Determination of Oxidative Stress and Cardiac Dysfunction after Ischemia/Reperfusion Injury in Isolated Rat Hearts

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Background: Oxidative stress due to reactive oxygen species (ROS) is thought to play a considerable role in ischemia/reperfusion (I/R) injury that impairs cardiac function. The present study examined oxidative damage in I/R injury and investigated the correlation between oxidative stress and impaired cardiac function after I/R injury of the isolated rat heart.

Methods: Hearts isolated from male Sprague-Dawley rats were mounted on a Langendorff apparatus. Hearts arrested using St. Thomas cardioplegic solution and then they were reperfused. The hearts were divided into three groups depending on the frequency (0-2) of I/R. After I/R, left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), positive maximum left ventricular developing pressure (max LV dP/dt) and coronary flow (CF) were measured. Creatine kinase (CK) was measured in the coronary effluent and 8-hydroxy-2’deoxyguanosine (8OHdG), a marker of oxidative DNA damage, was measured. Adenosine triphosphate (ATP) was measured from frozen myocardial tissue after experiment.

Results: We immunohistochemically demonstrated and quantified levels of 8-OHdG after I/R injury of the heart. The frequency of I/R injury and cardiac dysfunction significantly and negatively correlated. The ATP products were similar among the three groups. The incidence of ventricular arrhythmias was not by affected oxidative stress.

Conclusion: The frequency of I/R injury had more of an effect on 8-OHdG products and on impaired cardiac function with less myocyte damage than ischemic duration within 30 minutes of ischemia.

Keywords: oxidative stress, ischemia/reperfusion injury, 8-hydroxy-2’deoxyguanosine (8OHdG), cardiac dysfunction

Introduction

Reperfusion of ischemic areas after coronary interven-

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Hydroxyl or singlet oxygen radicals are responsible for hydroxylation at the C-8 position of 2'-deoxyguanosine to produce 8-hydroxy-2'-deoxyguanosine (8OHdG), which is a reliable marker of oxidative DNA damage.4-6) Nagayoshi et al. found that levels of urinary 8-OHdG were increased after reperfusion in acute myocardial infarction.7) Thus, 8OHdG is considered a reliable biomarker of oxidative damage due to I/R injury of the heart.

The aim of this study was to determine oxidative DNA damage in I/R injury of the heart and investigate the correlation between oxidative stress and impaired cardiac function in I/R injury.

Materials and Methods

The animals used in this study were handled in accordance with the Guidelines for Animal Experimentation at the University of the Ryukyus, and the Animal Care committee of this institution approved this experimental protocol. None of the rats had any clinically apparent infection.

Male Sprague-Dawley rats (SLC, Shizuoka, Japan) weighing from 300 to 430 mg (9–10 weeks old) were intraperitoneally anesthetized with pentobarbital (50 mg/kg) and heparinized with intravenous heparin sodium 1000 IU/kg. The hearts were rapidly excised, mounted onto the Langendorff perfusion system with ascending aortic cannulation, and then perfused in a retrograde fashion with normothermic (37°C) Krebs-Henseleit Solution (KHS: NaCl 120 mmol/L, KCL 4.8 mmol/L, MgSO4 1.2 mmol/L, CaCl2 1.25 mmol/L, KH2PO4 1.2 mmol/L, Glucose 11 mmol/L, NaHCO3 25 mmol/L) at a constant pressure of 100 cmH2O, and oxygenated with 95% O2 and 5% CO2 (partial pressure of O2 >600 mmHg). A thin-wall latex balloon was inserted into the left ventricle (LV) through the left atrium to monitor LV pressure (LVP). The balloon was filled with bubble-free saline and adjusted to an LV end-diastolic pressure (LVEDP) of 5–10 mmHg during initial equilibration. Balloon volume was maintained throughout the experiments. The LVP and LVEDP (mmHg) were measured using a pressure transducer (TP-2000, Nihon Kohden, Tokyo, Japan) attached to the aortic cannula, which was connected to an electromagnetic flow meter (MFV-3200, Nihon Kohden, Tokyo, Japan). Heart rate (HR) was counted using a cardiotachometer (AT-600G, Nihon Kohden, Tokyo, Japan) triggered by a pressure pulse. All hemodynamic parameters were continuously recorded using an 8-channel thermal-pen recorder (WT-685G, Nihon Kohden, Tokyo, Japan).

