Prevention of Postischemic Cardiac Injury by the Orally Active Iron Chelator 1,2-Dimethyl-3-Hydroxy-4-Pyridone (L1) and the Antioxidant (+)-Cyanidanol-3

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In this study, we investigated the role of oxygen-derived free radicals and iron in mediating myocardial injury during ischemia and reperfusion. Iron is of special interest because it may enhance tissue injury during ischemia and reperfusion by catalyzing the formation of highly reactive hydroxyl radicals (by modified Haber-Weiss or Fenton reactions). Rat hearts, perfused by the Langendorff method, were subjected to global ischemia (15 minutes at 37° C) and reperfusion. The effects of two iron chelators, 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and 5-hydroxy-2-hydroxymethyl-4-pyrone (kojic acid), and one antioxidant, (+)-cyanidanol-3, on contractile function, coronary flow, lactate dehydrogenase release, and lactate production were studied. The combination of these iron chelators is of special importance because L1 is known to prevent lipid peroxidation, induced by ADP/Fe2+ and NADPH in microsomes, in contrast to kojic acid. We found significant protection of contractile function (apex displacement) during reperfusion with 50 μM L1 and 20 μM (+)-cyanidanol-3 (p < 0.01, n = 6), whereas no protection was found with 50 μM kojic acid (n = 6). Measurements of lactate dehydrogenase release during reperfusion showed a protective pattern similar to that found for heart contractile function, although 50 μM kojic acid also showed a significantly lower lactate dehydrogenase release during the first 10 minutes of reperfusion. No differences in coronary resistance or lactate release were found between the various groups. Our findings indicate that iron and oxygen-derived free radicals are important in the pathogenesis of postischemic reperfusion injury probably because of the formation of hydroxyl radicals. During heart ischemia, administration of the orally active iron chelator L1 or the antioxidant (+)-cyanidanol-3 may be a promising approach in establishing postischemic cardiac protection. (Circulation 1989;80:158–164)

A growing amount of evidence indicates that oxygen-derived free radicals are important in the pathogenesis of cardiac tissue destruction during ischemia and reperfusion.1–4 Most of the evidence is indirect and based on studies that have reported protective effects of superoxide dismutase, catalase, mannitol, or other free radical scavengers.5–8 Recently, oxygen-derived free radicals have been directly identified in ischemic and postischemic cardiac tissue by electron-spin-resonance techniques.9,10 The actual source of oxygen-derived free radicals in ischemic heart tissue is still not known, but several sources have been postulated in the literature, for example, the mitochondrial electron transport chain,11 the enzyme xanthine oxidase,2 activated polymorphonuclear cells,12,13 auto-oxidation of catecholamines,14 and the biosynthesis of prostaglandins.15 Generation during ischemia and reperfusion of superoxide (O2•−) or its dismutated product, hydrogen peroxide (H2O2), is toxic for cardiac tissue.16,17 This toxicity, however, can be markedly increased when a transition metal is present that can catalyze hydroxyl radical formation from superoxide and H2O2 (Haber-Weiss reaction16,19). Iron is considered the most important transition metal present in cardiac tissue. Normally, all iron is stored in ferritin in which it is unable to catalyze hydroxyl radical formation. In earlier studies, we showed that superoxide mobilizes iron from ferritin.20 Liberation of iron during ischemia by this mechanism may well be responsible for the formation of hydroxyl radicals and enhanced cardiac
injury. Because of their high reactivity, hydroxyl radicals are regarded to be very toxic for biologic tissues. Upon generation, they react rapidly with various molecules such as lipids, proteins, or DNA, thereby destroying their molecular structure. Moreover, hydroxyl radicals can initiate lipid peroxidation that may lead to membrane damage and cell death.\(^{21}\) To substantiate the role of hydroxyl radicals in mediating ischemic and postischemic cardiac injury, we perfused rat hearts under normoxic and postischemic reperfusion conditions with the antioxidant (+)-cyanidanol-3 and with the iron chelators L1 and kojic acid. The naturally occurring flavonoid (+)-cyanidanol-3 is a relatively nontoxic compound with an oral LD\(_{50}\) in the rat of more than 16 g/kg.\(^{22}\) This flavonoid interacts powerfully with many free radical generating systems, in which it neutralizes superoxide and hydroxyl radicals.\(^{22-24}\) For this reason, (+)-cyanidanol-3 has been used in clinical trials to treat patients with acute viral hepatitis.\(^{25}\) The iron chelators L1 and kojic acid were used in the present investigation because of the contrasting effects of both compounds on lipid peroxidation induced by ADP/Fe\(^{3+}\) and NADPH in microsomes.\(^{26}\) Mostert et al.\(^{26}\) showed that iron complexed to L1 is unable to catalyze lipid peroxidation in microsomes in contrast to iron complexed to kojic acid. These two iron chelators are part of a new, recently developed generation of iron chelators that form water soluble, colored complexes with iron at physiological pH.\(^{27-29}\) The molecular structures are presented in Figure 1. Three molecules of each chelator form a complex with one iron atom. The iron-binding constants of L1 and kojic acid are 10\(^{35}\) and 10\(^{27}\), respectively.\(^{30}\)

