Greater susceptibility of failing cardiac myocytes to oxygen free radical-mediated injury

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Abstract

Objective: Oxygen-derived free radicals can produce myocardial cellular damage, which might contribute to the ischemia-reperfusion injury and to heart failure (HF). However, the effects of oxygen radicals on myocyte structure have not been examined in the failing heart.

Methods: We examined the susceptibility of intact cardiac myocytes isolated from control (n=16) and rapid pacing (240 bpm, 4 wks)-induced HF (n=8) dog hearts to an exogenous hydroxyl radical (·OH), generated from H 2 O 2 and Fe 3+-nitrilotriacetate. The production of ·OH was monitored by electron spin resonance with 5,5'-dimethyl-1-pyroline-N-oxide (DMPO) as a spin trap.

Results: The magnitude of DMPO-·OH signals was not attenuated in the presence of either control or HF myocytes. ·OH induced a time-dependent decrease in myocyte length (i.e. hypercontracture). The time to the onset of hypercontracture and that to the submaximal hypercontracture after exposure was significantly shortened in HF. Activities of superoxide dismutase, catalase, and glutathione peroxidase was not decreased in HF.

Conclusions: HF myocytes were more susceptible to oxidative stress-induced cellular injury, which was not due to decreased antioxidant defense, but to the intrinsic properties of cells.

Keywords: Heart failure; Free radicals; Myocytes

1. Introduction

Reperfusion of ischemic myocardium may induce an injury to the tissue, which might result from a marked increase in the generation of reactive oxygen species (ROS) [1]. Recently, there is an evidence supporting that the formation of ROS is also increased in experimental [2,3] and clinical heart failure (HF) [4,5]. The excess amount of ROS can cause the oxidation of membrane phospholipids and exert harmful effects on preservation of functional integrity of the biological tissue. When ROS are produced within the heart, they may induce the contractile dysfunction and structural damage [6]. Therefore, ROS may play an important role in the progression and aggravation of HF. However, the current knowledge on the effects of ROS on myocardial structure is based on studies using normal cardiac preparations and no previous studies have examined the effects of ROS on the failing hearts. Thus, there are no data comparing the degree of ROS-mediated cardiac structural injury between normal and failing hearts.

Highly reactive hydroxyl radical (·OH), generated from superoxide anion (·O 2 ) and H 2 O 2 via the iron mediated Fenton reaction, is the ROS which cause cellular injury [6]. In addition, peroxynitrite (ONOO − ) is also an important endogenous generator of ·OH [7]. Previous studies using electron spin resonance (ESR) methods have demonstrated that ·OH and H 2 O 2 are generated in reperfused myocardium [1] as well as failing myocardium [8]. An exposure of freshly isolated cardiac myocytes to ·OH causes an irreversible distortion of their shape; i.e. hypercontracture [6,9]. Cardiac myocytes are an excellent model for studies of the effects of ROS on the myocardium.
at the cellular level [9]. It could obviate the potential problems in intact hearts of the confounding effects of ROS on systemic hemodynamics and coronary circulation.

The purpose of this study was to examine whether the susceptibility of cardiac myocytes to ROS-mediated injury might differ between control and HF. Therefore, we examined the loss of rod shape morphology of isolated adult canine left ventricular (LV) myocytes after the exposure to exogenously generated °OH in vitro as an index of oxidative injury and determined whether this index differs between control and rapid ventricular pacing-induced HF [10,11]. °OH was chosen for the experiments because it is shown to be the predominant ROS causing cellular injury [6] and has been demonstrated to be increased in the failing myocardium [8]. Further, to delineate the role of antioxidant reserve in ROS-mediated cardiac injury, we measured the activity of the scavenging enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) in control and HF myocardium.

2. Methods

2.1. Animal model of HF

Eight adult mongrel dogs with HF induced by rapid ventricular pacing [11] and 16 control dogs (15–25 kg body weight) were used. On the day of the study, ventricular pacing was stopped and LV contractile performance was evaluated by echocardiography [11]. The protocols were approved by the Committee on the Ethics of Animal Experiments, Kyushu University.

