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CYTOTOXICITY OF THE HYPOXANTHINE-XANTHINE OXIDASE SYSTEM ON V79 CELLS: COMPARISON OF THE EFFECTS OF SOD AND CuDIPS

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The hypoxanthine — xanthine oxidase system generates an extracellular flux of superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). Catalase but not superoxide dismutase (SOD) protects V79 cells exposed to the hypoxanthine — xanthine oxidase system, showing that H_2O_2 is the major reactive oxygen species involved in the cytotoxicity of such a system. In contrast to SOD, the lipophilic SOD like compound CuII (diisopropylsalicylate)$_2$ (CuDIPS) exhibits some protection at non cytotoxic concentration. It is also found that methanol partially protects cells exposed to the hypoxanthine-xanthine oxidase system. It appears that in our experimental conditions (temperature, ionic strength and pH) the protective effect afforded by methanol and CuDIPS is due to the inhibition of the xanthine oxidase activity.

KEY WORDS: Reactive oxygen species, cell lethality, superoxide dismutase, superoxide dismutase mimic, catalase.

INTRODUCTION

In eukaryotic cells, during mitochondrial electron transport, most of the oxygen is reduced to water by cytochrome oxidase. The oxygen reduction to water involves 4e− and 4H+. However in certain situations, especially UV irradiation,[1] under hyperbaric conditions,[2] during the metabolism of certain carcinogens[3] and at sites of inflammation where the oxidative burst of the phagocytic leukocytes occurs,[4] the oxygen can be partially reduced to generate the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH°), which are respectively the one, two and three univalent reduction intermediates of oxygen.

The generation of an extracellular flux of superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) causes cellular death. Catalase but not superoxide dismutase (SOD) protects the cells when they are exposed to acetaldehyde/xanthine oxidase[5] or to xanthine/xanthine oxidase.[6] However, the high molecular weight and the hydrophilic structure of SOD allow its enzymatic function in the bulk solution but not within the cellular membrane where the protonated form of $O_2^{\cdot -}$ ($HO_2^{\cdot}$) can initiate lipid peroxidation leading to cellular death.

In our work, we have studied the cytotoxicity of the hypoxanthine/xanthine oxidase system and compared the respective effects of SOD and of the lipophilic SOD like

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CuII (diisopropylsalicylate)$_2$ (CuDIPS), which unlike SOD is able to penetrate into the cellular membrane.[7,8]

MATERIALS AND METHODS

Chemicals

Hypoxanthine, xanthine oxidase (XO) from buttermilk grade I, catalase from bovine liver (free thymol), superoxide dismutase from bovine erythrocytes, horseradish peroxidase type VI (HRPO) and allopurinol were obtained from Sigma Chemical Co., (St. Louis, MO). CuII (3,5-diisopropylsalicylate)$_2$ was synthesized as previously described[9] from 3–5 diisopropylsalicylate (Janssen) and $CuCl_2$ by Dr Hocquaux in our laboratories (L'oreal – Aulnay-sous-Bois, France).

Predicted elementary analysis for CuDIPS calculated for $C_{26}H_{34}CuO_6$: C 61.72, H 6.73, O 18.99, Cu 12.56. Found: C 61.65, H 6.75, O 18.83, Cu 12.

Cells

Chinese hamster lung V79 fibroblasts were a gift of Dr Chouroulinkov (Villejuif, France). The cells were grown in Eagle's minimal essential medium (Gibco laboratories) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, L-glutamine and 10% foetal calf serum (FCS) which was inactivated by warming for half an hour at 56°C. The cells were maintained in a 5% CO_2 air-atmosphere at 37°C. Two passages per week were necessary. In these conditions, the duplication time was 11.5 hours.

Cytotoxicity assay

V79 cells were seeded in 60 mm Corning petri dishes (150 cells/dish) in complete medium. Twenty four hours later, the medium was removed, the cells were rinsed in prewarmed Dulbecco's phosphate buffer saline (BSS:137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4 – Gibco laboratories) added with 5 ml 100 μM hypoxanthine. All components were dissolved in BSS except CuDIPS dissolved in methanol. The final concentration of methanol was 0.5% v/v.

