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DO THE COPPER COMPLEXES OF HISTAMINE, HISTIDINE AND OF TWO H_2-ANTAGONISTS REACT WITH O_2^-?

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The effects of cimetidine, ranitidine, histamine and histidine, as well as of their copper complexes, have been examined in an enzymic and chemical O_2^- generated systems. Copper complexes like CuZnSOD inhibited both the reduction of cytochrome c and NBT^{2+} in xanthine-xanthine oxidase systems, but their inhibitory action was due to a certain extent to the copper-induced inhibition of xanthine oxidase. EDTA abolished the inhibitory effect of all copper complexes studied. Luminol chemiluminescence in $NADH_2$-PMS system was inhibited by CuZnSOD while it was enhanced by copper complexes. The copper-accelerating effect gradually increased up to about 1 μM Cu and decreased, reaching the control values up to 10 μM Cu. In the presence of low copper concentrations chemiluminescence was inhibited by CuZnSOD only, while in the presence of high copper concentrations it was inhibited by catalase and mannitol, but not by CuZnSOD. The ligands however, have been ineffective in the two O_2^- generated systems.

KEY WORDS: Copper complexes, luminol chemiluminescence, superoxide, xanthine oxidase, histamine, H_2- antagonists

INTRODUCTION

Oxygen-derived free radicals have been found to contribute to the development of gastrointestinal mucosal damage.[1-3] This explains the interest to reactions of active oxygen species with substances displaying an ulcerogenic or antiulcer activity. Cimetidine and ranitidine inhibit excessive acid secretion caused by histamine and currently are in worldwide clinical use for treatment of peptic ulcers. They are potent histamine H_2- receptor antagoinsts but it is possible that they also have other activities. Thus cimetidine decreases or even abolishes the inhibitory effect of histamine on reaction systems in which active oxygen species are generated.[4,5]

On the other hand it is known that a great many copper complexes of different compounds are potent antiulcer agents and has been suggested that one of the mechanisms of this effect is their ability to mimic SOD.[6,7]

It was reported that the cimetidine activity for inhibiting the acid secretion is dramatically enhanced by an addition of copper ions and that Cu(cimetidine)$_2$ exhibits a higher SOD-like activity than any previous complex.[8] By means of pulse radiolysis experiments it was discovered that only one hstidine complex $(CuHist_2H)^{3+}$ catalyzes the disproportionation of O_2^-.[9] Cu(histidine)$_2$ complexes formed in a buffer

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or plasma have a SOD-like activity in hypoxantine-xanthine oxdiase-cytochrome system, but with a high I_{50}.[10]

In connection with the data for the mechanism of SOD-like activity of copper ions and complexes in indirect SOD methods,[11-14] we examined the effects of histamine copper complexes, of its predecessor histidine and of two H_2-blockers – cimetidine and ranitidine – in two O_2^- generating systems. The present results have shown that these copper complexes, similarly to SOD, inhibit the reduction of cytochrome c and NBT^{2+} in a xanthine-xanthine oxidase system but contrary to SOD they activate luminol chemiluminescence in a $NADH_2$-PMS system.

MATERIALS AND METHODS

Materials

Cytochrome c, xanthine oxidase (20 U/ml), bovine Cu–Zn-superoxide dismutase (5000 U/mg), catalase (250 mg/12.5 ml) and $NADH_2$ were purchased from Böehringer, Mannheim; Luminol and Phenazine methosulfate (PMS) from A.g. Buchs, Switzerland; xanthine and nitro-blue tetrazolium (NBT^{2+}) from Koch-Ligh Laboratories Ltd, England; mannitol from Riedel-de-Haen ag seelse, Hannover; $CuCl_2$ from Reanal, Budapest, Hungary; cimetidine from Smith Kline French Pharmaceutical Co. Ltd.; ranitidine from Janssen Chimica, Belgium. $Cu(cimetidine)_2$ was prepared as described in[15] using $CuCl_2$. Per absorption maximums of 340 and 610 nm the preparation is identical with that of Greenway *et al.*[15]

$Cu(ranitidine)_2$ was received in a similar way but at a ratio 1:3 of copper to ligand.

$Cu(histamine)_2$ and $Cu(histidine)_3$ were prepared according to[16] with metal to ligand ratios of 1:10 and 1:2 respectively. All chemicals employed were of analytical grade and were used without further purification.