Experimental protocol

All hearts were perfused for 20 min to stabilize hemodynamics, and then baseline values of cardiac parameters were measured.

After initial equilibration of 20-min perfusion, the heart was arrested by injecting the aorta with 20 ml/kg, of St. Thomas cardioplegic solution at 4°C, and then placed in 30 ml of KHS at 37°C to prevent hypothermia-induced cardioprotection. The KHS in the chamber was then drained, and the heart was reperfused with aerobic KHS (partial pressure of O2 >600 mmHg) for 30 min at 37°C.

The hearts were divided into three groups (Fig. 1) depending on the frequency of I/R (total arrest time was 30 min and subsequent reperfusion proceeded for 30 min in each I/R group; total duration of I/R injury was thus 60 min): Non I/R underwent only 60 min perfusion (I/R-0 group, n = 8), or one arrest for 30 min with 30 min of reperfusion (I/R-1 group, n = 8), or two arrests for 15 min each followed by reperfusion for 15 min (I/R-2 group, n = 8). After 60 min of I/R injury, the hearts were post-perfused for a further 30 min. The temperature of all hearts was maintained at 37°C at all times of these experiments. During the perfusion period, the KHS was maintained at 37°C in tube glass surrounded hot water, and the hearts were immersed in 37°C KHS during the ischemic period. The coronary effluent was collected before (baseline, 20 min) and at 80, 90, 100 and 110 min after the experiment and stored -80°C for of creatine kinase (CK) measurement. At the end of the experiments, two hearts from each group were stored in Bouin’s solution for immunohistochemical detection of 8-OHdG and six others were frozen to quantify 8-OHdG and adenosine triphosphate (ATP). Values for LVDP, LVEDP, max LV dP/dt and CF were determined at baseline (20 min) and at 80, 90, 100 and 110 min.

Immunohistochemical detection of 8-OHdG

Hearts were immunohistochemically stained using the avidin-biotin-peroxidase complex (ABC) method.8) Hearts in Bouin’s solution were immersed in 50% and 70% ethanol to remove picric acid, dehydrated,
embedded in paraffin and cut into 3.5-μm sections for mounting on glass slides coated with poly-L-lysine. The specimens were deparaffinized, and then primary anti-8-OHdG monoclonal antibody (N45.1: Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) was added, and the slides were incubated overnight at 4°C. Slides were immersed in biotin-labeled rabbit anti-mouse IgG (Dako, Kyoto, Japan) second antibody overnight, followed by the avidin-biotin complex (Dako).9,10) Immunohistochemical findings were quantified using the 8-OHdG index using Image J version 1.42 freeware (National Institution of Mental Health, Bethesda, MD, USA) and Adobe Photoshop version 8.0.1 (Adobe Systems, San Jose, CA, USA) as follows:10)

\[
\text{8-OHdG index} = \frac{\sum \text{[8-OHdG staining area (μm}^2\text{)]}}{\text{total cell number}}.
\]

Color slides (35 mm) of four specific locations (anterior wall, lateral wall, inferior wall and septum of the LV) in each specimen were prepared. Color images were obtained as TIFF files and opened in gray scale using Image J. The total cell number was determined after setting an appropriate threshold for nuclei, and then the 8-OHdG staining area was calculated by resetting an appropriate threshold for nuclei expressing 8-OHdG. Total nuclei numbers and 8-OHdG staining area were calculated from all four specific sections in each group.

