Free Rad. Res., Vol. 27, pp. 627–636
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Nitroxide Radicals Prevent Metal-aggravated Reperfusion Injury in Isolated Rat Heart

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Accepted by Prof. JMC Gutteridge

(Received 19 June 1997; In revised form 30 July 1997)

The effects of Cu(II) and the stable nitroxide radical 4-OH-2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (TPL) on reperfusion injury following global myocardial ischemia have been studied using the isolated rat heart model in the Langendorff configuration. Hearts were equilibrated with Krebs-Henseleit buffer (KH-buffer) for 10 min and subjected to 18 min of normothermic global ischemia. After 20 min reperfusion, hemodynamic parameters recovered as follows: ventricular developed pressure (77%), dP/dt (71%) and -dP/dt (80%), heart rate (91%), and work index (70%). End-diastolic pressure was 16 mm Hg. When 10μM Cu-nitrilotriacetate or Cu-(histidine)$_2$ was included in the perfusate before, during, and following ischemia, the heart injury was more extensive and the work index only recovered to 17% of the preischemic value. The inclusion of 100μM TPL during reperfusion abolished the copper-induced sensitization. In the absence of copper, TPL did not provide any protection against ischemia-reperfusion damage to the heart. The inclusion of 100μM 1,4-dihydroxy-2,2,6,6-tetramethylpiperidine (TPL-H) during reperfusion, partially abolished the copper-induced sensitization. Since conversion between TPL and TPL-H takes place, the fact that both forms provide protection can increase their protective efficacy.

Keywords: Transition Metals, Oxidative damage, Ischemia, Antioxidants, Hemodynamic parameters

Abbreviations: DP, developed pressure; EDP, end diastolic pressure; TPL, 4-OH-2,2,6,6-tetramethylpiperidine-1-oxyl; TPL-H, 1,4-dihydroxy-2,2,6,6-tetramethylpiperidine; SOD, superoxide dismutase; NTA, nitrilotriacetate; KH-buffer, Krebs-Henseleit buffer; WI, work index; dP/dt and -dP/dt, rate of contractility and relaxation, respectively; EPR, electron paramagnetic resonance.

INTRODUCTION

Transition metals like iron and copper play a complex role in ischemia-reperfusion cardiac injury. On one hand, copper complexes were found to inhibit the oxidative stress. Hearts of spontaneously hypertensive rats were better preserved if they were perfused with KH-buffer containing 0.5 mM Cu(II)$_2$(aspirinate)$_4$.[1] On the other hand, redox-active transition metals catalyze $^{\cdot}$OH production through the Haber-Weiss

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reaction. The copper-induced increase of myocardial injury induced by reactive oxygen-derived species should be considered in view of reports that cardiomyopathy and arrhythmias are a component of Wilson's disease, the major recognized clinical syndrome of copper overload.[2–3] This is the first reason, why copper, rather than iron, has been chosen in this study. Secondly, in chemical, biochemical and cellular systems, copper has been demonstrated to be up to 60-fold more reactive than iron in catalyzing ·OH formation and associated oxidative damage.[4–5] Lastly, Powell *et al.* recently demonstrated that copper-loading of hearts increases severity of postischemic injury, release of lactate dehydrogenase and ·OH formation, demonstrating the importance of this metal in mediating oxidative injury to the postischemic heart.[6]

Various therapeutic strategies have attempted to reduce biological damage either by scavenging free radicals already formed or by intervening in their formation process. Varying degrees of protection have been obtained using ·OH scavengers,[7–8] spin traps,[9] chelators of iron and copper,[10–11] inhibitors of lipid peroxidation,[12] antioxidant enzymes such as superoxide dismutase and catalase[7–8] and zinc to displace redox-active metal ions.[4–5,13] The practical application of these compounds is limited, however, by their side effects, low solubility, pharmacokinetics and cell infiltrability, the requirement for preischemic loading of the tissue, and the incomplete degree of protection they provide even under the best conditions.

Nitroxides are stable cyclic radicals which readily enter cells and exhibit superoxide dismutase-mimicking activity,[14] oxidize reduced metals, and react with carbon- and oxygen-centered free radicals.[15] Such activities suggest a prime role for nitroxides in the prevention of biological damage induced by radicals. Furthermore, the low toxicity of nitroxides makes them good candidates for *in vivo* use. So far, nitroxides have been shown to prevent oxidative damage in cell cultures[14] and laboratory animals.[16–17]

This study focuses on the antioxidative activity of TPL in an experimental model of isolated rat heart and the elucidation of the mechanisms underlying its protective and potential adverse effects.