At time = 0, 50 μl of a xanthine oxidase solution ($\times$ 100) was added. After one hour incubation, the cells were rinsed once with prewarmed BSS (37°C), mixed with prewarmed complete medium (37°C) and left for one week in the incubator. The cells were then fixed with 80% ethanol for 15 minutes and stained with 10% Giemsa for 20 minutes. The colonies containing more than fifty cells were counted and the results are expressed as a percent of the control.

H_2O_2 assay

The quantity of hydrogen peroxide generated at 37°C by the hypoxanthine/XO reaction or by CuDIPS was measured by the method of Pick and Keisari.[10] Briefly at time = 0, 50 μl of a xanthine oxidase solution (250 mU/ml was added to 5 ml 100 μM hypoxanthine plus the component to be tested as mentioned above. At the indicated time, an aliquot of 800 μl was added to 200 μl BSS containing 100 μg phenol red and 10 U HRPO. The tubes were incubated for 5 minutes at room temperature (22°C) and

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brought to pH = 12.5 by addition of 10 μl 1 N NaOH. The absorption at 610 nm was determined using phenol red (100 μg/ml) and HRPO (10 U/ml) in 1 ml BSS plus 10 μl 1 N NaOH as blank. There is a linear relationship between absorbance at 610 nm and H_2O_2 concentration in the 1–60 μM range.

Oxygen consumption

The consumption of oxygen was determined in an oxygraph (Gilson Medical Electronic, France) equiped with a Clarke electrode. The measurements were performed at 37°C in air-saturated water. The chamber of the oxygraph contains 1.8 ml of hypoxanthine and of the component to be tested. The experiment was carried out in BSS. The solution was equilibrated for 10 minutes before the beginning of the assay.

At t = 0, 200 μl of xanthine oxidase (250 mU/ml) were added. The final concentration of hypoxanthine and of xanthine oxidase was 100 μM and 25 mU/ml, respectively.

RESULTS

The cytotoxicity of CuDIPS was studied in BSS containing 100 μM of hypoxanthine without xanthine oxidase. After one hour incubation, the cytotoxicity of CuDIPS increased with its concentration. At 1 and 20 μg/ml the percentages of cells surviving were 90% and 10% respectively. The concentration causing 50% cell death was ≃ 3 μg/ml (Figure 1). Catalase (100 μg/ml) completely restored the survival of the cells when it was added to the cultures prior to CuDIPS.

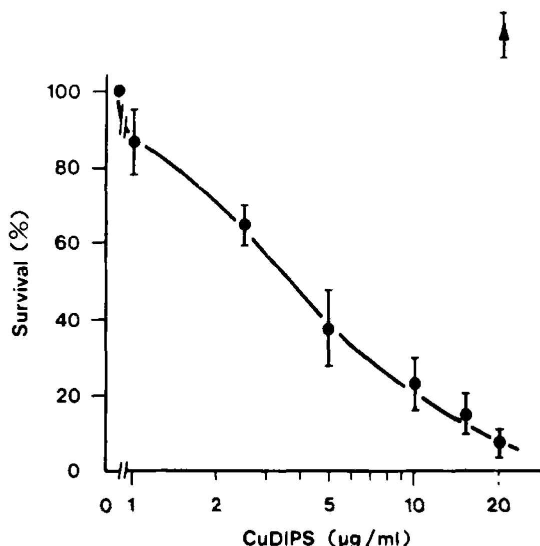

FIGURE 1 Cytotoxicity of CuDIPS on V79 cells. Cells were treated for 1 hour in BSS containing hypoxanthine (100 μM) in absence ● or in presence of catalase (100 μg/ml) ▲. Bars represent the S.E.M. of two or three independent experiments.

