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AN *IN VIVO*, *EX VIVO* AND *IN VITRO* COMPARATIVE STUDY OF ACTIVITY OF COPPER OLIGOPEPTIDE COMPLEXES *VS* Cu(II) IONS

MARIO CIUFFI[a,*], CRISTINA CELLAI[b], SERGIO FRANCHI-MICHELI[a], PAOLA FAILLI[a], LUCILLA ZILLETTI[a], MAURO GINANNESCHI[c], MARIO CHELLI[c], ANNA MARIA PAPINI[c] and FRANCESCO PAOLETTI[b]

[a]*Dipartimento di Farmacologia Preclinica e Clinica, Firenze, Italy*, [b]*Istituto di Patologia Generale, Firenze, Italy and* [c]*Dipartimento di Chimica Organica 'Ugo Schiff, e CNR (centro sintesi dei composti eterociclici e loro applicazioni), Università degli Studi di Firenze, Firenze, Italy*

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The tetrapeptide-Cu(II) complex H-(L-His-Gly)$_2$-OH/Cu(II), indicated as L-Cu(II), has been investigated, as compared to the Cu(II) inorganic salt $CuSO_4$, for its antioxidative and anti-inflammatory properties under a panel of experimental conditions. Both inorganic and organic Cu(II) compounds showed comparable activities *in vitro* and *ex vivo* by: (i) protecting, in a dose-dependent manner, rat brain homogenates from Fe(III)/ascorbate- or haemoglobin-induced lipid peroxidation; (ii) inhibiting the superoxide-mediated ferricytochrome *c* reduction by activated macrophages. $CuSO_4$ and L-Cu(II) also exhibited similar anti-inflammatory effects *in vivo* by reducing significantly the extent of carrageenan-induced edema in the rat paw. The activities of the two compounds diverged strikingly only in the xanthine/xanthine oxidase system at low phosphate buffer concentration. L-Cu(II) decreased the rate of NBT reduction by superoxide in a true SOD-like fashion without affecting urate production. Instead, Cu(II) ions caused the rapid xanthine oxidase inactivation thus inhibiting both urate and superoxide production; this effect might be ascribed to the superoxide-mediated generation of the strong oxidant Cu(III) and its interaction with the enzyme. The administration of Cu(II), whether complexed with linear oligopeptides or as an inorganic salt, to animals or tissue extracts, conferred protection against oxidation and ought, conceivably, to interact with endogenous biological molecules and form highly bioavailable complexes which serve, subsequently, as the real scavengers. Moreover, the claimed prominent scavenger activities of Cu(II)-oligopeptide complexes over inorganic copper ions could be realised only in very simple *in vitro* systems through mechanisms which, although of biochemical interest, are unlikely to be of physiopathological significance.

KEY WORDS: copper chelates, oligopeptide-Cu(II) complex, lipid peroxidation.

INTRODUCTION

Reactive oxygen species are known to interact with many of the cellular components and be widely involved in a variety of physiopathological processes [1, 2]. The first defence line of aerobic organisms against the formation and propagation of active oxygen derivatives is represented by superoxide dismutases (SODs), a family of metalloenzymes which catalyse the conversion of superoxide to molecular oxygen and H_2O_2 [3, 4].

In order to enhance cellular protection against free radical injury, SOD has also been tentatively employed as an anti-inflammatory agent [5, 6]. Unfortunately, the systemic use of SOD in therapy has been hampered so far by its short half-life in the circulation [7], poor capability to enter cell membranes [8, 9], antigenicity [10] and potential sensitivity to inactivation by H_2O_2 [11, 12]. To overcome these inconveniences several low molecular mass transition metal complexes with SOD-like properties have been developed and those with copper received special attention during past years [13–16] as convenient SOD substitutes.

Copper bound to histidine-containing peptides

* Corresponding author.

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(like in the active site of SOD) were assumed to catalyse the dismutation of superoxide by a redox reaction involving Cu(II) and Cu(I) [17, 18]. The role of histidine in these complexes should be that of increasing the potential due to the imidazole nitrogen bond which stabilised low-valent transition metals including Cu(I)-complexes but the matter is still controversial [18, 19].

