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INHIBITION BY SUPEROXIDE DISMUTASE-MIMETIC COPPER COMPLEXES OF PHORBOL ESTER-INDUCED RESPIRATORY BURST IN HUMAN GRANULOCYTES

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Abstract—Two superoxide dismutase-mimetic lipophilic copper complexes, $Cu(II)_2$(indomethacin)$_4$ [$Cu(II)_2$(indo)$_4$] and $Cu(II)_2$(3,5-diisopropylsalicylate)$_4$ [$Cu(II)_2$(3,5-DIPS)$_4$], were tested for their effects on the respiratory burst of intact human granulocytes and on xanthine oxidase, under conditions where superoxide and hydrogen peroxide were generated. The effect of the copper complexes on these enzyme systems (as opposed to their dismutase effect on superoxide) was determined by measuring oxygen uptake with an oxygen meter. It was found that, after a short delay, both systems were inhibited markedly by micromolar amounts of these complexes. This inhibition was prevented by treatment with EDTA or catalase if added prior to starting the reaction. Similar inhibitory effects were seen using copper sulfate. It appears that these lipophilic SOD-mimetic compounds can, in the presence of H_2O_2 and O_2^-, give rise to a species that can inhibit some component of the respiratory burst oxidase or protein kinase C in intact granulocytes and xanthine oxidase in solution. The observed decrease in O_2^- levels observed upon addition of these compounds is likely due to inhibition of the source and not to their SOD-mimetic properties.

Numerous low molecular weight copper complexes have been described which are reported to have a variety of biological activities including superoxide dismutase-like activity, i.e. the ability to convert O_2^- to O_2 and H_2O_2 [1–3]. Superoxide dismutase† activity is usually measured as a decrease in O_2^- concentration, either (1) by an indirect assay in which superoxide dismutase competes with cytochrome *c* or nitroblue tetrazolium (NBT‡) for reaction with O_2^- or (2) by a direct assay in which the decrease in O_2^- (generated by pulse radiolysis) is followed by absorbance [2]. Although numerous other reactions are known (reviewed in Refs. 1 and 4), these copper complexes have often been used assuming their effects to be due to their ability to dismute O_2^- [2, 5]. The reaction has usually been considered to be analogous to that of Cu,Zn superoxide dismutase (CuZnSOD) where the mechanism is

$$O_2^- + Cu(II)L \rightarrow O_2 + Cu(I)L$$

$$O_2^- + Cu(I)L + 2H^+ \rightarrow H_2O_2 + Cu(II)L.$$

Lipophilic copper complexes such as $Cu(II)_2$(indomethacin)$_4$ [$Cu(II)_2$(indo)$_4$] and $Cu(II)_2$(3,5-diisopropylsalicylate)$_4$ [$Cu(II)_2$(3,5-DIPS)$_4$] have been of biological interest because of their anti-inflammatory and anti-tumor promoting properties [1, 6–9]. They have also been reported to inhibit a number of functions in stimulated granulocytes such as oxygen uptake, NBT, cytochrome *c* and ESR spin-label reduction, chemiluminescence, myeloperoxidase activity, activation of carcinogens and inhibition of DNA strand breaks [10–14]. In all cases, it has been explicitly or implicitly stated that their effects were due to dismutation of O_2^-.

The "SOD-mimetic" activities of two copper complexes, $Cu(II)_2$(indo)$_4$ and $Cu(II)_2$(3,5-DIPS)$_4$ have now been investigated in more detail. Rather than examine their dismutase effects on O_2^- levels, we have studied their effects on two enzyme systems which produce O_2^-, the respiratory burst of intact human granulocytes and xanthine oxidase. O_2 uptake was used to follow activity. We now report that the combination of O_2^-, H_2O_2, and copper complex is strongly inhibitory both to the respiratory burst of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated granulocytes and to xanthine oxidase, suggesting that previous interpretations of the mechanism of action of the copper complexes may be incorrect in some experimental systems.