MATERIALS AND METHODS

Materials

4-OH-2,2,6,6-tetramethylpiperidine-1-oxyl (TPL), copper(II) chloride, nitrilotriacetic acid-disodium salt, histidine, chelating resin (iminodiacetic acid), conalbumin were obtained from Sigma Chemical Co. 1,4-dihydroxy-2,2,6,6-tetramethylpiperidine (TPL-H) was prepared by catalytic reduction of TPL using H_2 bubbling in the presence of the catalyst platinum for 45 min,[18] or by bubbling HCl gas through an ethanolic solution of TPL followed by drying. Because TPL-H is readily oxidized to TPL, it was kept frozen and fresh solutions were prepared daily right before the experiment.

Animals

Sprague-Dawley male rats (250–350 g) were used and allowed ad libitum access to standard laboratory stock diet and water. Rats were injected with sodium heparin (500 units, i.p.), 30 min prior to anesthesia with sodium pentobarbital (60 mg/kg, i.p). Hearts including a segment of ascending aorta attached were rapidly excised, put in ice-cold heparinized saline, and then orthogradely perfused in the Langendorff configuration. The aorta was ligated to a plastic cannula, rather than to a metal piece, and connected to a water-jacketed column which maintained the heart and perfusate at 37°C. The perfusate entered the hearts at a pressure of 85 cm H_2O.

Unless otherwise stated, perfusion was performed with freshly prepared "blood-free" modified Krebs-Henseleit (KH) buffer, containing 118 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glu-

cose and 1.2 mM KH_2PO_4. In certain experiments phosphate was omitted from the buffer. To remove adventitious redox-active iron the buffer was treated overnight with 50g/l conalbumin followed by dialysis and with 40g/l chelating resin (iminodiacetic acid) to remove adventitious redox-active copper.[19] Copper, when desired, was added together with 2-fold excess of NTA or histidine. Once in the water jacketed column, the perfusate was continuously gassed with 95% O_2/5% CO_2, maintaining pH7.4. The hemodynamic parameters of cardiac function were continuously monitored.

Experimental Protocol

The typical experiment included preischemic perfusion of 10–30 min, to allow stabilization of the hemodynamic parameters. Then, 18 min of *no-flow* normothermic global ischemia was produced by cutting off the flow to the aorta, followed by 20 min of reperfusion (Fig. 1). Unless otherwise stated, additives such as copper and/or TPL were added 10–20 min before ischemia and included in the perfusate throughout the whole experiment.

Indexes of Cardiac Function

Six indicators of cardiac function were determined: ventricular developed pressure (DP), rate of contractility and relaxation dP/dt and -dP/dt respectively, heart rate, end-diastolic pressure (EDP) and work index (WI), which equals to: DP × heart rate.

Electron Paramagnetic Resonance (EPR) Measurement

To determine nitroxide concentrations, samples were drawn by a syringe into a long capillary. The EPR spectra were recorded on a JEOL JES-RE3X ESR spectrometer working at X-band with center field set at 3292 G field, modulation amplitude 0.2 G, and 4 mW incident microwave power.

Statistical Analysis

All data are presented as mean ±SEM. Statistical analysis was performed using the Student test and, in the cases of abnormal distribution, Mann-Whitney test. $P < 0.05$ was considered significant.

