Iron-Load Increases the Susceptibility of Rat Hearts to Oxygen Reperfusion Damage

Protection by the Antioxidant (+)-Cyanidanol-3 and Deferoxamine

Antonius M.M. van der Kraaij, MD, Leendert J. Mostert, MSc,
Henk. G. van Eijk, PhD, and Johan F. Koster, PhD

To investigate whether iron is involved in the reperfusion syndrome by aggravating free radical injury, the hearts from iron-loaded and control rats were perfused under normoxic, anoxic, and reperfusion conditions. Normoxic perfusion revealed no change in coronary flow, contractility, or lactate dehydrogenase (LDH) release between these two groups. Under anoxic and reperfusion conditions, however, we found a significant increase of ventricle fibrillation (56% vs. 0%, p<0.01, n=9), a significantly lower recovery of contractility (21 ± 7.4% vs. 81 ± 6.6%, mean ± SEM; p<0.001), and a significant increase of LDH release (667 ± 142 vs. 268 ± 37 mU LDH/min/g wet wt, mean ± SEM; p<0.05). Administration of either 20 μM of the antioxidant (+)-cyanidanol-3 or 50 μM of the iron-chelator deferoxamine totally prevented the generation of ventricle fibrillation and normalized contractility to control levels in the iron-loaded group. Moreover, 20 μM (+)-cyanidanol-3 significantly lowered LDH release in this period (312 ± 67 mU), whereas deferoxamine had no protective effect on this LDH release (1,494 ± 288 mU). Normal hearts appeared to be protected by 20 μM (+)-cyanidanol-3 as well. In this group (n=6), a significantly higher recovery of contractility (97.1 ± 3.2% vs. 81 ± 6.6%, p<0.05) and a significantly lower release of LDH (110 ± 27 vs. 268 ± 37 mU, p<0.05) was found compared with the control group (n=9). No difference in superoxide dismutase or glutathione peroxidase activity was found between the groups. It is concluded that 1) iron-loaded rat hearts are more susceptible to anoxia and oxygen reperfusion damage; 2) iron load itself, under normoxic conditions, does not seem to be harmful; and 3) the antioxidant (+)-cyanidanol-3 is able to protect normal as well as iron-loaded rat hearts against anoxic and reperfusion damage. We suggest that iron plays an important role in the occurrence of tissue damage and ventricle fibrillation during anoxia and reperfusion, probably through the formation of hydroxyl radicals and/or perfferyl oxide. (Circulation 1988;78:442–449)

Several recent investigations have reported that oxygen-derived free radicals might play an important role in the pathogenesis of electron-ischemic and postischemic (reperfusion) cardiac injury.1–3 Indirect evidence supporting this hypothesis is based on studies that have reported protective effects of free radical scavengers such as superoxide dismutase (SOD), catalase, mannitol, and allopurinol4–7 on heart tissue during ischemia and reperfusion. Further evidence is based on the formation of malondialdehyde, an end product of lipid peroxidation, which parallels postischemic reperfusion damage.1 Moreover, oxygen free radicals have recently been identified directly in postischemic cardiac tissue with electro-spin-resonance techniques.8 The free radical superoxide and hydrogen peroxide, which are thought to be formed during ischemia and reperfusion,1–3,8 are, however, less toxic for myocardial tissue in the absence of a transition metal.9 Their reactivity is low, and they can easily be converted by SOD, catalase, and glutathione peroxidase. In the presence of catalytic amounts of transition metals such as iron, however, superoxide and hydrogen peroxide can be transformed into a highly reactive hydroxyl-radical, OH· (Haber-Weiss reaction).10