Methods

Superoxide dismutase activity was measured by enzymic and non-enzymic O_2^- generating systems. Xanthine–xanthine oxidase – cytochrome c[17] and xanthine–xanthine oxidase – NBT^{2+}[18] methods were applied according to conditions, described by the above mentioned authors with or without EDTA depending on the aim of the experiment. The $NADH_2$-PMS system[19] was optimized for the conditions of luminol chemiluminescence (CL) by I. Russanova and V. Savov (unpublished data). Light measurements were performed on LKB-Wallac 1251 Luminometer. In this system light emission – CL – increases with the rize of concentration of $NADH_2$ (up to 1 mM) and luminol (up to 500 μM), the optimum PMS concentration being 10 μM and optimum pH 8.5. A reaction mixture of 1 ml containing 200 μM $NADH_2$, 10 μM PMS, 500 μM luminol and 0.1 M phosphate buffer with pH 8.5 at 25°C was used. The kinetics of CL has a short (seconds) lag-period, a rapid exponential increase, the duration of which depends on intensity, and a slow (up to 20 min) fading. With such kinetics the effect of the examined substances was detected on the basis of light emission intensity at its peak. The chemiluminescence in this system is not inhibited by catalase and mannitol.

Xanthine oxidase activity was measured on the basis of uric acid formation followed at 292 nm in a reaction mixture for NBT^{2+} reduction from which NBT^{2+} was omitted.

RESULTS

Reduction of Cytochrome c and NBT^{2+} by Xanthine–xanthine oxidase O_2^--generated System

$CuCl_2$ as well as the copper complexes of cimetidine, ranitidine, histamine and histidine inhibited, similarly (but they are much less effective) to CuZnSOD, the reduction of cytochrome c and NBT^{2+} in xanthine–xanthine oxidase system. The inhibition was dose-dependent and the I_{50} concentrations were calculated on this basis (Table 1). EDTA (100 μM) abolished the inhibitory effect of all copper complexes studied. Ligands alone, in concentrations which corresponded to or exceeded those of the complexes have no effect (data not shown). Thus, it was concluded that the copper complexes' effect was due solely to the copper action, a fact which is known for a large number of copper complexes ([14] and the ref. therein). As Table 1 shows the I_{50} values of copper complexes ranged from 0.3 o 8.5 μM. This may be explained by the differences in their redox potentials in their stability towards other media components and in their dissociation. These values are consistent with those calculated for a large number of copper complexes with other ligands.[14] Our data do not serve to confirm the data, verifying a high SOD- like activity of $Cu(cimetidine)_2$ as well as its effectiveness in the presence of EDTA.[8] Comparing I_{50} for different methods may confuse as k_o [D] are different by a factor of about 10 for cyt-c 10 μM × 1.1 × 10^6 and NBT 27 μM × 5.88 × 10^4.

The effect of the copper complexes in indirect SOD assay systems has been considered as SOD-like activity, though it was demonstrated that copper complexes take part in reactions not only with O_2^- but also with other oxygen species.[12,13] Furthermore in these systems different side-reactions take place and this fact casts certain doubts on the ability of copper to catalyze the dismutation of O_2^- and confirms the suggestion that its SOD-like activity is a putative one.

The possible participation of copper in the reduction or reoxidation of cytochrome c and the effective competing of $NBT^{+\cdot}$ with O_2^- for Cu^+ have been discussed in

TABLE I

Effects of copper complexes on the reduction of NBT^{2+} and cytochrome °c in xanthine–xanthine oxidase O_2^- generated system as well as on the urate production of xanithine oxidase

Compound	NBT^{2+}-reduction[1] I_{50} (copper μM)	cyt.c-reduction[2] I_{50} (copper μM)	urate production[3]	
			concentration range μM	inhibition %
$CuCl_2$	0.3	3.2	0.3– 3.0	0–28
Cu-ranitidine	0.3	6.5	0.3– 6.5	10–32
Cu-cimetidine	3.0	8.5	0.3– 8.5	20–68
Cu-histamine	3.8	4.8	4.0– 5.0	30–45
Cu-histidine	3.8	4.0	4.0–10.0	13–15

