Pergamon

Biochemical Pharmacology, Vol. 47, No. 3, pp. 555–562, 1994.

0006-2952/94 $6.00 + 0.00

INDUCTION OF OXIDATIVE STRESS AND PROTECTION AGAINST HYDROGEN PEROXIDE-MEDIATED CYTOTOXICITY BY THE SUPEROXIDE DISMUTASE–MIMETIC COMPLEX COPPER–PUTRESCINE–PYRIDINE

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(*Received* 9 *July* 1993; *accepted* 22 *September* 1993)

Abstract—The low molecular weight Cu_2Zn_2-superoxide dismutase (SOD) active centre analogue copper–putrescine–pyridine (Cu-PuPy, *N,N'*-bis(2-pyridylmethylene)1,4-butanediamine(*N,N',N'',N'''*)-Cu(II)-diperchlorate) has been shown to dismutate superoxide with high efficiency. In the presence of glutathione it sustains the production of H_2O_2 via redox cycling. We investigated the influence of Cu-PuPy on the glutathione status and the clonogenic survival of Chinese hamster ovary (CHO) cells. At 0.05 mM Cu-PuPY was not toxic and exerted only a minor effect on cellular glutathione. At Cu-PuPy concentrations of 0.1–0.5 mM glutathione became increasingly oxidized and was depleted during treatment while toxicity dramatically increased. The time course of toxicity was unusual: after passing a minimum at 50 or 100 min (0.5 mM or 0.2 mM Cu-PuPy, respectively), clonogenic survival increased by two orders of magnitude in the following 50 min. On the other hand, Cu-PuPy protected cells effectively against toxic doses of hydrogen peroxide. We conclude that Cu-PuPy combines a prooxidant and an antioxidant mode of action that sequentially modify the survival response of CHO cells: initial production of hydrogen peroxide by Cu-PuPy-catalysed glutathione oxidation leads to the intracellular accumulation of potentially toxic radical intermediates that may be inactivated via superoxide dismutation upon further treatment with Cu-PuPy.

Key words: reactive oxygen species, superoxide dismutation, SOD–mimetic copper compex, hydrogen peroxide toxicity, glutathione oxidation

Among the pharmacological effects ascribed to SOD-like† copper complexes are anti-inflammatory, antiulcer, anticonvulsant, antineoplastic, antidiabetic and radioprotective activities (for review see Ref. 1). Attempts were made to assign these properties to the ability of the complexes to dismutate superoxide, but other reactions certainly also contribute to some of the observed effects. In contrast to SOD, where the access to the active site is specifically restricted to $O_2^{\cdot-}$ (superoxide) by electrostatic barriers, low molecular weight copper complexes with SOD activity may also react with competing reducing agents present in a biological environment. Thus, the cytotoxicity of thiosemicarbazonato copper complexes to Ehrlich ascites tumor cells was assigned to their reaction with GSH and the generation of activated oxygen species [2, 3].

Cu-PuPy [*N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate] mimics both structure and function of Cu_2Zn_2-SOD (EC 1.15.1.1). As in the native enzyme, the tetradentate ligand is capable of coordinating both Cu(I) and Cu(II) [4]. Due to its distinct stability [4], its membrane permeability [5] and its high dismutation rate constant [6] the complex fulfills essential requirements of a pharmacological substitute for SOD. *In vivo* Cu-PuPy exhibited a pronounced anticarcinogenic activity in rats with implanted Walker 256 carcinosarcoma [7]. It reduced tumor size, delayed metastasis and led to a significant increase in host survival. Recently, Steinkühler *et al.* [5] have shown that the addition of GSH to Cu-PuPy resulted in glutathione oxidation, O_2 consumption and H_2O_2 generation. The stoichiometry indicated that redox cycling of Cu-PuPy catalyses the formation of H_2O_2 by oxidation of GSH to GSSG (glutathione disulfide). Other predominant cellular reducing compounds were also tested for their ability to reduce Cu-PuPy *in vitro*. Ascorbate proved to be less efficient than GSH by one order of magnitude, whereas NADH and NADPH failed to reduce the complex [8].

In eukaryotic cells the tripeptide GSH is present in millimolar concentrations and thus is the most abundant cellular non-protein thiol compound. Its important role in the detoxification of xenobiotics, regulation of thiol status, reduction of peroxides and regeneration of antioxidants, and its function as radical scavenger has been emphasized many times

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† Abbreviations: apo-PuPy, *N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine; L-BSO, L-buthionine-*S,R*-sulfoximine; CHO, Chinese hamster ovary; Cu-PuPy, *N,N'*-bis(2-pyridylmethylene)-1,4-butanediamine (*N,N',N'',N'''*)-Cu(II)-diperchlorate; GSH, reduced glutathione; GSSG, oxidized glutathione; PCA, perchloric acid; PBS, phosphate-buffered saline; ProtSSG, protein–glutathione mixed disulfide; SOD, superoxide dismutase.

