Free Rad. Res. Comms., Vols. 12-13, pp. 691-696
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PROTECTION AGAINST FREE RADICAL-INDUCED AND TRANSITION METAL-MEDIATED DAMAGE: THE USE OF "PULL" AND "PUSH" MECHANISMS

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Free radicals have been incriminated in a variety of injurious processes including the toxicity of the herbicide paraquat and the damage following ischemia and reperfusion of different organs.

Based on the assumption that iron and copper could serve as mediators for the transformation of relatively low reactive species (such as superoxide radicals, hydrogen peroxide, ascorbate, and others) to the highly reactive species, in the site-specific metal-mediated mechanism, two new modes for intervention have been tried out. The first is the introduction of specific chelators that "pull" out redox-active and available metals, and by this reduce the apparent damage. Desferrioxamine was shown to protect bacterial cells and mammals against the poisonous effects of paraquat. Using the retrogradly perfused isolated rat heart, we have demonstrated that the chelator neocuproine, which effectively binds both iron and copper provides a major protection against hydrogen peroxide-induced cardiac damage and against ischemia/reperfusion-induced arrhythmias. Likewise, TPEN a heavy metal chelator, provides almost total (> 90%) protection against ischemia/reperfusion-induced arrhythmias.

The other mode of intervention is the use of redox-inactive metal ions that could compete for the binding sites of iron and copper, and by this "push" these metal ions out, lead to their displacement, and divert the site of free radical attack. Applying Zn(II) complexes provided a marked protection against metal mediated free radical-induced damage in the copper-mediated paraquat toxicity to *E. coli*, and in the arrhythmias induced by ischemia and reperfusion.

It is proposed that the complex zinc-desferrioxamine would be the ultimate protector being effective by both the "pull" and "push" mechanisms.

KEY WORDS: Chelation, desferrioxamine, zinc, neocuproine, TPEN, Zn-desferrioxamine.

ABBREVIATIONS: PQ^{+2}: paraquat, 1,1′-dimethyl,4,4′-dipyridinium dichloride; NTA: nitrilotriacetate; SOD: superoxide dismutase; TPEN: N,N,N′N′-tetrakis (2-pyridylmethyl)-ethylenediamine; Neocuproine: 2,9-dimethyl-1,10-phenanthroline; *P*: peak ventricular systolic pressure; DETAPAC: diethylenetriamine pentaacetic acid.

INTRODUCTION

The classical modes for intervention in free radical induced biological damage include the use of chemical scavengers and protecting enzymes.[1] These modes interfere with the deleterious processes by either competing for the injurious free radicals[2] or by compromising the production of these highly reactive species.[3,4] In view of the essential mediatory role of transition metals in the production of deleterious free radicals the use of chelators for protection has been proposed. Specific chelators for transition metals, like desferrioxamine, could tightly bind iron and copper and modulate the redox potential of these metal ions. These functions explain the observed protection afforded by such a "pull" out mechanism

Recently, we have employed a new mode for intervention in such processes. This

is based upon the similarity between the ligand chemistry of zinc on the one hand, and iron and copper on the other. When zinc ion, which is redox-inactive, is introduced into the system, it could compete for the binding sites of transition metals, displace these ions, divert the site of free radical production, and provide protection to the system under investigation.[1]

It is proposed that the ultimate mode for protection should include the use of zinc-desferrioxamine complex whose stability constant is 10^{14}.[5] Metal-free desferrioxamine is a randomly oriented linear molecule, consisting of several subunits, which does not penetrate easily into cells. Upon metal binding the desferrioxamine within its complex with zinc assumes a well-defined and organized globular structure.[5] This structural alteration is expected to render the zinc-desferrioxamine complex more permeable into cells than the metal-free species. It is visualized that within the cell, zinc-desferrioxamine would readily exchange with available iron or copper ("pull" mechanism) to yield ferrioxamine or copper-desferrioxamine complex, respectively. By this, controlled levels of zinc would be liberated and could act to further protect by "pushing" out additional redox-active metal ions from their binding sites. It is suggested that this combination of "pull" and "push" mechanisms could efficiently prevent the site-specific metal-mediated oxidative damage.

EXPERIMENTAL SYSTEMS

Paraquat toxicity

Following our demonstrations that either iron or copper are essential mediators for the manifestation of paraquat toxicity in bacteria[6-8] and animal[9] models, we have shown that chelation could eliminate the poisonous effect of paraquat in bacterial cells[6-8] (Figure 1) and could markedly decrease these effects in mammalian systems.[9]

