts of OH that are comparable to those that occur during ischemia/reperfusion.\(^{6}\) The concentrations in the organ bath of H\(_2\)O\(_2\) and Fe-NTA were 0.75 mmol/L and 10 \(\mu\)mol/L, respectively. H\(_2\)O\(_2\) was infused directly into the organ bath to ensure that radical formation took place as closely as possible. To avoid photobleaching, radical exposure was performed in a darkened room.

**Experimental Protocols**

Muscles were mounted in the setup as described previously.\(^{14}\) Trabeculae were stretched to a diastolic force of 2 to 5 mN/mm\(^2\). After contractile parameters had stabilized (at 1 Hz and 37°C), preparations were exposed to OH for 2 minutes. Contractility was monitored until diastolic force reached its maximum (typically 30 minutes). At that point, preparations were detached from the setup and immediately transferred into skinning solution. Sixteen to twenty-four hours later a calcium concentration response curve was measured in these fibers, and the fibers were subsequently frozen for Western blot analysis.

Measurements of calcium sensitivity and force development of the myofilaments were performed on detergent-skinned trabeculae with solutions as described previously.\(^{14-16}\) Sarcomere length was standardized by laser-diffraction (He/Ne laser). The MDL-28.170 experiments (see following section) were done after slight modification and relocation of the setup. Data for the semiquantitative determination of sarcomere-length, there are slight differences in basic calcium EC\(_{50}\) values. However, within the experimental groups compared directly, the same standardized sarcomere length and setup were always used.

For protein composition analysis, trabeculae (0.5 to 1.0 mg) were homogenized in 100 \(\mu\)L of (in mmol/L) 50 HEPES (pH 7.4), 150 KCl, 0.1 EGTA, 1 DTT, 1 benzamidine, 1 PMSF, and 1 \(\mu\)g of each chymostatin, leupeptin, papain, and aprotinin. Protein determination was performed by BCA (Pierce). Protein (15 \(\mu\)g) was applied to a 12.5% SDS-gel. After electrophoresis and semidry blotting on polyvinylidene difluoride (PVDF)-membrane, blots were blocked with 5% nonfat dry milk in TBS/0.1% Tween20 overnight, and incubated with monoclonal antibodies against TnT (1:2000, clone JLT-12, Sigma) or against TnI (1:4000, clone 8I-7, Sigma) for 2 hours at room temperature. In light of a possible cross-reactivity of anti-TnT with GAPDH,\(^{17}\) additional blots with GAPDH antibodies were performed. A positive GAPDH signal has been seen in OH-exposed as well as in control preparations, whereas JLT-12 staining showed an additional band only in the OH-exposed samples. Antigen-antibody complexes were visualized by peroxidase-conjugated anti-mouse antibodies using enhanced chemiluminescence (Pierce). Western blotting was done with fresh samples from the skinned fiber experiments because we observed that the troponin degradations were very unstable and no longer detectable after 2 cycles of freezing and thawing.

**Pharmacological Interventions**

To investigate the mechanisms of troponin T degradation and its possible contribution to the increased diastolic force, calpain I was inhibited with the tripeptide MDL-28.170\(^{18}\) (gift of Aventis Bridge-water, New Jersey). Preparations were incubated with 1 \(\mu\)mol/L for 20 minutes, which has been shown to inhibit calpain I effectively in neuronal tissue.\(^{19}\)

Other pharmacological interventions were used to specifically block the possible sources of intracellular calcium overload. The SR was inhibited by 10 \(\mu\)mol/L cyclopiazonic acid (Calbiochem) and 1 \(\mu\)mol/L ryanodine (Sigma). Effectiveness of the SR-block was confirmed by absence of rapid cooling contractures. Rapid cooling contractures as an relative indicator of SR-Ca\(^{2+}\) content were performed as described previously.\(^{20}\)

L-type Ca\(^{2+}\) current was inhibited by verapamil (1 \(\mu\)mol/L; Sigma). OH exposure was started when contractile parameters had restabilized after addition of verapamil.

