Quantification of Hydroxyl Radical and Its Lack of Relevance to Myocardial Injury During Early Reperfusion After Graded Ischemia in Rat Hearts

Genzou Takemura, Tomoya Onodera, and Muhammad Ashraf

To elucidate the pathophysiologic role of the hydroxyl radical (·OH) during the postischemic reperfusion of the heart, we measured the ·OH product in the coronary effluent from isolated perfused rat heart during a 30-minute reperfusion period after various ischemic intervals of 5, 10, 15, 20, 30, and 60 minutes. Salicylic acid was used as the probe for ·OH, and its derivative, 2,5-dihydroxybenzoic acid (2,5-DHBA), was quantified using high-performance liquid chromatography with ultraviolet detection. 2,5-DHBA was negligible in the effluent from nonischemic hearts, but a significant amount was detected from the hearts rendered ischemic for 10 minutes or longer. The peak of 2,5-DHBA was seen within 90 seconds after the onset of reperfusion in every group. The accumulated amount of 2,5-DHBA was maximal in the group with 15-minute ischemia (6.73 ± 1.04 nmol/g wet heart wt after 30 minutes of reperfusion); it decreased as the ischemic time was prolonged and was 2.38 ± 0.84 nmol/g wet wt after 30 minutes of reperfusion in the group with 60-minute ischemia. In the model of 15-minute ischemia/30-minute reperfusion, there was no correlation between the accumulated amount of 2,5-DHBA and functional recovery (±dP/dt, heart rate, and coronary flow), lactate dehydrogenase release, and morphological damage. Although treatment with 0.5 mM deferoxamine, an iron chelator, significantly decreased 2,5-DHBA (from 6.73 ± 1.04 to 2.29 ± 0.80 nmol/g wet wt after 30 minutes of reperfusion, p < 0.01), it failed to reduce the postischemic myocardial injury in the group with 15-minute ischemia. The results suggest that ·OH production is influenced by the preceding ischemic interval and that ·OH does not exert an immediate direct effect on postischemic damage during early reperfusion in the isolated perfused rat heart, although a possibility remains that the small portion of ·OH trapped by salicylic acid may not be intimately associated with myocardial injury.

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KEY WORDS • ischemia • reperfusion • hydroxyl radical • salicylic acid

Production of oxygen-derived radicals and metabolites has been linked to the deleterious effects of reperfusion on the ischemic heart.1–4 It has become possible to detect free radicals in the postischemic reperfused heart using electron paramagnetic resonance spectroscopy (EPR). Recent studies using EPR have revealed that the peak of the production of radicals appears in the early phase of reperfusion.5–9 EPR is undoubtedly a powerful tool in studying the relation between free radicals and the evolution of ischemia/reperfusion injury. However, EPR spin trapping has its limitations: the spin adducts of the commonly used spin trap 5,5-dimethyl-1-pyrroline N-oxide are metabolized and unstable in vivo;10,11; high concentrations (>20 mM) of the spin trap α-phenyl N-tert-butyl nitrene (PBN) are toxic in vivo preparations;8 and their sensitivity is relatively low.12 Recently, however, to avoid toxicity, Mergner et al13 introduced a high concentration of PBN (60 mM) to the venous effluent rather than directly to the ischemic reperfused heart to measure radicals. In spite of these shortcomings, attempts to quantify radicals by applying direct EPR or EPR spin trapping have been made.5,8 Although the toxicity of the free radicals to the heart has been reported, most of these previous studies were performed without determining the actual dose–effect relation.14–18 Therefore, the role of free radicals in cellular injury remains controversial.

Among the oxygen-derived free radicals, the hydroxyl radical (·OH) is highly reactive and may play a critical role in postischemic myocardial damage during reperfusion.19,20 Previously, we have demonstrated that ·OH can be measured in the reperfused heart after 30 minutes of ischemia by using high-performance liquid chromatography (HPLC) of hydroxylation products of salicylic acid used as a trapping agent.21 Since this method is principally the same as that of Floyd and colleagues22,23 but is modified by using conventional ultraviolet instead of electrochemical detection, it can detect the stable derivatives of salicylic acid as indicators of highly reactive ·OH in a comparatively stable fashion.