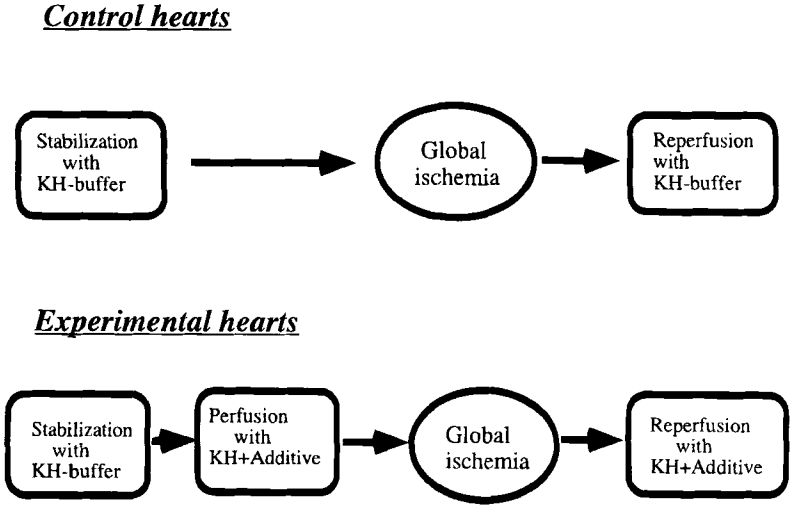


FIGURE 1 *Schematic Representation of Schedule for Normoxia, Ischemia and Reperfusion of Isolated Rat Hearts.*

RESULTS

The Effect of Copper on Reperfusion Injury

In control experiments, hearts were subjected to ischemia (18 min) and reperfusion (20 min) using metal-free perfusate. Under such conditions most of the heart function, impaired during 18 min ischemia, was restored upon reperfusion, as shown in Figs. 2 & 3. The addition of 10μM Cu-NTA or 40μM Cu-bis-histidine to the perfusate, significantly aggravated the reperfusion injury, as manifested by an increase in EDP and a decrease in the recovery of DP, dP/dt and -dP/dt, heart rate, and WI. Figure 2 displays the mean (±SEM) of the recovered hemodynamic parameters as % of their respective pre-ischemic values, whereas Figure 3 shows the EDP value measured following reperfusion. Control experiments showed that unless the heart was subjected to ischemia/reperfusion, copper exerted no adverse effect on the cardiac function whether or not phosphate was included in the perfusate under normoxia (data not shown).

To test whether Cu-induced aggravation of reperfusion injury requires long exposure to the metal, the experiment was repeated (n = 6) wherein 10μM Cu-NTA was included in the perfusate only at the first 5 min of reperfusion. The mean recovery, presented as percent of the pre-ischemic value (shown in parentheses) ±SEM, of the hemodynamic parameters after reperfusion was: DP = 13 ± 5% (104.4 ± 3.7 mmHg), dP/dt = 23 ± 8% (2228 ± 27 mmHg/sec), -dP/dt = 35 ± 13% (1557 ± 62 mmHg/sec), Heart rate = 56 ± 20% (214 ± 7.6 min^{-1}), WI = 16 ± 6% (2.19 ± 0.1 · 10^4 mmHg · min^{-1}) while EDP was 61 ± 4 mmHg. Comparing these results with those shown in Figures 2 and 3 shows that the recovery of the hemodynamic parameters of this group was similar to that observed when copper was included throughout the entire experiment.

