Copper Loading of Hearts Increases Postischemic Reperfusion Injury

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We studied the role of copper as a potential mediator of postischemic reperfusion injury in the isolated, perfused rat heart. Hearts were equilibrated with Krebs-Henseleit buffer for 10 minutes and then loaded with copper by way of perfusion with buffer containing 20 μM copper(II)-bis-histidial for 30 minutes. Control hearts were perfused with Krebs-Henseleit buffer alone during the loading period. Hearts then were washed with buffer for 10 minutes and subjected to 20 minutes of normothermic global ischemia followed by 30 minutes of reperfusion. Atomic absorption spectroscopy revealed a 67% increase in total copper content in loaded hearts by the end of the wash. By the end of the 30-minute period of reperfusion, control hearts demonstrated a 50–60% recovery of myocardial function as determined by peak systolic pressure, contractility, and heart rate. In contrast, copper-loaded hearts exhibited virtually no functional recovery within the 30-minute time period. Using salicylate as a probe, we determined that peak and duration of -OH formation appears to be increased in copper-loaded hearts during reperfusion. Furthermore, efflux of lactic dehydrogenase was significantly increased in copper-loaded hearts. Our results clearly demonstrate that increasing cardiac content of copper results in enhanced postischemic reperfusion injury associated with increased formation of •OH, thus suggesting an important catalytic role for this transition metal. (Circulation Research 1991;69:881–885)

Studies have suggested that redox-active transition metals act as catalysts for formation of oxygen-derived free radicals during genesis of postischemic reperfusion injury.¹ The majority of these studies have implicated iron, while ignoring the catalytic role of copper. This probably has evolved as a result of studies demonstrating the existence of a pool of iron bound to low molecular weight substances that appears to increase during ischemia and reperfusion² and other studies indicating a protective effect of the chelator desferrioxamine.³,⁴ It is not clear if heart contains a similar pool of copper, although two studies do suggest that cerebrospinal fluid⁵,⁶ contains a significant fraction of redox-active copper. Considering that heart has a significant content of copper (~5 μg/g), albeit less than iron (~60 μg/g),⁷ it is conceivable that such a pool exists to allow for transfer of metal between storage proteins, such as ceruloplasmin, and copper-dependent enzymes, such as superoxide dismutase. In light of studies demonstrating that copper is from 10- to 60-fold more potent than iron in catalysis of Fenton reactions (Cu²⁺+H₂O₂→Cu³⁺+OH⁻+OH⁻) in biological⁸ and chemical⁹ systems, even a very small fraction in a redox-active form could be a very significant mediator of •OH formation. In the present study, we attempted to increase the size of this pool by copper loading of the isolated heart preparation with the ultimate aim of assessing postischemic reperfusion injury.

Materials and Methods

Animals and Reagents

Male Sprague-Dawley rats (250–350 g) were obtained from Charles Rivers Laboratories, Inc., Wilmington, Mass., or Taconic Farms, Inc., Germantown, N.Y. All rats were allowed at least 3 days of in-house acclimatization before experimental use. During this time, all animals were allowed ad libitum access to standard laboratory food and water. Sodium salicylate, 2,5-dihydroxybenzoic acid (2,5-DHBA), copper(II) chloride, and histidine were obtained from Sigma Chemical Co., St. Louis, Mo. Acetic acid (high-performance liquid chromatography [HPLC] grade), methanol (HPLC grade), and HPLC water were obtained from J.T. Baker, Phillipsburg, N.J. Sodium heparin and sodium pentobarbital were obtained from the hospital pharmacy.
Therapeutic grade 95% O₂–5% CO₂ was obtained from General Welding Supply Co., Westbury, N.Y. All other chemicals were of reagent grade and were obtained from standard sources.

Perfused Heart Preparation

Rats were injected with sodium heparin (500 units i.p.) 30 minutes before being anesthetized with sodium pentobarbital (60 mg/kg i.p.). Hearts were removed rapidly and placed in ice-cold heparinized saline. The hearts then were perfused orthogradely through the coronary arteries at a constant pressure of 95 cm H₂O as previously described.¹⁰

Perfusate

The perfusate was a modified Krebs-Henseleit buffer consisting of (mM) NaCl 118, KCl 6.1, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, HEPES 1.0, and glucose 11.1. The perfusate was gassed with 95% O₂–5% CO₂ for oxygenation and maintenance of pH at 7.4. Perfusion temperature was maintained at 37°C. Copper (20 μM) was added to the perfusate as the bis-histidyl complex (1:2, Cu:His).

