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The Superoxide Dismutase-Mimic Copper-Putrescine-Pyridine Suppresses Lipid Peroxidation in CHO Cells. Implications for its Prooxidative and Antioxidative Mechanisms of Action

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Copper-Putrescine-Pyridine (Cu-PuPy) is a membrane-permeable complex which efficiently dismutates superoxide. In excess of 0.1 mM it is highly cytotoxic and oxidizes cellular GSH with concomitant production of H_2O_2. Here we show that treatment of CHO cells with 0.2 mM Cu-PuPy (0–200 min) leads to an accumulation of H_2O_2. Organic hydroperoxides which are also formed at low levels in the presence of Cu-PuPy, increase significantly after removal of the copper complex. We conclude that Cu-PuPy acts as an oxidant until cellular GSH is depleted. However, by interfering with radical chain propagation reactions, it suppresses lipid peroxidation and thus substitutes for consumed physiological antioxidants in a later stage of treatment. This consistently explains our previous, seemingly paradox, finding that longer Cu-PuPy treatments may be significantly less toxic than shorter ones.

Key Words: Copper complex, copper toxicity, hydrogen peroxide toxicity, lipid peroxidation, superoxide dismutation.

Abbreviations: CHO, Chinese hamster ovary; Cu-PuPy, *N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate; GSH, glutathione; GSSG, glutathione disulfide; SOD, superoxide dismutase (EC 1.15.1.1.).

INTRODUCTION

The stable, membrane permeable copper complex Cu-PuPy (Figure 1) catalytically dismutates superoxide at a comparable rate ($k_2 = 6 \cdot 10^8\ M^{-1}s^{-1}$) as Cu_2Zn_2-SOD.[1-3] Cu-PuPy may also be reduced by GSH ($k_2 = 1 \cdot 10^2\ M^{-1}s^{-1}$), thus entering a redox cycle which consumes O_2 and yields H_2O_2 and GSSG.[4] We have previously shown that Cu-PuPy exhibits a marked cytotoxicity towards CHO cells at concentrations exceeding a threshold of 0.1 mM.[5] The observed oxidation and loss of cellular GSH is compatible with the notion that H_2O_2 plays a primary role in Cu-PuPy cytotoxicity. We have further observed a surprising gain in clonogenic survival when cytotoxic Cu-PuPy

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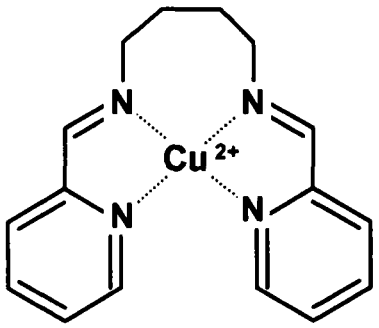

FIGURE 1 Structure of Cu-PuPy

treatments were extended over a specific period of time. Thus a 100 min treatment with 0.2 mM Cu-PuPy reduced cell survival to 10^{-6}, while a treatment for 150 min was significantly less toxic (10^{-3}).

Another clue to its interaction with cell survival was the finding that Cu-PuPy prevents usual consequences of oxidative injury, i.e. oxidation and loss of NAD(H) and NADP(H) and a deterioration of the cellular status of ATP. This was supposed to be due to the observed inactivation of glutathione reductase and presumably also of poly(ADP-ribose) polymerase by the copper complex.[6,7] It implies that in spite of the significant oxidative stress, cells remain metabolically intact and are thus amenable to repair by Cu-PuPy.

The unusual dependence of cell survival on the duration of treatment, and the fact that the addition of toxic doses of H_2O_2 did not further increase Cu-PuPy toxicity in a combined treatment[5] impose stringent mechanistic constraints on the interpretation of the prooxiative and antioxidative reactivities of Cu-PuPy. We concluded that 1. Cu-PuPy induces reversible damage by formation of H_2O_2, until cellular GSH is consumed; and 2. that Cu-PuPy prevents the delayed irreversible loss of clonogenicity by inactivating accumulated, H_2O_2-derived, reactive oxygen species. It is important to realize that the observed survival gain requires a time lag (i.e. a kinetic separation) between initial formation of damage and its repair. Hence, any direct interception of H_2O_2 (for instance catalase- or peroxidase-like reactivities), an inhibition of the Fenton reaction, or a scavenging of hydroxyl radical by Cu-PuPy could not account for this effect.