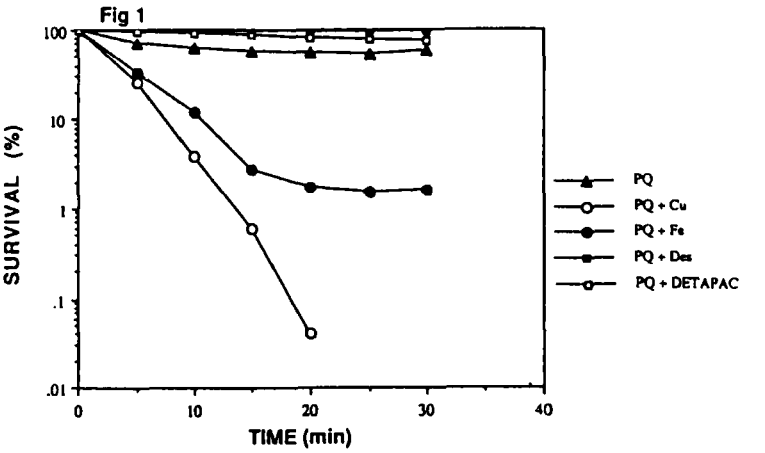

FIGURE 1 Effect of transition metals, iron and copper, and chelators, desferrioxamine and DETAPAC on paraquat-induced bacterial inactivation. All incubation systems contain 1×10^7 *E. coli* B cells/ml and glucose (0.5% w/w) in phosphate buffer (1.0 mM, pH 7.4). ▲ paraquat (0.25 mM); ○ paraquat (0.25 mM) together with copper(II) (1 μM); ● paraquat (0.25 mM) together with iron(II) (50 μM); ■ paraquat (0.25 mM) together with desferrioxamine (100 μM); □ paraquat (0.25 mM) together with DETAPAC (10 μM).

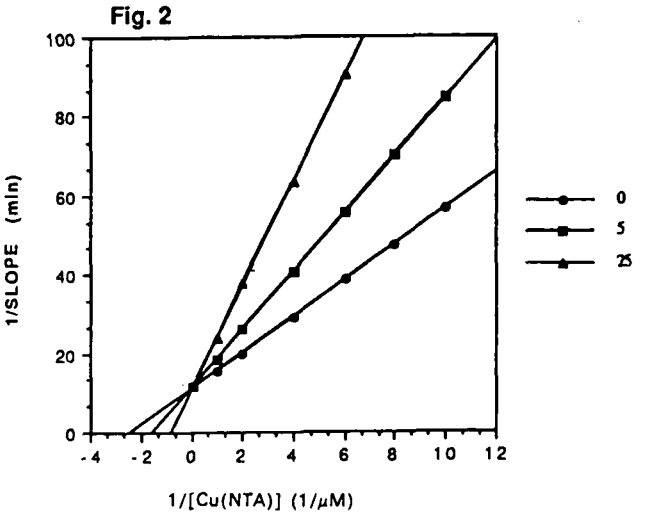

FIGURE 2 Double-reciprocal plots (inital killing rate)$^{-1}$ versus (Cu(II)-NTA)$^{-1}$ showing the relationship of Zn(II)-NTA and Cu(II)-NTA to PQ-induced bacterial inactivation. ● no Zn(II)-NTA added; ■ 5 μM Zn(II)-NTA; ▲ 25 μM Zn(II)-NTA.

Desferrioxamine, which is a clinically approved and widely used drug, is an effective agent in the system[9,10] that affords its protection by this "pull" out mechanism.

The introduction of zinc-NTA into the copper-mediated paraquat-induced bacterial inactivation has led to a competitive inhibition of the toxic effects of paraquat and to a corresponding cellular protection. Figure 2 shows the competitive mode of this protection. From the *slopes* of the bacterial survival curves, with paraquat and copper-NTA, in the absence and in the presence of two concentrations of zinc-NTA, the Lineweaver-Burk graph is evaluated. The intercepts for the three lines representing 1/slope versus 1/[Cu-NTA] for [Zn-NTA] = 0, 5 and 25 μM, are identical. This indicates that zinc competes for the copper binding site, and by this provides the observed protection against the toxicity of this herbicide. The study of other zinc complexes, including zinc-desferrioxamine, in bacterial model and in mammalian systems is underway.

Cardiac damage following ischemia and reperfusion

The effect of desferrioxamine on injurious processes to the heart has been studied.[11-14] We have recently investigated two amphiphilic chelators, which effectively bind iron and copper, and easily penetrate into cells. The model used involves regional ischemia, which is achieved by occlusion of the left main artery (LAD), of the isolated rat heart in the Langendorf configuration. Monitoring a variety of hemodynamic parameters, we could show that neocuproine, 2,9-dimethyl-1,10-phenanthroline, provides a marked protection to the reperfused heart (Table 1).[15] This protection can be seen from the extension (more than doubling) of the duration the heart spends in normal sinus rhythm, and the concomitant shortening in the duration of the ventricular fibrillation (Table 1). Likewise, the recovery, in the reperfusion phase, of the peak systolic pressure, P, and of $+dP/dt$ and $-dP/dt$ in the presence of neocuproine is markedly better than in control group (Table 1).