MATERIALS AND METHODS

Copper complexes

$Cu(II)_2$(indomethacin)$_4$ [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole acetate] and $Cu(II)_2$(3,5-diisopropylsalicylate)$_4$ were prepared by the method of Sorenson [6]. Both complexes were green and were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all reactions was 0.1% (v/v) or less.

Cell isolation

Granulocytes were isolated from freshly drawn

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† Enzymes: catalase (EC 1.11.1.6); superoxide dismutase (EC 1.15.1.1); and xanthine oxidase (EC 1.2.3.2).

‡ Abbreviations: $Cu(II)_2$(indo)$_4$ $Cu(II)_2$(indomethacin)$_4$; $Cu(II)_2$(3,5-DIPS)$_4$, $Cu(II)_2$(3,5-diisopropylsalicylate)$_4$, CuZnSOD, Cu,Zn superoxide dismutase; DMSO, dimethyl sulfoxide; NBT, nitroblue tetrazolium; and TPA 12-*O*-tetradecanoylphorbol 13-acetate.

blood of healthy volunteers. They were prepared as described elsewhere [15]; in brief, granulocytes and erythrocytes were separated from lymphocytes on a density gradient and erythrocytes were removed by ammonium chloride and hypotonic lysis. Final cell samples, which were >97% granulocytes, were resuspended at 2×10^6/mL in balanced salt solution [BSS: 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose (pH 7.4)] and kept on ice until used.

Oxygen uptake

Uptake of dissolved O_2 from solution was measured with a Yellow Springs Instrument model 53 oxygen monitor, modified to allow automatic switching between two cells at 1-min intervals.

Monitoring of granulocyte reactions. All experiments were carried out at 37° in a 3-mL reaction volume containing 2×10^6 cells/mL. Test compounds were added at −5 min, the copper complex being the last addition. The generation of O_2^- was then started by the addition of TPA in DMSO to give final concentrations of 20 nM and 0.1%, respectively. Calculations of O_2 consumption assume an initial O_2 concentration of 224 μM at 37°.

Monitoring of xanthine oxidase reactions. Oxygen uptake was measured at room temperature in a 3-mL reaction volume containing 50 mM sodium phosphate buffer (pH 7.8), 1 mM xanthine and test compounds as specified (the copper complex being the second to last addition). Xanthine oxidase (10 mU/mL) was added to start the reaction (0 time). Calculations of oxygen uptake assume an initial O_2 concentration of 255 μM at 24°.

Spectrophotometry

The conversion of xanthine to urate catalysed by xanthine oxidase was measured at 295 nm in a Perkin–Elmer Lambda 5 recording spectrophotometer. The reaction volume of 1 mL comprised 50 mM sodium phosphate buffer, pH 7.8, 50 μM xanthine and test compounds as specified. Xanthine oxidase (1 mU/mL) was added to start the reaction. Calculations of enzyme activity are based on E_{295} urate = 11,000 M^{-1} cm^{-1} [16].

Chemicals

Xanthine oxidase (grade I) and catalase (9,300 U/mg) were from the Sigma Chemical Co., St. Louis, MO; higher specific activity catalase (65,000 U/mg) was from Boehringer Mannheim. Where specified, heat-inactivation of catalase was carried out at 65° for 16 hr, conditions demonstrated to destroy 98% of the catalatic activity. TPA was supplied by LC Services Corp., Woburn, MA. All other chemicals were analytical grade.

RESULTS

Inhibitory effect of copper complexes on granulocytes

$Cu(II)_2(indo)_4$ and $Cu(II)_2(3,5\text{-DIPS})_4$ have been used frequently in biological systems as lipophilic "SOD-mimetic" compounds, that is, their biological or biochemical effect has usually been presumed to be due to their ability to dismute O_2^-. To investigate the possibility that such complexes might have a direct effect on the respiratory burst of intact granulocytes (the ability of the cells to generate O_2^-, O_2 uptake rather than O_2^- production was used as a measure of the respiratory burst. Representative results are shown in Figs. 1 and 2, and a summary of several experiments using these complexes and, for comparison, $CuSO_4$, is presented in Table 1. When 10 μM $Cu(II)_2(indo)_4$ was added to 2×10^6 granulocytes/mL 5 min prior to stimulation with TPA, a marked inhibition of the respiratory burst (measured as oxygen uptake) was observed