**Measurement of 8-OHdG levels from extracted DNA in rat heart**

We extracted from rat hearts using the DNA Extractor TIS Kit (Wako Pure Chemical, Osaka, Japan). Hearts were homogenized in 3 ml of cold lysis buffer, and then the homogenate was centrifuged at 600 × g at 4°C for 15 min to precipitate the nuclear fraction. Nuclear pellets were washed twice with lysis buffer then suspended in 300 μL of enzyme reaction solution, 1 μL of RNase and 3 μL of oxidation inhibitor. The mixture was incubated at 37°C for 10 min, and then 15 μL of protein digestion solution was added. After 60-min incubation at 37°C, the mixture was centrifuged at 10,000 × g for 5 min at room temperature and then the supernatant was mixed with 300 μL of sodium iodide and 600 μL of alcohol in a microcentrifuge tube and centrifuged at 10,000 × g for 10 min at room temperature. The pellet was resuspended in 1 ml of 70% ethanol and centrifuged at 10,000 × g for 5 min at room temperature. The concentration of DNA precipitated in DDW buffer was calculated from the absorbance at an OD of 260 nm.11,12)

We determined 8-OHdG using a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG...
Check; Japan Institute for the Control of Aging, Fukuroi, Japan). The DNA was mixed with 8-OHdG monoclonal antibody, incubated at 4°C overnight and rinsed with 250 μL of washing solution. Enzyme-labeled secondary antibody (100 μL) was incubated with the DNA at room temperature for 60 min and rinsed with 250 μL of washing solution, followed by a 15-min incubation at room temperature with 100 μL of reconstitution enzyme substrate. The reaction was stopped by adding 100 μL of reaction termination solution and then absorbance was measured at 450 nm.\textsuperscript{13)

**Determination of myocardial energy metabolites**

Frozen myocardial tissues were lyophilized for 6 h and homogenized with 0.6 M/L perchloric acid. The homogenate was centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was assayed. Adenosine triphosphate (ATP) was determined using firefly luminescence with an ATP monitoring agent (LL-100-2; Toyo Ink, Tokyo, Japan) and a lumiphotometer (Minilumat LB9506; Berthold GmbH and Co KG, Galmabacher, Germany). Values for ATP are expressed in μmol/L.

**Determination of cardiac enzymes in the coronary effluent**

The extent of cardiac enzyme release was measured in coronary effluent samples. CK was measured using a JCA-BM8060 Auto analyzer (JEOL, Tokyo, Japan). Enzyme activity is expressed as international units per liter (IU/L).

**Statistical analysis**

Data were statistically analyzed using SPSS statistical software (version 17.0; SPSS Inc., Cary, NC). The distribution normality of all variables was tested using the Shapiro-Wilks normality test. All values are presented as means ± SEM. Comparisons among three groups at multi time points were analyzed by the one-way analysis of variance as appropriate, followed by paired or unpaired Student \( t \) tests with Tukey and Games-Howell multiple comparison tests. Differences with \( p < 0.05 \) were considered statistically significant. Variances between predictor variables and cardiac dysfunction were determined by multiple regression. We used final LVDP as an outcome variable, and frequency of I/R injury, global ischemic time, amount of 8-OHdG, LVEDP and CF as predictor variables. The \( p \) value from the \( t \) test for the regression slope of predictor variables was used to determine the probability of the analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>I/R-0 (n = 8)</th>
<th>I/R-1 (n = 8)</th>
<th>I/R-2 (n = 8)</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, g</td>
<td>1.34 ± 0.04</td>
<td>1.33 ± 0.04</td>
<td>1.39 ± 0.03</td>
<td>n.s</td>
</tr>
<tr>
<td>Age, week</td>
<td>9-10</td>
<td>9-10</td>
<td>9-10</td>
<td>n.s</td>
</tr>
<tr>
<td>HR (baseline), bpm</td>
<td>264.3 ± 10.6</td>
<td>293.8 ± 11.7</td>
<td>280 ± 13.9</td>
<td>n.s</td>
</tr>
<tr>
<td>LVEDP (baseline), mmHg</td>
<td>5.9 ± 1.5</td>
<td>6.5 ± 1.7</td>
<td>6.0 ± 1.3</td>
<td>n.s</td>
</tr>
<tr>
<td>LVDP (baseline), mmHg</td>
<td>89.7 ± 5.2</td>
<td>84.1 ± 4.9</td>
<td>84.2 ± 4.1</td>
<td>n.s</td>
</tr>
<tr>
<td>max dP/dt (baseline), mmHg/s</td>
<td>2970.0 ± 115.6</td>
<td>2904.7 ± 152.1</td>
<td>3066 ± 122.0</td>
<td>n.s</td>
</tr>
<tr>
<td>Coronary flow, ml/min</td>
<td>14.4 ± 0.5</td>
<td>14.8 ± 0.3</td>
<td>15.9 ± 0.7</td>
<td>n.s</td>
</tr>
<tr>
<td>LDH (baseline), IU/L</td>
<td>1.75 ± 0.42</td>
<td>2.0 ± 0.37</td>
<td>1.13 ± 0.35</td>
<td>n.s</td>
</tr>
<tr>
<td>CK (baseline), IU/L</td>
<td>2.25 ± 0.62</td>
<td>2.00 ± 0.68</td>
<td>2.50 ± 0.62</td>
<td>n.s</td>
</tr>
</tbody>
</table>