However, as also pointed out by Czapski and Goldstein [20] for a correct evaluation of activities of SOD-mimetic compounds several questions should be addressed concerning mainly their stabilities, interaction with other molecules, bioavailability and eventually mechanisms of action in relation to the environment in which they were tested. All these items are unlikely to be faced collectively due to the fact that attention is often focused on *in vitro* experimental systems, necessary to derive fine and reliable information on the given drug but, actually, not sufficient to cover the full range of its potentialities.

To this aim, the L-histidylglycyl-L-histidylglycine-Cu(II) complex [L-Cu(II)] was synthesised [21] and tested further under *in vitro*, *ex vivo* and *in vivo* conditions in comparison with Cu(II) ions, whose activities were often neglected in the literature. Results presented herein concern mainly, but not exclusively, the properties of this particular Cu(II)-tetrapeptide complex taken as the representative member of histidine-containing Cu(II) coordination compounds and exhibiting *in vitro* high SOD activity in front of a low peroxidative potential [18, 19]. Indeed, L-Cu(II) was found to exert both antioxidant and anti-inflammatory activities but these were largely reproduced by replacing L-Cu(II) with Cu(II) ions. Some important differences, however, could be noticed in very simplified systems for superoxide production such as the xanthine/xanthine oxidase (X/XO) assay where the presence of either the organic or inorganic Cu(II)-complex led to different chain reactions.

MATERIALS AND METHODS

Materials

Rat haemoglobin, 1,1,3,3,tetramethoxypropane, bovine serum albumin, carrageenan, ketamine-HCl, ferricytochrome *c*, *N*-formyl-methionyl-leucyl-phenylamine, xanthine, xanthine oxidase and nitroblue tetrazolium were from Sigma Chemical Co., MO, USA. Bovine erythrocyte CuZn-superoxide dismutase and beef liver catalase were products of Boehringer Manneheim, Germany. RPMI 1640 and Hank's solution were purchased from GIBCO BRL, Paisley, Scotland, UK. All other reagents were of the highest analytical grade commercially available.

Measurement of lipid peroxidation

Experiments carried out *in vitro* involved the induction of lipid peroxidation in rat (Wistar, 150–200 g; Morini, Italy) corticocerebral homogenate, in the absence (control) or in the presence of either Cu(II) ions, oligopeptides L and AcL [H-(L-His-Gly)$_2$-OH and Acetyl-(L-His-Gly)$_2$-OH, respectively] or their Cu(II) complexes, L-Cu(II) and AcL-Cu(II) [H-(L-His-Gly)$_2$-OH/Cu(II) and Acetyl-(L-His-Gly)$_2$-OH/Cu(II), respectively] with concentrations varying from 10^{-5} to 10^{-4} M. For *ex vivo* studies, lipid peroxidation was induced in cortex homogenates of rats which had been previously injected i.p. with equivalent doses of the $CuSO_4 \cdot 5H_2O$ (3.4 mg kg^{-1}), L-Cu(II) (8 mg kg^{-1}) or AcL-Cu(II) (8.6 mg kg^{-1}) and killed 2 h later. Sham-operated rats (control) received an equal volume of distilled water. In both *in vivo* and *ex vivo* experiments, the brains from decapitated rats were sectioned along a horizontal plane at the level of the corpus callosum to obtain cortex samples. These were homogenised in 50 mM phosphate buffer pH 7.4 (10% w/v) and subjected to lipid peroxidation by the addition of either $FeCl_3$ (10^{-5} M) and ascorbic acid (10^{-3} M) or haemoglobin (10^{-5} M). Tissue extracts were incubated at 37°C in a shaking water bath for 30 min. The extent of lipid peroxidation was assessed by measuring the levels of conjugated dienes and thiobarbituric reactive substances (TBARS) as follows.

Conjugate-diene assay [22]

Tissue homogenates (0.1 ml) were extracted with four volumes of a mixture of chloroform and methanol (2:1), then centrifuged for 10 min at 1900 ***g*** and aliquots of the chloroform phase were evaporated under an argon stream. The total lipid extract was redissolved in 1.5 ml of cyclohexane, and a spectrum was scanned between 220 and 350 nm (against a cyclohexane blank) in a Perkin-Elmer Spectrophotometer. The digitised raw spectra were corrected in order to eliminate the background due to Rayleigh scattering and the amounts of conjugated dienes were estimated at 232 nm by the difference between the sample and control absorbance values using a molar absorption coefficient of 2.52×10^4 M^{-1} cm^{-1}.