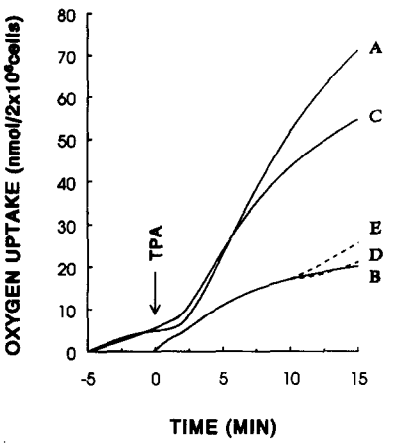

Fig. 1. Inhibition of TPA-stimulated respiratory burst in granulocytes by $Cu(II)_2(indo)_4$. (A) Control or catalase or EDTA or $Cu(II)_2(indo)_4$ + 100 μM EDTA at −5 min (all produced very similar results); (B) $Cu(II)_2(indo)_4$ at −5 min; (C) $Cu(II)_2(indo)_4$ plus catalase at −5 min; (D) $Cu(II)_2(indo)_4$ at −5 min plus catalase at +10 min; and (E) $Cu(II)_2(indo)_4$ at −5 min plus EDTA at +10 min. Where indicated, $Cu(II)_2(indo)_4$ was added at 10 μM. Other details are given in Materials and Methods.

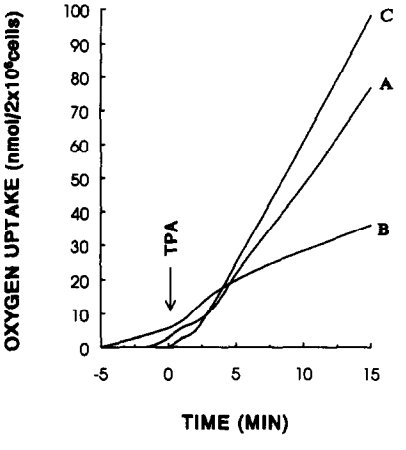

Fig. 2. Inhibition of TPA-stimulated respiratory burst in granulocytes by $Cu(II)_2(3,5\text{-DIPS})_4$. (A) Control; (B) 5 μM $Cu(II)_2(3,5\text{-DIPS})_4$; and (C) $Cu(II)_2(3,5\text{-DIPS})_4$ plus either catalase or EDTA at −5 min Other details as in Fig. 1.

Table 1. Summary of the inhibitory effect of $Cu(II)_2(indo)_4$, $Cu(II)_2(3,5\text{-DIPS})_4$ and $CuSO_4$ on granulocyte-mediated oxygen uptake

	(nmol O_2 consumed)	Percent inhibition	N
1. Control (with DMSO)	51.3 ± 4.3	0	14
2. + $Cu(II)_2(indo)_4$	15.7 ± 2.1	69.4	8
3. + $Cu(II)_2(indo)_4$ + catalase	66.7 ± 17.5	−34.7	4
4. + $Cu(II)_2(indo)_4$ + EDTA	53.9 ± 27.7	5.1	2
5. + $Cu(II)_2(3,5\text{-DIPS})_4$	27.4 ± 4.7	46.6	4
6. + $Cu(II)_2(3,5\text{-DIPS})_4$ + catalase	61.9 ± 8.0	−25.0	3
7. + $Cu(II)_2(3,5\text{-DIPS})_4$ + EDTA	63.7 ± 10.6	−12.1	2
8. Control (without DMSO)	44.4 ± 8.5	0	3
9. + $CuSO_4$	13.9 ± 5.4	68.7	2
10. + $CuSO_4$ + catalase	53.8	−4.5	1
11. + $CuSO_4$ + EDTA	35.4	32.2	1