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In the present study, we used this method to determine 1) the amount of \( \cdot \text{OH} \) produced in the reperfused heart after various durations of ischemia and 2) the relation between \( \cdot \text{OH} \) production and cardiac function, metabolites released, and morphology. In addition, we also used an iron chelator, deferoxamine (DFX), as an inhibitor of \( \cdot \text{OH} \) production to elucidate the mechanism of \( \cdot \text{OH} \) production and its significance in the pathogenesis of postischemic injury during reperfusion.

Materials and Methods

Heart Preparation

Male Sprague-Dawley rats weighing 200–250 g were anesthetized by intraperitoneal injection of 30 mg/kg pentobarbital. After intraperitoneal injection of 500 USP units/kg heparin sodium, hearts were removed and retrogradely perfused through the aorta in a noncircuiting Langendorff apparatus with Krebs-Henseleit buffer as previously described.\(^2\) The buffer was saturated with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \) (pH 7.4, 37°C) for 1 hour. The millimolar composition of the perfusion buffer was NaCl 118, KCl 4.7, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, CaCl\(_2\) 2.5, NaHCO\(_3\) 25, and glucose 5.5. To minimize the effect of iron contamination on the production of \( \cdot \text{OH} \), the buffer was treated with Chelex resin-100 (Bio-Rad Laboratories, Richmond, Calif.) as previously described.\(^2\) MgSO\(_4\) and CaCl\(_2\) were added after Chelex treatment. The perfusion bottles were placed above the heart with an overflow to ensure a mean coronary perfusion pressure of 65 mm Hg.

Left ventricular pressure was measured by the method described by Grupp and Grupp: a catheter was inserted through a pulmonary vein into the left atrium and advanced into the left ventricle, forced through the apex, and connected to a pressure transducer (Gould P23Db). The first derivative of left ventricular pressure (\( \pm \text{dP/dt} \)) was obtained from a differentiator (model 7P20, Grass Instrument Co., Quincy, Mass.). Coronary effluent was collected and weighed for determination of coronary flow. Heart rate, left ventricular pressure, and \( \pm \text{dP/dt} \) were monitored on a Grass 7D polygraph.

At the end of the experiments, the left ventricular free wall was immediately immersed in 2.5% buffered glutaraldehyde, minced into 1-mm-thick transmural pieces, and processed for light and electron microscopy.

Experimental Protocol

After equilibration for 15 minutes, the hearts were initially perfused with the buffer containing 1 mM salicylic acid (Sigma Chemical Co., St. Louis, Mo.) for 20 minutes. The pH of the buffer was readjusted after the addition of salicylic acid. Then the hearts were subjected to a global ischemia by turning off the flow switch for 5, 10, 15, 20, 30, or 60 minutes (\( n = 16 \) for 15-minute ischemic hearts and \( n = 8 \) each for other ischemic hearts) and were reperfused with the buffer containing 1 mM salicylic acid for 30 minutes with buffer containing 1 mM salicylic acid after equilibration.

In separate experiments, we added 0.5 mM DFX (CIBA, Summit, N.J.) to evaluate the effect of iron chelation on the formation of \( \cdot \text{OH} \) and on the tissue injury. This concentration of DFX has been shown to be sufficient to prevent an iron-catalyzed Haber-Weiss reaction (Hoe et al\(^2\)). DFX was applied to the 15-minute ischemia/30-minute reperfusion model (\( n = 10 \)) and was administered from the onset of reperfusion to the end of the experiment.

We perfused ischemic (15-minute ischemia/30-minute reperfusion, \( n = 6 \)) and nonischemic (50-minute reperfusion, \( n = 6 \)) hearts with buffer free of salicylic acid. These experiments were done to determine the possible effect of salicylic acid on the heart without ischemia and on the heart with ischemia/reperfusion.

Measurement of \( \cdot \text{OH} \)

One milliliter of the effluent was treated with 40 \( \mu \text{l} \) of 100 mM 2,4-dihydroxybenzoic acid (2,4-DHBA), which is an internal standard, and 50 \( \mu \text{l} \) of 1N HCl and extracted with 10 ml HPLC grade diethyl ether on a vortex mixed for 90 seconds. The diethyl ether layer was separated and was evaporated to dryness in a water bath at 40°C. The residue was dissolved in 50 \( \mu \text{l} \) of 1N HCl and 32.5 \( \mu \text{mol} \) mobile phase, and 20 \( \mu \text{l} \) of the solution was injected into the HPLC apparatus.\(^3\) The HPLC apparatus consisted of two solvent delivery modules (model 110B, Beckman Instruments, Inc., Fullerton, Calif.), a Beckman 166 programmable detector module, and a Beckman 406 Analog Interface Module connected to an IBM-PC computer. The column used was a Hibar RT (Lichrosorb-RP-18, 10 \( \mu \text{m} \), 25 cm \( \times \) 4 mm) attached to a precolumn. The effluent was 80% 0.03 M citric acid–0.03 M acetic acid buffer (pH 3.6) and 20% methanol at a flow rate of 1.0 ml/min. The detector was set at a wavelength of 315 nm.\(^3\)

