Cu-ATSM, an Intracellular-Accessible Superoxide Dismutase (SOD)-Like Copper Complex: Evaluation in an Ischemia-Reperfusion Injury Model

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We have reported a stable superoxide dismutase (SOD)-like copper complex, Cu-ATSM, which shows high membrane permeability and distribution to the brain or heart. In this study, we evaluated the protective effects of Cu-ATSM on superoxide-mediated tissue damage caused by ischemia-reperfusion using an isolated perfused rat heart model. Lipid peroxidation levels in the Cu-ATSM-treated group were lower than those in the non-treated group. Furthermore, released creatine phosphokinase into the perfusate, a marker of tissue damage, was reduced by Cu-ATSM treatment. These results indicated the possibility of Cu-ATSM being an effective SOD-like drug for the treatment of superoxide-mediated damage, such as ischemia-reperfusion injury.

Keywords ischemia-reperfusion; copper complex; superoxide dismutase

Superoxide and superoxide-derived reactive radicals cause cell damage in some pathological states, such as ischemia-reperfusion injury, aging, and inflammation.1–3 It has been supposed that superoxide production occurs mainly in intracellular spaces, such as mitochondria.4–6 Most native superoxide dismutases (SODs) exist in intracellular spaces; Cu,Zn-SOD is found in the cytosolic fraction, and Mn-SOD is in mitochondria. These facts suggest that an increase in SOD activity in the intracellular space or granules is required to reduce superoxide-mediated damage. To reduce superoxide-mediated damage, numerous investigators have studied a variety of SOD-like drugs, classified into two categories. One category is a SOD-containing macro-molecular enzyme complex, such as polymer-conjugated SOD, liposome-enveloped SOD and recombinant-human SOD. The other is a low-molecular weight SOD-like complex containing a metal, such as Cu, Mn and Fe.7–10 Although some attempts to extend half lives of SOD-drugs in blood have been made, a SOD-drug which is delivered into the intracellular space and accesses superoxide generation sites has not been reported. Thus, we have attempted to develop such a SOD-like drug and propose a copper-dithiosemicarbazone complex as a candidate.11

Cu-Diacetyl-di(N4-methylthiosemicarbazone) (Cu-ATSM, Fig. 1) is a neutral, compact, lipophilic Cu-complex with high membrane permeability and blood-brain barrier permeability, which also shows high SOD-like activity. Further, it is likely to be stable even in a biological environment.11 In order to evaluate the possibilities of Cu-ATSM as an intracellular accessible SOD-like drug, we assessed the effects of Cu-ATSM on superoxide-mediated tissue damage caused by ischemia-reperfusion using perfused rat heart.

MATERIALS AND METHODS

Chemicals 2-Thiobarbituric acid (TBA) was obtained from Nacalai Tesque, Inc., Japan. Sodium malondialdehyde (MDA) metabsulphite was obtained as described previously,12 and used for the preparation of an MDA standard solution. Creatine phosphokinase (CPK) used for quantification was purchased from Oriental Yeast (Osaka, Japan). Cu-ATSM was synthesized following the methods of Gingras, et al.,13 and chemical purity was confirmed by elementary analysis and mass spectrometry. Anal. Caled for C76H84CuN8S2: C, 25.84; H, 4.38; N, 26.10. Found: C, 29.81; H, 4.38; N, 25.88. MS m/z: 321 (M+). All other chemicals used were of analytical grade.

Perfused Rat Heart Preparation The preparation of perfused rat heart was carried out according to the Langendorff's technique.14 Male Wistar rats (350–450 g) were injected intraperitoneally with 500 i.u. of heparin. After 20 min, the heart was removed following stunning, and quickly placed into cold perfusate. The aorta was attached to a stainless steel cannula and the heart was perfused at a rate of 6 ml/min with Krebs–Ringer's bicarbonate (KRB) buffer containing 118.5 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM magnesium sulfate, 2.5 mM calcium chloride, 1.2 mM potassium dihydrogen phosphate, 25.0 mM sodium bicarbonate, and 12.8 mM glucose at 37 °C. The buffer was continuously bubbled with a gas mixture containing 95% oxygen and 5% carbon dioxide.