**Methods**

**Animals and Perfusion Protocol**

Twenty-seven male Wistar rats that were 20 weeks of age (body weight, 200–250 g) were divided into five groups: control group (n=6), cyanidanol group (n=6), two groups for iron chelation with L1 (n=6) and kojic acid (n=6), and one group for combined perfusions with L1 plus cyanidanol (n=3).

After a short anesthesia with di-ethylether, the hearts were quickly removed from the body and placed in ice-cold Tyrode’s buffer to stop contractility. Immediately after cessation of contractility, the hearts were cannulated in the aorta and perfused by the method of Langendorff.\(^{31}\) The perfusions were carried out at 37°C and pH 7.4, with a modified Tyrode’s buffer, containing (mM) 128 NaCl, 4.7 KCl, 1.3 CaCl\(_2\), 20.2 NaHCO\(_3\), 0.4 NaH\(_2\)PO\(_4\), 1 MgCl\(_2\), and 11 glucose saturated with 95% O\(_2\)-5% CO\(_2\). At the end of an initial equilibration period of 15 minutes, contractile function and coronary flow were defined 100%. Samples of the perfusate were collected during this stabilization period and during reperfusion to determine lactate dehydrogenase (LDH) and lactate release. Total ischemia (15 minutes) was induced by closing the tap between the perfusion apparatus and the heart. Tissue temperature was maintained in this period by immersing the hearts in Tyrode’s buffer at 37°C. We saturated this buffer with 95% N\(_2\)-5% CO\(_2\) to minimize oxygen diffusion at the epicardial surface. The hearts were reperfused for 15 minutes with oxygenated perfusate. During ischemia, contractile function was not measured to avoid technical complications. The hearts were perfused, apart from the ischemic period, with a constant pressure of 80 cm H\(_2\)O. Coronary flow was measured indirectly in the aorta as a retrograde aortic flow (Transflow 601 system, Skalar, Delft, The Netherlands). Contractile function was recorded as apex displacement by a Harvard heart/smooth muscle transducer (Type 386, Edenbridge, UK). A total load of 1 gram was hooked to the apex of the heart.\(^{32}\) We defined contractile function as heart frequency (beats/min) multiplied by amplitude (mm). This product was considered a valid parameter for heart function because Stam and DeJong showed a good correlation between this index and left ventricular systolic pressure in rat hearts. All compounds tested were administered throughout the whole experiment. Based on dose-dependent experiments, we decided to perfuse with 50-\(\mu\)M concentrations of L1 and kojic acid. This concentration had no influence on contractile function or coronary resistance and is sufficient to chelate all possible free iron.

**Chemicals and Determinations**

(+)-Cyanidanol-3 was obtained from Zyma Nyon, Switzerland, and 5-hydroxy-2-hydroxymethyl-4-pyran (kojic acid) was obtained from Aldrich (Gillingham, UK). 1,2-Dimethyl-3-hydroxy-4-pyridone (L1) was prepared by the method of Kontoghiorghes.\(^{34}\) These compounds were dissolved in Tyrode’s buffer to obtain 50-\(\mu\)M solutions of L1 and kojic acid and a 20-\(\mu\)M solution of cyanidanol. The cyanidanol-containing buffer was protected against light by covering the perfusion apparatus with aluminum foil. Lactate was measured by the method of Hohorst,\(^{35}\) and LDH was measured with a Boe-
hringer commercial kit (Mannheim, FRG). All other chemicals were of analytical grade.