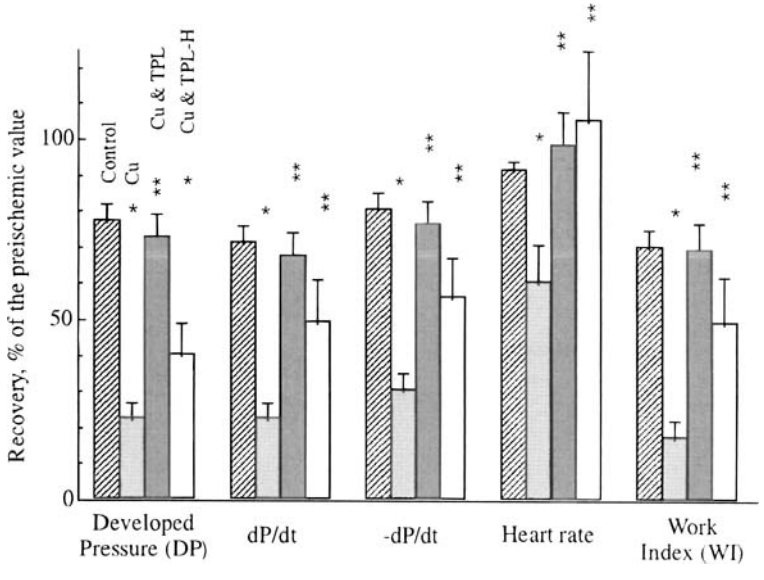


FIGURE 2 *Aggravation by Copper of Impairment of Post-ischemic Hemodynamic Parameters of Isolated Rat Heart and the Protection by Nitroxide and Hydroxylamine.* Isolated rat hearts were perfused with KH-buffer in the absence and the presence of 10 µM Cu alone or Cu with 100 µM nitroxide (TPL) or hydroxylamine (TPL-H), subjected to 18 min of global ischemia and then reperfused for 20 min. The perfusate was continuously gassed with 95% O_2/5% CO_2, maintaining pH7.4. The hemodynamic parameters DP, dP/dt, -dP/dt, heart rate and work index were measured, the recovery was presented as % of their respective preischemic values and expressed as mean ± SEM from 7–20 independent experiments. * significantly lower than control. ** significantly higher than Cu-treated group.

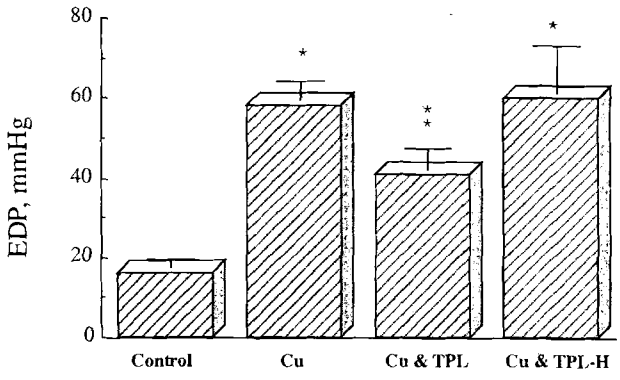


FIGURE 3 *The Effect of Nitroxide (TPL) and Hydroxylamine (TPL-H) on End-Diastolic Pressure (EDP).* Isolated rat hearts were perfused with KH-buffer in the absence and the presence of 10 μM Cu alone or Cu with 100 μM nitroxide (TPL) or hydroxylamine (TPL-H), subjected to 18 min of global ischemia and then reperfused for 20 min. The perfusate was continuously gassed with 95% O_2/5% CO_2, maintaining pH7.4. The end-diastolic pressure (EDP) was measured and expressed as mean ± SEM from 7–20 independent experiments. * significantly higher than control. ** significantly lower than Cu-treated group.

This further indicates that the critical injury takes place during the first 5 min of reperfusion.

The Effect of TPL on Reperfusion Injury

TPL in the perfusate in the absence of copper did not provide any protection against ischemia/reperfusion damage (data not shown). On the other hand, TPL prevented the Cu-induced aggravation of reperfusion injury. Statistically, the recovery of normal function of hearts perfused with Cu in the presence of 100–200μM TPL was significantly higher (1-tail t-test), than that of hearts perfused with Cu alone (Fig. 2). Accordingly, EDP was significantly lower in the presence of TPL than that measured in hearts treated with copper alone.

To check if TPL fully protects against post-ischemic damage or only against the Cu-induced aggravation of reperfusion injury, the recovery of hemodynamic parameters of hearts treated with Cu+TPL was compared with that determined for control. No statistically significant difference (2-tail t-test) was found (see Fig. 2). The EDP measured for the Cu+TPL treated group was significantly higher (1-tail Mann-Whitney), than that found for the control group. It can be concluded, that TPL modifies the Cu-induced aggravation of reperfusion injury but does not fully prevent the damage.

The Effect of TPL-H

Nitroxides, such as TPL, are rapidly reduced in cells to their respective hydroxylamines. To check if the reduced form of TPL contributes to the protective effect, TPL-H was substituted for the nitroxide in the perfusate. Since air can gradually oxidize TPL-H, the hydroxylamine was added to the perfusate immediately before reperfusion. TPL-H at 100μM decreased, though less effectively than TPL, the Cu-induced aggravation of reperfusion injury as reflected by the improved recovery of dP/dt, -dP/dt, heart rate, and WI. Yet, the values of DP and EDP did not differ from those measured for Cu-treated group (Figs. 2 & 3).