Determination of TBARS [23]

A volume of 0.1 ml of brain cortex homogenate was mixed with 0.5 ml of thiobarbituric acid (1% w/v) in 0.05 M NaOH followed by 0.5 ml of 25% HCl (v/v). The mixtures were placed into test tubes sealed with screw caps and then boiled for 10 min. After cooling, the chromogen was extracted with 3 ml of *n*-butanol and the organic phase was separated by centrifugation at 2000 ***g*** for 10 min. Changes in absorbance were monitored at 532 nm and O.D. values were expressed either as percentages of control or converted to nanomoles of malondialdehyde (MDA) per milligram of protein using a curve with

1,1,3,3,tetramethoxypropane as the standard reference.

Protein assay

The concentration of proteins in tissue extracts was determined according to Lowry *et al.* [24] using bovine serum albumin as the standard.

Induction of paw edema by carrageenan [25]

Male Wistar rats (150–200 g) were selected randomly, divided into two groups (four to five rats for each group), and starved for 18 h prior to treatment. Drugs, $CuSO_4$ (3.4 mg kg^{-1}) or L-Cu(II) (8 mg kg^{-1}), in equimolar doses, were administered i.p. 30 min before the injection of 50 μl of carrageenan suspension (1% in 0.9% NaCl) in the plantar tissue of the right hind paw of anaesthetised rats (ketamine HCl 20 mg kg^{-1}, i.p.) to induce edema. Uninjected rat paw served as controls. The paw thickness was measured by water displacement plethysmography, immediately prior to injection and after 15, 30, 60, 90, 120 and 150 min from carrageenan injection. The results were expressed as percentages of increase in thickness as compared to the uninjected paw.

Induction of macrophage oxidative burst and measurement of superoxide-mediated ferricytochrome c *reduction*

The SOD-inhibitable reduction of ferricytochrome *c* by activated macrophages was employed to compare *in vitro* scavenger activities of L-Cu(II) and Cu(II) ions. Lung macrophages were harvested from guinea pigs (Nossam, Italy) according to the procedure of Myrvik *et al.* [26]. Briefly, the tracheas of anaesthetised (pentothal, 80 mg kg^{-1} i.p.) animals were intubated and 60 ml of 0.9% NaCl solution were injected in aliquots of 5 ml. Cells were collected by centrifugation (700 ***g*** for 10 min) of the fluids and submitted to hypotonic lysis with 0.87% NH_4Cl to eliminate erythrocyte contamination. Mononuclear cells (10^6 cells) were then incubated in culture dishes for 2 h in RPMI 1640 medium at 5% CO_2 and 37°C to allow cell attachment and spreading. The plates were then washed with Hanks' balanced salt solution and macrophages were incubated for 1 h with 10 nM chemotactic *N′*-formyl-methionyl-leucyne-phenylalanine (fMLP) and ferricytochrome *c* (1 mg ml^{-1}) in the absence (control) and presence of either $CuSO_4$, the ligand or its Cu-adduct (10^{-5} and 10^{-4} M). Changes in absorbance were detected at 550 nm and the values, expressed as nanomoles of superoxide/10^6 cells, were calculated using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}.

Xanthine/xanthine oxidase assay

The assay was carried out basically according to the procedure of Beauchamp and Fridovich [27] in either 10 or 2 mM phosphate buffer pH 7.4 at 25°C in the presence of 47 μM xanthine and 0.1 mM nitroblue tetrazolium (NBT) as also reported by Bonomo *et al.* [28]. The reaction was started by the addition of a diluted XO solution (final concentration in cuvette was approx. 4.5 μg of protein corresponding to 5.4 mU of enzyme) that caused a change in absorbance, due to NBT reduction, of 0.024 O.D. min^{-1} at 560 nm and 25°C and followed over a period of 5 min. The measurement of uric acid production [29] in the X/XO system was carried out under the same conditions as above (apart from NBT solution that was omitted and replaced by an equal volume of buffer) in the presence of an increasing concentration of either Cu(II) ion or Cu(II)-oligopeptide complexes within the range of 0–1.5 mM. Increases in absorbance were monitored at 293 nm and 25°C for 5 min after XO addition. CuZn-SOD and catalase have been also used in these experiments at the concentration of 0.1 μg and 10 μg ml^{-1} in cuvette, respectively.