**Statistical Analysis**

Data are presented as the mean±SEM unless indicated otherwise. All comparisons were made with analysis of variance by Bonferroni’s method. Significance was considered at \( p < 0.05 \).

**Results**

**Effects of Cyanidanol, L1, and Kojic Acid on Contractile Function During Normoxia and Reperfusion**

Throughout the experiment, the hearts were perfused with either 50 \( \mu M \) L1, 50 \( \mu M \) kojic acid, 20 \( \mu M \) cyanidanol, or 50 \( \mu M \) L1 plus 20 \( \mu M \) cyanidanol, and the effects upon contractile function were compared with control hearts (Figures 2 and 3). As mentioned in “Methods,” these concentrations were derived from dose-dependent experiments and had no influence on heart contractile function. After 15 minutes of normal perfusion, contractile function was 12,365±4,013 for control hearts, 12,018±2,259 for cyanidanol hearts, 12,468±3,644 for L1 hearts, 12,891±3,253 for kojic acid hearts, and 12,247±765 mm/min for L1 plus cyanidanol hearts (mean±SD, \( n = 6 \); except the L1 plus cyanidanol group, which was \( n = 3 \)). After 15 minutes of total ischemia, during reperfusion, contractile function was restored significantly higher in the L1 group compared with the control group (66.2±5.8% vs. 20.9±4.6% at 45 minutes, mean±SEM, \( *p < 0.05 \), \( *p < 0.01 \), \( n = 6 \)), whereas no differences were found in contractile function between control hearts and hearts perfused with kojic acid.

**Figure 2.** Plot of contractile function during normoxia (0–15 minutes) and reperfusion of control hearts (○) and hearts perfused with 50 \( \mu M \) L1 (□) or 50 \( \mu M \) kojic acid (▲). At 15 minutes, contractile function was defined 100% and was 12,365±4,013 mm/min (mean±SD) for control hearts, 12,468±3,644 for hearts perfused with L1, and 12,891±3,253 for hearts perfused with kojic acid. During reperfusion, contractile function was restored to levels significantly higher in the L1 group compared with the control group (66.2±5.8% vs. 20.9±4.6% at 45 minutes, mean±SEM, \( *p < 0.05 \), \( *p < 0.01 \), \( n = 6 \)), whereas no differences were found in contractile function between control hearts and hearts perfused with kojic acid.

**Figure 3.** Plot of contractile function during normoxia (0–15 minutes) and reperfusion of control hearts (○) and hearts perfused with 20 \( \mu M \) cyanidanol (▼) or 20 \( \mu M \) cyanidanol plus 50 \( \mu M \) L1 (▲). After 15 minutes, contractile function was defined 100% and was 12,365±4,013 mm/min (mean±SD) for control hearts, 12,018±2,259 for cyanidanol hearts, and 12,247±765 for L1 plus cyanidanol hearts. During reperfusion, contractile function was restored significantly higher in the cyanidanol group (60.6±7.1%, \( n = 6 \)) and cyanidanol plus L1 group (67.7±8.4%, \( n = 3 \)) compared with the control group (20.9±4.6%, \( n = 6 \), mean±SEM, \( *p < 0.05 \), \( *p < 0.01 \)).
**Effects of Cyanidanol, L1, and Kojic Acid on LDH Release, Lactate Release, and Coronary Flow During Reperfusion**