Since TPL-H is readily oxidized, the possibility that its protective effect results from traces of TPL, present in the perfusate, was examined. In a parallel chemical experiment the extent of TPL-H oxidation, under conditions similar to those used in the Langendorff system, was examined by EPR spectrometry. 100μM TPL-H was incubated in perfusate, saturated with O_2/CO_2 (95:5), and samples were taken every 1 min and scanned for TPL signal. The results demonstrated, that less than 9μM of TPL-H were oxidized during 20 min. When the effect of 10 μM TPL on Cu-induced aggravation of reperfusion injury in the isolated heart was studied, no protection was observed (data not shown).

Phosphate Effect

To minimize effects of adventitious transition metals, which generally contaminate phosphate salts,[10,19] several experiments were conducted using phosphate-free KH buffer. By large, the function of hearts perfused with or without phosphate was similar. However, in the absence of phosphate

in the perfusate the heart exhibited enhanced sensitivity to insults, such as ischemia. In fact, the normal function of isolated hearts treated with phosphate-free perfusate lasted for shorter periods, possibly due to a progressive depletion of cellular P_i and subsequently an impairment of oxidative phosphorylation. Interestingly, the inclusion of 50–200 μM TPL in phosphate-free perfusate during normoxia impaired the heart function within 10 min (data not shown). Therefore, for studying the TPL effect on ischemia/reperfusion injury in the absence of phosphate, TPL (100μM) was added to the perfusate only at the onset of reperfusion rather than 10 or 20 min before ischemia. Under such experimental conditions, the protective effect of TPL against copper-induced potentiation of ischemia/reperfusion injury was similar to that exerted by TPL in KH buffer supplemented with phosphate.

Nitroxide Reaction With Copper

The possibility that the protective activity of nitroxides against copper-induced aggravation of reperfusion injury derives from their interaction with Cu(II) ions, was tested. An excess of $CuSO_4$ was added to 20μM TPL and the EPR signal of the nitroxide was recorded. Neither the shape nor the intensity of the EPR signal of TPL was affected by Cu(II), thus excluding the possibility of reaction or complex formation.

To check for a reaction of the nitroxide with copper(I), 20μM TPL was mixed with tetrakis (acetonitrile)copper(I) hexafluorophosphate. This salt is water-soluble, yet, despite the strict anoxia maintained during preparation of the solution and while adding TPL, the solution always contained some Cu(II). Figure 4 displays the EPR spectrum of TPL measured under anoxia in the absence and the presence of tetrakis(acetonitrile)copper(I) hexafluorophosphate (nominally 50 μM). The signal which immediately disappeared upon exposure to Cu(I), was restored upon subsequent exposure of the reaction mixture to air (Fig. 4) or after an addition of 1 mM $K_3Fe(CN)_6$.

DISCUSSION

The Role of Metals

The effect of transition metals, such as copper or iron on reperfusion injury is well recognized. Traces of soluble iron or copper can catalyze the transformation of $O_2^{\cdot-}$ to hydroxyl radical *via* the metal-catalyzed Haber-Weiss reaction. It has been previously shown using a similar model, that iron at 10μM increased injury to isolated rat hearts.[20]

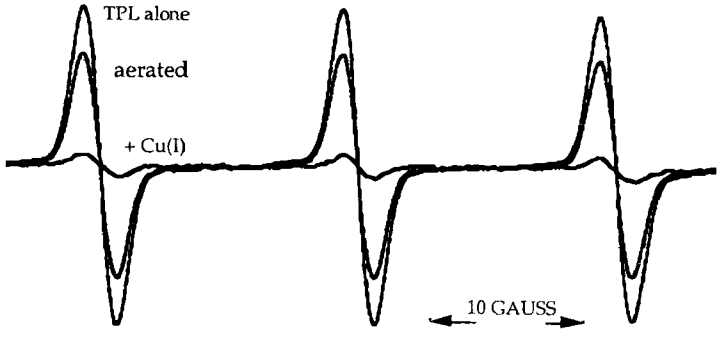


FIGURE 4 *The Reduction of TPL by Cu(I).* The EPR spectrum of 20μM TPL in the absence and the presence of 50μM tetrakis (acetonitrile)copper(I) hexafluorophosphate, prepared, mixed and recorded under anoxia, using a JEOL JES-RE3X ESR spectrometer working at the X-band with center field set at 3292 G, 0.2 G modulation amplitude, and 4 mW incident microwave power. Later, air was allowed into the solution and the spectrum was recorded again.