Reverse-mode sodium-calcium exchange was inhibited by KB-R7943, an isoiourea derivative that selectively blocks reverse mode of the sodium-calcium exchanger.\(^{21}\) KB-R7943 (gift of R&D Laboratories, Osaka, Japan) was dissolved in DMSO and 20 \(\mu\)mol/L was added immediately after finishing OH exposure. In control experiments, equivalent amounts of DMSO were used. The ability of KB-R7943 to inhibit sodium-calcium exchanger reverse-mode activity in the present setting was confirmed by using the procedure described previously.\(^{21}\) Briefly, after blockade of the SR, the extracellular sodium was replaced by tetramethyl-amonium. This resulted in a Ca\(^{2+}\) influx via sodium-calcium exchanger reverse mode and an increase of diastolic force from 2.3±0.2 to 13.2±1.8 mN/mm\(^2\) within 40 seconds. KB-R7943 reduced this increase (2.0±0.2 to 7.5±1.9 mN/mm\(^2\); n=14 KB-R7943, n=16 control; P<0.05 KB-R7943 versus control), indicating effective sodium-calcium exchanger reverse-mode inhibition.

**Verification on Rat Myocardium**

In rat myocardium, a proteolytic degradation of TnI is reported.\(^{22}\) To verify whether our protocol produced similar results regarding the development of (1) a diastolic dysfunction and (2) a proteolytic degradation of TnI to those previously reported, we repeated a subset of experiments with rat myocardium to study these 2 parameters. Wistar rats (Harlan, Børchen, Germany) (either sex, 180 to 300 g; fed ad lib) were anesthetized with halothane 10 minutes after heparinization (1000 IU sodium heparin IP). All experimental conditions and procedures were similar, except for the Ca\(^{2+}\)-concentration (1.5 mmol/L) and stimulation frequency (2 Hz) in the experiments on intact trabeculae.

**Electron Microscopic Histology**

We used electron microscopy (EM) to examine the trabeculae after exposure to OH radicals to determine whether irreversible injury occurred. We compared trabeculae before, during peak diastolic dysfunction, and 30 minutes after peak diastolic dysfunction (recovery). Preparations were fixed in situ the experimental setup. EM sections were prepared using standard techniques.

**Data Analysis and Statistics**

Contractile parameters were recorded and analyzed using customized programs.\(^{7}\) The sigmoidal force-calcium relationship of the skinned fibers was fit by a nonlinear fit procedure to a modified Hill equation.\(^{16}\) For statistical analysis, each EC\(_{50}\) point is treated as a measured variable. Both Student’s t test for paired and nonpaired data are used where applicable. Values (double sided) for P<0.05 were considered to be significant. Data are expressed as mean±SEM.
Results

Influence of Hydroxyl Radicals on Contractility of the Intact Preparation

In accordance with previous reports, hydroxyl radical exposure of rabbit trabeculae led to a rapid increase of diastolic force and a loss in developed force. In the rabbit, hydroxyl radical exposure for 2 minutes resulted in an increase of diastolic force from 3.9 ± 0.4 to 36.3 ± 11.4 mN/mm² (n = 9, P < 0.05; Figure 1). Developed force decreased from 7.8 ± 1.2 to 3.3 ± 0.8 mN/mm² (n = 9, P < 0.05). In a separate set of experiments, contractile parameters were observed after OH exposure until a new steady-state level was reached. In accordance with previous work, this new steady-state level is marked by a slightly elevated diastolic force and unaltered developed force compared with preinterventional values; diastolic force increased initially to 714 ± 218% of its value before OH exposure and returned approximately 1 hour after OH exposure to a new steady-state level of 237 ± 59% (n = 4, P < 0.05). Developed force stabilized in these experiments at 80 ± 25% (P = NS versus preinterventional level). In another set of experiments, preparations were treated similarly but not exposed to hydroxyl radicals; contractile parameters of these control preparations remained stable over the equivalent period of time (n = 9).

Effect of Hydroxyl Radicals on Cardiac Myofilaments

At peak diastolic dysfunction, preparations were rapidly removed from the setup and skinned. After skimming of the preparations, a calcium concentration response curve was recorded. Hydroxyl radical–exposed preparations had an increased calcium sensitivity (EC₅₀ = 2.69 ± 0.15 × 10⁻⁶ versus 3.27 ± 0.24 × 10⁻⁶ mol/L in the control group; n = 9/group, P < 0.05; Figure 2). Maximal force development of the fibers was unaltered (22.7 ± 3.1 versus 27.0 ± 3.1 mN/mm² in control preparations; n = 9, P = NS). This calcium sensitization was associated with a degradation of TnT, whereas TnI was unaffected (Figures 2A and 2B). The degradation band of TnT had a MW around 39 kDa.