Statistical analysis

Data were reported as the average values ± SEM. Student's t-test (suitably computed by means of the paired or grouped data method) was used and a probability value of $P < 0.05$ was regarded as statistically significant.

RESULTS

The potential scavenging activities of Cu(II)-complexes were first evaluated *in vitro* by measuring their effects on induced lipid peroxidation in rat corticocerebral homogenates. Basal levels of peroxidated products were greatly enhanced upon the addition of Fe(III) and ascorbate to the homogenates (Fig. 1) to promote peroxidation by a Fenton-type reaction. However, induced increases in TBARS were abolished when homogenates had been also added with 10^{-4} M Cu(II)-containing compounds including namely L-Cu(II), its diastereomer H-(D-His-Gly-L-His-Gly)-OH/Cu(II), the derivative of L-Cu(II) [AcL-Cu(II)] (acetylated on terminal NH_2 to improve the liposolubility of the copper) and, interestingly, $CuSO_4$. The tetrapeptide ligands alone and its acetylated derivative yielded no significant variation in comparison to control.

Further investigation on the antioxidative effects of Cu(II)-peptide complexes *vs* $CuSO_4$ have been carried out mainly on L-Cu(II). This choice was justified by the fact that, among the several Cu(II)-peptide complexes, L-Cu(II): (i) showed high SOD-like activity in front of low peroxidative potential [19]; (ii) it has been well characterised for its physico–chemical properties at different pH values

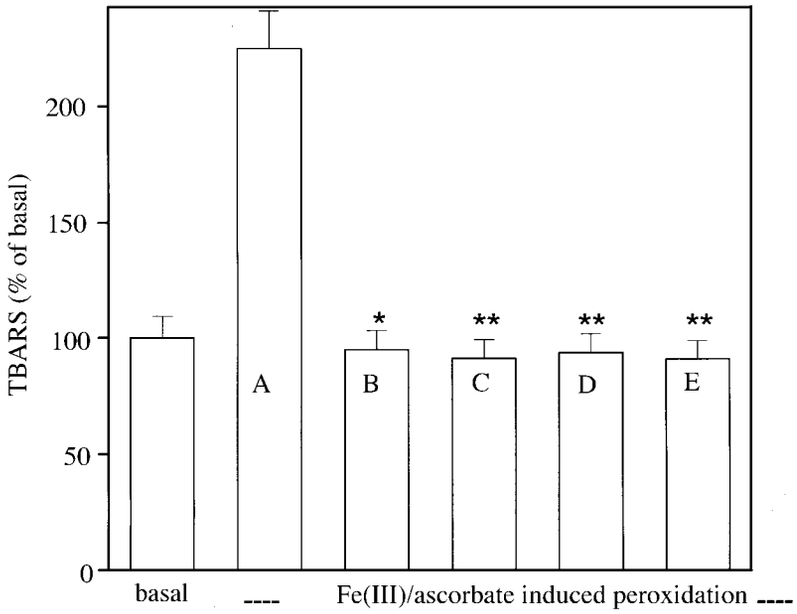

Fig. 1. *In vitro* effects of $CuSO_4$ and Cu(II)-oligopeptide complexes (10^{-4} M) on the induced formation of TBARS in rat corticocerebral homogenates incubated with or without copper compounds. (A), control (no additions); (B), + $CuSO_4$; (C), + L-Cu(II); (D), + diastereomer L-Cu(II); (E), + AcL-Cu(II). Values ± SEM were expressed as percentages of basal TBARS levels (O.D. mean = 0.240 at 532 nm) in uninduced homogenates. $^*P < 0.05$; $^{**}P < 0.02$ *vs* iron-induced control homogenate (no additions).

by means of spectroscopic and potentiometric techniques (manuscript in preparation).