(for review see Refs 9, 10). Cu-PuPy-mediated oxidation of GSH to GSSG would thus not only produce toxic H_2O_2 but could further aggravate cell damage since the detoxification of H_2O_2, or of organic peroxides and free radical species that are known to originate from H_2O_2, would be impaired by a decrease in GSH. Since Cu-PuPy is an effective SOD mimic and therefore intrinsically protective against oxidative damage, we were intrigued by the consequences that the diverse modes of action of this copper complex would have on antioxidative metabolism and the survival of cells.

MATERIALS AND METHODS

Chemicals. Cu-PuPy was synthesized as described [6]. Elemental analysis gave C: 36.50%; H: 3.33%; N: 10.08%; O: 26.50% (calculated: C: 35.73%; H: 3.56%; N: 10.42%; O: 25.29%). Copper-free PuPy was accordingly synthesized in the absence of the copper salt and not checked further for purity. Catalase from bovine liver (17,600 U/mg, EC 1.11.1.6), Cu_2Zn_2-SOD from bovine erythrocytes (EC 1.15.1.1), horseradish peroxidase type VI (EC 1.11.1.7), 3-methyl-2-benzothiazolinone hydrochloride, 3-(dimethylamino)benzoic acid, L-BSO, GSH, GSSG, bathophenanthroline disulfonic acid, iodoacetic acid, 1-fluoro-2,4-dinitrobenzene were obtained from Sigma Chemie (Deisenhofen, Germany). Dulbecco's PBS, McCoy's 5A medium, serum, trypsin, antibiotics and BME-vitamins were purchased from Gibco BRL (Eggenstein, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Tissue grade polystyrene culture flasks were purchased from Greiner (Frickenhausen, Germany). A gas mixture (calibration grade) of 95% N_2 and 5% CO_2 was prepared by Linde AG (Unterschleißheim, Germany).

Subculture and treatment of cells. CHO cells were grown in McCoy's 5A medium, supplemented with 10% (v/v) newborn calf and 5% (v/v) fetal calf serum, 50 mg/L penicillin, 50 mg/L streptomycin and 100 mg/L neomycin sulfate. Cells were maintained at 37° in a 5% CO_2 atmosphere and subcultured every 2 or 3 days. Subculture was performed by detachment of the exponential cells with 0.25% trypsin (without EDTA) and appropriate dilution with fresh growth medium. Routine control of growth parameters yielded population doubling times of 14–15 hr and plating efficiencies of 70–95%. For experiments, cells were allowed to reach confluency, which required a medium change and further incubation for a total of 48 hr. Cells were then trypsinized and seeded at densities of 3×10^6 cells/3 mL medium into T_{25} flasks (25 cm^2 growth area). After 2 hr in the incubator, when cells were firmly attached, treatment was started by replacing the medium with Cu-PuPy-containing medium (37°) and returning the flasks to the incubator. The treatment medium had been prepared immediately before by adding appropriate amounts of a sterile 20 mM Cu-PuPy solution in water. Depletion of glutathione to 5–10% of control values was achieved by treating cells with 2.5 μM L-BSO for 18 hr before and during Cu-PuPy treatment. L-BSO treatment alone reduced the surviving fraction of cells only insignificantly (0.94 ± 0.08). For an acute block of glutathione synthesis, 5 mM L-BSO was added to the cells 10 min before and was present during the whole Cu-PuPy treatment. Sterilization of additions was accomplished by filtering the stock solution through a 0.2 μM cellulose acetate filter (Schleicher & Schuell FP030). To study the role of oxygen for the clonogenic survival of Cu-PuPy-treated cells, treatments were performed in hypoxic medium and in an atmosphere of 95% nitrogen and 5% CO_2. Hypoxic medium was obtained by purging serum-free medium for 4 hr with the sterile gas mixture. For hypoxic after-treatments Cu-PuPy incubation was terminated by replacing the treatment medium with warm hypoxic medium while flushing the flasks with the gas mixture. The closed flasks were then placed for 2 hr at room temperature in a polypropylene box which was continuously flushed with the gas mixture.

The diameter of untreated trypsinized cells was distributed with a mean of 12.4 μm and a standard deviation of 1.4 μm as revealed by Coulter counter measurements. Quoted cellular concentrations are based on an assumed mean solvent volume of 10^{-12} L/cell.

Clonogenic survival assay. Treatments with Cu-PuPy were terminated by removal of treatment medium and rinsing the cell layer with 2×3 mL of ice-cold trypsin. Trypsinization was controlled in a microscope and finished after 3–3.5 min by addition of ice-cold growth medium. Cells were suspended thoroughly by suction with a pipette, diluted and plated for colony development into four to six replicate flasks in appropriate dilutions. Plating efficiency was 70–90% under these conditions. Colony development was detected and the surviving fraction determined as described [11].