2.2. Isolation of cardiac myocytes

After the completion of in vivo LV function studies, the animals were sacrificed with a lethal dose of α-chloralose and the heart was quickly excised. Cardiac myocytes were isolated from the LV free wall as described previously [11]. In brief, a wedge of LV free wall was dissected free of the heart and was perfused by a branch of the left circumflex coronary artery with nominally calcium-free buffer of the following composition (mmol/l): NaCl 140.0, KCl 4.8, MgSO₄ 2.4, NaH₂PO₄ 1.2, NaHCO₃ 2.5, HEPES 12.0, and glucose 12.5 gassed with 100% O₂. The image of the cell 2 mm in diameter was acquired via CCD camera and recorded on a video image analysis system. The myocytes were resuspended in buffer containing 1.25 mmol/l Ca²⁺ for 1 h at room temperature before assessing their shape.

2.3. Myocyte morphology

Only cardiac myocytes with the following characteristics were analyzed: single, rod-shaped cells unattached to adjacent cells. We considered it critically important to be certain that the cellular damage due to isolation procedures may not affect myocyte morphological viability during exposure to °OH. Therefore, three indexes of cell viability were examined for each isolation performed; 1) the percentage of quiescent rod-shaped cells in 10 randomly chosen 1 mm fields, 2) the percentage of rod-shaped cells in 10 randomly chosen 1 mm fields excluding 0.4% trypan blue, and 3) the percentage of rod-shaped cells responded to the electrical field stimulation [11]. To determine the percentage of cells responded to the electrical field stimulation, the myocytes were stimulated to contract between a pair of platinum wire electrodes by 0.25-Hz, 100-μA direct-current pulses of alternating polarity. The single, rod-shaped myocytes, unattached to either adjacent cells or debris, which contracted with each electrical stimulation pulse and were quiescent between stimuli, were defined as ‘responder cells.’

2.4. ESR measurement of °OH generation

To quantify the amount of °OH generated, electron spin resonance (ESR) spectra were recorded by a spectrometer (JES-RE-1X; JEOL) operating at X-band (9.43 GHz) with a spin trap, 5,5'-dimethyl-1-pyroline-N-oxide (DMPO; 80 mmol/l) [9]. °OH was generated from H₂O₂ (1–10 mmol/l) in the presence of Fe³⁺-nitrilotriacetate (NTA; 20 μmol/l) at room temperature. Quantitation of the DMPO-OH signal was performed by comparing the amplitude of the observed signal to a standard Mn²⁺/MgO marker.

2.5. °OH-mediated cardiac myocyte injury

Isolated myocytes were placed in a chamber on the stage of an inverted microscope (Olympus) and superfused with the Krebs buffer (pH 7.4, 35°C) of the following composition (mmol/l): NaCl 140.0, KCl 4.8, MgSO₄ 2.4, NaH₂PO₄ 1.2, NaHCO₃ 2.5, HEPES 12.0, CaCl₂ 1.25, and glucose 12.5 gassed with 100% O₂. The image of the cell was acquired via CCD camera and recorded on a video tape continuously during the experiment [9]. Rest length of rod shaped cells, determined along its longitudinal axis,
was measured before and during exposure to \(^\cdot\)OH generating system, \(\text{H}_2\text{O}_2\) and \(\text{Fe}^{3+}\)-NTA. The cell length at different incubation time points was divided by that at time zero (before addition of the radicals and/or drugs). Data from 15 to 30 cells were obtained for each experiment. We employed the time to the onset of hypercontracture and that to the submaximal hypercontracture (transition from the rod shaped morphology to the hypercontracted or rigor form) after exposure to \(\cdot\)OH as an index of myocyte susceptibility to ROS.

2.6. Antioxidant enzyme activities

The enzymatic activities of SOD, catalase, and GSHPx were measured in the remaining myocardial tissue obtained from control and HF dogs [12–14].