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\text{O}_2^+ + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^\cdot
\]
This radical is very toxic for biological tissue because it reacts immediately with lipids, proteins, or DNA and destroys their molecular structure. Reaction with polyunsaturated fatty acids, for example, can easily induce lipid peroxidation, which might lead to membrane damage and cell death.11 Almost all cellular iron is located in ferritin. Recently, we have shown that superoxide is able to mobilize iron from ferritin and that ferritin can be the physiological iron donor for the initiation of lipid peroxidation.12 In the present study, we investigated whether an iron-dependent mechanism of cell damage might be responsible for the reperfusion syndrome. Therefore, the amount of total iron in rat hearts (stored in ferritin) was increased by injecting the rats once a week with imferon (iron dextran) for a period of 6 weeks and by adding an iron supplement (FeSO4) to their food. After this treatment, we studied these hearts under normoxic, anoxic, and reperfusion conditions by measuring contractility (apex displacement), coronary flow, and LDH release. To substantiate the hypothesis that iron and free radicals are involved in the reperfusion syndrome, the effects of the iron chelator deferoxamine and the antioxidant (+)-cyanidanol-313,14 were studied. (+)-Cyanidanol-3 is of special interest because this drug has already been used in clinical trials for treating alcoholics. Finally, we measured SOD and glutathione peroxidase activities to exclude a possible iron-dependent impairment of the defense system against free radicals.

Materials and Methods

Animals

Thirty male Wistar rats, 12 weeks old, were randomly divided into two groups to obtain an iron-loaded group (15 animals) and a control group (15 animals). Iron load was accomplished by injecting 0.5 ml Imferon (iron dextran, 50 µg Fe³⁺/ml; Fisons, Leusden, The Netherlands) in the animals’ gluteus muscles once a week for a period of 6 weeks and by adding a supplement (FeSO₄ · 7H₂O; 7.5 µg Fe²⁺/g standard food) to their food during this period. The injections were administered after a brief anesthesia with diethylether. The control group was injected with dextran (10% solution) under the same conditions. No differences in body weight or behavior between the two groups during this period were observed. The perfusion experiments were performed 1 week after the last injection, at which time their body weight was 200–250 g.

Perfusion Protocol

The hearts were perfused according to Langendorff after using diethylether again as an anesthetic. The perfusion was carried out at 37°C, pH 7.4. We used a modified Tyrode’s buffer containing (mM) NaCl 128, KCl 4.7, CaCl₂ 1.3, NaHCO₃ 0.2, NaH₂PO₄ 0.4, MgCl₂ 1, and glucose 11 saturated with 95% O₂-5% CO₂. Perfusion pressure was held constant at 80 cm H₂O. Coronary flow was measured indirectly in the aorta as a retrograde aortic flow (transflow 601 system, Skalar, Delft, Holland).

Figure 1. Effects of anoxia and reperfusion on the contractility of iron-loaded rat hearts (○—○, n=9), control hearts (□—□, n=9), and control hearts in the presence of (+)-cyanidanol-3 (20 µM) (■—■, n=6) expressed as percentage of the preanoxic (basal) value. 100% contractility (frequency x amplitude) equals 15,896±744 (mean±SEM) in the iron-loaded group, 16,359±579 in the control group, and 16,871±355 in the (+)-cyanidanol-3-protected group. Recovery of contractility during reperfusion was significantly lower in the iron-loaded group (at 80 minutes: 21.0±7.4% [mean±SEM], *p<0.001) and significantly higher in the (+)-cyanidanol-3-protected group (97.1±3.2%, *p<0.05) compared with the control group (81.0±6.6%).
We measured contractility as apex displacement with a Harvard heart-smooth muscle transducer (model 386, Edenbridge, UK) that was hooked to the apex of the heart. Contractility was calculated by multiplying heart frequency (beats/min) by amplitude (mm). After 15 minutes of initial perfusion, just before starting anoxic perfusion, coronary flow and contractility were measured and defined as 100%. Anoxia was induced by perfusing the hearts under the same conditions with Tyrode’s buffer saturated with 95% N₂-5% CO₂. After 45 minutes of anoxia, we reperfused with the oxygen-saturated Tyrode’s buffer for a period of 20 minutes. The two compounds (50 μM deferoxamine or 20 μM cyanidanol) were administered throughout the whole experiment, which took 80 minutes.