Measurement of cellular copper binding. Cell layers were washed with PBS and extracted with 200 μL 10% PCA. The acid-insoluble layer remaining in the flask was solubilized with 200 μL of 0.5 N NaOH. Both extracts were analysed for total copper by flameless atomic absorption spectrophotometry on a Perkin–Elmer Zeeman 3030 spectrometer, using Cu-EDTA as standard.

Protein determination. Protein was assayed by the Lowry procedure [12], with bovine serum albumin as standard. The protein content of untreated cells was 0.11 ± 0.01 mg/10^6 cells ($N = 28$).

H_2O_2 *determination.* We used the chromogenic method of Ngo and Lenhoff [13] for H_2O_2 measurements. The assay is based on the H_2O_2-dependent, horseradish peroxidase-catalysed coupling of methylbenzothiazolinone and dimethylaminobenzoic acid to a purple indamine dye. The rate of its formation, measured at 590 nm, is proportional to the concentration of H_2O_2. Cu-PuPy strongly interfered with this assay. Also, direct photometric determination of H_2O_2 was not feasible in the presence of Cu-PuPy due to strong absorption of the copper complex in the UV region.

Determination of glutathione. Cell layers were washed twice with ice-cold PBS and immediately covered with 100 μL of cold 10% PCA, which contained 1 mM bathophenanthroline disulfonic acid as metal chelator and 150 μM γ-glutamyl-glutamate as internal standard. After 10 min at −20° the thawed

PCA extract was collected. The cell residue attached to the flask was dissolved in 0.5 mL 0.5 N NaOH and used for protein quantification, or was further processed for the determination of ProtSSG. In this case, the residue was washed twice with 0.5 mL cold ethanol, scraped off and treated with dithiothreitol to release glutathione from protein [14]. The reaction mixture was then brought to 10% PCA, and bathophenanthroline disulfonic acid and γ-glutamyl-glutamate were added. Derivatization, separation by HPLC and simultaneous analysis of GSH and GSSG in PCA extracts was performed as described by Fariss and Reed [14]. In this method, free thiol groups are protected by carboxymethylation with iodoacetic acid. Then, amino groups are reacted with 2,4-dinitrofluorobenzene to yield UV-absorbing dinitrophenyl derivatives. These are applied to a reverse-phase ion exchange column (μBondapak Amine, Waters) and eluted with a sodium acetate gradient in a water–methanol–acetic acid solvent at pH 4.5. The dinitrophenyl derivatives of GSH and GSSG are detected at 365 nm and quantified by relating peak areas to the internal standard.

Recovery of GSH and GSSG in the presence of high amounts of Cu-PuPy was tested in assays containing γ-glutamyl-glutamate, GSH and GSSG (150 nmol each), 100 nmol bathophenanthroline disulfonic acid and 60 nmol Cu-PuPy. We found no hint of GSH oxidation when PCA was immediately added after Cu-PuPy. If acidification by PCA was delayed, GSH oxidized to GSSG and γ-glutamyl-glutamate was gradually lost. Its disappearance (10% after 30 min, 100% after 200 min) was not dependent on the presence of GSH or GSSG. Upon prolonged incubation we would also expect the formation of a Cu–glutathione complex [5, 8]. In any case more than 95% of total glutathione could be recovered after 200 min in the presence of Cu-PuPy. Thus, the regular conditions of extraction and immediate derivatization as described above allow the quantitative determination of glutathione but recovered GSSG may be partly derived from a copper–glutathione complex. Also, we do not expect significant intracellular *post mortem* oxidation of GSH by Cu-PuPy during extraction or derivatization.

Statistical treatment of data. Unless otherwise indicated, means with their standard errors are given in the text or graphs. When different cell dilutions were combined for the determination of surviving fractions, the replicates were internally weighted according to their colony counts (sampling variance), otherwise an equal weight was assigned to each flask. Mean surviving fractions and their standard errors were calculated in the logarithmic scale (geometric means). The positive slope b of a linear regression was tested against zero by comparing the quotient $t = b/s_b$ with a t-distribution of n-2 degrees of freedom. Independent time course experiments were not averaged in order to retain time resolution. Instead, the maximal deviation that was observed between replicate points is given.