**Results**

**Table 1** shows no significant differences in heart weight, baseline (at 20 min) LV parameters (HR, LVEDP, LVDP and max LV dP/dt), CF and cardiac enzymes (CK) in coronary effluent among the three groups.

**LV functions**

Figure 2a–2c shows changes in the time courses of LVDP, max LV dP/dt and LVEDP in all three groups. Both LVDP and max LV dP/dt that immediately deteriorated to almost null in all I/R groups during the period of arrest (data was not shown), rapidly returned to baseline or above at the start of the post I/R period (at 80 min), and then gradually deteriorated. During post I/R for 30 min, LVDP and max LV dP/dt were lower in I/R-2 hearts than I/R-1 hearts. The LVDP was 62.4 ± 3.3 mmHg in I/R-2 hearts versus 90.2 ± 3.8 mmHg in I/R-1 hearts at 110 min (\( p < 0.05 \), Fig. 2a). Max LV dP/dt values in I/R-2 and in I/R-1 hearts at 110 min were 2660.0 ± 128.4 and 3247.1 ± 150.1 mmHg/s, respectively (\( p < 0.05 \), Fig. 2b). The LVEDP value was elevated more in I/R-1, than in I/R-0 and I/R-2 hearts post-I/R injury (Fig. 2c). At 110 min, LVEDP was significantly higher in I/R-1 than in I/R-0 hearts (19.6 ± 5.4 vs. 2.5 ± 0.5 mmHg; \( p < 0.05 \)). During
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the post I/R period, non-sustained ventricular tachycardia (VT) or ventricular fibrillation (VF) developed in 7 (87.5\%) of 8 hearts in the I/R-1 group, but in neither of the I/R-0 and I/R-2 groups.

Coronary flow

Figure 2d shows CF (ml/min) in three groups. CF was significantly decreased both in I/R-1 (8.4) and I/R-2 (9.1) hearts compared with I/R-0 hearts (11.6) at the end of procedure, whereas I/R-1 and I/R-2 hearts did not significantly differ \((p = 0.87)\).

Immunohistochemical detection of 8-OHdG

Immunohistochemical findings revealed significantly more 8-OHdG (brown) in nuclei from I/R-injured rat hearts than in nuclei (blue) from hearts that had not received I/R injury (Fig. 3a–3c). Furthermore, the 8OHdG index significantly increased depending on the frequency of I/R injury (Fig. 4).