**Chemicals and Determinations**

Deferoxamine (desferal) was purchased from CIBA-GEIGY, Switzerland, and (+)-cyanidanol-3 [(+)-catechin] was purchased from Zyma, Nyon, Switzerland. Both compounds were dissolved in Tyrode’s buffer. The cyanidanol-containing buffer was protected against light by covering the perfusion apparatus with aluminum foil. All other chemicals used were analytical grade. SOD activity was determined with the cytochrome c reduction inhibition method of McCord. The SOD concentration was expressed in micrograms per milligram protein with bovine erythrocyte SOD (lot no. 10594720-11, Boehringer, Mannheim, FRG) as a standard. Glutathione peroxidase activity was determined according to the method of Paglia and Valentine. One unit corresponds to one micromole NADPH oxidation per minute. LDH was measured with a Boehringer commercial kit. Total iron was determined, after acidic destruction of the heart, by adding 10 mM Ferene S and using an extinction coefficient, at 595 nm, of 35,500/M/cm.

**Statistical Analysis**

Data are presented as the mean ± SEM. All comparisons were made with one-way analysis of variance, except the occurrence of ventricular fibrillation, which was compared with Fisher’s exact test for dichotomy. p<0.05 was considered to be significant.

**Results**

**Effects of Iron Load on Contractility, Coronary Flow, and LDH Release**

During the initial equilibration period no differences were measured in contractility (Figure 1), flow (Figure 2), or LDH release (Figure 3) between the iron-loaded (n=9) and control group (n=9). In the iron-loaded group, six rat hearts developed ventricular fibrillation (VF) during anoxia or reperfusion (56%), whereas no VF was found in the control hearts (p<0.01, Fisher’s exact test). These VFs started in five hearts at 21, 24, 32, 33, and 36 minutes of anoxic perfusion and in one heart after 12 minutes of reperfusion. Only two hearts (32 and
36 minutes) spontaneously recovered from fibrillation during early reperfusion at 3- and 1-minute reperfusion times, respectively. Contractility was regarded zero during VF (Figure 1). After 20 minutes' reperfusion, contractility returned to 81 ± 6.6% in the control group and 21 ± 7.4% in the iron-loaded group (n = 9, mean ± SEM, p<0.001) (Figure 1). If the hearts, which were fibrillating throughout reperfusion, are not taken into account, the contractility is restored to 41 ± 6.1% (n = 5, mean ± SEM, p<0.01 vs. control, n = 9), and if all fibrillating hearts including those that recovered during early reperfusion are not taken into account, contractility is restored to 47.3 ± 4.9% (n = 3, mean ± SEM, p<0.05 vs. control, n = 9; figures not shown). Coronary flow revealed no difference between the iron and control group in the first 10 minutes of anoxia (Figure 2). After these 10 minutes, however, the flow in the iron-loaded group dropped compared with the control group. After 30 minutes of anoxia
FIGURE 5. Effects of (+)-cyanidanol-3 (20 μM) (─, n=3) and deferoxamine (50 μM) (▼—▼, n=3) on the coronary flow of iron-loaded rat hearts during anoxia and reperfusion in relation to unprotected iron-loaded rat hearts (●—●, n=9). 100% coronary flow equals 9.8±0.7 ml/min in the unprotected iron-loaded group, 9.3±0.5 ml/min in the (+)-cyanidanol-3 group, and 9.1±0.2 ml/min in the deferoxamine group.

and during early reperfusion, this drop becomes significantly different compared with the control group (p<0.05). If all fibrillating hearts are not taken into account, no difference in coronary flow is found between these two groups (figures not shown), so VF itself in the iron-loaded group might well have been responsible for this drop in coronary flow.

LDH release in the effluent during reperfusion was significantly higher in the iron-loaded group (p<0.05, Figure 3). After 2 minutes reperfusion, LDH release was maximal in both groups and 268±37 mU/min/g wet wt in the control group (n=9) versus 667±142 in the iron-loaded group (mean ± SEM, n=9, p<0.05). If all fibrillating hearts again were not taken into account, LDH release remained significantly different from control group (795.5±124.9 mU, n=3, figures not shown).