Experimental Protocol

The protocol consisted of an initial 10-minute equilibration period with Krebs-Henseleit buffer alone, followed by perfusion with buffer containing 20 μM copper for 30 minutes. Control hearts were perfused with buffer alone during the loading phase. Metal loading was followed by a 10-minute wash period with Krebs-Henseleit buffer alone and then 20 minutes of normothermic global ischemia followed by 30 minutes of reperfusion.

Indexes of Cardiac Function

Four indicators of cardiac function were used in this study. Coronary flow was determined by a timed collection of coronary effluent. Heart rate was calculated from the RR interval of the electrocardiogram. Left ventricular peak systolic pressure was determined by placement of a latex balloon (0.1 ml) into the left ventricle as previously described.¹¹ Contractility was calculated as the maximum rate of rise of the pressure curve or +dP/dtₘₚₜ.

Chemical Analysis

Detection of ·OH. Postischemic ·OH production was evaluated using salicylate as a probe as previously described.¹² In these experiments, sodium salicylate (100 μM) was included in the perfusate starting with the wash period. The particular dihydroxybenzoate assayed was the 2,5-derivative (2,5-DHBA).

Analysis of copper. Copper content of the myocardium was analyzed using atomic absorption spectroscopy (SpectRAA-10, Varian Associates, Inc., Palo Alto, Calif.) after acid digestion of heart tissue.

Lactic dehydrogenase. Lactic dehydrogenase activity in pulmonary effluent was determined using the method described by Bergmeyer et al¹³ and is expressed in Racker units.

Statistical Analysis

Analysis of differences of cardiac functional parameters and lactic dehydrogenase release were analyzed with a repeated measures analysis of covariance (RMANOVA) in which the within factor was time and the covariate was the experimental value at the end of the wash period. Results involving formation of ·OH were analyzed using a natural logarithmic transformation of the data to decrease variation followed by a repeated measures analysis of variance (RMANOVA). Statistical difference at one time point was determined using a t test of the transformed data. All statistics were performed with the SPSS/PC+ (SPSS Inc., Chicago) statistical analysis package.

Results

Copper Loading

Perfusion of hearts with 20 μM copper-bis-histidyl for 30 minutes resulted in a 67% increase (p<0.02, t test) in total copper content (Figure 1). Of interest is the finding that control hearts perfused with Krebs-Henseleit buffer for the same time period demonstrated a 25% increase (p<0.02, t test) in total copper content (Figure 1), probably as a result of contamination of the buffer with trace amounts of metal.

Cardiac Functional Recovery

As demonstrated by Figure 2, overall return of cardiac function after 20 minutes of normothermic global ischemia is severely affected by preloading with copper. Preischemic values of both control and copper-loaded hearts were as follows: heart rate:
The most severely affected functions were pressure development (Figure 2B) and contractility (Figure 2C). Although control hearts demonstrated a significant (p<0.01, RMANOVA) time-dependent recovery of peak systolic pressure (Figure 2B), virtually no recovery (p<0.02, RMANOVA) was observed in the copper-loaded hearts. A similar recovery pattern was observed for contractility as determined by +dP/dt\(_{\text{max}}\) (Figure 2C). Although it is apparent that recovery of heart rate (Figure 2A) is delayed in the copper-loaded hearts, the overall difference between the two groups was not significant (p=0.09, RMANOVA).

Preischemic coronary flow for control and copper-loaded hearts was 15.4±0.4 and 13.8±0.9 ml/min, respectively. There were no overall differences in reestablishment of coronary flow between the two groups (maximum posts ischemic recovery for control and copper-loaded hearts was 90% and 75%, respectively) (data not shown).