[1]Reaction mixtures contained 100 μM xanthine, 27 μM NBT^{2+} 9 mU/ml xanthine oxidase and 1 mM potassium phosphate, pH 6.8, at 25°C. The mean value of ΔA_{560} without additions was 0.016.
[2]Reaction mixtures contained 50 μM xanthine, 10 μM cytochrome c, 19 mU/ml xanthine oxidase, 27 μg/ml catalase and 1 mM potassium phosphate, pH 6.8, at 25°C. The mean value of ΔA_{550} without additions was 0.025.
[3]Reaction mixtures as in 1 without NBT^{2+}. The mean value of ΔA_{292} without additions was 0.022. The rates of the three reactions were measured for four minutes without and with additions. Results are expressed as the mean value of four experiments.

detail.[14] In addition the significance of $OH^{\cdot}$ for the reoxidation of cytochrome c and the reaction of $NBT^{+\cdot}$ with Cu^{+} was indicated.[11]

As Table 1 shows, the inhibition of xanthine oxidase from copper complexes was of significant importance for their effects in the twoO_2^{-}-generated systems.

Luminol Chemiluminescence in $NADH_2$-PMS-O_2^{-}-Generated Systems

The chemiluminescence (CL) in $NADH_2$-PMS-luminol system was not inhibited by catalase or mannitol (data not shown) but was inhibited by CuZnSOD in a dose-dependent manner (Figure 1A). Contrary to SOD, the copper ions and complexes in concentrations of 0.2 to 1.0 μM activated the luminol CL (Figure 1B). Ligands alone had no effect in concentrations, corresponding to these of the complexes but in higher concentrations they inhibited slightly (data not shown). Since it is obvious that the effect is due to copper, the effect of $CuCl_2$ was analyzed in more detail.

With the increase of copper concentrations up to 1 μM the CL intensity roze tremendously (Table 2) and the period of time necessary for reaching CL peak increased from 30 sec up to 2.0 min. Any further increase of copper concentrations up to 10 μM lead to a decrease in the activating effect and CL intensity went down to the control values. Also CL peak reaching time was shortened to 30 seconds. The activating effect of copper in concentrations from 0.2 to 10.0 μM was with a different sensitivity to CuZnSOD, catalase and mannitol. Thus up to 1.0 μM copper, its activating effect was reduced by CuZnSOD and up to 0.6 μM copper, it was not affected by catalase (Table 2) or 4 mM mannitol (data not shown). At copper con-

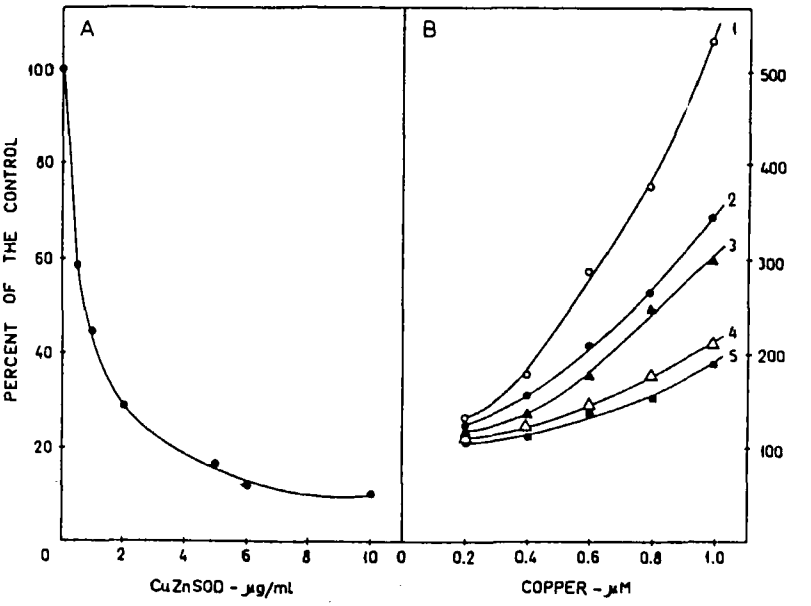

FIGURE 1 Chemiluminescence of luminol in $NADH_2$-PMS-O_2^{-}-generated systems. A – Inhibition by CuZnSOD. Reaction mixtures contained 200 μM $NADH_2$, 10 μM PMS, 500 μM luminol, 0.1 M potassium phosphate pH 8.5, at 25°C plus the indicated concentrations of CuZn-superoxide dismutase; B – Activation by copper. Reaction mixtures as in A plus the indicated concentrations of copper: (1) $CuCl_2$; (2) Cu(ranitidine)$_2$; (3) Cu(cimetidine)$_2$; (4) Cu(histidine)$_2$; (5) Cu(histamine)$_2$.