Effects of (+)-Cyanidanol-3 and Deferoxamine on Iron- Loaded Rat Hearts During Anoxia and Reperfusion

Administration of (+)-cyanidanol-3 (20 μM, n=3) to the perfusion fluid completely protected the iron-loaded rat hearts during anoxia and reperfusion. We found no VF, whereas 56% was found in the unprotected iron-loaded group. In the reperfusion period, contractility was restored to control levels (87.7±9.0%) and significantly different from the unprotected iron-loaded hearts (21±7.4%, mean ± SEM; p<0.001, Figure 4). Perfusion with (+)-cyanidanol-3 showed a complete normalization of LDH release to control levels (312.3±117.0 vs. 268±37 mU), which is also significantly lower compared with the unprotected iron-loaded group (667±142, Figure 6). Coronary flow in the iron-loaded hearts in the presence of cyanidanol revealed a pattern comparable with control hearts (Figures 2 and 5).

Administration of deferoxamine (50 μM, n=3) protected the iron-loaded hearts against VF. Also, no VF was found in this group compared with 56% in the unprotected iron-loaded group. During reperfusion, contractility was restored to 68.0±6.1% (Figure 4). Although this recovery was not as high as in the cyanidanol (87.7%) or control group (81%), it was significantly higher than in the unprotected iron-loaded group (p<0.01, Figure 4). Measurements of coronary flow, which are exhibited in Figure 5, during anoxia with deferoxamine show no drop in coronary flow, which was seen with iron load alone. In the presence of deferoxamine, a higher LDH release was found during early reperfusion (Figure 6), after 10 minutes of reperfusion, however, LDH release became lower compared with the iron-loaded group although still higher than
in the presence of (+)-cyanidanol-3. Deferoxamine did not influence the LDH assay. At the moment, we do not have an explanation for this initial release of LDH and, as far as we know, this has never been reported before.

Effects of (+)-Cyanidanol-3 and Deferoxamine on Normal Rat Hearts During Anoxia and Reperfusion

The protective effects of deferoxamine on reperfusion injury in normal heart tissue have been reported extensively in the literature.\(^{18,19}\) As far as we know, no reports have been presented on the effects of (+)-cyanidanol-3 on normal heart tissue during anoxia and reperfusion. We, therefore, studied these effects more extensively. The antioxidant (+)-cyanidanol-3 (20 μM) protected normal rat hearts under the same conditions against anoxic and reperfusion injury. During anoxia, contractility was the same in the (+)-cyanidanol-3–protected (n=6) and control groups (n=9) (Figure 1). After 15 minutes of reperfusion, however, a significantly higher recovery of contractility with 20 μM (+)-cyanidanol-3 was found (97±3.2% vs. 81±6%, Figure 1). During early reperfusion, contractility recovered more rapidly and with less rhythmic disturbances in the (+)-cyanidanol-3 group. Also, LDH release during reperfusion was significantly lower in the (+)-cyanidanol-3–protected group (110±27 vs. 268±37 mU, p<0.05; Figure 3), whereas coronary flow was significantly higher during early reperfusion (Figure 2). This might be related to contractility, which is restored earlier and with less rhythmic disturbances in these hearts.

Measurements of Total Iron and Activities of Superoxide Dismutase and Glutathione Peroxidase

Table 1 clearly reveals that administration of iron to the rats results in an increment of total iron content in the heart tissue. To exclude the possibility that the mechanism of anoxic and reperfusion injury is due to an impaired defense system against free radicals, SOD and glutathione peroxidase activities were measured. Table 1 clearly