The detection limit for 2,5-DHBA was 2 pmol at a signal-to-noise ratio of 3 and that for 2,3-DHBA was 10 pmol. A linear relation existed between 2 pmol and 20 nmol for 2,5-DHBA and between 10 pmol and 20 nmol for 2,3-DHBA. We used 2,5-DHBA rather than 2,3-DHBA because of its greater sensitivity for quantification of \( \cdot \text{OH} \) production.

2,5-DHBA was expressed in the unit of amount (nanomoles per gram wet heart weight per minute) and of concentration (nanomolar per gram per minute) at each sampling point, and the accumulated amounts (nanomoles per gram after 30-minute reperfusion) and mean concentration (nanomolar per gram after 30-minute reperfusion) were calculated by integrating the areas underneath the individual time course curves for 30-minute reperfusion.

Measurement of Lactate Dehydrogenase in the Effluent

Coronary effluent was used for measurement of lactate dehydrogenase (LDH), an indicator of myocardial tissue injury. This was assayed by a coupled-enzyme spectrophotometric technique using a Sigma assay kit. The accumulated amount of LDH release during 30 minutes of reperfusion (units per gram wet heart weight after 30-minute reperfusion) was obtained by integrat-
ing the area underneath the individual time course curve for 30 minutes of reperfusion.

**Morphological Examination**

Tissue from the midventricular wall was cut into 1.0-mm pieces, immersed in 2.5% buffered glutaraldehyde for 4 hours, rinsed in 0.17 M sodium cacodylate buffer (pH 7.3), and postfixed in 1% buffered osmium tetroxide for 1 hour. The tissue pieces were dehydrated in ethanol and propylene oxide and embedded in Spurr medium. One-micron-thick sections were cut with an LKB ultramicrotome, mounted on a glass slide, and stained with 1% toluidine blue. The representative blocks from each group were thin sectioned for transmission electron microscopy. These sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope.

A semiquantitative estimate of cell damage was carried out on 1-μm-thick sections. Three randomly chosen blocks from each heart were examined for quantitation of cell damage without prior knowledge of the treatment. Approximately 500 cells were analyzed in each heart, and the degree of cell damage was assigned to each cell. The degree of cell damage was determined by the following classification: normal—compact myofibers with uniform staining of nucleoplasm, well-defined rows of mitochondria between the myofibrils, and no separations of opposing intercalated disc; mild damage—same as above, except some vacuoles were present adjacent to the mitochondria; severe damage—reduced staining of cytoplasmic organelles, clumped chromatin material, wavy myofibers, and granularity of cytoplasm. The cells with contraction band necrosis were added in this category. Ultrastructural features of the above classification were confirmed by transmission electron microscopy.

**Statistical Analysis**

Data were expressed as mean±SEM. Group comparisons were done by analysis of variance with multiple comparisons or t test when appropriate. Spearman’s analysis was used for determining the correlation between the parameters. A difference of p<0.05 was considered significant.

**Results**

Functional recovery of the postischemic hearts estimated after 30 minutes of reperfusion was decreased as the ischemic interval increased (Figure 1). The percent recovery of ±dP/dt was approximately 50% in the hearts subjected to 20-minute ischemia, and no left ventricular pressure was measured in the hearts with 60-minute ischemia. The percent recovery of coronary flow, however, was maintained at approximately 40% in the hearts with 60-minute ischemia.

**The Amount of 2,5-DHBA Produced During Reperfusion After Graded Ischemia**

During postischemic reperfusion, 2,5-DHBA and 2,3-DHBA were detected (Figure 2). Figure 3 shows the time course of amount and concentration of 2,5-DHBA detected for each ischemic interval. 2,5-DHBA was negligible in the effluent from nonischemic hearts, and it was also insignificant in the effluent from the hearts with 5-minute ischemia. Significantly higher amounts, however, were detected from the hearts exposed to more than 10 minutes of ischemia. During 30 minutes of reperfusion, we observed the peaks for both amount and concentration of 2,5-DHBA within 90 seconds after the onset of reperfusion in every group.