RESULTS

Cellular binding of copper

We determined total cellular copper by atomic absorption spectrometry. Copper was bound by cells more effectively when added as Cu-PuPy than as $CuSO_4$. At an extracellular copper concentration of 0.2 mM initial binding rates were 90 ± 8 μM/min for Cu-PuPy compared to 1.8 ± 0.6 μM/min for $CuSO_4$. In the copper measurements we differentiated between the PCA-soluble and the PCA-insoluble fractions of cells. According to generally accepted assumptions, the acid-soluble fraction contains water-soluble low molecular weight molecules, whereas the acid-insoluble pool comprises macromolecules like proteins and DNA as well as lipid. After 50 min of Cu-PuPy incubation an equilibrium is reached with similar concentrations of extracellular copper and intracellular, acid-soluble copper. The high degree of binding of copper from Cu-PuPy-containing medium to the acid-insoluble fraction was remarkable. Thus, in cells treated with 0.2 or 0.5 mM Cu-PuPy a maximal accumulation of 9.3 ± 0.8 mM copper (calculated over the total cell volume) was observed after 100 min. Upon washing and further incubation for 100 min in Cu-PuPy-free medium, this value decreased to 5.1 ± 0.3 mM.

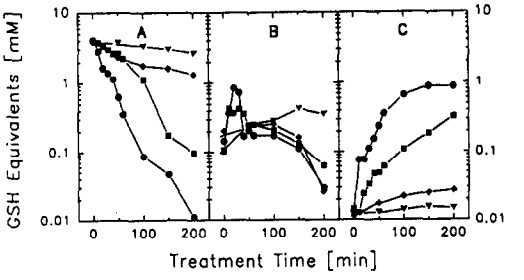

Fig. 1. Status of intracellular glutathione during treatment with 0.05 mM (▼), 0.1 mM (◆), 0.2 mM (■) and 0.5 mM (●) Cu-PuPy. (A) GSH, (B) GSSG, (C) ProtSSG. The maximal deviation between three similar experiments was 10% (GSH), 23% (GSSG), 12% (ProtSSG).

Effect of Cu-*PuPy on cellular glutathione*

Cu-PuPy-treated CHO cells lose glutathione at rates that are approximately proportional to the concentration of the copper complex in the medium. The glutathione concentrations shown in Fig. 1 were initially standardized to cellular protein and thus compensated for loss by membrane leakage. Protein loss was not detectable except in cells treated with 0.5 mM Cu-PuPy for 150 or 200 min (30 or 48% loss). Time periods for 50% depletion of free glutathione (GSH and GSSG), estimated from three independent experiments, were 162 ± 30, 70 ± 3 and 22 ± 1 min for 0.1, 0.2 and 0.5 mM Cu-PuPy, respectively.

The increase in intracellular GSSG (Fig. 1B) and the pronounced oxidation of proteins by formation of mixed disulfides with GSH (Fig. 1C) are indicative of the severe oxidative stress exerted by Cu-PuPy at concentrations above 0.1 mM. In untreated cells, 0.12 ± 0.02 nmol glutathione was bound to 1 mg protein. A small increase in protein oxidation was observed at Cu-PuPy concentrations as low as

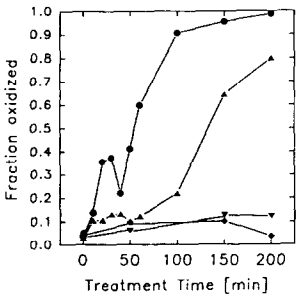

Fig. 2. Relative oxidation of intracellular glutathione during treatment with 0.05 (▼), 0.1 (◆), 0.2 (■) and 0.5 (●) mM Cu-PuPy. The oxidized fraction was calculated according to (2 GSSG + ProtSSG)/(GSH + 2 GSSG + ProtSSG). One of three similar experiments. The maximal deviation was 15%. The discontinuity between 30 and 60 min was reproducibly observed in the treatments with 0.2 and 0.5 mM Cu-PuPy.

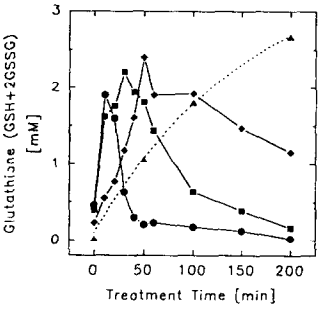

Fig. 3. Net balance of synthesis and loss of glutathione (GSH + 2 GSSG) during treatment with 0.0 (▲, dashed line), 0.05 (▼), 0.1 (◆), 0.2 (■) and 0.5 (●) mM Cu-PuPy. The curves show the difference in intracellular glutathione between cells with uninhibited glutathione synthesis and cells treated in the presence of 5 mM L-BSO. Single experiments.

0.05 mM, and in cells treated with 0.5 mM Cu-PuPy for 150–200 min we found an impressive increase to 8 nmol ProtSSG/mg protein. These cells were virtually devoid of GSH. Figure 2 summarizes the oxidative effects of Cu-PuPy on the glutathione system. The fraction of GSSG comprises all species that may be recovered as GSH (or GSSG) in the HPLC method, i.e. GSH, GSSG, ProtSSG, and presumably GSH or GSSG which may be complexed to copper. The interesting rebound effect of GSH oxidation around 50 min of Cu-PuPy treatment was reproducibly observed. Possible explanations for this effect are a delayed induction of GSSG export or a transient decrease in oxidation, brought about by either decreased stress or by increased effectivity of GSSG reduction. Increased biosynthesis of GSH (see below) could not alone account for this effect.