TABLE 2
Effects of CuZnSOD and catalase on the copper-induced activation of luminol chemiluminescence in $NADH_2$-PMS-O_2^--generated system

	no additions		plus CuZnSOD		plus catalase	
Copper	max light emission (mV)	activation %	max light emission (mV)	inhibition %	max light emission (mV)	inhibition %
No	23.2 ± 0.86	0	9.4 ± 0.40	60	24.7 ± 1.78	0
0.2	30.2 ± 0.86	30	13.0 ± 0.20	57	32.2 ± 2.17	0
0.4	42.4 ± 2.12	83	19.1 ± 2.42	55	43.8 ± 1.06	0
0.6	66.8 ± 6.46	188	39.4 ± 0.61	41	31.9 ± 1.21	52
0.8	87.5 ± 4.04	277	52.5 ± 1.67	30	31.3 ± 1.97	64
1.0	123.6 ± 3.38	433	92.8 ± 2.44	25	38.9 ± 0.25	69
3.0	64.6 ± 0.15	178	53.6 ± 1.00	17	18.1 ± 0.58	72
5.0	48.7 ± 1.16	110	46.2 ± 2.32	5	14.6 ± 0.42	70
7.0	40.0 ± 0.25	72	38.8 ± 1.52	0	10.2 ± 0.20	75
9.0	30.2 ± 2.10	30	32.3 ± 1.57	0	7.6 ± 0.20	75
10.0	27.9 ± 0.30	20	28.2 ± 1.82	0	6.2 ± 0.32	80

Reaction mixtures contained 200 μM $NADH_2$, 10 μM PMS, 500 μM luminol and 0.1 M potassium phosphate, pH 8.5, at 25°C either without additions or plus 2 μg/mg CuZnSOD or 20 μg/ml catalase. Light intensity was measured in its maximum (between 0.30–2.0 min after mixing the reagents) and was expressed as the mean ± SE of six to ten experiments. All light measurements were corrected for background of 0.5 mV.

centrations higher than the above-mentioned the activating effect of copper was not influenced by CuZnSOD any more but began to be reduced by catalase and mannitol. This indicated that at low copper concentrations its activating effect was O_2^--dependent and independent of H_2O_2 and $OH^\cdot$ and conversely at higher concentrations of copper the effect was dependent on H_2O_2 and $OH^\cdot$ and indepndent of O_2^-. The possibility could not be excluded that the absence of a CuZnSOD effect was due to the inhibition of the enzyme by H_2O_2.

DISCUSSIONS

In direct methods for SOD activity copper and copper complexes catalyze the disproportionation of O_2^-.[13] However with indirect systems at low O_2^- concentrations and in the presence of other substances in the medium the effect of the copper complexes differs from that of SOD. The copper complexes examined here, inhibit the reduction of cytochrome c and NBT^{2+} but to a large extent the effect is due to side-reactions. And what is more is that in o-dianisidine photo-oxidation assay[11] and in the $NADH_2$-PMS-luminol system SOD and the copper complexes have opposite effects. In these relatively simple systems copper engages in a reaction not only with O_2^- but with other active oxygen species and this is demonstrated by the catalase and mannitol effects. Furthermore it catalyzes reactions with components of the medium so that the final results depends on its composition. At realistic biological conditions the possibilities for the taking place of side-reactions increase manifold. So that with regard to active oxygen species and the damages caused by them to biological structures the final effect of copper can be protective or intensifying-sensibilizing.[13] Evidently copper complexes cannot be an analogue to SOD. So that the assumption that the SOD activity of copper complexes acts as a mechanism for their antiulcer and

anti-inflammatory action[6,7] cannot be correct. The other mechanisms, discussed by the same author are more probable. In this respect the inhibiting effect of copper complexes on xanthine oxidase, which is a significant O_2^- generator in the organism, is of great interest for future research works. Certain data exist that allopurinol, which is a specific inhibitor of this enzyme, protects the mucose from lesions.

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