Our previous studies were hampered by the lack of a suitable method for quantification of hydroperoxides or of thiobarbituric acid reactive substances in the presence of Cu-PuPy, and therefore inferences on their role in the toxic mechanism of Cu-PuPy remained indirect. Here we were able to determine hydroperoxides by their ability to selectively oxidize Fe^{2+}-salts in dilute acids. The resulting Fe^{3+} forms a highly absorbing complex with xylenol orange.[8] In contrast to iodometric or peroxidase-coupled assays, this assay was sufficiently sensitive and proved to be robust to interferences with Cu-PuPy in the specific experimental setting.

MATERIALS AND METHODS

Materials

Cu-PuPy was synthesized as described in ref.[1] Catalase (19,900 U/mg, EC 1.11.1.6) from bovine liver, allopurinol, xylenol orange, sorbitol, butylated hydroxytoluene and ammonium ferrous sulfate were obtained from Sigma Chemie (Deisenhofen, Germany). Cell culture medium, bovine sera and other medium additions were purchased from Gibco BRL (Eggenstein, Germany). All other reagents were obtained from Merck (Darmstadt, Germany). Tissue grade polystyrene culture flasks were from Greiner (Frickenhausen, Germany). Solutions of reagents were prepared with doubly quartz-distilled, pyrolysed water.

Culture and Treatment of Cells

Conditions of subculture and treatments were exactly the same as in our previous investigations.[5–7] In short, CHO cells were grown and treated in McCoy's 5A medium, supplemented

with 10% newborn calf and 5% fetal calf serum, 50 mg/l penicillin, 50 mg/l streptomycin, and 100 mg/l neomycin sulfate. For treatments, confluent cell monolayers were trypsinized and cells seeded at densities of $1 \cdot 10^6$ cells/ml in T_{25} flasks ($3 \cdot 10^6$ cells, for analysis of cell-bound hydroperoxides) or in T_{175} flasks ($30 \cdot 10^6$ cells, for analysis of hydroperoxides in the medium). After two hours in the incubator, when cells were firmly attached, the medium was replaced by treatment medium, to which Cu-PuPy (20 mM) had been added immediately before to give a final concentration of 0.2 mM. All incubations, including Cu-PuPy treatments, were performed at 37°C and 5% CO_2 in a water-saturated atmosphere.

Water-soluble Hydroperoxides

The original FOX1 recipe[8] consists of 1 mM xylenol orange, 2.5 mM ammonium ferrous sulfate, 1 M sorbitol and 250 mM H_2SO_4 in water. Sorbitol efficiently scavenges alkoxyl radicals and converts them to hydrogen peroxide and hydroperoxyl radicals, which propagate the ferrous oxidation step. Thus it acts as a chain amplifyer and increases the yield of ferrous ion per mol H_2O_2.[8] Since Cu-PuPy interfered with this amplification reaction in a concentration-dependent manner, sorbitol was omitted here from the assay at the expense of a reduced sensitivity (Figure 2).

Four 900 µl medium aliquots received either water, catalase (5 µl 2 mg/ml), (H_2O_2 (45 µl, 10 mM) or catalase + H_2O_2, and were adjusted to 950 µl with water, mixed and reacted for 10 sec. Samples were then vortexed with 100 µl of modified FOX1-reagent and incubated at room temperature. After 30 min the samples were centrifuged and the absorbances read at 560 nm. The four readings allowed compensation for the minor nonspecific absorbance increase in samples containing 0.2 mM Cu-PuPy (0.045 AUFS/30 min) and assured specific quantification of H_2O_2 with a sensitivity of 0.5 µM.

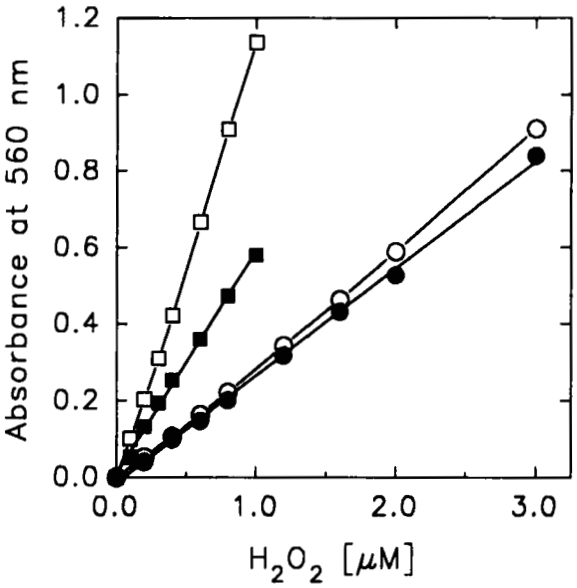

FIGURE 2 Quantitation of H_2O_2 in culture medium in the absence (open symbols) and the presence (closed symbols) of 0.2 mM Cu-PuPy, using the FOX1 assay with (□,■) or without sorbitol (○,●) as chain amplifier. Single experiment.