O_2 uptake is expressed as nmol per 10 min per mL ± SEM (or range for N = 2). Percent inhibition was calculated in each individual experiment with respect to a parallel reaction mixture lacking the copper compound. Where indicated, the concentration of $Cu(II)_2(indo)_4$ was 10 μM, $Cu(II)_2(3,5\text{-DIPS})_4$ was 5 μM, catalase was 1860 U/mL (200 μg/mL), EDTA was 100 μM, and $CuSO_4$ was 20 μM. DMSO was present at a final concentraton of 0.1% in 1–7. Reagents were added at −5 min, and reactions were started by the addition of 20 nM TPA at 0 min.

(Fig. 1B compared to 1A). This inhibition was prevented completely by prior addition of EDTA (Fig. 1A) or catalase (Fig. 1C). EDTA or catalase added 10 min after TPA had little or no effect (Fig. 1D and 1E). Similarly, 5 μM $Cu(II)_2(3,5\text{-DIPS})_4$ also markedly inhibited oxygen uptake and the inhibition was prevented by prior addition of catalase or EDTA (Fig. 2).

Inhibition of xanthine oxidase by copper complexes

The activity of xanthine oxidase was monitored by the uptake of O_2 to form O_2^- and H_2O_2. As can be seen in Fig. 3, 0.5 μM $Cu(II)_2(3,5\text{-DIPS})_4$ complex markedly inhibited oxygen uptake (D compared to A), but only after a lag time of 3–5 min. The inhibition was much reduced by addition of 200 μg/mL catalase (Fig. 3B) or 100 μM EDTA (Fig. 3C) if added together with $Cu(II)_2(3,5\text{-DIPS})_4$. Similar results were obtained using $Cu(II)_2(indo)_4$ (data not shown).

A summary of the inhibitory effects on xanthine oxidase of the two copper complexes and $CuSO_4$ is presented in Table 2. All three compounds behaved similarly. All caused inhibition when added 5 min prior to xanthine oxidase and inhibition was largely prevented by simultaneous addition of catalase or EDTA. However, when catalase or EDTA was added 10 min after xanthine oxidase, no reversal of the inhibition was seen (data not shown). Protection by catalase against $CuSO_4$-mediated inhibition was not simply due to the added protein since (i) heat-inactivated catalase failed to protect, and (ii) 7-fold less protein having equal catalase activity (enzyme from Boehringer Mannheim) was equally protective (data not shown).

As an independent measure of xanthine oxidase activity, the formation of urate from xanthine was measured spectrophotometrically at 295 nm, in parallel with an oxygen uptake experiment. As shown in Fig. 4, both assays demonstrated similar inhibition by $CuSO_4$ and prevention of inhibition by prior addition of catalase and EDTA.

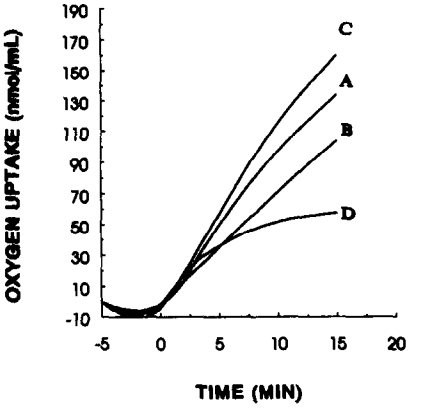

Fig. 3. Effect of $Cu(II)_2(3,5\text{-DIPS})_4$ on xanthine/xanthine oxidase-stimulated oxygen uptake. Where indicated, $Cu(II)_2(3,5\text{-DIPS})_4$ was added at 0.5 μM. (A) Xanthine oxidase alone; (B) xanthine oxidase plus catalase, either alone or with $Cu(II)_2(3,5\text{-DIPS})_4$, at −5 min; (C) xanthine oxidase plus EDTA, either alone or with $Cu(II)_2(3,5\text{-DIPS})_4$ at −5 min; and (D) xanthine oxidase plus $Cu(II)_2(3,5\text{-DIPS})_4$ at −5 min. In all cases, the reaction was started at 0 min by the addition of xanthine oxidase. Results of a single, representative experiment are shown. Other details are given in Materials and Methods.