Antiperoxidative activities of either the L-Cu(II) or $CuSO_4$ have been compared in dose–response experiments where the Fe(III)/ascorbate-induced formation of conjugated dienes in corticocerebral homogenates was measured (Fig. 2). The addition of increasing amounts (from 0 to 10^{-4} M) of the two copper solutions to tissue extracts caused a proportional decrease in the levels of induced conjugated dienes. Inhibition curves were not identical since $CuSO_4$ seemed slightly less effective than L-Cu(II); these differences, however, proved to be not statistically significant. Full protection against peroxidation occurred at the concentration of 10^{-4} M, but an appreciable inhibition was exerted even at 10^{-5} M. Neither the sulfate anion nor the tetrapeptide ligand alone or its acetylated derivative yielded significant variations over control values.

Ex vivo experiments were also performed to measure the bioavailability of either $CuSO_4$ or L-Cu(II) and AcL-Cu(II). Equimolar amounts of these compounds were injected i.p. into rats 1 h prior to killing; thereafter, rat cortical homogenates were prepared and incubated with iron compounds to trigger lipid peroxidation. The system Fe(III)/ascorbate, as compared to haemoglobin, showed itself to be a better inducer of both conjugated dienes and TBARS in control homogenates. However, induced lipid peroxidation was decreased significantly in both groups of treated *vs* untreated animals to reach in some cases levels which were below the basal values (Table I). Values concerning the acetylated complex were quite comparable to those of L-Cu(II) (data not shown).

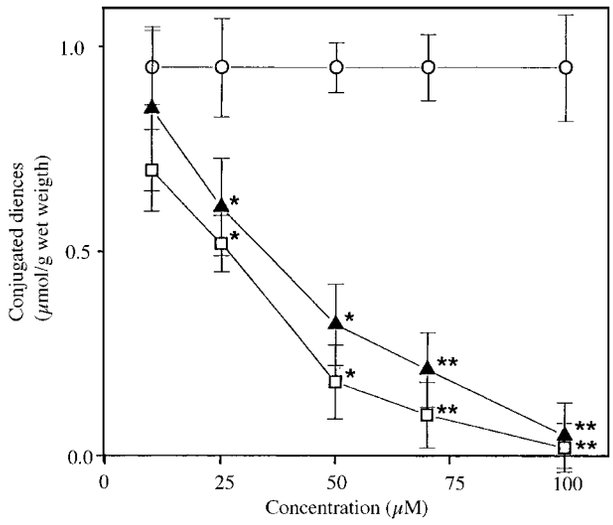

Fig. 2. Conjugated diene formation in rat corticocerebral homogenates following iron-induced lipid peroxidation in the absence or in the presence of increasing concentrations of $CuSO_4$ (▲) or L-Cu(II) (□). Data were the means ± SEM of four separate experiments (each assay has been carried out in triplicate). Basal levels of conjugated dienes in uninduced homogenates (O.D. mean = 0.189 ± 0.01) were subtracted. $^*P < 0.005$; $^{**}P < 0.001$ *vs* control iron-induced homogenate (○) (no copper compounds added).

The same protocol of L-Cu(II) and $CuSO_4$ administration was also employed to evaluate *in vivo* potential anti-inflammatory properties of the two com-

Table I
***Ex vivo* effects of $CuSO_4$ (3.4 mg kg^{-1} i.p.) and L-Cu(II) complex (8 mg kg^{-1} i.p.) on either Hb or Fe(III)-induced formation of conjugated dienes and TBARS in rat corticocerebral homogenates**

	Control rats (8)	*$CuSO_4$-treated rats (6)*	*L-Cu(II)-treated rats (7)*
Lipid conjugated diene (μmol g^{-1} wet wt.)†			
Homogenate + Hb	0.36 ± 0.12	0.12 ± 0.04*	0.08 ± 0.04*
Homogenate + Fe(III)	1.04 ± 0.08	0.51 ± 0.06*	0.40 ± 0.07*
TBARS (μmol MDA mg^{-1} protein)			
Homogenate	2.72 ± 0.05	2.79 ± 0.06	2.90 ± 0.07
Homogenate + Hb	3.85 ± 0.15	2.17 ± 0.08*	2.00 ± 0.12*
Homogenate + Fe(III)	5.45 ± 0.11	3.33 ± 0.90*	3.48 ± 0.10*

*$P < 0.001$ significant *vs* controls (Student's t-test: grouped data evaluation).