Methanol-soluble Hydroperoxides

At the end of Cu-PuPy treatments cell monolayers ($3 \cdot 10^6$ cells) were thoroughly washed with ice-cold saline and overlaid with 950 µl FOX2-reagent.[8] FOX2 consisted of 100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene and 25 mM H_2SO_4. One flask received 50 µl water, the other, as standard, 50 µl H_2O_2 (10 mM). After 10 min at room temperature the extracts were centrifuged to remove flocculated material. Absorbances were read after a total reaction time of 30 min. Under the treatment conditions used, Cu-PuPy concentration is maximally 0.2 µM in the final FOX2 assay mixture, and it was shown that background absorbance remained constant (0.076 ± 0.003 AUFS, $N = 8$) when cells were treated with 0–0.5 mM Cu-PuPy for 25 min. After substracting this background, the quantity of hydroperoxides was calculated in relation to the internal standard H_2O_2, which has the same apparent extinction coefficient as organic

peroxides such as cumyl or *n*-butyl hydroperoxides.[8] Recovery of H_2O_2, determined by spiking FOX2 samples of Cu-PuPy-treated cells (0–0.5 mM) with additional 50 µl H_2O_2 after the first reading, gave 93 ± 3% (N = 14).

Statistics

Means are given with their associated standard errors in the text or (as error bars) in the graphs. Half-lifes were determined by a nonlinear least squares fit of the data to an exponential decay curve.

RESULTS AND DISCUSSION

Formation of H_2O_2 During Treatment with 0.2 mM Cu-PuPy

CHO cells have a high enzymatic capacity to eliminate H_2O_2. Thus, while 100 µM H_2O_2 added to cell-free medium decayed exponentially with a half-life of 77 ± 9 min (N = 5), its half-life was reduced to 5.5 ± 0.1 min (N = 9) in the presence of cells. Since our previous investigations suggested a role for H_2O_2 in the cytotoxic mechanism of Cu-PuPy, it was of interest to test whether detectable amounts of H_2O_2, generated during Cu-PuPy catalysed oxidation of GSH, would actually survive in the presence of cellular antioxidant enzymes. Cu-PuPy itself proved to be remarkably inactive towards H_2O_2: in phosphate-buffered saline, pH 7.4, containing 8 mM Cu-PuPy, the half-life of 60 mM H_2O_2 was 183 ± 16 min (N = 8).

The results confirm the accumulation of micromolar quantities of extracellular H_2O_2 during Cu-PuPy treatment (Figure 3), suggesting that glutathione peroxidase and catalase cannot cope with the intracellular generation of H_2O_2. As discussed in more detail in ref.,[6] the irreversible inactivation of glutathione reductase by Cu-PuPy on the one hand curtails production of H_2O_2, but presumably also triggers the eventual complete breakdown of antioxidant defences by loss of cellular GSH. Thus, the kinetic properties of glutathione peroxidase predict that its activity rapidly declines when GSH levels fall below 10^{-4} M.[9] Such cytoplasmatic GSH concentrations are attained at 150 min or even earlier, considering that the measurements also comprised the slower depleting mitchondrial GSH pool.[6] Furthermore, this enzyme is known to be irreversibly inactivated when exposed to hydroperoxides in the absence of GSH. Similar effects are observed for SOD and catalase.[10] The time course shown in Figure 3 is compatible with the notion that H_2O_2 production ceases after about 100 min of treatment, and that accumulated H_2O_2 is then not further removed by glutathione peroxidase and catalase but that it slowly decreases by non-enzymatic reactions.