Measurement of 8-OHdG

The amounts of 8OHdG in I/R-0, I/R-1 and I/R-2 were 1.69 ± 0.08, 1.99 ± 0.11 and 2.16 ± 0.12 pg/\(\mu\)g DNA, respectively. The value was significantly higher in I/R-2 than in I/R-0 hearts \((p < 0.05)\), whereas I/R-1 and I/R-2 hearts did not significantly differ \((p = 0.974, \text{Fig. 5})\).

Measurement of ATP

The ATP products were similar among the three groups (I/R-0, 3.84 ± 0.34; I/R-1, 3.42 ± 0.53; I/R-2, 3.58 ± 0.50)
μmol/L; Fig. 6a). The ATP products did not significantly differ among the three groups.

**Release of cardiac enzyme**

Level of CK was higher in I/R-1 than in I/R-0 and I/R-2 hearts at any time after I/R injury, but no significant differences were observed among the three groups at any time point (Fig. 6b).

**Discussion**

We immunohistochemically detected significantly more 8-OHdG, a marker of oxidative DNA damage, in myocyte and endothelial cell nuclei from rat hearts treated with, than that without I/R injury. Significantly more 8-OHdG was produced in I/R-2 than in I/R-0 hearts (p < 0.05). It
means the presence of oxidative DNA damage in I/R injury of hearts. Immunohistochemical and quantitative detection of the 8-OHdG from I/R injured rat hearts confirmed that oxidative DNA damage increased depending upon the frequency of I/R injury rather than the duration of arrest (global ischemic), when the duration of total arrest and reperfusion were both 30 min. Impaired cardiac function, suggested by final LVDP and max LV dP/dt depression, also depended on the frequency of I/R injury rather than arrest time. A close negative correlation with a correlation coefficient of $-0.816$ was found between LVEDP at 110 min and the frequency of I/R injury ($p < 0.05$) and also between max LV dP/dt at 110 min and frequency of I/R injury (-0.425, $p < 0.05$). However, the amount of 8-OHdG did not correlate with either LVDP (-0.192, $p = 0.26$) or max LV dP/dt (-0.091, $p = 0.351$). In terms of myocyte damage from oxidative stress, ATP products and CPK elevation did not significantly differ among three groups whereas significantly more 8-OHdG was produced in I/R-2 than I/R-0 hearts. It suggested that oxidative stress did not affect myocyte damage within 30 min ischemia. Coronary flow was significantly decreased more in both I/R-1 and I/R-2 hearts than in I/R-0 hearts, but we could not confirm a significant correlation between I/R-1 and I/R-2 hearts. Coronary flow significantly correlated with LVEDP at 110 min (correlation coefficient, 0.407; $p < 0.05$).

Reperfusion-induced arrhythmias such as VT and VF developed only in I/R-1 hearts, and seemed to correlate with ischemic time and elevated LVEDP rather than frequency of I/R injury.

Myocardial dysfunction (stunning) can develop after reperfusion of a global ischemic myocardium during cardiac surgery or in the setting of regional ischemia and reperfusion from PCI, thrombolysis, unstable angina and stress- or exercise-induced angina. Stunning is an important causative factor in the development of ischemic cardiomyopathy, where repeated episodes of myocardial ischemia and reperfusion might lead to the development of heart failure.\textsuperscript{14,15)} Reperfusion of the ischemic heart might result in ROS generation\textsuperscript{2)} and could be associated with myocardial “stunning” after reversible I/R injury.\textsuperscript{16,17)} Mitochondrial DNA damage is induced by ROS, which leads to the generation of more ROS,\textsuperscript{18)} and perhaps a burst of ROS production. Therefore, the large amount of ROS produced in myocytes after I/R injury might be mediated in part by myocardial dysfunction (stunning).