Role of Myofilaments

Inhibition of the calcium-dependent protease calpain I by MDL-28.170 prevented degradation of TnT and preserved calcium-responsiveness of the myofilaments (Figure 3B). Calcium EC₅₀ was 1.97 ± 0.16 × 10⁻⁶ mol/L (n = 9) in the OH-exposed group and 2.38 ± 0.11 × 10⁻⁶ mol/L (n = 10) after hydroxyl radical exposure in presence of MDL-28.170 (P < 0.05; Figure 3A). However, in the intact experiment, diastolic force still increased in presence of MDL-28.170 by 460%, which was not significantly different from the increase observed in the hydroxyl radical–exposed control group. This suggests that the proteolytic myofilament alterations do not significantly contribute to the acute diastolic dysfunction.

Role of SR Function

To investigate the role of SR function for the development of the contracture, influence of OH was tested on contracting trabeculae with intact and with pharmacologically blocked SR. This SR-block by 10 μmol/L CPA and 1 μmol/L ryanodine resulted in complete elimination of the rapid cooling contracture amplitude from 9.2 ± 1.6 to effectively 0 mN/mm², indicating that a complete functional knockout of the SR had been achieved. Application of hydroxyl radicals to preparations with intact SR increased diastolic force by 540 ± 93% (from 4.9 ± 1.0 to 26.1 ± 7.4 mN/mm²; n = 12). In the SR-Block group, hydroxyl radical exposure resulted in a very similar increase of diastolic force by 539 ± 77% (from 3.4 ± 0.3 to 18.6 ± 3.9 mN/mm²; n = 15, P = NS). Thus, the contribution of the SR to the acute diastolic dysfunction is also highly unlikely.

Role of the L-Type Calcium Channel

Another possible source of intracellular calcium overload could be found in dysfunction of the L-type calcium channel. To examine this possibility, the L-type calcium channel was blocked by 100 μmol/L CPA and 1 μmol/L ryanodine. Application of hydroxyl radicals to preparations with intact L-type calcium channels increased diastolic force by 537 ± 127% (from 4.5 ± 1.5 to 31.0 ± 7.7 mN/mm²; n = 12). In the L-Block group, hydroxyl radical exposure resulted in a very similar increase of diastolic force by 530 ± 130% (from 3.4 ± 0.3 to 18.6 ± 3.9 mN/mm²; n = 15, P = NS). Thus, the contribution of the L-type calcium channel to the acute diastolic dysfunction is also highly unlikely.
blocked by incubating trabeculae with \(10^{-6}\) mol/L verapamil. This resulted in a decrease of developed force from 17.8±4.2 to 6.0±1.2 mN/mm². After stabilization of contractile parameters, hydroxyl radical exposure was performed. Diastolic force increased by 680% (from 2.4±0.2 to 16.0±3.2 mN/mm²), which was not significantly different from the increase of diastolic force in hydroxyl radical–exposed control trabeculae (n=8). This indicates that the L-type calcium channel is not mainly responsible, if at all, for the occurring calcium overload.

**Role of Sodium-Calcium Exchange**

To elucidate the role of the sodium-calcium exchanger as a possible source of the hydroxyl radical–induced intracellular calcium overload, its reverse mode was blocked with 20 \(\mu\)mol/L KB-R7943. KB-R7943 was applied when hydroxyl radical exposure was finished to ensure that the possible protective effects of KB-R7943 are not due to a potential scavenger capacity of the molecule or its solvent. Amplitude of the hydroxyl radical–induced contracture was significantly reduced in presence of KB-R7943. Peak diastolic force was 7.7±1.2 in the control group (equivalent concentration of the KB-R7943 solvent DMSO, n=10) and 3.5±0.5 mN/mm² in the KB-R7943-group (n=7, \(P<0.01\); Figure 4).