We used L-BSO to determine the effect of Cu-PuPy treatment on the biosynthesis of glutathione. L-BSO is a selective, irreversible inhibitor of γ-glutamylcysteine synthetase (EC 6.3.2.2), which completely blocks glutathione biosynthesis at the used non-toxic concentration of 5 mM [15]. The curves in Fig. 3 were obtained by subtracting glutathione levels of cells that were treated with Cu-PuPy in the presence of L-BSO from the levels in Cu-PuPy-treated cells with functional biosynthesis. Assuming equal glutathione loss in L-BSO-treated and uninhibited cells, the curves represent the glutathione levels which are due to new glutathione synthesis. We estimate the glutathione turnover of untreated CHO cells to be 1.4 mM/hr (Fig. 3, broken line), which corresponds to a glutathione half-life of 2 hr. This is in accordance with the glutathione turnover in other cultured cells like rat hepatocytes [16]. As Fig. 3 shows, Cu-PuPy-treated cells respond with a burst of glutathione synthesis, which is followed by a phase where loss of glutathione predominates over its synthesis. The inverse correlation between the time of its maximal net synthesis and the Cu-PuPy concentration indicates a dose-dependent induction of glutathione synthesis.

Effect of Cu-*PuPy on the clonogenic survival of cells*

The survival of cells exposed to oxidative stress may depend considerably on the cellular density during treatment [11]. Among the influential factors could be differential dilution of diffusable toxic species like $O_2^{\cdot-}$ and H_2O_2, leakage of protective enzymes from damaged cells or cell–cell interactions. To minimize such artifacts we have routinely treated cells at a constant density of 10^6 cells/mL medium (0.12×10^6 cells/cm^2).

Cell survival was only slightly affected by Cu^{2+} (added as $CuSO_4$), by the copper-free ligand apo-PuPy or by ClO_4^-, the counter ion of the complex (added as $NaClO_4$). After 200 min of treatment with 0.2 mM of these additions, the respective surviving fractions were 0.8, 0.9 and 0.7.

Figure 4 shows that 0.05 mM Cu-PuPy is not toxic. This specific curve was obtained from an experiment with a comparatively low plating efficiency of 0.7. The apparent rise in the surviving fraction to values above 1.0 in this treatment is statistically significant (linear regression slope > 0: $P < 0.05$) and clearly is due to an improvement in plating efficiency to values close to 1.0. Cu-PuPy exhibits marked threshold toxicity with rapid survival reduction above 0.1 mM. The most striking effect of Cu-PuPy, always observed at treatments with 0.2 or 0.5 mM, is the increase in the survival curve by 2–3 orders of magnitude within 50 min of an initial survival minimum. This counter-intuitive effect means that cell survival profits from continuation of treatment.

Prolonged incubation at a Cu-PuPy concentration of 0.2 or 0.5 mM produced increasing amounts of cell debris that could not be removed from the flasks by trypsin. However, the survival gain after the preceding minimum was not due to an artifact of selective trypsinization, since the length of time of trypsin treatment was sufficient to detach all of those cells that were still clonogenic and the loss of cells, clonogenic or not, was less than 25% under the most toxic conditions investigated. We observed the same survival kinetics in experiments with 0.2 mM Cu-PuPy (30–200 min, not shown), where the

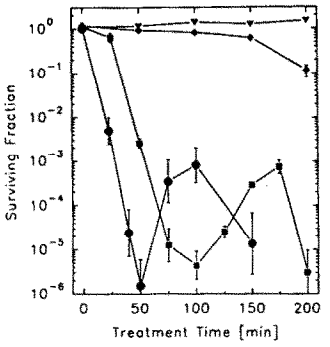

Fig. 4. Clonogenic survival as response of cells to treatment with 0.05 (▼), 0.1 (◆), 0.2 (■) and 0.5 (●) mM Cu-PuPy. Data are from separate experiments. Shown are typical curves as were similarly obtained in three (0.05, 0.1 mM) or more experiments (0.2, 0.5 mM). If shown, error bars represent experimental standard errors from three to six replicate flasks, otherwise the error was smaller than symbol size. The maximal deviation observed between experiments was 2 orders of magnitude. It was observed in treatments with 0.5 mM Cu-PuPy and was mainly due to a time shift of approximately 10 min in the survival curves.