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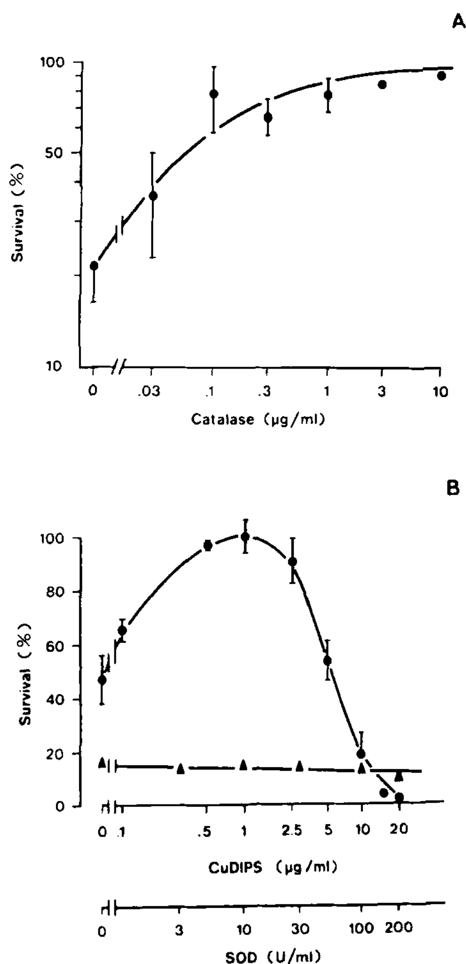

FIGURE 2A Effect of catalase on the cytotoxicity of the hypoxanthine-xanthine oxidase system on V79 cells. Cells were treated in BSS with hypoxanthine (100 μM) and xanthine oxidase (2.5 mU/ml) for 1 hour. Catalase was added just prior to the addition of xanthine oxidase. Bars represent the S.E.M. of two or three independent experiments.

FIGURE 2B Effect of CuDIPS on the cytotoxicity of the hypoxanthine-xanthine oxidase system on V79 cells. Cells were treated in BSS with hypoxanthine (100 μM) and xanthine oxidase (2.5 mU/ml) for 1 hour. SOD (3–200 U/ml) ▲ and CuDIPS (0.1–20 μg/ml) ● were added just prior to the addition of xanthine oxidase. Bars represent the S.E.M. of two or three independent experiments.

Since in the 1–20 μg/ml range CuDIPS neither generates nor catalyses the degradation of H_2O_2 (data not shown), the possibility cannot be dismissed that a complex catalase-CuDIPS has been formed, inhibiting the cytotoxicity of CuDIPS.

The hypoxanthine/XO system generates $O_2^{\cdot -}$ and H_2O_2 directly by the addition of one or two electrons to O_2 with the formation of xanthine and urate at the end of the reaction. Some H_2O_2 is also formed by the spontaneous dismutation of $O_2^{\cdot -}$.

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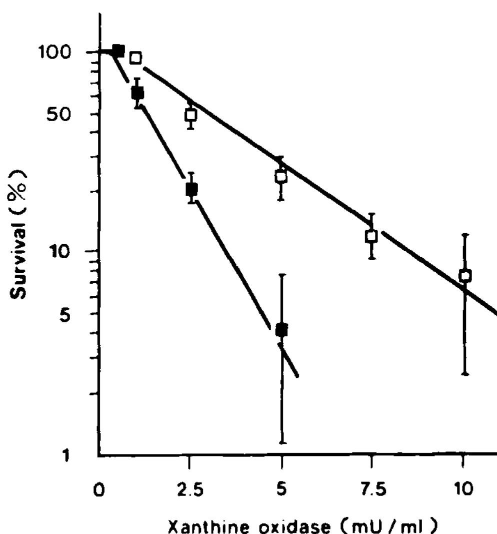

FIGURE 3 Effect of methanol on the cytotoxicity of the hypoxanthine-xanthine oxidase system on V79 cells. Cells were treated in BSS with hypoxanthine (100 μM) and various concentrations of xanthine oxidase for 1 hour in absence ■ or in presence □ of 0.5% (v/v) methanol. Bars represent the S.E.M. of two or three independent experiments.

The effects of SOD, CuDIPs and catalase were investigated individually using the hypoxanthine (100 μM)/XO (2.5 mU/ml) system.

Catalase showed a protective effect at a very low concentration: at 0.03 μg/ml the cells were partially protected, they were completely protected at concentrations above 1 μg/ml (Figure 2A).