TABLE I
The effects of neocuproine and TPEN on hemodynamic parameters in the isolated perfused rat heart with transient coronary artery occlusion

Experimental Parameter	Control	Neocuproine (40 μM)	TPEN (10 μM)
Recovery of P, peak systolic pressure, in the reperfusion phase (% of pre-ischemic P)	23 ± 25	61 ± 37	82 ± 6
Recovery of $+dP/dt$ (% of pre-ischemic value)	21 ± 30	56 ± 33	81 ± 6
Recovery of $-dP/dt$ (% of pre-ischemic value)	17 ± 24	52 ± 29	73 ± 9
Incidence (%) of ventricular fibrillation at the end of 180 sec reperfusion	63	13	0
Duration of ventricular fibrillation (sec) in the first 180 sec of reperfusion	108 ± 75	49 ± 48	0
Duration of normal sinus rhythm (sec) in the first 180 sec of reperfusion	53 ± 53	115 ± 48	180 ± 0
Recovery of coronary flow (% of pre-ischemic flow) during the first 180 sec of reperfusion	88 ± 43	86 ± 38	89 ± 17

TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl)-ethylenediamine, has proven in our studies to be an even better protector against cardiac injury.[16] Using the same system, TPEN completely prevented the arrhythmias following the reperfusion. While in the control group (without chelators), >90% of the hearts fibrillate during the reperfusion phase, in the presence of TPEN, at concentration as low as 7.5 μM, they are totally protected. Other physiologic parameters (Table I) have indicated an analogous dramatic protection by TPEN in this model.

Similar protective results by neocuproine have been observed when the hearts were subjected to a different oxidative insult – inclusion of 240 μM of hydrogen peroxide in the perfusate, rather than occulsion of the LAD.[15] In other experimental models, TPEN has proven to be a highly effective protecting agent. These include reperfusion damage following 18 min of global ischemia and 3 hours of cardiac arrest with cardioplegic perfusate at 37°C.[15-20] The protection afforded by each of these chelators is in complete accord with the "pull" mechanism of protection.

In the same experimental model, when the perfusing medium contained zinc-histidine complex ($Zn(His)_2$) a marked protection was observed, as well. This protec-

TABLE II
The effects of zinc-histidine complex ($Zn(His)_2$) on hemodynamic and biochemical parameters in the isolated perfused rat heart with transient coronary artery occlusion

Experimental Parameter	[Zn] μM: 0 / [His] μM: 0	12.5 / 25	25 / 50	50 / 100	100 / 200	200 / 400	0 / 400
Duration of normal sinus rhythm in the first 5 min of reperfusion (sec)	6	49	104	128	207	255	28
Duration of ventricular fibrillation in the first 5 min of reperfusion (sec)	261	188	154	122	49	8	232
Recovery of P (% of pre-ischemic value)	< 20	n.d.	51	53	76	68	n.d.
Recovery of $+dP/dt$ (% of pre-ischemic value)	< 20	n.d.	38	52	71	64	n.d.
Protection against leakage of LDH to the perfusate (%)	0	n.d.	n.d.	n.d.	n.d.	65	22

tion can be seen from the impressive dose-dependent extension of the duration of the normal sinus rhythm during the reperfusion phase and from the concomitant concentration-dependent shortening of the duration of ventricular fibrillation (Table II). The protection to the heart is also indicated by the improved recovery of P, $+dP/dt$ and $-dP/dt$ in the experimental groups, when compared to the controls (no zinc-histidine complex and no histidine present). The decreased leakage of lactate dehydrogenase (LDH) from the cells to the perfusate, in the presence of $Zn(His)_2$, but not in the presence of histidine alone, provides an additional biochemical indicator for the protection to the heart by this "push" mechanism (Table II). The effect of the combination zinc-desferrioxamine is currently being studied.

DISCUSSION

The protective effect by the "pull" mechanism using a variety of substances that could function as chelators for iron and copper, in a broad spectrum of biological processes, in which free radicals have been implicated as causative agents, have been demonstrated.[1] The complex formed between such a chelator and iron or copper is often rather innocuous due to the removal of the metal from its biological target, so that even if the chelate is redox-active, protection could be expected.

An additional example for the protection by the "push" mechanism which is provided by zinc ions is the dramatic decreased levels of single- and double-strand breaks in iron- or copper-mediated and ascorbate-driven breakage in purified DNA.[21]

A major effort has been recently initiated in order to examine the expected beneficial effects of the zinc-desferrioxamine complex, in a variety of systems. Considering the high stability constant of zinc-desferrioxamine complex,[5] the predicted off-rate of zinc from this complex could be expected to be very low, and the resulting exchange rate could have proven too small to be of any biological relevance. Nevertheless, chemical experiments in our laboratory have confirmed that iron and copper indeed readily displace the zinc from the zinc-desferrioxamine complex.

The observed protection by Desferal-Mn(IV) complex[22] could be analogously explained. It could be visualized that the Desferal-Mn(IV) complex highly facilitates the infiltrability of Apo-desferrioxamine into cells, and there the zinc within Desferal-MN(IV) would readily exchange with available iron to yield ferrioxamine. These combined "pull" and "push" mechanisms would probably be more efficient in providing the observed protection than the SOD-mimicking activity of this complex.

Acknowledgements

This work has been supported by research grants from the Gesselschaft fur Strahlen und Umweltforschung, Neuherberg, Federal Republic of Germany; the National institute of Health (Grant ES-04296); and the Council for Tobacco Research (U.S.A.) (# 1900). The research and technical assistance of Ms. E. Gelfand in the preparation of this manuscript is gratefully acknowledged.

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Accepted by Prof. G. Czapski