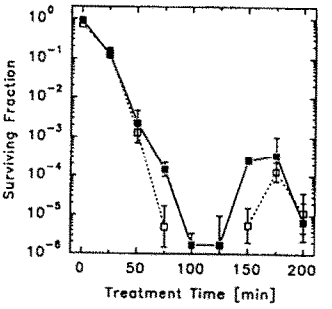

Fig. 5. Effect of hypoxia after treatment on the toxicity of 0.2 mM Cu-PuPy. Treatment of cells with Cu-PuPy was terminated after the indicated time and followed by incubation for 2 hr at room temperature in the absence of Cu-PuPy under oxic (■) or hypoxic (□) conditions. Single experiments.

trypsinization step was omitted. This excludes a differentially sensitizing effect of trypsin. Since in these experiments virtually the same number of non-clonogenic cells was present in the period following treatment, a differentially protective effect of lysed cells on residual clonogenic cells is rendered implausible.

A small protective effect of catalase (200 U/mL) against 0.2 mM Cu-PuPy was observed in one of three performed experiments, but the surviving fractions after 100 min of treatment were only increased by a factor of 3–6. The addition of SOD (100 U/mL) had no detectable effect at all. These findings are not unexpected since both enzymes are only effective against extracellular H_2O_2 or $O_2^{\cdot-}$. Also the presence of catalase in the phase after treatment did not improve the colony yield.

When treatments were performed in used medium (from 24 hr incubations of confluent cells) or in medium that had been acidified to pH 6.6 with HCl, toxicity of 0.2 mM Cu-PuPy was slightly reduced. In both cases, surviving fractions were improved by a factor of 2–4, but again the survival kinetics were not qualitatively affected. The increased survival seen with used medium was not due to the consumption of glucose or of precursors for glutathione or pyridine nucleotide synthesis, since supplementation of glucose, cystine or of BME-vitamins did not revert the effect (not shown). Glutathione-deficient cells, obtained by pre-incubation with 2.5 μM L-BSO, were equally sensitive towards 0.2 mM Cu-PuPy as glutathione-proficient cells during the initial toxic period, but after 100 min the surviving fractions fell below the detection limit of 10^{-7}.

The Cu-PuPy catalysed oxidation of GSH is known to consume oxygen [5]. Therefore, to test if the inflection of the survival curve could be due to reoxygenation following treatment-induced hypoxia, we performed a variety of experiments under hypoxic conditions, i.e. in N_2-equilibrated medium under a N_2 atmosphere, or we regularly agitated the flasks during treatments to assure constant oxygenation. None of these modifications had an influence on the magnitude or the kinetics of Cu-PuPy toxicity (not shown). In contrast, when euoxic treatments were followed by hypoxic incubation for 2 hr, survival was significantly reduced during the inversion phase (Fig. 5). Taken together, these data exclude a reoxygenation effect and indicate that oxygen is either not a major determinant or not a limiting factor for the observed time course of toxicity, but that oxygen is required by a delayed repair process.

To test whether Cu-PuPy itself protects against damage by H_2O_2, we co-incubated cells for a fixed time period (50 min) at a fixed initial H_2O_2 concentration (1.5 mM) with variable concentrations of Cu-PuPy or the copper-free ligand apo-PuPy. The half-life of H_2O_2 under these conditions was 5.2 ± 0.1 min in the absence of Cu-PuPy (H_2O_2 determination failed in the presence of Cu-PuPy). H_2O_2 alone reduced cell survival to 6×10^{-3}. To our surprise we found that in the presence of 0.2 or 0.5 mM Cu-PuPy any additional toxic effect of H_2O_2 was absent (Fig. 6). Also, apo-PuPy had a considerable protective effect at 0.5 mM.

DISCUSSION

Cu-PuPy mediates the cellular binding of up to 10 mM copper. Cells treated with 0.2 or 0.5 mM Cu-PuPy in excess of 100 min have a disk-shaped, definitely stiff appearance which is unaffected when cells are trypsinized. For comparison, cells treated with equally toxic doses of H_2O_2 (surviving fractions less than 10^{-3}) are already rounded off before detachment or take this shape during trypsinization. We suppose that the observed rigidity of Cu-PuPy-treated cells is due to an accumulation of Cu-PuPy in the cell membrane. This is in accordance with the data of Steinkühler *et al.* [5], who found that,

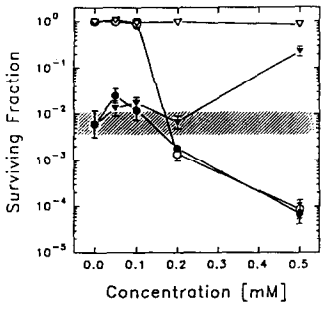

Fig. 6. Survival interaction of Cu-PuPy or apo-PuPy with H_2O_2. Cells were incubated for 50 min with the indicated concentrations of Cu-PuPy or apo-PuPy in the presence of 1.5 mM H_2O_2. H_2O_2 alone (dashed area), Cu-PuPy alone (○), Cu-PuPy + H_2O_2 (●), apo-PuPy alone (▽), apo-PuPy + H_2O_2 (▼). Two others experiments with different H_2O_2 doses were performed and gave qualitatively similar results.