The comparison between SOD and CuDIPS showed that SOD in the 3–200 U/ml range did not protect the cells whereas the protection afforded by CuDIPS increased with the concentration up to 1 μg/ml. At this concentration (1 μg/ml) the protection was complete (Figure 2B). For a CuDIPS concentration above 1 μg/ml, its protective effect decreased as its concentration increased. At the higher concentrations of CuDIPS (10–15–20 μg/ml), the survival fraction fell below the value of the group exposed to hypoxanthine/XO plus 0.5% methanol without CuDIPS.

Methanol was the vehicle for CuDIPS, its final concentration in BSS was 0.5% (v/v). At this concentration, methanol seemed to partially protect the cells exposed to the hypoxanthine (100 μM)/XO (2.5 mU/ml) system. With or without 0.5% methanol, the percentages of cells surviving were 45% and 15% respectively (Figure 2B). This protective effect of methanol was confirmed by the comparison of the two xanthine oxidase dose-response curves which were carried out with or without 0.5% methanol (Figure 3).

In order to establish whether the observed effects of methanol and of CuDIPS on the hypoxanthine/XO system were due to the scavenging effect of methanol for the hydroxyl radical and to the scavenging effect of CuDIPS for the superoxide anion or due to the inhibition of xanthine oxidase activity, we decided to investigate the effects of methanol and CuDIPS on (i) the appearance of H_2O_2 (ii) the oxygen consumption

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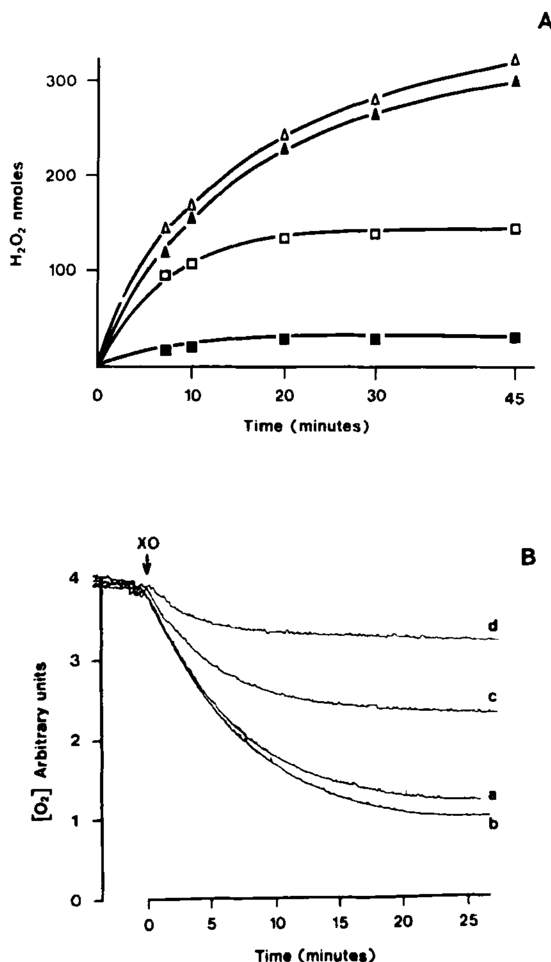

FIGURE 4A Effects of SOD, CuDIPS and methanol on the kinetics of H_2O_2 production by the hypoxanthine (100 μM) and xanthine oxidase (2.5 mU/ml) at 37°C. No addition ▲; with SOD (200 U/ml) △; with methanol (0.5%) □; with CuDIPS (2.10^{-6} M) ■.

FIGURE 4B Effects of SOD, CuDIPS and methanol on the oxygen consumption during the hypoxanthine-xanthine oxidase reaction at 37°C. At time = 0, 25 mU/ml xanthine oxidase was added (arrow) to the BSS containing 100 μM hypoxanthine (a); 100 μM hypoxanthine plus 200 U/ml SOD (b); 100 μM hypoxanthine plus 0.5% methanol (c); 100 μM hypoxanthine plus 0.5% methanol and 2.10^{-6} M CuDIPS (d).

by the hypoxanthine/XO system. Figure 4A shows that hydrogen peroxide production by hypoxanthine/XO system increased with the incubation time. The amount of H_2O_2 was 300 nmol after 45 minutes. Allopurinol 20 μM greatly inhibited the formation of hydrogen peroxide as would be expected for an inhibitor of xanthine oxidase (data not shown). The addition of SOD (200 U/ml) to the system slightly increased the yield of H_2O_2 whereas the production of H_2O_2 dropped to 50% with methanol (0.5%) and to 5% with CuDIPS (1 μg/ml). The accumulation of H_2O_2 with time reached a

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plateau from 10–20 minutes with methanol and CuDIPS. When SOD or no modifier was added to the hypoxanthine/XO system, the plateau was not observed in the period of time studied.