Lactic Dehydrogenase Release

As demonstrated in Figure 3, release of lactic dehydrogenase from copper-loaded hearts was as much as four times higher than from control hearts, particularly during the first 10–15 minutes of reperfusion. It is apparent that copper-loaded hearts exhibited a higher baseline (T\(_0\)) release of lactic dehydrogenase; however, even when this was taken into account, the overall difference between the two groups was highly significant (p<0.02, RMANOVA).

Formation of \(-\text{OH}\)

As demonstrated in Figure 4, both peak and duration of formation of \(-\text{OH}\), as detected by salicylate hydroxylation, appear to be increased in copper-loaded animals. However, because of a high degree of variability in the results, the overall difference between the groups was not significant (p=0.10, RMANOVA of ln-transformed data), although the difference at T\(_2.5\) was significant (p<0.05, t test of
In-transformed data). Furthermore, a significant ($p<0.05$, RMANCOVA) time-dependent increase in 2,5-DHBA was observed in both groups.

**Discussion**

The results presented in this study demonstrate that copper loading of hearts worsens postischemic reperfusion injury. Postischemic recovery of contractile function was virtually nonexistent in hearts loaded with 20 μM copper for 30 minutes. In addition, release of lactic dehydrogenase during reperfusion was greatly increased in metal-loaded hearts, suggesting enhanced tissue damage. These results represent very significant findings, as they suggest a possible catalytic role for copper. One possible mechanism for the enhanced cardiac injury may be increased formation of ·OH. During postischemic reperfusion, a burst of ·OH formation has been demonstrated\(^1\), that until now had been associated with iron-mediated Fenton chemistry.\(^3\) But copper is known to be a very efficient catalyst of Fenton reactions, being from 10- to 60-fold more potent than iron.\(^8,9\) A second-order rate constant, $k$, of 76 M$^{-1}$sec$^{-1}$ has been calculated for the iron-catalyzed Fenton reaction.\(^1\) If copper is substituted for iron, $k$ is increased two orders of magnitude\(^1\) to $4.7 \times 10^5$ M$^{-1}$sec$^{-1}$. Therefore, even a relatively small increase in the pool of reactive copper could result in a significant increase in ·OH formation. The results of the present research demonstrate an increase in total copper. It is likely that some of this copper has remained in a redox-active form and thus explains the observed increase in ·OH formation.

Other evidence for a catalytic role for copper can be inferred from previous studies that have examined the role of iron as a mediator of tissue injury during the ischemic and postischemic periods. Desferrioxamine (DFO), a metal chelator, has been demonstrated to improve postischemic function and metabolism in numerous models.\(^3,4\) Yet, when the relative association constants of this chelator for iron and copper ($K_{Fe-DFO} = 10^{11}$; $K_{Cu-DFO} = 10^4$)\(^5\) are considered, it is possible that both metals are being affected. Although iron binding to desferrioxamine is much stronger than copper, concentrations of this chelator are generally still more than adequate to bind copper that is present. For just this reason, desferrioxamine has been used to treat potential copper-overload situations.\(^15\) Furthermore, neocuprine, a copper chelator, has been demonstrated to decrease reperfusion arrhythmias in the isolated rat heart.\(^16\)

That copper can result in cardiac tissue oxidative injury is not without precedent. It has recently been recognized that there can be myocardial complications of Wilson's disease.\(^17,18\) Furthermore, the increased baseline release of lactate dehydrogenase from copper-loaded hearts is indicative of tissue injury, albeit probably minor, without the need for ischemia. This finding is consistent with a recent study demonstrating that perfusion of isolated hearts with 1 mM copper chloride results in ultrastructural changes resembling the oxygen- or calcium-paradox type.\(^19\) These effects may be related to the ability of copper(II) to "steal" electrons from heme proteins,\(^20,21\) resulting in copper(I), which can then catalyze formation of ·OH. Under the reducing conditions present during ischemia and reperfusion, copper can be expected to be a very efficient source of electrons needed to react with H$_2$O$_2$ to form ·OH.

In the present study, evidence has been presented demonstrating that the copper-loaded heart is more severely damaged during ischemia and subsequent reperfusion. The increased damage appears to be related to increased formation of oxygen-derived free radicals. Thus, these studies suggest that copper is an important catalyst of free radical formation and form the basis for further studies to better define the role of this metal as a potential mediator of postischemic reperfusion injury.

**References**

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