Measurement of Myocardial Hemodynamic Function Myocardial function of the perfused hearts was monitored throughout the experiments. A glass cannula, connected to a pressure transducer (Spectramed Medical Products (S Pte., Ltd., DTX PLUS) by a polyethylene tube and filled with perfusate, was inserted via the left atrium into the

![Cu-ATSM](Cu-diacetyl-di(N4-methylthiosemicarbazone))

Fig. 1. Structural Formula of Cu-ATSM

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left ventricle. The left ventricular systolic pressure was continuously measured using Biophsylograph 180 system (NEC SAN-EL Instruments, Japan), and the heart rate and first derivative of left ventricular systolic pressure \( dP/dt \) were recorded. Coronary perfusion pressure was monitored by a transducer connected to the perfusion apparatus.

**Experimental Protocol** The prepared heart was perfused for 40 min to stabilize. During that time, pre-ischemic sampling of the perfusate from the heart was performed at an interval of one minute, and then the heart was exposed to complete ischemia for 20 min by clamping of the left arterial and aortic cannula. At the end of the ischemic period, reperfusion was started with the oxygenated perfusate for 80 min, and the perfusate eluted from the heart was collected at an interval of 1 min.

For the administration, 0.76 \( \mu g/ml \) of Cu-ATSM solution was prepared with the perfusate containing 0.05\% dimethyl sulfoxide (DMSO). In the treated group, the hearts were administered 500 \( \mu l \) of the solution into the aortic input line via a six-direction valve injector. The administration were carried out at 10 and 5 min before stopping the flow. Dose was determined considering its \textit{in vitro} IC\(_{30}\) value.\(^{11}\) In the control group, the perfusate containing 0.05\% DMSO was administered in the same manner.

**Assessment of Ischemia-Reperfusion-Induced Tissue Damage** Myocardial damage caused by ischemia-reperfusion was estimated by enzyme release from the perfused hearts and by lipid peroxidation. CPK was selected as a marker enzyme, and lipid peroxidation was assessed by measurement of the formation of TBA reactive material. All collected perfusates were kept at 0\(^\circ\)C, and used for the CPK assay. At 80 min after reperfusion, the heart was weighed, homogenized in saline, and immediately used for the lipid peroxidation assay.

**Assay of Released CPK** CPK activity in the released perfusate was measured according to the previous method.\(^{15}\) In brief, the formation of creatine phosphate from ATP and creatine was followed by determination of the acid-molybdate labile phosphate of creatine phosphate. Released enzyme activity was expressed as milli-

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**Fig. 2. Typical Hemodynamic Parameter Changes during the Perfusion Experiment**

maxLVP, maximum left ventricular pressure; aveLVP, average left ventricular pressure; Perfusion. P, perfusion pressure; \( dP/dt \), maximum of the first derivative of left ventricular pressure.
units per ml perfusate.

**Assay of TBA-Reactive Substrates Formation** Formation of TBA-reactive substrates was determined as described previously. A standard curve was prepared using malondialdehyde (MDA), and lipid peroxidation was expressed as MDA equivalents per gram heart.

**Statistical Analysis** Statistical analysis was performed using the Student's t test. A p-value less than 0.05 was regarded as significant.

**RESULTS**

Figure 2 shows a typical pattern of hemodynamic parameters plotted against perfusion time in a non-treated heart. Most of the hearts showed a pattern similar to this graph.

Table I shows the effects of ischemia-reperfusion on the hemodynamic values of perfused hearts. Data represent the % of pre-ischemic levels at 60 min after reperfusion. In order to assess myocardial function, the maximum and average left ventricular systolic pressure (maxLVP and aveLVP), the maximum dP/dt (maxdP/dt), heart rate (HR), and coronary perfusion pressure (Perfusion. P) were recorded as the hemodynamic parameters. In both the control and the Cu-ATSM treated group, the mean values of the hemodynamic parameters, except for Perfusion. P, were almost 100% of the pre-ischemic values. However, Perfusion. P values increased around two-fold compared to the pre-ischemic values in both groups.

Figure 3 shows the time course of CPK release from the perfused hearts. During the pre-ischemic period, CPK was released at a level of about 20 mU/ml in the control group and in the Cu-ATSM treated group. However, after the ischemic period, the CPK release increased significantly compared to pre-ischemic values. In the Cu-ATSM treated group, the release of CPK into the perfusate during the post-ischemic period was lower than those of the controls over the same time. The total of the measured CPK release during the reperfusion period in the Cu-ATSM treated group was 1545 ± 113, which was significantly lower than that of the control group, 2399 ± 317 (mU/ml).