The accumulated amount of 2,5-DHBA and mean concentration of 2,5-DHBA are illustrated in Figure 4. After 30 minutes of reperfusion, the respective accumulated amounts and mean concentrations were 0.02±0.02 nmol/g and 0.07±0.07 nM/g in the nonischemic group, 1.74±1.02 nmol/g and 3.54±2.05 nM/g in the 5-minute ischemic group, 4.80±1.45 nmol/g and 15.40±7.63 nM/g in the 10-minute ischemic group, 6.73±1.04 nmol/g and 18.18±3.53 nM/g in the 15-minute ischemic group, 5.89±1.71 nmol/g and 24.84±8.60 nM/g in the 20-minute ischemic group, 4.03±1.25 nmol/g and 25.61±10.78 nM/g in the 30-minute ischemic group, and 2.38±0.84 nmol/g and 20.28±5.27 nM/g in the 60-minute ischemic group. The accumulated amount of 2,5-DHBA was significantly higher in the groups rendered ischemic for 10, 15, 20, and 30 minutes than in the nonischemic group. Detection was maximal in the hearts ischemic for 15 minutes and decreased as the ischemic period prolonged. Meanwhile, there were no significant differences in the mean concentrations of 2,5-DHBA among the 15-, 20-, 30-, and 60-minute ischemic groups, although the values were significantly higher than that found in the nonischemic group (Figure 4).

The accumulated amount of 2,5-DHBA during the first 15 minutes of reperfusion period in the groups with 0-, 5-, 10-, 15-, 20-, 30-, and 60-minute ischemia was 0.02±0.02, 0.92±0.52, 3.37±0.77, 4.18±0.62, 3.54±0.80, 2.55±0.65, and 1.44±0.44 nmol/g, respectively. The maximal value was also seen in the group with 15-minute ischemia. There was no correlation between the 2,5-DHBA amount found during the first 15 minutes of reperfusion and the parameters of myocardial injury (data not shown).

**Correlation Between the Amount of 2,5-DHBA and Myocardial Injury**

The relation between the amount of 2,5-DHBA and the functional recovery, LDH release, and morphological changes was determined after 30 minutes of reperfusion in the 15-minute ischemic hearts, in which the accumulated amount of 2,5-DHBA was maximal. Two of 16 hearts in this group showed sustained ventricular...
fibrillation during the entire reperfusion period and were eliminated from the correlation analysis. The accumulated amount of LDH release was significantly greater in the group with 15-minute ischemia than in the nonischemic control group (2.994±0.413 versus 1.069±0.203 units/g after 30 minutes of reperfusion, p=0.003), and the percentage of the cells with either severe damage alone or cells with mild and severe damage was significantly higher compared with the nonischemic group (Table 1 and Figure 5).

**Figure 2.** Panel A: Chromatogram of the effluent before ischemia. The peaks of 2,5-dihydroxybenzoic acid (2,5-DHBA) and 2,3-DHBA are not noted. Panel B: Chromatogram of the effluent at 60 seconds after the onset of reperfusion following 15 minutes of ischemia. The peaks of 2,5-DHBA and 2,3-DHBA are clearly observed.

**Figure 3.** Time course of the amount and concentration of 2,5-dihydroxybenzoic acid (2,5-DHBA) during the 30-minute reperfusion period after various ischemic intervals (5, 10, 15, 20, 30, and 60 minutes). The left y axis indicates the unit of the amount, the right y axis indicates the unit of the concentration, and the x axis shows the time course in each panel. In every case, both the peaks of amount and concentration are seen in the early reperfusion period (≤90 seconds).
As shown in Figure 6, no correlation was noted in any parameters of myocardial injury with the accumulated amount of 2,5-DHBA (Figure 6).