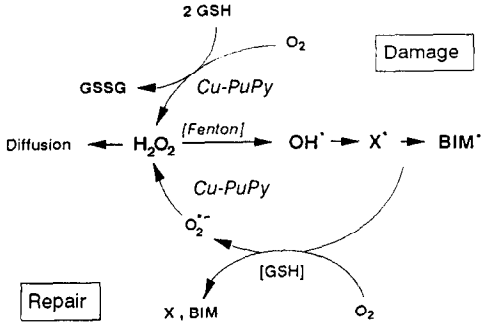

Fig. 7. Suggested reaction scheme of Cu-PuPy as oxidant and antioxidant. H_2O_2 produced via redox cycling of Cu-PuPy with GSH is converted to ·OH radicals. Their fast reaction with organic molecules (X) leads to the accumulation of less reactive, longer living radicals that may further damage biologically important molecules (BIM) like DNA, protein, lipids. During this process, GSH is oxidized and eventually depleted from the cells. A part of the accumulated radicals may directly react with O_2 to give $O_2^{\cdot-}$. Other radicals may transfer their unpaired electron via residual GSH to O_2. These reactions are driven by the removal of $O_2^{\cdot-}$ via Cu-PuPy-catalysed dismutation. In this way, two radical molecules are converted to one molecule of H_2O_2 and one molecule of water. Competition of the accumulated radicals with Cu-PuPy for residual GSH would further shift the process towards detoxification since it inhibits the production of H_2O_2 and increases the fraction of Cu-PuPy engaged in the dismutation reaction.

compared to Cu-PuPy dissolved in PBS, Cu-PuPy entrapped in liposomes was significantly less accessible for reduction by ascorbate even after disruption of liposomes by 0.2% Triton X-100. A lipid-bound pool may be a reservoir for continued replenishment of cytoplasmatic Cu-PuPy after removal of extracellular Cu-PuPy.

Under severe oxidative stress the capacity of cells to re-reduce GSSG in the GSSG reductase reaction may become insufficient. Accumulated GSSG may be exported or react with protein sulfhydryls via a thiol exchange reaction. Among the consequences of protein oxidation are conformational changes, protein degradation and the inhibition of enzyme activities which may deregulate metabolic pathways [17]. Our data demonstrate the strong influence of Cu-PuPy on the status of glutathione. Depletion of glutathione and minor oxidation were already observed at the non-toxic Cu-PuPy concentration of 0.05 mM. Cu-PuPy at concentrations above 0.1 mM further increased glutathione depletion and forcibly enhanced glutathione and protein oxidation. As is also evident from L-BSO treatments, glutathione depletion *per se* does not necessarily impair cell survival. Instead, our data suggest a close correlation between glutathione oxidation and cytotoxicity.

H_2O_2 formed by catalysed oxidation of GSH is certainly a main determinant of the toxicity of Cu-PuPy. It is a precursor of the reactive hydroxyl (·OH) radical, which damages biologically important molecules either directly or via radical chain reactions. Specific protection against H_2O_2 is accomplished by glutathione peroxidase, which requires GSH as cosubstrate, and to a lesser extent by peroxisomal catalase [9]. The decomposition of H_2O_2 to ·OH (Fenton reaction) proceeds with traces of reduced transition metals (Fe^{2+}, Cu^{1+}) that are present in a biological environment. Imlay and Linn [18] showed that functional cellular metabolism is required for an ongoing Fenton reaction. They suggested that NADH is the cellular electron source for the necessary regeneration of reduced metal and that SOD may interfere with this process.

Nitroxide SOD mimics have been shown to protect cells against damage by H_2O_2 [18, 20]. Since H_2O_2 concentrations were not influenced, a "catalase-mimetic" activity was excluded. Instead, this effect was attributed to either scavenging of H_2O_2-derived free radical species by these compounds, or to interference with the Fenton reaction. Since it was also observed under hypoxic conditions, where no superoxide radicals are expected, protection by $O_2^{\cdot-}$ dismutation was considered unlikely by the authors.

Inverting survival time courses like those in Fig. 4 are unusual and have not come to our attention before. They give important clues and impose stringent constraints on the modes of Cu-PuPy reactivity. As a strictly irreversible process, cell survival is a monotonously decreasing function of time. Mechanistically, time-dependent survival curves that pass through a minimum indicate that treatment times do not coincide with the times of cell death and require an accumulation of reversible damage. Termination of treatment would either have to prevent its repair or induce its conversion to lethal damage. To be sure, such discontinuity also requires kinetic separation of the formation of potential damage and its repair or lethal conversion, i.e. the modifying process must not act immediately on the formation process. Therefore, mechanisms like the selection of a pre-existing resistant subpopulation, catalase-like degradation of H_2O_2, inhibition of the Fenton reaction or scavenging of

OH by Cu-PuPy cannot explain such survival kinetics.