**Effects of OH Exposure on Rat Myocardium**

Trabeculae from rat hearts displayed an increase of diastolic force from 3.8±0.6 to 21.7±6.4 mN/mm² after hydroxyl radical exposure (n=14, \(P<0.05\) versus control; Figure 1), whereas developed force decreased from 7.9±2.1 to 2.7±0.7 mN/mm² (n=14, \(P=NS\) versus control). Calcium sensitivity of the rat skinned fiber was not affected by OH exposure in rat myocardium (Figure 5A). Maximal force development was significantly reduced in OH-treated preparations (31.9±4.4 versus 20.4±1.4 mN/mm²; \(P<0.05\); Figure 5B). Western blot analysis revealed intact TnT (not shown), but degraded TnI (Figure 5C) as expected in light of results from previous studies.

**EM Histology**

EM analysis revealed membrane damage neither during peak contractile dysfunction nor after recovery (30 minutes later). Also, no structural changes were observed in the myofilaments. Interestingly, the only clear difference was the appearance of the mitochondria, which were normal in fresh preparations (Figure 6A) and swollen during peak contractile dysfunction (Figure 6B), but not after recovery (Figure 6C).

**Discussion**

The response of contracting cardiac trabeculae to OH is marked by a characteristic acute increase in diastolic force. This observation is in close agreement with previous reports. In rabbit myocardium, hydroxyl radical exposure leads to a degradation of TnT, associated with an increased calcium sensitivity of the myofilaments. However, inhibition of the calcium-dependent protease calpain I abolishes this TnT degradation and preserves calcium sensitivity, but it does not diminish acute diastolic dysfunction after hydroxyl radical exposure significantly. Also, neither SR function nor blockade of the L-type calcium channel influences the severity of the diastolic dysfunction. In sharp contrast, inhibition of reverse-mode activity of sodium-calcium exchange almost completely preserves diastolic function. These data indicate that \(Ca^{2+}\) influx through reverse-mode \(Na^{+}-Ca^{2+}\) exchange represents the main mech-
anism underlying diastolic calcium overload and dysfunction after OH exposure

Hydroxyl Radical Exposure and Myofilament Responsiveness

We observed that in hydroxyl radical–exposed rabbit myocardium TnT is degraded, whereas TnI is intact. This was associated with increased calcium sensitivity, whereas maximal force development was unaffected. The observation of the association between TnT degradation and increased calcium sensitivity is consistent with the current models of the structure and function of the troponin complex. In the present work, degradation of TnT in the rabbit was prevented by MDL-28.170, a membrane-permeating inhibitor of calpain I. Despite prevention of TnT–degradation with MDL-28.170, the acute diastolic dysfunction still developed. This indicates that TnT degradation does not significantly contribute to the acute increase of diastolic force after hydroxyl radical exposure. Degradation of TnT most likely results from increased intracellular calcium concentration that leads to an activation of calpain I.

The present study is the first study directly investigating the influence of oxidative stress on the myofilaments in rabbit myocardium. Previously, numerous studies have been performed in stunned rat myocardium. In those studies, degradations of troponin I were found and have been shown to be calpain I dependent. This difference to previous work presents the following questions: (1) is the observation of TnT degradation due to the use of rabbit instead of rat myocardium; or (2) is TnT degradation independent of the species characteristic for hydroxyl radical–induced myocardial damage, whereas TnI degradation occurs preferentially in stunned myocardium? To resolve these questions, additional experiments in rat myocardium were conducted using the identical protocol; the results demonstrate that in hydroxyl radical–exposed rat myocardium TnI is proteolyzed, whereas TnT is intact. Maximal force development of the rat skinned fiber is reduced, but calcium sensitivity is unaltered. The TnI proteolysis, as well as the present functional observations, is compatible with previous work. The present data indicate that OH alone can induce a molecular and functional phenotype of the myofilaments that shows parallels to that of stunned myocardium.