Administration of an iron chelator, such as deferoxamine, improved the postischemic myocardial function,[11] diminished separation of myofilaments and mitochondrial swelling[21] and preserved membrane phospholipids in the ischemic heart.[22]

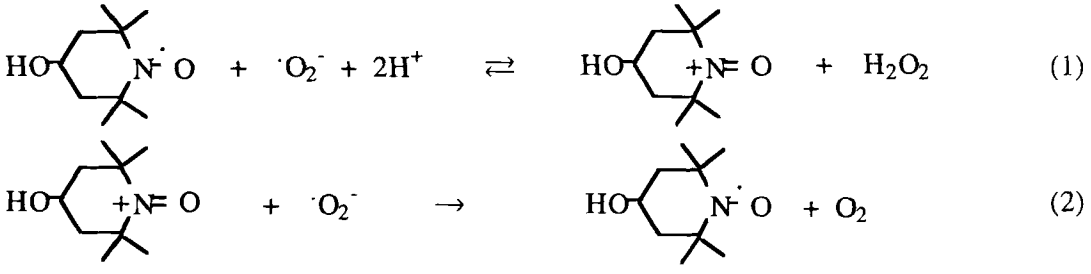
The effect of copper is more complex. The possibility that the properties of a metal can be altered by the complexing or chelating agent, has been previously discussed by many investigators. Chelates, such as $Cu(II)_2(3,5\text{-}DIPS)_4$ were found to lower reperfusion injury in a cat mesenteric model.[23] The protective effect of $Cu(II)_2(3,5\text{-}DIPS)_4$ was attributed to its SOD-mimetic activity.[23] Pharmacologic doses of copper complexes showed antisecretory effect and thus prevent lowering of gastric pH below 6.[24] Furthermore, also EDTA-Cu(II) demonstrated SOD-mimic activity.[25] On the other hand, it has been demonstrated, that copper-loading of hearts increases severity of reperfusion injury, suggesting the importance of this metal as a mediator of oxidative injury to the postischemic heart.[6,10] Obviously, copper can act either as a pro-oxidant or an antioxidant, depending on the ligand and the test-system, thus explaining apparent previous discrepancies between reports of protective and adverse activities of copper.

The present results confirm previous conclusions[6] that loading hearts with copper worsens reperfusion injury. In the present study copper was added chelated to NTA or histidine. These ligands bind copper strongly enough to keep it in the solution, yet enable its coordination to cellular components and, thus, its participation in the process of site-specific injury.

The Effect of Nitroxide and Hydroxylamine

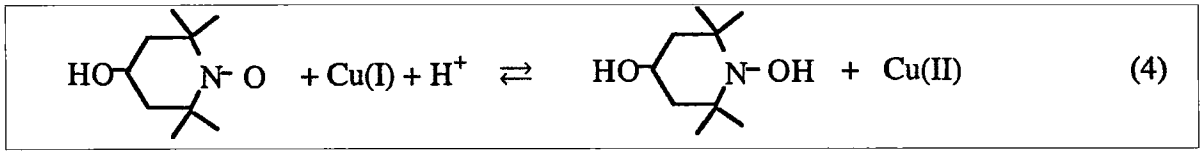
As shown in Figures 2 and 3, TPL and to a lesser extent TPL-H, prevented the Cu-induced aggravation of reperfusion injury, as reflected by the improved recovery of the hemodynamic performance of the heart during the reperfusion phase.

Nitroxides constitute a unique class of antioxidants. The mechanisms, which underlie the protective effect, most likely include SOD-mimicking activity of nitroxides. Unlike SOD, nitroxides can enter the cell and act as intramolecular SOD-mimic which removes both extra- and intracellular superoxide, through an exchange between two oxidation states, namely: nitroxide and oxo-ammonium cation.[26]



$$2{\cdot}O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (3)$$

In the present case, it is more likely that the protective effect of TPL results from its reaction with Cu(I) ions. Cu(II) is metabolically reduced to Cu(I) in the cells, particularly under ischemic conditions. TPL can oxidize Cu(I) to Cu(II) as evidenced by the loss of the EPR signal of the nitroxide (Fig. 4). The restoration of the signal by $K_3Fe(CN)_6$ or air further indicates the formation of TPL-H from TPL through the oxidation of Cu(I):



Thus, through reaction 4, TPL can pre-empt the Fenton reaction and the formation of secondary ·OH radicals.