The Effect of DFX on -OH Production and Myocardial Injury

The effect of DFX on the formation of -OH was evaluated in the group with 15-minute ischemia/30-minute reperfusion. DFX itself did not cause any change in myocardial function, LDH release, and morphology when it was perfused to the intact hearts for 30 minutes (n=3, data not shown). DFX significantly reduced both the amount and concentration of 2,5-DHBA at most sampling periods; also, the accumulated amount was reduced from 6.73±1.04 to 2.29±0.80 nmol/g after 30 minutes of reperfusion, and the mean concentration was reduced from 18.18±3.53 to 5.41±1.88 nM/g after 30 minutes of reperfusion (Figure 7). However, the functional recovery, LDH release, or morphology was not affected (Tables 1 and 2). Sustained ventricular fibrillation was noted in one of 10 hearts treated with DFX (10%), and the incidence of fibrillation was not different from that seen in the 15-minute ischemic group without DFX treatment (12.5%). The heart with ventricular fibrillation was excluded from the analysis of myocardial injury.

Influence of Salicylic Acid on Nonischemic Heart and on Postischemic Myocardial Injury

Perfusion of 1 mM salicylic acid-containing buffer for 50 minutes did not affect the function of nonischemic control hearts (Table 3).

As shown in Table 3, the functional recovery was not improved in the ischemic group perfused with salicylic acid-containing buffer. One of the hearts without salicylic acid experienced fibrillation and was excluded from the analysis.

Discussion

Methodological Considerations

We recently reported a modified HPLC method, which was originally described by Floyd and colleagues,22,23 to measure -OH products in the coronary effluent from the reperfused rat heart after 30-minute ischemia.22 This method is based on the chemical reaction of salicylic acid with -OH, yielding 2,5-DHBA, 2,3-DHBA, and catechol as its derivatives in an approximate proportion of 40%, 49%, and 11%, respectively.28 It is reported that salicylic acid with a concentration of 1 mM traps approximately 10% of the theoretically possible -OH formed in vivo.22 Also, its hydroxylation product, 2,5-DHBA, can be quantified by HPLC, thus making it possible to determine the amount of -OH actually produced. On the other hand, with EPR it is difficult to discriminate between the trapped radicals. The present method can quantify 2,5-DHBA at the picomole level, which is 1,000-fold more sensitive than EPR, as previously reported.21 Thus, this method might be superior to EPR for quantitative estimates of -OH because of its higher specificity and sensitivity to this radical species, although its usage is limited only to -OH detection.

Recently, Halliwell et al29 raised an issue regarding the specificity of the salicylic acid method for the

**TABLE 1.** Semiquantitative Estimate of Morphological Damage After 30 Minutes of Reperfusion in 15-Minute Ischemic Hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Normal (mean±SEM)</th>
<th>Mild (mean±SEM)</th>
<th>Severe (mean±SEM)</th>
<th>Mild+severe (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonischemic control</td>
<td>8</td>
<td>97.3±0.7</td>
<td>2.3±0.7</td>
<td>0.4±0.2</td>
<td>2.7±1.9</td>
</tr>
<tr>
<td>15-minute ischemia/30-minute reperfusion</td>
<td>14</td>
<td>68.0±3.0</td>
<td>26.3±2.6</td>
<td>5.7±0.8</td>
<td>32.0±3.0</td>
</tr>
<tr>
<td>15-minute ischemia/30-minute reperfusion treated with DFX</td>
<td>9</td>
<td>61.8±1.2</td>
<td>30.3±1.6</td>
<td>8.0±1.0</td>
<td>38.3±1.3</td>
</tr>
</tbody>
</table>

n: Number of hearts; DFX, deferoxamine. Values are mean±SEM.
The hearts (two of 16 hearts with untreated ischemia/reperfusion and one of 10 hearts with DFX) that showed sustained ventricular fibrillation were eliminated from the analysis.

*p<0.05 compared with the corresponding value for the control group by t test.
Time Course and Amount of •OH Production

First, we evaluated the time-dependent production of •OH during the course of reperfusion after inducing ischemia ranging from 0 to 60 minutes. The peak of •OH formation, both the amount and concentration, appeared very early (30–90 seconds) after the onset of reperfusion. This conclusion is in agreement with other previous studies in which oxygen-derived free radicals were detected using EPR, and this study further provides more accurate quantitative analysis of •OH production.