DISCUSSION

Low molecular weight copper complexes have long been investigated for their anti-inflammatory and "SOD-mimetic" activity, i.e. their purported

Table 2. Summary of the inhibitory effect of $Cu(II)_2(indo)_4$, $Cu(II)_2(3,5\text{-DIPS})_4$ and $CuSO_4$ on xanthine oxidase-mediated oxygen uptake

	(nmol O_2 consumed)	Percent inhibition	N
1. Control (with DMSO)	96.7 ± 8.1	0	17
2. + Catalase	72.5 ± 5.4	25.0	4
3. + EDTA	125.3 ± 4.9	−29.6	8
4. + $Cu(II)_2(indo)_4$ (1 μM)	32.9 ± 3.7	66.0	9
5. + $Cu(II)_2(indo)_4$ + catalase	58.4 ± 10.8	19.4	2
6. + $Cu(II)_2(indo)_4$ + EDTA	138.6 ± 8.6	−10.6	3
7. + $Cu(II)_2(3,5\text{-DIPS})_4$ (0.5 μM)	41.7 ± 5.3	56.9	12
8. + $Cu(II)_2(3,5\text{-DIPS})_4$ (1 μM)	10.9 ± 9.4	88.7	2
9. + $Cu(II)_2(3,5\text{-DIPS})_4$ (1 μM) + catalase	76.8 ± 21.0	−5.9	2
10. + $Cu(II)_2(3,5\text{-DIPS})_4$ (1 μM) + EDTA	116.7 ± 17.6	6.9	2
11. Control (without DMSO)	110.0 ± 15.6	0	4
12. + $CuSO_4$ (2 μM)	41.4 ± 5.7	62.4	4
13. + $CuSO_4$ + catalase	55.3 ± 0.3	11.9	3
14. + $CuSO_4$ + EDTA	113.6	−18.2	1

O_2 uptake is expressed as nmol per 10 min per mL ± SEM (or range for N = 2). Percent inhibition was calculated in each individual experiment with respect to a parallel reaction mixture lacking the copper compound. Where indicated, the concentration of catalase was 1860 U/mL (200 μg/mL) and EDTA was 100 μM. DMSO was present at a final concentration of 0.1% in 1–10. Copper compounds were added at −5 min. Reactions were started by the addition of xanthine oxidase at 0 min.

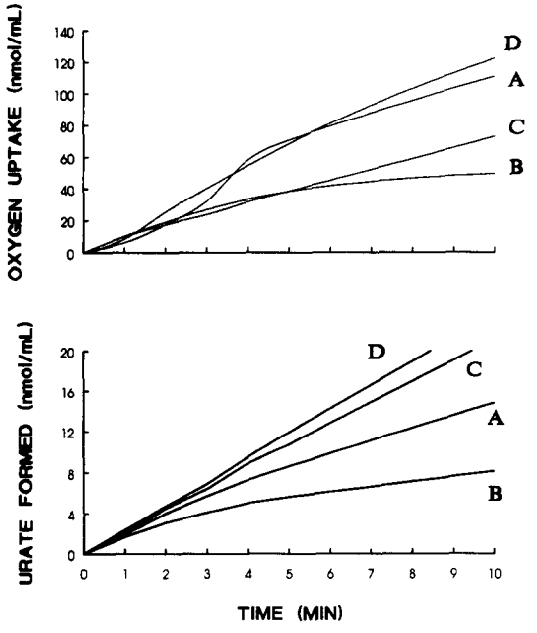

Fig. 4. Inhibition of xanthine oxidase by $CuSO_4$. (Upper panel) Oxygen uptake. (A) Control; (B) 2 μM $CuSO_4$; (C) catalase (200 μg/mL) with or without 2 μM $CuSO_4$; and (D) EDTA (100 μM) with or without 2 μM $CuSO_4$. (Lower panel) Generation of uric acid from xanthine. (A) Control; (B) 0.2 μM $CuSO_4$; (C) catalase (200 μg/mL) with or without 0.2 μM $CuSO_4$; and (D) EDTA (100 μM) with or without 0.2 μM $CuSO_4$. Indicated reagents were added 5 min prior to the addition of xanthine oxidase (10 mU/mL for upper panel; 1 mU/mL for lower panel). Other details as in Fig. 3.