Additional mechanisms contribute toward protection because TPL-H, which cannot oxidize Cu(I), inhibited the copper-potentiated reperfusion injury (Figs. 2 & 3). The possibility that TPL-H itself has no protective activity and protection is due to traces of TPL, is excluded as 10μM TPL failed to protect. Yet, the hydroxylamine can neither oxidize Cu(I) nor remove superoxide radicals. It has been shown, using the same model, that cyclic hydroxylamine protected against reperfusion injury. This effect was attributed to the antioxidative effect of hydroxylamine against lipid radicals (L·, LO·, LOO·) and its ability to reduce them.[27–28] It is possible that a similar mechanism is operative also in the present experimental model.

CONCLUSIONS

The present research shows that: a) copper markedly potentiates the post-ischemic injury, primarily during the first 5 min of reperfusion; b) nitroxide antioxidants, such as TPL, protect the isolated rat heart from ischemia-reperfusion injury; c) nitroxides do not prevent cardiac damage to heart perfused with metal-free solution. Rather, it fully abolishes the copper-induced injury; d) TPL-H, the reduced form of TPL, also demonstrates protective activity, although lower than that of TPL; e) the oxidation of the reduced metal seems to be the major mechanism underlying the protective activity of the nitroxide; f) because exchange between TPL and TPL-H takes place, the fact that both forms provide protection, can increase their protective activity.

Acknowledgments

This study was supported by research grants from the Faculty of Medicine, The Hebrew University of Jerusalem, Israel (A.S. & M.C.), by the Austrian Organization of the Friends of the Hebrew University of Jerusalem, by the Fond zur Forderung der Wissenschaftlischen Forschung (FWF), Vienna (M.C.), and by grant #309/931 from the Israel Science Foundation of the Israel Academy of Science (A.S.).

References

[1] Fantholahi, M., Azodi, M., Dollwet, H. and Ely, D. (1988). The beneficial role of copper aspirinate in rat heart preservation, *Ohio Journal of Science*, **88**, 19–20.
[2] Factor, S., Cho, S., Sternleib, I., Scheinberg, I. and Goldfisher, S. (1982). The cardiomyopathy of Wilson disease, *Virchows Archives*. **397**, 301–311.
[3] Kuan, P. (1987). Cardiac Wilson's disease, *Chest*. **91**, 579–583.
[4] Chevion, M. (1988). A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals, *Free Radicals in Biology and Medicine*. **5**, 27–37.
[5] Korbashi, P., Katzhendler, J., Saltman, P. and Chevion, M. (1989). Zinc protects Escherichia coli against copper-mediated paraquat-induced damage, *Journal of Biological Chemistry*, **264**, 8479–8482.
[6] Powell, S., Hall, D. and Shih, A. (1991). Copper loading of hearts increases postischemic reperfusion injury, *Circulation Research*. **69**, 881–885.
[7] Woodward, B. and Zakaria, M. (1985). Effect of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart, *Journal of Molecular and Cellular Cardiology*. **17**, 485–493.
[8] Manning, A., Bernier, M., Crome, R., Little, S. and Hearse, D. (1988). Reperfusion-induced arrhythmias: a study of the role of xanthine oxidase-derived free radicals in the rat heart, *Journal of Molecular and Cellular Cardiology*, **20**, 35–45.
[9] Hearse, D. and Tosaki, A. (1987). Reperfusion-induced arrhythmias and free radicals: studies in the rat heart with DMPO, *Journal of Cardiovascular Pharmacology*. **9**, 641–650.
[10] Applebaum, Y., Kuvin, J., Borman, J., Uretzky, G. and Chevion, M. (1990). The protective role neocuproine against cardiac damage in isolated perfused rat hearts, *Free Radical Biology & Medicine*, **8**, 133–143.

[11] Ambrosio, G., Zweier, J., Jacobus, W., Weisfeldt, M. and Flaherty, J. (1987). Improvement of postischemic myocardial function and metabolism induced by administration of deferoxamine at the time of reflow: The role of iron in the pathogenesis of reperfusion injury, *Circulation*. **76**, 906–915.