Second, the accumulated amount of 2,5-DHBA was significant in the groups rendered ischemic for 10 minutes and longer. The amount was significantly higher in 15-minute ischemia than in 60-minute ischemia, and it decreased as the ischemic interval was increased. Since the “no-reflow phenomenon” is reported to occur in the rat heart after prolonged global ischemia, the washout rate of the coronary effluent should be taken into account when the amount of •OH product is compared among the groups with different ischemic intervals. Thus, the mean concentration of 2,5-DHBA during reperfusion was also calculated. The concentration of 2,5-DHBA did not simultaneously increase with the ischemic intervals but was similar among the groups with 15–60-minute ischemia. In the EPR studies, Kramer et al reported the progressive increase in oxygen radical formation with longer durations of ischemia (20, 30, and 40 minutes). Zweier et al

Detection of •OH. They reported the possibility that 2,5-DHBA, but not 2,3-DHBA, could be formed from the reaction of salicylic acid with the cytochrome P-450 system. In the present study, we observed both 2,5-DHBA and 2,3-DHBA but used the former because of its greater sensitivity for the detection of •OH. We also occasionally observed only 2,5-DHBA, but in these experiments, the values were near the lower limit of 2,5-DHBA detection. Therefore, the absence of 2,3-DHBA in these cases was attributed to the lower sensitivity of 2,3-DHBA in the present system. A possibility still exists that 2,5-DHBA detected in the coronary effluent originated from sources other than •OH attack on salicylic acid. The treatment of the heart with deferoxamine inhibited the formation of 2,5-DHBA, suggesting that at least a major portion of 2,5-DHBA formed is due to the •OH attack on salicylic acid.

Since salicylic acid makes the stable derivatives by trapping •OH, it may theoretically be considered as a scavenger of •OH. Therefore, we compared the effect of salicylic acid on functional recovery in control ischemic and treated ischemic hearts. Salicylic acid failed to exert any beneficial effect on the postischemic injury. This suggests that 1) salicylic acid does not have a significant scavenging effect because it can trap only a small portion (≈10%) of produced •OH or because •OH trapped by salicylic acid in the effluent may not be intimately associated with the myocardial injury, or 2) elimination of •OH does not seem to reduce the postischemic injury, as discussed below.
reported a decrease in the radical formation in tissue after longer periods of ischemia (60 minutes) and reperfusion. Although we also noted a pattern of -OH formation (decrease after prolonged ischemia) in the unit of amount similar to that found by Zweier et al, we failed to show that pattern in the unit of concentration. This discrepancy might be ascribed to the relatively lower concentration of salicylic acid associated with different kinetic factors32 as well as different kinds of trapped radicals. Thus, measurement of 2,5-DHBA in tissue may resolve this issue; the time course of the 2,5-DHBA amount found in tissue during the early phase of reperfusion, when -OH is maximally observed in the effluent, may be useful in elucidating the kinetics of -OH formation more clearly. Measurements of radicals in tissue and -OH in effluent (this study) suggest that an optimal time window of ischemia in the formation of maximal -OH may exist in the posts ischemic heart.

Role of -OH During Early Reperfusion After Ischemia

-OH is believed to be one of the most reactive species of free radicals and to play a critical role in posts ischemic myocardial injury during reperfusion.19,20 However, there is still no direct evidence that -OH promotes or mediates posts ischemic damage; it may act as a facilitator, i.e., promote the conditions for uncontrolled entry of various ions affecting cellular structure and function. Previous studies have reported that exogenously produced -OH caused the damage to endothelial and myocardial cells.14,16-18 -OH used in those studies was generated from an enzyme-dependent reaction or electrolysis. However, -OH was not quantified in those studies; thus, without quantitative data it was not possible to establish the dose--effect relation. Therefore, there remains a possibility that the dose used in the previous studies was unphysiologically high. An overdose of an agent is often harmful even when the agent is beneficial in a small dose. We recently reported that a mixture of 100 μM ferrous chloride, 300 μM hydrogen peroxide, and 1 mM ascorbic acid, one of the commonly used methods for -OH generation,13,34 could produce 42.7 μM 2,5-DHBA after 2 minutes in Krebs-Henseleit buffer.31 This concentration was approximately 10,000-fold more than the maximal concentration of 2,5-DHBA detected in rat heart in the present study.

To further elucidate the actual pathophysiological role of -OH in posts ischemic reperfused rat heart, we investigated the relation between the amount of 2,5-DHBA and different parameters of cardiac injury. In 15-minute ischemic/30-minute reperfused rat hearts, there was no correlation between the amount of 2,5-DHBA detected and functional recovery, LDH release, or morphological damage. According to the previous studies,14,16-18 exogenous -OH is harmful to the heart, but our results imply that -OH may not be produced in sufficient amounts to cause the damage.