ability to dismute superoxide [3, 6, 11]. Agents such as $Cu(II)_2(indo)_4$ and $Cu(II)_2(3,5\text{-DIPS})_4$ have been used frequently in biological systems and observed effects have often been ascribed to SOD-like activity [7–9, 11–13, 17], although other mechanisms have been suggested [1]. Most previous studies which have observed inhibitory or cytotoxic effects of copper complexes have attributed this to SOD-mimetic activity of the intact complex or of released copper [10–12, 17, 18]. However, the possibility of biological effects caused by mechanisms other than the dismutation of O_2^- needs to be considered. It has been shown that lipophilic copper complexes and copper sulfate both inhibit cytochrome P450 catalysed reactions [19]. Copper sulfate also has been shown to have a potentiating effect on radiation-induced inactivation of penicillinase in the presence of O_2^-; this effect is enhanced markedly if H_2O_2 is also present [20]. These authors propose that copper(II) is complexed to the protein, reduced to copper(I) by O_2^-, and then OH· is generated by H_2O_2 in a site-specific Fenton reaction. $Cu(II)_2(3,5\text{-DIPS})_4$ as well as $CuSO_4$ inhibit TPA-stimulated protein kinase C [21]. A lipophilic copper complex has been shown to be cytotoxic for K652 erythroleukemia cells; this effect is largely blocked by catalase, but not by inactivated catalase [18]. Thus, actions other than dismutation of O_2^- can be attributed to lipophilic complexes.

Our data indicate that lipophilic copper complexes as well as copper sulfate can inhibit both the respiratory burst of intact human granulocytes and xanthine oxidase; this inhibition can be prevented by catalase, reminiscent of the observations discussed above [18, 20]. Interestingly, earlier authors failed to find an inhibitory effect of SOD-mimetic copper complexes on either xanthine oxidase or the respiratory burst of granulocytes. The inhibition may

have been overlooked because, in both cases [12, 22], the reactions were followed for less than 3 min after addition of the copper compound. Our data are consistent with an earlier report [10] that there is a lag period of about 5 min before the inhibition is evident.

Protection by prior addition of EDTA is expected, since an excess of EDTA is known to remove copper from its complexes with indomethacin or DIPS [2]; copper(II)-EDTA is not SOD-mimetic [23]. The lag in onset of inhibition by the copper complexes suggests that redox cycling of the copper in the complex is needed. The lag may be a period in which the ligand is destroyed, as postulated for superoxide dismutase itself [24] and for other metal chelates [25, 26].

The precise mechanism of inhibition by these copper complexes of the respiratory burst of granulocytes and xanthine oxidase remains uncertain. The respiratory burst oxidase is a multi-component, membrane bound enzyme system which can be activated and inhibited at many different sites by a very large number of diverse agents [27]. Toxicity to tumor cells by these and other SOD-mimetic compounds has been reported and was proposed to be due to their ability to dismute O_2^- and raise H_2O_2 levels [17, 28, 29]. More recently, cytotoxicity has been proposed to be due to intracellular redox cycling of the copper complex to generate intracellular O_2^- and H_2O_2 [18]. We favor the mechanism proposed by Samuni *et al.* [20] discussed above since we observed inhibition both in a cell-free system (xanthine oxidase) and in a cellular system (granulocytes). In any case, our observations indicate that caution is warranted in the interpretation of experiments using these copper complexes, particularly where they are used as SOD-mimetic agents.

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