<table>
<thead>
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<th>Total iron (μg/g heart)</th>
<th>Superoxide dismutase (μg/mg protein)</th>
<th>Glutathione peroxidase (mg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td>50.8±11.0</td>
<td>1.77±0.30</td>
<td>285.9±26.4</td>
</tr>
<tr>
<td>Iron-loaded (n=9)</td>
<td>112.4±24.1*</td>
<td>1.85±0.28</td>
<td>270.5±24.5</td>
</tr>
<tr>
<td>Iron-loaded plus 50 μM</td>
<td>107.2±22.7†</td>
<td>2.26±0.21</td>
<td>267.5±8.0</td>
</tr>
<tr>
<td>deferoxamine (n=3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron-loaded plus 20 μM</td>
<td>108.6±17.0†</td>
<td>2.20±0.11</td>
<td>285.3±39.3</td>
</tr>
<tr>
<td>cyanidanol (n=3)</td>
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*\(p<0.01\), †\(p<0.05\).
shows that the activities of these two enzymes are equal in all four groups.

Discussion

The mechanism by which free radicals might be formed in anoxic, ischemic, or posts ischemic heart tissue is poorly understood. Several hypotheses have been postulated in the literature. McCord suggested in 1985 that xanthine dehydrogenase is converted during ischemia to xanthine oxidase by calcium-activated proteases. The formation of this enzyme might be responsible for the generation of superoxide, \( \text{O}_2^- \), which is directly formed when hypoxanthine is converted by this enzyme. Others have demonstrated a minor role of this mechanism in rabbits because of the absence of this enzyme in this animal. They have suggested that the ability of allopurinol, an inhibitor of xanthine oxidase, to protect rabbit hearts during ischemia must be due to a mechanism other than inhibition of this enzyme. Other sources of free radicals generated in ischemia in vivo might be polymorphonuclear cells and monocytes, activated under ischemic conditions, auto-oxidation of catecholamines, or formation of free radicals in impaired mitochondria. Experiments in vitro have shown that the toxicity of superoxide, \( \text{O}_2^- \), and hydrogen peroxide is largely diminished for biological tissues in the absence of iron. In the presence of iron, however, these radicals can be transformed into highly reactive hydroxyl radicals, \( \text{OH}^\cdot \) (Haber-Weiss reaction), which are regarded to be very toxic for biological tissues. Furthermore, it was shown that ferritin, the iron storage protein present in every cell, can be the free iron donor (\( \text{Fe}^{2+} \)) during \( \text{O}_2^- \) generation. Our experiments show that iron load itself, over a 6-week period, is not toxic. We found no differences in body weight or behavior between iron-loaded and control animals; moreover, under normoxic perfusion conditions, contractility, flow, and LDH release were the same in both groups. During anoxia and reperfusion, however, iron-loaded rat hearts turned out to be much more vulnerable, measured by a significant increase of VF, lower recovery of contractility, and higher release of LDH. The iron-loaded rat hearts were protected with the antioxidant (+)-cyanidanol-3 and the iron chelator deferoxamine, although LDH release with deferoxamine was higher compared with control levels and in contrast with protecting effects on VF and contractility. Because of equal SOD and glutathione peroxidase activities in both groups, it can be concluded that the occurring damage is not due to an impaired defense mechanism against free radicals but probably to a larger supply of \( \text{OH}^\cdot \) radicals formed in the presence of increased iron amounts. During anoxia, mainly contractility is disturbed as shown by a significant increase of VF, whereas during reperfusion cell necrosis becomes more prominent as shown by a higher LDH release and lower recovery of contractility. Protection of normal as well as iron-loaded rat hearts during anoxia and reperfusion by (+)-cyanidanol-3 has never been reported before and might be a promising progress in establishing protective measures in ischemia. Although we cannot exclude that the long-term storage of iron damages the cell directly, it is shown by our data that iron-loaded rat hearts are much more susceptible during anoxia and reperfusion. This gives strong evidence that besides the generation of free radicals, the availability of transition metals, such as iron, are indispensable for mediating anoxic or ischemic damage. The use of iron chelators, which cannot induce lipid peroxidation, might therefore be a promising approach in preventing ischemic myocardial injury.

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References


**KEY WORDS**
- anoxia
- iron
- hydroxyl radical
- free radicals
- ischemia
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