In addition, proteolysis of troponin complex caused by OH may be species dependent. Several recent studies have addressed the possible species variation after stunning versus other stimuli that produce TnI degradation. Lack of TnI degradation in swine and dog has been observed, similar to the lack on TnI degradation in the rabbit in our model. In addition, a dissociation between TnI proteolysis and stunning has been reported for rat myocardium, and in a recent preliminary presentation by van Eyck and colleagues, it was shown that TnI proteolysis was not essential for myocardial stunning in the dog. Interestingly, TnT degradation was observed in their model of open-chest dogs. Although the majority of these reports point toward the fact that myofilament alterations that occur during ischemia may predominantly be in TnI in small rodents and in TnT in larger rodents and mammals, it is important to note that TnI degradation has

![Figure 5](http://circres.ahajournals.org/)(A) In contrast to rabbit myocardium, calcium sensitivity of skinned fibers from hydroxyl radical exposed rat myocardium is not altered (A), whereas maximal force development is significantly reduced by 33% (B). Western blot analysis revealed a degradation of troponin I (C). **P<0.05.

![Figure 6](http://circres.ahajournals.org/)(B) Electron microscopic sections of a fresh trabecula (A), fixed at the peak of diastolic contracture (B), or 30 minutes thereafter (C). Although no apparent membrane damage was observed, we did see an alteration in the appearance of mitochondria. At peak contracture, these appeared swollen, whereas after recovery (C) this seemed to be reversed to baseline (A).
been observed in the myocardium of bypass patients.\textsuperscript{32} Because of the various models used, a straightforward species comparison using identical models would be required to resolve this issue.

Although from a biochemical point of view it is possible that calpain I proteolyses TnI as well as TnT,\textsuperscript{25} the mechanisms of the species dependency remain open and are important to be addressed in future studies. However, this does not impact on the central conclusion of the present study, ie, that myofilament alterations are not involved in the acute diastolic dysfunction; in presence of calpain I this myofilament proteolysis can be inhibited, but the acute diastolic dysfunction is still present. Furthermore, the conclusion that myofilament alterations do not contribute to the acute part of the diastolic dysfunction is underlined by a comparison of the data from rabbit and rat myocardium; although it could be conceivable that the elevated calcium sensitivity in preparations from the rabbit contributes to the rise of diastolic tension, the decreased maximal force development and unchanged calcium sensitivity in rat myocardium would rather result in decreased diastolic force, which is clearly not the case. Thus, despite the differences between the species regarding myofilament degradation, this does not account for the acute diastolic calcium overload after OH exposure, the mechanism of which was the goal of, and dissected, in this study. Future studies may be addressed to questions regarding if, and how, the myofilament-specific proteolysis may play a prominent role in the latter phases of OH-induced cardiac dysfunction and toward the exact nature of these myofilament alterations.

It has been shown in detail previously,\textsuperscript{7} and also by this study, that contractile parameters of rabbit myocardium are partially restored approximately 1 hour after hydroxyl radical exposure and 30 minutes after the peak of the OH-induced contracture, respectively. This new contractile steady state is characterized by a more than 2-fold increase of diastolic force compared with values before hydroxyl radical exposure. It can be speculated that the shift of calcium sensitivity is responsible for the increased diastolic force in the new "steady state." This hypothesis is supported by previous observations that the diastolic force in rat myocardium, where no calcium sensitizing shift is observed, fully returns to baseline.\textsuperscript{1}

### Hydroxyl Radicals and Calcium Overload

Potential mechanisms causing the calcium overload that have been postulated include dysfunction of the SR, dysfunction of the L-type calcium channel, leaks in the sarcolemma, and increased calcium influx via reverse mode of the sodium–calcium exchanger.\textsuperscript{3,20,33} The role of the SR in this context may appear controversial. It is known that the activity of the SR calcium ATPase is reduced by oxygen free radicals. Xu and coworkers\textsuperscript{33} presented evidence that the ATP binding site of the SR calcium ATPase is attacked by oxygen free radicals. Also, in intact myocardial preparations, a clear attenuation of the strongly positive force-frequency relation of rabbit myocardium after hydroxyl radical exposure has been shown.\textsuperscript{7} These studies clearly indicate that SR function is impaired by oxygen free radicals, but the question of whether SR damage is responsible for the diastolic dysfunction remained unresolved. In the present study, rabbit preparations with intact and with pharmacologically blocked SR were exposed to OH and the resulting diastolic dysfunction was virtually identical in both groups. Thus, although SR dysfunction plays a significant role in the non-acute cardiac dysfunction of OH exposure, the SR does not seem to significantly contribute to the development of this acute, diastolic dysfunction.