However, inhibition of the Fenton reaction by sequestration of Fenton-active metal ions is a likely explanation for protection against H_2O_2 by the copper-free ligand apo-PuPy. This would also imply that metal ions, when complexed to PuPy, are less efficient in driving the decomposition of H_2O_2 to reactive products. Indeed our experiments with H_2O_2 coincubation demonstrate that the copper complex of PuPy, i.e. Cu-PuPy, is not Fenton active.

Time-dependent sensitivity towards accumulated reversible damage could in principle be brought about by cell cycle progression into a phase of lower sensitivity or of higher repair capacity. To explain our curves, treatment would have to induce sensitive cells to progress within 50 min to a phase where they are by 2–3 orders of magnitude more resistant, or alternatively, would have to inhibit cells in a resistant stage from entering a sensitive phase. Furthermore, termination of treatment would have to prevent these effects. Such cell cycle effects are supposed to cause the increased survival of irradiated cells that are prohibited by suboptimal growth conditions after irradiation from proceeding through the cycle. An explanation of this "potential lethal damage repair" is, that arrested cells may repair damaged DNA and are prevented from entering mitosis while their chromosomes are damaged [21]. A similar mechanism may explain the slightly reduced toxicity of Cu-PuPy in used or acidic medium. However, our data show that 0.2 mM Cu-PuPy protects completely against the toxic bolus of 1.5 mM H_2O_2 in a 50-min treatment. This finding would imply that all of the surviving cells have entered a protected cycle stage by this time, and is not compatible with the survival gain observed during prolonged Cu-PuPy treatment. Also, it is difficult to conceive how an induced modification of cell cycle kinetics could produce the close dependence of Cu-PuPy dose on the inflection times of the survival curves.

Recently, Winterbourn [22] put forward an attractive hypothesis on the role of SOD in a general antioxidative mechanism. She proposed that free radicals enter a protective cycle by transferring their unpaired electron to oxygen either directly, if chemically reducing, or via GSH as an intermediate, to give $O_2^{\cdot-}$. The function of SOD is to drive reversible electron transfers to O_2 thermodynamically by removal of product and to prevent damage by reverse electron transfers from $O_2^{\cdot-}$ to biomolecules. Overall, such a mechanism would be protective, since two radicals are converted via dismutation to two non-radical products and one molecule of H_2O_2, which could be enzymatically removed or otherwise enter a new cycle of radical generation. Without further assumptions, this hypothesis can explain our observed protection against H_2O_2-induced cell injury by the SOD-mimetic property of Cu-PuPy. It can account for the unusual time dependence of cell survival since protection would not act directly on H_2O_2 but on a pool of H_2O_2-derived, repairable radicals. This hypothesis also predicts that repair depends on the availability of oxygen. From our observation that oxygen contributes to protection in the phase after Cu-PuPy treatment one can infer that repair profits from residual intracellular Cu-PuPy, though at the moment we have no simple explanation for the fact that during proper Cu-PuPy treatment hypoxia had no observable effect. Our data are further compatible with a supportive role of GSH in the repair process, since L-BSO-treated cells do not recover from damage. On the other hand, GSH concentrations of 0.1 mM that are present in the recovery phase of glutathione-proficient cells may still suffice for considerable protection. Also the repair efficiency of $O_2^{\cdot-}$-dismutation would continually increase due to decreasing competition of GSH for Cu-PuPy (Fig. 7).

In our CHO cell system we observe a marked threshold for both glutathione oxidation and cell survival at a Cu-PuPy concentration of 0.1 mM. The capacity of tumor cells to inactivate H_2O_2 and free radicals is often impaired with respect to non-malignant cells [5, 23, 24]. Such cells are expected to be killed by much lower Cu-PuPy concentrations than cells with a higher antioxidative buffer capacity. Also, both the toxic mechanism of Cu-PuPy as a catalyst of glutathione oxidation and the assumed protective mechanism of Cu-PuPy as a driving force for the removal of accumulated radicals require oxygen. It may be hypothesized that in an environment with limiting oxygen supply, damage is in temporal advantage over subsequent protection. Such mechanisms may contribute to the observed specific toxicity of Cu-PuPy and similar copper complexes towards tumor cells.

Acknowledgements—We are grateful to Prof. Dr Ulrich Weser and Dr Hans-Jürgen Hartmann for helpful discussion and to Dr Wolfgang Beisker for performing cell cycle analyses.

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