For oxygen consumption (Figure 4B) the inhibition of xanthine oxidase activity by 0.5% methanol and 1 μg/ml of CuDIPS was of the same order of magnitude as the activity of the enzyme assayed by H_2O_2 determination.

DISCUSSION

The results presented in this paper show that hydrogen peroxide is the major constituent involved in the cytotoxicity of the hypoxanthine/XO system. Catalase at very low concentration protects the cells whereas superoxide dismutase does not protect the cells exposed to the hypoxanthine/XO system. In contrast to SOD CuDIPS a lipophilic SOD like compound exhibits a protective effect at non cytotoxic concentrations.

H_2O_2 and $O_2^{\cdot-}$ are both generated in the extracellular space by the hypoxanthine/XO system, but $O_2^{\cdot-}$ does not diffuse into the cell as readily as H_2O_2.[11]

$O_2^{\cdot-}$ reduces extracellular traces of metal ions which, in turn, reduce H_2O_2 to $OH^{\circ} + OH^-$ increasing the flux of HO° in the proximity of the cell. Thus, it was expected that the addition of SOD, which is not able to penetrate the cell membrane, exhibits some protective effect when the cells are exposed to an extracellular flux of $O_2^{\cdot-}$. But, in our conditions as previously reported by other authors using an acetaldehyde/XO system,[5] or a xanthine/XO system,[6] the addition of SOD does not protect the cells. However, the addition of SOD to cell cultures can protect the cells, especially when the extracellular flux of $O_2^{\cdot-}$ is generated by the activation of the NAD(P)H-oxidase enzyme of the phagocytic cells.

This apparent discrepancy may depend on the extracellular environment of the cells. In the plasma, $O_2^{\cdot-}$ can reduce iron bound to ferritin, then the reduced iron is removed from ferritin and reacts with H_2O_2 in a Fenton reaction to generate OH°.[12] Furthermore, plasma contains ascorbate and sulfhydryl (RSH) compounds which exhibit some protective effect against "oxidative stress" and become ineffective after their oxidation by $O_2^{\cdot-}$. Thus SOD protects blood cells by its ability to inhibit both the reduction of iron and the oxidation of reducing agents.[13]

Unlike $O_2^{\cdot-}$, its protonated form HO_2° is an uncharged molecule which theoretically can cross the cell membrane, but its high reactivity with the phospholipids of the membrane does not allow such a diffusion. If HO_2° is involved in the cytotoxicity of the hypoxanthine/XO system, it would be expected that SOD would not protect the cells because it cannot penetrate the cell membrane, whereas a lipophilic SOD-like may protect the cells, which is the case.

However the protective effect of CuDIPS cannot be linked to its SOD-like activity since, in our experimental conditions, it inhibits the activity of the xanthine oxidase assayed by H_2O_2 production or by oxygen consumption. These data are not in agreement with other results which have shown that CuDIPS does not inhibit xanthine oxidase.[14]

However, some copper complexes are known to inhibit certain enzyme activities. CuDIPS has been reported to inhibit the mutagenicity of dimethylbenz(a)anthracene[15,16] and of benzo(a)pyrene.[17] These effects seem to be mediated by the inhibition of P-450 reductase activity.[16] Furthermore, the cytochrome-P-450 catalysed hyd-

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roxylation was inhibited by a copper complex (copper-tyrosine) and was associated with an inhibition rather than with a stimulation of the formation of hydrogen peroxide, the product of dismutation of the superoxide radicals generated as the result of the decay of oxycytochrome-P-450.[18]

These data show that, besides its SOD like activity, the biological effects of CuDIPS could be mediated by the inhibition of some enzymatic systems.

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