We might have underestimated the “burst” of -OH production by integrating the 2,5-DHBA amount during the entire reperfusion period, in which maximum burst occurred within 90 seconds followed by decline during the remaining reperfusion period. Thus, to more accurately evaluate the -OH burst effect, we also calculated the accumulated amount of 2,5-DHBA during the first
15 minutes of the reperfusion period. The maximal value was still seen in the group with 15 minutes of ischemia, which was significantly higher than that in the group with 60 minutes of ischemia. Furthermore, there was no correlation between the 2,5-DHBA amount formed during the first 15 minutes of reperfusion and the parameters of myocardial injury.

To further elucidate the role of -OH, DFX was given to the heart at the onset of reperfusion to suppress -OH production, and cardiac injury was evaluated. DFX inhibited -OH formation in the treated hearts but failed to reduce cardiac injury. The failure of DFX to attenuate myocardial injury despite an inhibition of -OH production strengthens the conclusion of this study that there is a lack of correlation between -OH formation and myocardial injury in the setting of ischemia/reperfusion.

Several previous studies have reported conflicting results on the effectiveness of DFX on postischemic myocardium.35-44 Those studies have many differences in the experimental models (rat, rabbit, or dog with regional or global ischemia), protocols (pretreatment or posttreatment with different dosages or different ischemia and reperfusion intervals), and methods of evaluation of cardiac injuries (arrhythmia, function, metabolism, or infarct size). Among these studies, Bolli et al43 and Williams et al44 did confirm the effectiveness of DFX treatment at the onset of reperfusion on the reduction of free radical generation using EPR. Both of these groups reported the beneficial effect of DFX on functional and/or metabolic recovery, implying the importance of iron and -OH in postischemic myocardial injury during reperfusion. Those reports appear to be in conflict with our present data. However, there are still major differences in the methods between their studies and ours: 1) They used EPR for the detection and thus did not specify the detected species of free radicals. 2) The animal species and ischemic protocols were different (regional ischemia for 15 minutes in dogs by Bolli et

### Table 2. Effect of Deferoxamine on the Functional Recovery, Lactate Dehydrogenase Release, and Morphology After 30 Minutes of Reperfusion in 15-Minute Ischemic Hearts

<table>
<thead>
<tr>
<th></th>
<th>Without DFX (n=14)</th>
<th>With DFX (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Functional recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+dP/dt</td>
<td>90.9±5.5</td>
<td>95.4±3.0</td>
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<tr>
<td>% of baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mm Hg/sec</td>
<td>2,002±67*</td>
<td>2,047±47*</td>
</tr>
<tr>
<td>−dP/dt</td>
<td>87.6±5.5</td>
<td>81.8±5.2</td>
</tr>
<tr>
<td>% of baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mm Hg/sec</td>
<td>−1,477±92*</td>
<td>−1,493±70*</td>
</tr>
<tr>
<td><strong>Heart rate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of baseline</td>
<td>86.9±5.5</td>
<td>78.3±3.3</td>
</tr>
<tr>
<td>bpm</td>
<td>229±9*</td>
<td>251±11*</td>
</tr>
<tr>
<td><strong>Coronary flow</strong></td>
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<tr>
<td>% of baseline</td>
<td>73.9±3.0</td>
<td>74.0±4.0</td>
</tr>
<tr>
<td>ml/min</td>
<td>14.7±0.7*</td>
<td>15.0±0.9*</td>
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<tr>
<td><strong>LDH release after REP (units/g)</strong></td>
<td>2.994±0.412</td>
<td>3.338±0.533</td>
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<tr>
<td><strong>Morphology (% of cells)</strong></td>
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<tr>
<td>Mildly+severely damaged</td>
<td>32.0±3.0</td>
<td>38.3±1.3</td>
</tr>
<tr>
<td>Severely damaged</td>
<td>5.7±0.8</td>
<td>8.0±1.0</td>
</tr>
</tbody>
</table>

DFX, deferoxamine; bpm, beats per minute; LDH, lactate dehydrogenase; REP, 30 minutes of reperfusion. Values are mean±SEM.

Hearts with ventricular fibrillation were eliminated from the analysis. No significant differences were seen in any comparisons (analyzed by t test).

*Raw baseline values.