2.7. Statistical analysis

Data are expressed as means±standard error of the mean (S.E.M.). An unpaired Student’s \(t\) test was used to compare values between control and HF. An ANOVA with repeated measures was used to compare the time-dependent changes of cell length during the exposure to \(\cdot\)OH between control and HF. Differences were considered statistically significant at \(P<0.05\).

3. Results

3.1. LV contractile function

Chronic rapid pacing caused a significant increase in LV end-diastolic dimension, LV end-systolic dimension, and a decrease in LV ejection fraction (Table 1). For the HF dogs, LV peak positive \(dP/dt\) was depressed and LV end-diastolic pressure was increased compared to control values.

3.2. \(\cdot\)OH generating system

With \(\text{H}_2\text{O}_2\) (10 mmol/l) or \(\text{Fe}^{3+}\)-NTA (20 \(\mu\)mol/l) alone, no significant DMPO-OH signals were detected. On mixing \(\text{H}_2\text{O}_2\) and \(\text{Fe}^{3+}\)-NTA, a prominent 1:2:2:1 quartet signal was observed characteristic for DMPO-OH (Fig. 1). Both mannitol (100 mmol/l) and catalase (50 U/l) completely prevented the formation of these signals (Fig. 1), but SOD (5 U/l) had no such effects, indicating that \(\cdot\)OH was indeed generated. This was further confirmed by the finding that the iron chelator, desferrioxamine (0.5 mmol/l), significantly attenuated the spin signals (Fig. 1). In order to determine whether myocytes could not quench \(\cdot\)OH, the magnitude of DMPO-OH signal was measured in the presence of myocytes (10\(^5\) cells/ml) in the reaction mixture. Even in the presence of normal or HF cells, a DMPO-OH signal was again observed (Fig. 1). The magnitude of DMPO-OH ESR signals in the presence of

<table>
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<th>Characteristic of rapid pacing-induced heart failure model*</th>
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<tr>
<td>Control</td>
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<tr>
<td>Body weight (kg)</td>
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<tr>
<td>LV end-diastolic dimension (mm)</td>
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<td>LV end-systolic dimension (mm)</td>
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<td>LV ejection fraction (%)</td>
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<td>LV peak +dP/dt (mmHg/sec)</td>
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<td>LV end-diastolic pressure (mmHg)</td>
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* Values are given by the means±S.E.M. Statistical comparisons are by unpaired \(t\) test. HF, heart failure; LV, left ventricular.

**, \(P<0.01\) for difference from control.

Fig. 1. ESR spectra recorded in the presence of 80 mmol/l DMPO of A: \(\text{H}_2\text{O}_2\) (10 mmol/l) and \(\text{Fe}^{3+}\)-NTA (20 \(\mu\)mol/l); B: \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA in the presence of mannitol (100 mmol/l); C: \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA in the presence of catalase (5 mmol/l); D: \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA in the presence of desferrioxamine (0.5 mmol/l); E: \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA in the presence of control myocytes (10\(^5\) cells/ml); F: \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA in the presence of HF myocytes (10\(^5\) cells/ml). A prominent 1:2:2:1 quartet signal of DMPO-OH was observed with \(\text{H}_2\text{O}_2\)+\(\text{Fe}^{3+}\)-NTA.
myocytes was slightly lower than that in the absence of cells, which, however, did not reach statistical significance. Importantly, the degree of cell-induced quenching was comparable between control and HF (Table 2). Further, the hyperfine coupling constants of DMPO-OH signals (aH and aN) were not altered even in the presence of the cells and were also identical between control and HF (Table 2).

3.3. \(^{\text{OH}}\)-mediated myocyte hypercontracture

The average yield of rod-shaped cells was comparable between control and HF (Table 3). Virtually almost all rod-shaped cells excluded trypan blue, regardless of whether the cells were obtained from control or HF. In addition, there were no significant differences in the ratio of responded cells by the electrical field stimulation to all rods. Thus HF myocytes exerted similar viability as normal control cells at baseline conditions. Longitudinal cell length from the HF hearts were significantly longer than that from control hearts (Table 3).