Table II shows TBA-reactive substrates 80 min after reperfusion. Data are expressed as MDA nmol per g heart. The Cu-ATSM treated group showed low MDA values compared to the control group, indicating that lipid oxidation in the Cu-ATSM group was lower than that in the control.

**DISCUSSION**

It has been suggested that myocardial ischemia and reperfusion interfere with long-term myocardial function,

![Graph](image)

**Fig. 3.** Changes of CPK Activity in the Heart Perfusate during the Perfusion Experiment

Each point represents the mean of released CPK (mU/ml) and S.E.M. for 5 hearts. Open circle, Cu-ATSM treated hearts; open triangle, control hearts.

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**Table I.** Average Hemodynamic Parameter Changes with Ischemia-Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>maxLVP</th>
<th>aveLVP</th>
<th>maxdP/dt</th>
<th>HR</th>
<th>Perfusion. P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94.9</td>
<td>137.2</td>
<td>84.4</td>
<td>89.9</td>
<td>182.9</td>
</tr>
<tr>
<td></td>
<td>(21.1)</td>
<td>(45.0)</td>
<td>(15.7)</td>
<td>(13.7)</td>
<td>(28.2)</td>
</tr>
<tr>
<td>Treated</td>
<td>107.7</td>
<td>118.2</td>
<td>108.4</td>
<td>91.2</td>
<td>219.5</td>
</tr>
<tr>
<td></td>
<td>(23.8)</td>
<td>(26.8)</td>
<td>(26.8)</td>
<td>(2.7)</td>
<td>(24.6)</td>
</tr>
</tbody>
</table>

a) Each hemodynamic parameter value at 60 min after reperfusion was compared to that of pre-ischemia, and the post-ischemia/pre-ischemia ratio was revealed as a %. Data represent the mean and S.E.M. for 5 hearts.

**Table II.** TBA-Reactive Substrates in Reperfused Hearts

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/g heart)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.11 ± 0.40</td>
</tr>
<tr>
<td>Treated</td>
<td>1.03 ± 0.25</td>
</tr>
</tbody>
</table>

a) Data represent the mean and S.E.M. for 5 hearts. b) The value was significantly lower than that of the control group (p < 0.05).
metabolism, and ultrastructure.\textsuperscript{17,18} Numerous investigators have indicated that post-ischemic myocardial dysfunction is mediated by oxygen-derived free radicals with reperfusion.\textsuperscript{1} In fact, direct evidence for free radical generation has been exhibited in the heart perfusion model, used in this study.\textsuperscript{19–21} Furthermore, it has been suggested that oxygen-derived free radicals, when generated with ischemia-reperfusion, cause severe damage in cardiac myocytes, endothelial cells, and cell membranes, and significant myocardial dysfunction occurred as a result.\textsuperscript{22}

CPK plays an important role in cardiac myocyte energy metabolism. When myocytes are damaged, such as by myocardial infarction, CPK is released into the systemic circulation. Thus, CPK release reflects damage of cardiac myocytes or membrane. In our experiment, as shown in Fig. 2, most of the hemodynamic parameters, except for perfusion pressure, recovered to near pre-ischemic levels (Table I). Perfusion pressure values increased to around twice the pre-ischemic levels, indicating damage of coronary blood vessels. CPK release immediately increased with reperfusion, and was maintained at the higher level throughout the study. These findings indicate that cellular components, such as membranes, enzymes, nucleotides, etc., were damaged by the oxygen-derived radicals in the experimental model used, and it is necessary to evaluate the induced damage by changes of metabolism or cellular components.\textsuperscript{3,23,24}

Oxygen-derived free radicals, mainly superoxide and hydroxyl radical, attack cell membranes, and then lipid peroxidation is initiated.\textsuperscript{25,26} We assessed lipid peroxidation with ischemia-reperfusion by monitoring the formation of TBA-reactive substrates. Compared to Cu-ATSM treated hearts, the control hearts showed high levels of lipid peroxidation. This suggests that Cu-ATSM reduced superoxide-mediated lipid peroxidation. Additionally, the measurement of released CPK after reperfusion indicated the protective effects of Cu-ATSM against damage to myocytes and/or membranes. Thus, it was demonstrated that Cu-ATSM reduced superoxide-mediated tissue damage in the used ischemia-reperfusion model. Those protective effects might be contributed to by its SOD activity and high intracellular accessibility.

In conclusion, it seems that Cu-ATSM can reduce superoxide, even in a biological environment. Also, the propriety of an SOD-like compound to be delivered into the intracellular space was indicated.

REFERENCES