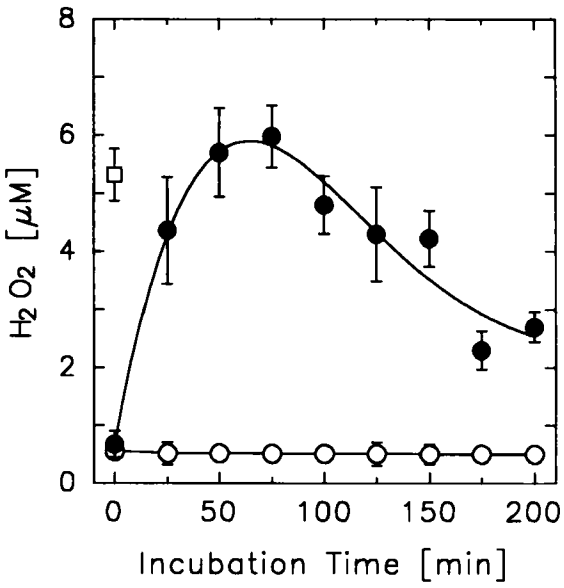

FIGURE 3 H_2O_2 content of culture medium containing 0.2 mM Cu-PuPy in the presence (●) and absence of CHO cells (○). Also shown is the recovery of 5 µM H_2O_2 (□) added to the treatment medium immediately before analysis. Results of four independent experiments. Other (not catalase-sensitive) hydroperoxides remained below the detection limit.

Suppression of Lipid Peroxidation by Cu-PuPy

Figure 4 shows that cellular organic hydroperoxides are produced in minor amounts during

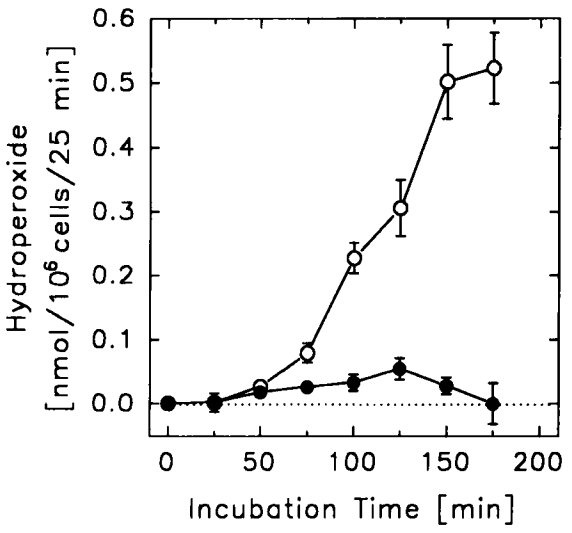

FIGURE 4 Formation of methanol-soluble hydroperoxides during continuous incubation of CHO cells with 0.2 mM Cu-PuPy and after its removal. At the indicated times t_i, cells were extensively washed free of Cu-PuPy and analysed for hydroperoxides immediately (●), or further incubated for 25 min in Cu-PuPy-free medium before analysis (○). For both curves, the formation rates were calculated from the concentration differences according to $r(t_i) = c(t_{i+25}) - c(t_i)$. Shown are results of three independent experiments.

Cu-PuPy treatment, and that their formation is significantly enhanced upon removal of the copper complex and further incubation for 25 min. It may reasonably be assumed that these methanol-extractable species are primarily lipid hydroperoxides, since any diffusible, water soluble hydroperoxides (i.e. H_2O_2) should have been removed from the cells by the extensive washing with saline. We therefore conclude that Cu-PuPy triggers and simultaneously suppresses lipid peroxidation. An inhibition of peroxidative radical propagation reactions by the complex is independently corroborated by its interference with the sorbitol-dependent amplification in the original FOX1 assay procedure.

Organic hydroperoxides are comparatively as reactive as H_2O_2 and may be slowly reduced to chain-initiating alkoxyl radicals.[11] But unlike the highly diffusible H_2O_2, they will be retained within cells upon washing. Therefore, they appear to qualify ideally as the required molecular species that 'store' damage for the time after removal of Cu-PuPy.

The complex is remarkably stable in a biological environment.[1,2,4] This suggests that only a minor proportion of the complex will undergo copper exchange reactions with serum albumin in the medium or with intracellular high-affinity copper-binding ligands, and that Cu-PuPy will secure a significantly enhanced cellular superoxide dismutating activity throughout the time course of our experiments.