Both control and HF myocytes maintained rod-shaped morphology for more than 30 min when superfused with normal buffer. When normal myocytes were superfused with H\(_2\)O\(_2\) and Fe\(^{3+}\)-NTA, which induced the rapid generation of \(^{\text{OH}}\) as described above (Fig. 1), rest cell length decreased gradually (Fig. 2). The degree of hypercontracture response was dependent on the increasing concentrations of H\(_2\)O\(_2\) (3–10 mmol/l) and therefore a concentration of 10 mmol/l H\(_2\)O\(_2\) was used in all subsequent experiments. The initial decrease of cell length was recognized 10 min after exposure and thereafter cells shortened further to a square hypercontracture state after 20–30 min. Cell remained in this hypercontracture state during the duration of experiment (30 min) and did not recover to the normal rod shape even after the washout. In the presence of mannitol (100 mmol/l) or catalase (50 U/ml), hypercontracture was completely inhibited (Fig. 2), confirming that \(^{\text{OH}}\) was indeed responsible for this injury. Mannitol or catalase itself did not affect the morphology of cells. When the time course of \(^{\text{OH}}\)-mediated hypercontracture was compared between control and HF, HF cells exerted a shortened survival time from rod shape into hypercontracture (Fig. 3A). The time to the onset of hypercontracture and that to the submaximal hypercontracture were significantly shortened in HF (Fig. 3B and 3C).

### 3.4. Antioxidant enzyme activities

There was no significant difference between control and HF in myocardial SOD and catalase activity (Fig. 4). In contrast, myocardial GSHPx activity was slightly, but significantly, increased in HF (87.1 ± 5.7 vs. 103.0 ± 5.6 nmol/mg protein; \(P < 0.05\)).

### 4. Discussion

The present study demonstrated that HF myocytes were more susceptible to exogenous oxidant stress than normal cells. Importantly, the greater susceptibility of HF myocytes to oxygen radical-mediated injury is attributable to their intrinsic properties, but is not to diminished antioxidant capacity.

We employed a freshly isolated myocyte model to study ROS-mediated cardiac injury as it allows us to investigate their direct toxic effects on cell viability independent of systemic hemodynamics and coronary circulation. Additionally, it assures that the measured effects of ROS are intrinsic to the myocyte itself and are not due to the effects on other types of cells such as endothelial, smooth muscle, and interstitial cells within an intact heart. This cellular injury resulted from a combination of H\(_2\)O\(_2\) and the more reactive \(^{\text{OH}}\) generated via the Fenton reaction, which was confirmed by ESR studies [6,9]. In addition, \(^{\text{OH}}\) can be generated endogenously via ONOO\(^{–}\). Isolated cardiac myocytes exposed to \(^{\text{OH}}\) showed a time-dependent rigor

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**Table 2**

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<tr>
<th>Characteristics of DMPO-OH signals in the presence and absence of isolated cardiac myocytes(^{a})</th>
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<tr>
<td>Control ((n=5))</td>
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<td>(–)</td>
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<td>Signal amplitude (% of (–))</td>
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<td>Hyperfine coupling constants (G)</td>
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\(^{a}\) Each data were consisted of the preparation performed. (–), the absence of myocytes; (+), the presence of myocytes.
shortening of cell length and eventually an irreversible hypercontracture. Myocyte hypercontracture might induce cytolysis in the myocardial tissue because the forces causing cellular rigor could lead to a mutual disruption of sarcolemma of the adjacent cells.