### Table 3. Influence of Salicylic Acid on Myocardial Function

<table>
<thead>
<tr>
<th></th>
<th>Nonischemic group</th>
<th>Ischemic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variables (%)</td>
<td>(n=8)+</td>
<td>(n=14)+</td>
</tr>
<tr>
<td>(+ n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+dP/dt</td>
<td>102.5±3.8</td>
<td>99.8±0.9</td>
</tr>
<tr>
<td>% of baseline</td>
<td>90.9±5.5</td>
<td>90.8±1.2</td>
</tr>
<tr>
<td>−dP/dt</td>
<td>98.8±4.2</td>
<td>99.2±0.7</td>
</tr>
<tr>
<td>% of baseline</td>
<td>87.6±5.5</td>
<td>84.2±2.2</td>
</tr>
<tr>
<td>Heart rate</td>
<td>96.9±2.2</td>
<td>98.8±0.7</td>
</tr>
<tr>
<td>Coronary flow</td>
<td>94.9±3.4</td>
<td>98.5±1.0</td>
</tr>
</tbody>
</table>

* Presence of salicylic acid–containing buffer; −, absence of salicylic acid–containing buffer. n, number of hearts. Values are mean±SEM. In the nonischemic group, functional changes were assessed after 50 minutes of perfusion with and without salicylic acid–containing buffer. In the ischemic group, functional changes were assessed after 15 minutes of ischemia and 30 minutes of reperfusion with and without salicylic acid–containing buffer. Hearts with ventricular fibrillation were eliminated from the analysis. No significant difference was seen in any comparisons (analyzed by t test).
al and global ischemia for 30 minutes in rabbits by Williams et al. 3) They did not perform the correlation analysis between the amount of free radical formation and cardiac injury. The failure of DFX in the protection of postischemic injury might also be explained in part by the fact that the functional deterioration in the model with 15-minute ischemia/30-minute reperfusion was too small to have much room for improvement. Furthermore, the rationale for the use of DFX in this investigation was to inhibit the generation of \( \cdot \text{OH} \). In the present study, the model with 15-minute ischemia/30-minute reperfusion was found to be one of the groups that produced the maximal amount of \( \cdot \text{OH} \), and this study was mainly focused on the issue of \( \cdot \text{OH} \) involvement in postischemic injury. Additional studies may be necessary to evaluate the effects of DFX both in the reversible and in the irreversible ischemic model. Therefore, those different factors might have a strong bearing on the effectiveness of DFX reported in those studies.

The evidence is overwhelming supporting the “free radical hypothesis” of postischemic dysfunction after reversible ischemia (myocardial “stunning”), whereas contradictory data exist about the radical hypothesis of irreversible injury in the postischemic reperfused heart (for reviews, see Bollir and Reimer et al[6]). We confirmed in our model a significant rise in LDH release and in morphological damage to cells, which could be recognized as irreversible, despite good functional recovery. At present there is no clear definition of reversible or irreversible ischemic damage of the whole heart. In fact, no systematic study has been conducted to determine the fate of the globally ischemic heart after prolonged reperfusion; thus, the present experimental model cannot be categorized as either reversible or irreversible.

Mechanism of \( \cdot \text{OH} \) Formation

DFX is a highly specific iron chelator and has been reported as an inhibitor of iron-dependent \( \cdot \text{OH} \) generation. In the present study, DFX effectively reduced the amount of 2,5-DHBA. Thus, the mechanism of \( \cdot \text{OH} \) reduction by DFX may be explained by the prevention of the iron-catalyzed Haber-Weiss reaction. Although it is not clear whether the reaction occurs in biological systems, the present results support the possible in vivo formation of \( \cdot \text{OH} \) via the Haber-Weiss reaction.

In the postischemic reperfused heart, superoxide anion and hydrogen peroxide are thought to be the precursors of \( \cdot \text{OH} \) and are believed to originate from the vascular endothelial cells by the action of xanthine and xanthine oxidase, in the mitochondrial electron transport chain and in the infiltrating neutrophils. In the isolated heart perfused with buffer, it is unlikely that sufficient numbers of neutrophils would remain to participate in the formation of radicals. Thus, since the present study evaluated the amount and role of \( \cdot \text{OH} \) burst in the isolated rat heart during early reperfusion after ischemia, the result cannot necessarily extend to in vivo studies. The studies using in vivo animal models are expected to provide additional data for elucidating the role of \( \cdot \text{OH} \) in ischemic damage.

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