[12] Nishida, T., Morita, S., Miyamoto, K., Masuda, M., Tominaga, R., Kawachi, Y. and Yasui, H. (1996). The effect of lazaroid (U74500A), a novel inhibitor of lipid peroxidation, on 24-hour heart preservation. A study based on a working model using cross-circulated blood-perfused rabbit hearts, *Transplantation*, **61**, 194–199.

[13] Powell, S., Saltmann, P., Uretzky, G. and Chevion, M. (1990). The effect of zinc on reperfusion arrhythmias in the isolated perfused rat heart, *Free Radicals in Biology and Medicine*. **8**, 33–46.

[14] Samuni, A., Krishna, C. M., Mitchell, J. B., Collins, C. R. and Russo, A. (1990). Superoxide reaction with nitroxides, *Free Radical Research Communication*, **9**, 241–249.

[15] Belkin, S., Mehlhorn, R., Hideg, K., Hankovsky, O. and Packer, L. (1987). Reduction and destruction rats of nitroxide spin probes, *Archives of Biochemistry and Biophysics*. **256**, 232–243.

[16] Rachmilewitz, D., Karmeli, F., Okon, E. and Samuni, A. (1994). A novel antiulcerogenic stable radical prevents gastric mucosal lesions in rats, *Gut*, **35**, 1181–1188.

[17] Beit-Yannai, E., Zhang, R., Trembovler, V., Samuni, A. and Shohami, E. (1996). Cerebroprotective effect of stable nitroxide radicals in closed head injury in the rat, *Brain Research*, **717**, 22–28.

[18] Rosen, G., Finkelstein, E. and Rauckman, E. (1982). A method for the detection of superoxide in biological systems, *Archives of Biochemistry and Biophysics*, **215**, 367–378.

[19] Powell, S., Hall, D., Aiuto, L., Warnir, R., Teichberg, S. and Tortolani, A. (1994). Zinc improves postischemic recovery of isolated rat hearts through inhibition of oxidative stress., *American Journal of Physiology*. **266**, H2497–H2507.

[20] Karwatowska-Prokopczuk, E., Czarnowska, E. and Beresewicz, A. (1992). Iron availability and free radical induced injury in the isolated ischemic-reperfused rat heart, *Cardiovascular Research*. **26**, 58–66.

[21] Badylak, S., Simmons, A., Turek, J. and Babbs, C. (1987). Protection from perfusion injury in the isolated rat heart by postischemic deferoxamine and oxypurinol administration, *Cardiovascular Research*, **21**, 500–506.

[22] Liu, X., Prasad, M., Engelman, R., Jones, R. and Das., D. (1990). Role of iron on membrane phospholipid breakdown in ischemic-reperfused rat heart. *American Journal of Physiology*, **259**, H1101–H1107.

[23] Hernandes, L., Grisham, M. and Granger, D. (1987). Effects of Cu-DIPS on ischemia-reperfusion injury. ed. (J. Sorenson) In: Sorenson, JRJ, Ed; Biology of copper complexes. Clifton, New Jersey: Humana Press. pp. 201–214.

[24] Sorenson, J. R. J. (1987). A physiological basis for pharmacological activities of copper complexes: An hypothesis. ed. (J. Sorenson) Biology of copper complexes. Clifton, New Jersey. Humana Press. pp. 3–10.

[25] Willingham, W. M. and Sorenson, J. R. J. (1987). pH-dependent superoxide dismutase-mimetic reactivity of copper(II)ethylenediaminetetraacetate. ed. (J. Sorenson) Biology of copper complexes. Clifton, New Jersey. Humana Press. pp. 467–473.

[26] Krishna, M., Grahame, D., Samuni, A., Mitchell, J. and Russo, A. (1992). Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide, *Proceedings of the National Academy of Sciences*, USA, **89**, 5537–5541.

[27] Komarov, P., Morgunov, A., Bilenko, M. and Zhdanov, R. (1992). Anti-ischemic effect of 1-hydroxy derivatives of nitroxyl bioantioxidants, in Bioactive spin labels, ed. (R. Zhdanov) Springer-Verlag, Berlin. pp. 491–506.

[28] Nilsson, U., Carlin, G. and Bulind-Fellenius, A. (1990). The hydroxylamine OXANOH and its reaction product, the nitroxide OXANO, acts as complementary inhibitors of lipid peroxidations, *Chemical-Biological Interactions*, **74**, 325–342.