LDH release in the effluent was significantly higher in the control group compared with the L1 group after 7 minutes of reperfusion ($p<0.05$, $n=6$; Figure 4). The highest level of LDH release in the L1 group occurred between 5 and 7 minutes of reperfusion. LDH release in the control group was maximal after 10 minutes reperfusion. Perfusions with kojic acid resulted in a significantly lower LDH release at 7 and 10 minutes reperfusion compared with control hearts (Figure 4). After 15 minutes of reperfusion, LDH release in this group was still increasing to become no longer significantly different from control levels. We do not have a good explanation for this delayed LDH release because no differences in the coronary flow were measured between the various groups (Table 1) and because contractile function in kojic acid hearts remained stable at the time of the elevated LDH release. Figure 5 shows LDH release in cyanidanol and L1 plus cyanidanol perfused rat hearts in relation to control hearts. A significantly lower LDH release with cyanidanol alone was found compared with control levels ($p<0.05$, $n=6$). Reperfusion in the presence of the combination of cyanidanol plus L1 showed a comparable protection, indicating that no additional effect occurred. To assure that a comparable ischemia was induced under the various conditions, lactate release was measured. More than 80% of total lactate release, measured during reperfusion, was released in the first 5 minutes of reperfusion. Figure 6 shows this release. No differences were encountered among the five groups, which indicates that the amount of ischemia was the same in all five groups. Finally, coronary flow during reperfusion is displayed in Table 1. A comparable pattern was found during reperfusion in the five separate groups, suggesting that the compounds of interest, as well as contractile function or cellular necrosis, had no influence on postischemic coronary resistance in our perfusion experiments.

**Discussion**

In this study, we found that administrations of the iron chelator L1 and the antioxidant (+)-cyanidanol-3 protect postischemic cardiac tissue. Heart perfusions with these substances resulted in a higher recovery of contractile function and a lower release of LDH during postischemic reperfusion. Moreover, the administration of a second iron chelator, kojic acid, had no effect under the same conditions. The differences observed between these iron chelators can be explained by the opposite effects each has in catalyzing the modified Haber-Weiss or Fenton reactions. The property of L1, to inhibit hydroxyl radical formation in vitro, may well be

![Figure 4. Plot of lactate dehydrogenase (LDH) release during reperfusion measured in heart effluents of control hearts (○, $n=6$), hearts perfused with 50 µM L1 (□, $n=6$), and hearts perfused with 50 µM kojic acid (▲, $n=6$). LDH release was significantly lower in the L1 group after 7 minutes of reperfusion and in the kojic acid group at 7 and 10 minutes compared with the control group (*$p<0.05$).](image)

**Table 1.** Coronary Flow During Postischemic Reperfusion as Percentage of Preischemic Level (100%)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control ($n=6$)</th>
<th>Cyanidanol ($n=6$)</th>
<th>L1 ($n=6$)</th>
<th>Kojic acid ($n=6$)</th>
<th>Cyanidanol+L1 ($n=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>93±8.9</td>
<td>77±7.5</td>
<td>80±18.9</td>
<td>95±24.0</td>
<td>97±13.5</td>
</tr>
<tr>
<td>4</td>
<td>119±18.9</td>
<td>134±23.5</td>
<td>151±36.4</td>
<td>135±19.6</td>
<td>138±14.5</td>
</tr>
<tr>
<td>7</td>
<td>141±26.3</td>
<td>169±17.7</td>
<td>157±37.9</td>
<td>158±22.0</td>
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</tr>
<tr>
<td>10</td>
<td>142±23.8</td>
<td>157±20.9</td>
<td>148±29.5</td>
<td>157±7.3</td>
<td>141±19.8</td>
</tr>
<tr>
<td>13</td>
<td>124±21.3</td>
<td>122±24.4</td>
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<td>133±16.1</td>
<td>122±19.3</td>
</tr>
<tr>
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<td>114±24.7</td>
<td>124±33.7</td>
<td>129±12.5</td>
<td>117±16.1</td>
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<td>110±24.5</td>
<td>119±32.0</td>
<td>122±13.3</td>
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</tr>
</tbody>
</table>

Data are mean±SD.
responsible for the observed heart protection during postischemic reperfusion. Our findings that equal concentrations of another iron chelator, kojic acid, with structural similarities to L1, had no protective effect, can be explained by the fact that iron complexed to kojic acid is still able to catalyze hydroxyl radical formation.\textsuperscript{26} The contrast between these two chelators strengthens our suggestion that the binding of iron is responsible for postischemic protection and not a possible scavenging capacity.

Protection of heart function and tissue necrosis by the antioxidant (+)-cyanidanol-3 is consistent with the hypothesis that oxygen-derived free radicals are involved in the pathogenesis of myocardial injury during ischemia and reperfusion.\textsuperscript{1-4} The capacity of (+)-cyanidanol-3 to neutralize superoxide\textsuperscript{22} may well account for the protection observed. It must be emphasized, however, that by scavenging superoxide, iron is not released from ferritin by this radical. It is therefore not surprising that by using the combination of (+)-cyanidanol-3 and L1, no additional protection is seen.