There are several possible mechanisms responsible for the greater susceptibility of HF myocytes to oxidative stress. First possibility is the decrease of ROS scavenging capacity and the resultant increase of oxyradicals within HF cells. If antioxidant capacity is reduced in HF, it would be well expected that the net amount of ROS would become excess when myocytes are exposed to exogenous ROS [2]. However, this possibility is unlikely since 'OH was not quenched in the presence of intact myocytes and, more importantly, the 'OH measured in the presence of equal amount of myocytes did not differ between control and HF (Fig. 1). Further, the activities of myocardial antioxidant enzymes were not reduced in HF (Fig. 4). This is in contrast with the finding of several animal studies [2], but is consistent with a recent study demonstrating no decrease in the gene expression, protein levels and activities of antioxidant enzymes in the end-stage failing human hearts [15]. Another possible mechanism is the greater membrane damage of HF myocytes during the cell isolation procedures. However, this possibility is also unlikely given that there were no significant differences in the cell viability indexes of myocyte preparations between control and HF (Table 3). Furthermore, in accordance with the previous studies [16], trypan blue was excluded even at the state of hypercontracture, indicating that the integrity of cellular membrane was maintained throughout the course of studies. Thus, the distinctive consequences of 'OH on cell viability appear to reflect the intrinsic abnormalities of HF myocytes. The rapid development of hypercontracture in HF might be due to intracellular ATP deficiency. Exposure of myocytes to ROS has been shown to lead to the depletion of ATP [17,18] and the occurrence of ROS-induced myocyte hypercontracture correlated with the decrease of ATP [6]. Therefore, it is possible that myocardial ATP content depletes more rapidly in HF than
exclude the contribution of specific alterations in the function of Ca^{2+} regulatory proteins in the ROS-induced myocyte hypercontracture. These mechanisms include enhanced SR Ca^{2+} release, enhanced Na^{+}–Ca^{2+} exchange, and Ca^{2+} influx from voltage dependent Ca^{2+} channels [19,20]. Thus further studies to examine the effects of ROS on Ca^{2+} transients, especially the rate of relaxation, using the myocytes under electrical field stimulation are warranted to delineate the role of Ca^{2+} homeostasis in the development of ROS-mediated cellular Ca^{2+} overload and hypercontracture. Further, the studies to examine the effects of the drugs which possibly interfere with Ca^{2+} homeostasis such as inhibitors of SR Ca^{2+}-ATPase, Na^{+}/H^{+}, or Na^{+}/Ca^{2+} exchangers on ROS-mediated myocyte injury are also warranted.

\OH is predominant ROS that can cause the oxidation of membrane phospholipids, proteins, and DNAs. Thus \OH is harmful against maintaining the functional integrity of the biological tissue. Further, our recent studies have demonstrated the production of \OH is increased in the failing myocardium [8]. Taken together, greater susceptibility of failing cardiac myocytes to \OH may promote myocyte shape change and have significant impact on the LV contractile function in HF. Previous studies demonstrated enhanced production of nitric oxide (NO) in the failing heart [21,22]. \O_2 and NO can react to the powerful prooxidant, ONOO\^−, which can form \OH [7]. Therefore, ONOO\^− might be also involved in the development of HF.

It remains speculative whether the concentrations of \OH used in this in vitro study are comparable to those occurring in vivo situations especially because of high concentrations of H_2O_2 in the present study (10 mmol/l). However, these concentrations were chosen based on the similar previous studies examining the effects of \OH on the shape of isolated myocytes [6]. Further, the amount of \OH produced in this generating system, the combination of H_2O_2 and Fe^{3+}-NTA, has been shown to be roughly comparable to that produced within the intact heart during ischemia and reperfusion [1,23,24]. Therefore, our in vitro studies appear to be applicable to the ability of failing myocytes to withstand the acute challenge of ischemia-reperfusion. However, caution should be necessary when extrapolating these results to the failing heart because the degree of oxidant stress that is involved in HF is probably much lower than the range that might be expected with ischemia-reperfusion and the rapid nature of the hypercontracture response during exposure to \OH might not be consistent with the chronic time course of myocardial dysfunction seen in HF.

The present study provides the first demonstration of increased susceptibility of failing myocytes to oxidative stress. It is possible that an endogenous generation of H_2O_2 and \OH, occurring on the reperfusion of ischemic heart [1] and also in failing heart [3,8], could similarly induce myocyte injury, which may contribute to further progression of HF.
Acknowledgements

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