The most reliable biomarker of oxidative DNA damage and repair in aging,\textsuperscript{4,5)} carcinogenesis,\textsuperscript{19)} and cardiovascular\textsuperscript{20–22)} and cerebrovascular\textsuperscript{23)} diseases is 8-OHdG. Here, we immunohistochemically demonstrated the presence of 8-OHdG in myocytes at all times tested in the I/R-injured rat hearts, and quantitative immunohistochemical measurement showed that levels were significantly increased in hearts with treated I/R injury. These findings suggested
that oxidative DNA damage occurs in rat hearts with any type of I/R injury (Fig. 1). To evaluate the correlation between oxidative stress and duration of global ischemic or frequency of I/R injury, we measured 8-OHdG in extracted DNA using ELISA. Many sensitive methods for the detection of 8-OHdG have been established, and electrochemical detector (ECD)-equipped high-performance liquid chromatography (HPLC) in particular has become the most popular, despite requiring an expensive apparatus and considerable sample preparation. It is suggested that assays using antibodies and ELISAs would be useful for accurately measuring 8-OHdG in biological materials. Evans et al. demonstrated a close correlation between 8-oxodG measured by HPLC-EC and ELISA. Thus, we used an ELISA to measure the absolute amount of 8-OHdG in DNA extracted from the rat heart, and found that it increased dependently upon the frequency of I/R injury. However, the frequency of I/R injury and cardiac dysfunction significantly correlated, whereas the amount of 8-OHdG did not.

It seemed that the high CK level was affected by LVEDP, but no significant correlation was found between the CK level and LVEDP. Oxidative stress did not appear to influence the release of cardiac CK enzymes or ATP products according to the amount of myocyte damage, and the amounts of ATP products were similar in all three groups. It may mean that oxidative stress affected reversible cardiac dysfunction after I/R injury within 30 min ischemia.

Coronary flow provided useful indirect information about flow through the microvasculature. Coronary microvascular dysfunction after coronary recanalization reduces coronary flow reserve, which seems to revert spontaneously. Higashi et al. reported that excessive oxidative stress is involved, at least in part, in impaired endothelium-dependent vasodilation in patients with renovascular hypertension. Nitric oxide (NO) is thought to be an endothelium derived vasodilator that plays a complementary role in coronary vasoregulation. Nitrate and nitrite (NOx) are delivered into the coronary circulation as bioactive NO to preserve endothelial function. We previously confirmed that the NOx level in the coronary effluent of isolated rat heart clearly decreases after I/R injury. Our results suggested that impaired cardiac function after I/R injury was mainly affected by microvascular dysfunction, which decreased CF. However, we could not identify a significant correlation between CF and cardiac dysfunction.

Reperfusion of the heart after a period of ischemic injury might lead to potentially lethal arrhythmias. The most important causes of sudden death following spontaneous restoration of antegrade flow remain VT and VF. Oxygen-derived free radicals might play a key role in the genesis of ventricular arrhythmias. However, we did not identify a significant correlation between oxidative stress and the development of reperfusion-induced arrhythmia.

The present study found the Oxidative stress in the rat hearts treated with I/R injury, by expression of 8-OHdG either immunohistochemically or quantitatively, as a marker of Oxidative DNA damage. Oxidative cardiac dysfunction caused by I/R injury is potentially reversible with less myocyte damage within 30 min ischemia. We also found that the frequency of I/R injury was affected more by 8-OHdG products and impairments of cardiac function than ischemic duration when the total durations of arrest and reperfusion were equal, within 30 minutes of cardiac arrest. Cardiac dysfunction also seemed to be affected by microvascular dysfunction resulting in decreasing CF, but we could not demonstrate a correlation between the frequency of I/R injury and CF. There was no correlation between oxidative stress and reperfusion induced arrhythmia in this study. In the clinical aspects, our study might be use of considering antioxidative agents for cardiac I/R injury.

Our conclusion is that 8-OHdG, as a marker of oxidative DNA damage, was demonstrated in I/R injury of rat heart and the frequency of I/R injury had more of an effect on 8-OHdG products and on impaired cardiac function than ischemic duration, within 30 minutes of ischemia. However, more study groups with different ischemic and reperfusion times are needed to confirm our conclusions more clearly.

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Disclosure Statement

There is no disclosure statement.
References