The results of the present study are consistent with experiments in which protection with another iron chelator, deferoxamine, was reported.\textsuperscript{37,38} Badylak et al.\textsuperscript{37} showed protection of rat hearts by deferoxamine after 60 minutes of total ischemia. In that study, they reported significant lower creatinine phosphokinase release and less coronary resistance during reperfusion. Unfortunately, no parameter for heart function was assessed in this study. We found, using a similar Langendorff model, no restoration of heart function at all in rat hearts after 60 minutes of total ischemia at 37°C. We have estimated that the point of no return of heart function as a result of ischemia under these conditions is between 20 and 30 minutes (unpublished observations). In our opinion, 60 minutes of total ischemia at 37°C is too long to establish possible cardiac protection by drugs. Further protective evidence of deferoxamine was reported by Ambrosio et al.\textsuperscript{38} They found that administration of deferoxamine at the time of postischemic reflow was able to protect rabbit hearts after 30 minutes of total ischemia at 37°C. A higher recovery of myocardial function and less LDH release was observed in this study. These results are in contrast to the findings of Myers et al.,\textsuperscript{39} who reported no protection of deferoxamine in rabbit hearts after 2 hours of global ischemia at 27°C. In this study, they hypothesized

**Figure 5.** Plot of lactate dehydrogenase (LDH) release during reperfusion measured in heart effluents of control hearts (○, n=6), hearts perfused with 20 μM cyanidanol (◇, n=6), and with 20 μM cyanidanol plus 50 μM L1 (●, n=6). LDH release was significantly lower in the cyanidanol group after 7 minutes of reperfusion (*p<0.05) compared with the control group, and was the same as the LDH release in the cyanidanol plus L1 group, although no significant difference was found probably because of the low number of animals (n=3).

**Figure 6.** Bar graph of lactate production during the first 5 minutes of reperfusion in control hearts, hearts perfused with 20 μM cyanidanol (CY), 50 μM L1 (L1), 50 μM kojic acid, and 20 μM cyanidanol plus 50 μM L1 (CY+L1). No significant differences were observed, indicating that the amount of ischemia was comparable.
that iron-catalyzed hydroxyl radical formation can still be important because intracellular iron pools may be inaccessible to deferoxamine.

In several studies performed by Hearse et al,40,41 83% ventricular fibrillation was encountered in control rat hearts after 10 minutes of ischemia. In the present study, we found no ventricular fibrillation in any heart. This marked difference may be explained by the fact that regional ischemia was established by Hearse et al in contrast to total ischemia in our experiments. Generation of reentry circuits is known to be responsible for the induction of ventricular fibrillation (for review, see Manning and Hearse42). Heterogeneity of tissue injury is a critical progenitor of these reentry circuits. Our contrasting findings may therefore be explained by the use of different forms of ischemia.

As far as we know, no studies have been published reporting (post)-ischemic cardiac protection with iron chelators other than deferoxamine. Considering the contrasting results on the effectiveness of deferoxamine administration, our findings are important in establishing the role of iron during ischemia and reperfusion. In recent experiments in our laboratory, we showed that iron-loaded rat hearts are more susceptible to reperfusion injury after 45 minutes of anoxia.43 This study indicates that iron loading itself in cardiac tissue can accelerate tissue destruction during anoxia and reperfusion probably by the formation of hydroxyl radicals. In the present study, we have shown that ischemic rat hearts can be protected during posts ischemic reperfusion by the administration of the iron chelator L1 and the antioxidant (+)-cyanidanol-3. Moreover, we found that the iron chelator kojic acid, the complex of which is unable to block hydroxyl radical formation in vitro,26 had no protective effect. For these reasons, we conclude that iron and oxygen-derived free radicals are important in mediating posts ischemic cardiac injury probably because of the formation of hydroxyl radicals. The protection achieved by the orally active iron chelator L1, which has already been used in clinical trials to treat patients with β-thalassemia,28 may be a promising and easily assessable approach in establishing (post)-ischemic cardiac protection in patients in the near future.

References


**KEY WORDS** ischemia • free radicals • iron • iron chelating agents • rat hearts • lipid peroxidation • reperfusion injury • (+)-cyanidanol-3
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