In several studies, calcium channel antagonists prevented myocardial stunning.\textsuperscript{9,34,35} These data led to the hypothesis that increased open probability of the L-type calcium channel is the source of intracellular calcium overload in radical exposed myocardium.\textsuperscript{3} At least because of the negative results of in vivo studies and clinical trials, the contribution of L-type calcium channel to oxygen free radical–induced myocardial damage is still under discussion.\textsuperscript{10,23,36} Studies in which calcium antagonists had beneficial effects\textsuperscript{9,34,35} were carried out in ischemia/reperfusion models, and it has been proposed that the time of administration of the compound may be important for protection from myocardial stunning.\textsuperscript{34,35} Calcium antagonists are most effective if applied before or during the ischemic period or in the very beginning of reperfusion. This led to the conclusion that calcium antagonists are able to prevent the ischemic injury of myocardium, but fail in reperfusion injury where oxygen free radicals do occur.\textsuperscript{3,35} This is in accordance with the data from the present study; blockade of the L-type calcium channel, even with high concentrations of verapamil, did not influence hydroxyl radical–induced myocardial dysfunction.

There are indications that the intracellular sodium concentration is elevated in cardiomyocytes exposed to increased levels of oxidative stress.\textsuperscript{37} A decrease of the transmembrane sodium gradient would at least impair calcium extrusion by Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger in the forward mode and possibly lead to a calcium influx by activation of sodium–calcium exchanger reverse mode. After exclusion of other sources of hydroxyl radical–induced intracellular calcium overload, this calcium entry route seems to be likely. The lack of appropriate pharmacological compounds has hampered a direct investigation of the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger reverse mode in multicellular preparations. Recently, the isothiourea derivative KB-R7943 has been shown to selectively inhibit the reverse mode of the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger.\textsuperscript{38} KB-R7943 has been shown to prevent digitalis-induced calcium overload of cardiomyocytes\textsuperscript{21} and was also effective in preventing reoxygenation-induced injury in cardiomyocytes\textsuperscript{39} and isolated papillary muscles.\textsuperscript{40} The data from the present study show that KB-R7943 could prevent hydroxyl radical–induced acute diastolic dysfunction. KB-R7943 was not administered until after hydroxyl radical exposure was finished, so that potential scavenger properties of the KB-R7943 molecule can be excluded to cause the protective effect of the compound. The hypothesis that OH induced the intracellular calcium overload by activation of the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger reverse mode is confirmed by the present study. In addition, these findings exclude that OH-induced contractile dysfunction is due to an unspecified oxidative sarcolemmal injury. An unspecified membrane injury would also lead to a calcium overload of the...
cells, but such an unspecific injury could then not be prevented by specific inhibition of calcium influx via the Na\(^+\)/Ca\(^{2+}\) exchanger. This conclusion is enforced by the present results from the electron microscopy, and additionally underlined by the fact that OH-exposed trabeculae have the potential for significant recovery.

Limitations of the Study
Although the present study answered the question of how the acute diastolic dysfunction after OH injury is mediated, it remains undetermined whether and how the alterations of myofilament proteins myofilament impact contractile behavior over an extended period. Although we speculate that it is likely that these alterations may determine the sustained contractile dysfunction, this was deemed beyond the goal of the present study. In line with this limitation, the exact modification of the contractile proteins affected (ie, specifically TnT) remains to be determined, as is the question of how these myofilament alterations are dependent on the species. In addition, future studies may be directed toward the relevance of mitochondrial swelling as observed in our EM sections. Detection of sarcomere length was not exactly similar in different protocols, resulting in slight changes in EC\(_50\). However sarcomere length detection was identical within the individual protocols.

Conclusions
The data from the present study demonstrate that OH causes a well-defined, specific, and reproducible acute diastolic dysfunction. Although myofilament function is affected via a calpain I-mediated process, myofilament proteolysis seems to neither influence nor cause the acute diastolic dysfunction. This acute diastolic dysfunction results from calcium overload, which is mediated mainly, if not exclusively, by activation of reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange. Myofilament alterations, impairment of SR function, and dysfunction of L-type calcium channel are excluded as mechanisms relevant for the acute increase of diastolic force after hydroxyl radical exposure.

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