It is therefore tempting to search for mechanisms where superoxide could play a toxic role under the specific experimental conditions. Superoxide is able to drive the decomposition of H_2O_2 by keeping Fenton-active metals in their reduced state. The kinetic arguments against hydroxyl radical being a target for protection by Cu-PuPy do not immediately rule out that inhibition of Fenton-like reactions by the complex may become important at a later stage of treatment. Thus one might conceive a situation where the production of chain-initiating species would depend on the re-reduction of consumed metals by superoxide. It is, however, unlikely that such a situation will develop in the presence of the complex since cellular reductants other than superoxide, like NADH or NADPH, can perform this role equally well,[12,13] and notably, these are available in constant amounts throughout Cu-PuPy treatment.[6,7] It has been reported that superoxide, in its protonated form, is capable to directly initiate lipid peroxidation by abstracting hydrogen from unsaturated fatty acids[14] and, more facile, from preexisting lipid hydroperoxides.[15] As a lipid soluble SOD-mimic, Cu-PuPy will presumably prevent these reactions, but as discussed, initiation reactions are expected to proceed via uninhibited Fenton pathways.

There is, however, no evidence for enhanced superoxide formation during Cu-PuPy treatment: a delayed generation of superoxide during purine catabolism, brought about by oxidative conversion of xanthine dehydrogenase to an oxidase[16] would provide an attractive mechanism for protection by Cu-PuPy, but is unlikely since no

degradation of purine nucleotides could be observed during treatment[6] and the enzyme inhibitor allopurinol (100 μM) did not affect cell survival (not shown). Further, the unchanged cellular status of adenylates and their high energy charge[6] suggest that mitochondrial function is not impaired, and thus argues against an increased formation of superoxide during respiration. And last but not least, the fact that the complex completely prevents the cytotoxicity of added H_2O_2 in a short (50 min) treatment[5] provides another, crucial, argument against a contribution of superoxide to the toxicity of a Cu-PuPy treatment.

However, catalysed dismutation of superoxide may not just serve to prevent superoxide toxicity. Rather, its influence on the equilibrium of reactions involving superoxide may be of similar biological importance. The recently published 'radical sink'-theory[13] is based on this concept and proposes that SOD kinetically drives electron transfers from reducing radicals onto oxygen by removing superoxide. Overall, spontaneous electron flow is thus reverted and oxygen used as terminal antioxidant. GSH would assist in this process as a scavenger of oxidizing radicals to yield the reducing $GSSG^{\bullet-}$ radical, which reacts with oxygen to give GSSG and superoxide. Such a general antioxidative role of SOD would equally apply to the efficient SOD-mimic Cu-PuPy. The efficiency of this process will certainly be compromised by the observed decrease of cellular GSH, but it should be kept in mind that GSH depletion will in parallel attenuate production of oxidants.

Another application of the above concept is also worth to be seriously considered. It relies on the fundamental reversibility of the reported hydrogen transfer[15] from lipid hydroperoxides to superoxide: $LOOH + HOO\bullet \leftrightarrow LOO\bullet + H_2O_2$. Superoxide dismutation will clearly promote the reverse reaction and thus, ironically, support a role for H_2O_2 as reductant. The net result, including dismutation, is the irreversible reaction: $2\,LOO\bullet + H_2O_2 \rightarrow 2\,LOOH + O_2$. In principle such inhibition of chain propagation appears applicable to the specific conditions of our experiments, where significant amounts of H_2O_2 and a lipid soluble dismutation catalyst are present. Its actual efficiency however remains to be proven.

In conclusion, the available data suggest the following scenario. The prooxidative reactivity of Cu-PuPy, which depends on the oxidation of cellular GSH and which yields H_2O_2, causes depletion of GSH. When cellular antioxidants are consumed and glutathione peroxidase is inactive, organic hydroperoxides will accumulate. In this phase, the prooxidative reactivity of Cu-PuPy comes to a halt and its antioxidant reactivity, directed against peroxidative propagation reactions, gains in importance. Latently damaged, but still viable, cells now profit from further incubation with Cu-PuPy. If the complex is withdrawn at this stage, peroxidation reactions initiated from accumulated hydroperoxides will propagate unhindered and irreversibly damage cells.

This general scenario offers a consistent explanation for the counterintuitive survival gain in prolonged Cu-PuPy treatments. The experimental data are compatible with the notion that the protectivity of Cu-PuPy is due to its dismutation of superoxide, but they cannot rule out alternative mechanisms, for instance a stoichiometric scavenging of chain-propagating alkyl or peroxyl radicals by the reduced, Cu(I) form of the complex. Clearly, further studies are required to elucidate the precise antioxidative reactivities of Cu-PuPy. Independent findings, where catalytic dismutation of superoxide affords protection under conditions which exclude a toxic role of superoxide will be awaited with great interest.

Acknowledgement

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