†Expressed as the difference between the average values of the respective corticocerebral homogenates without Fe(III) or Hb (extinction mean values of control, Cu(II) and L-Cu(II)-treated rats were: 0.172 ± 0.003, 0.0179 ± 005; and 0.165 ± 0.004, respectively).

Each brain cortex was tested in triplicate and values were the means ± SEM. The numbers of rat brain cortices analysed were indicated in parentheses.

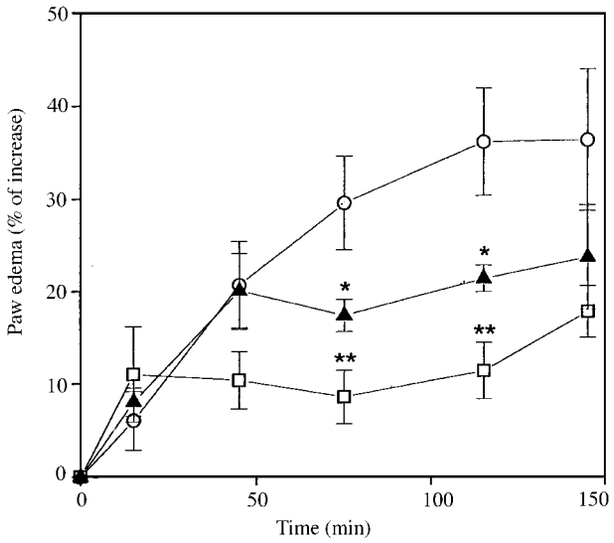

Fig. 3. Time-course of $CuSO_4$ (▲) and L-Cu(II) (□) inhibitory effects on carrageenan-induced paw-edema in the rats. Each value ± SEM represented the percentage of increase in paw thickness of carrageenan-injected *vs* the uninjected rats ($n = 4$). *$P < 0.05$; **$P < 0.02$ *vs* untreated (○) rats (Student's t-test: grouped data evaluation).

Table II
Effects of $CuSO_4$, L (ligand) and L-Cu(II) on the O_2^- production by either unstimulated or fMLP-stimulated macrophages

		Superoxide production†	
		Unstimulated cells	*fMLP-stimulated cells*
Control		1.74 ± 0.37	16.62 ± 0.22
$CuSO_4$	10^{-5} M	1.25 ± 0.39	2.08 ± 0.51*
	10^{-4} M	1.36 ± 0.45	1.82 ± 0.43*
L	10^{-5} M	2.18 ± 0.43	14.59 ± 0.81
	10^{-4} M	2.27 ± 0.21	16.66 ± 0.13
L-Cu(II)	10^{-5} M	1.73 ± 0.56	2.40 ± 0.29*
	10^{-4} M	1.19 ± 0.78	1.24 ± 0.17*

*$P < 0.001$ significant *vs* controls (Student's t-test: grouped data evaluation).

†Expressed as nmoles O_2^- produced by 10^6 cells; each value represented the mean ± SEM of at least four experiments.

pounds. The time-course of paw edema formation in untreated rats, upon carrageenan injection, showed that the plantar swelling increased progressively to reach a plateau within 2 h (Fig. 3). Instead, edema development in treated rats was reduced by approx. 50% or less. Anti-inflammatory effects occurred with some delay (more pronounced in the case of $CuSO_4$) and then maintained throughout the middle phase of induced inflammation.

In line with the potential anti-inflammatory activities of both organic and inorganic copper-compounds, these proved to inhibit cytochrome *c* reduction by superoxide anion in an *in vitro* system where rat alveolar macrophages were stimulated with fMLP. The burst of superoxide anion by activated macrophages was strongly quenched or even abolished in the presence of each Cu(II)-compound at the concentration of 10^{-5} or 10^{-4} M, respectively (Table II). No scavenging effect occurred in the presence of the tetrapeptide ligand alone.

SOD-like effects of $CuSO_4$ and L-Cu(II) have been eventually assessed in the X/XO system of superoxide production. This assay served to measure the rate of NBT reduction by superoxide anions and, alternatively, to monitor the generation of urate that was the other product of the X/XO reaction. Results in Fig. 4 showed that, indeed, both the Cu(II)-compounds were capable of inhibiting NBT reduction by superoxide in a dose-dependent manner, although $CuSO_4$ appeared more effective than L-

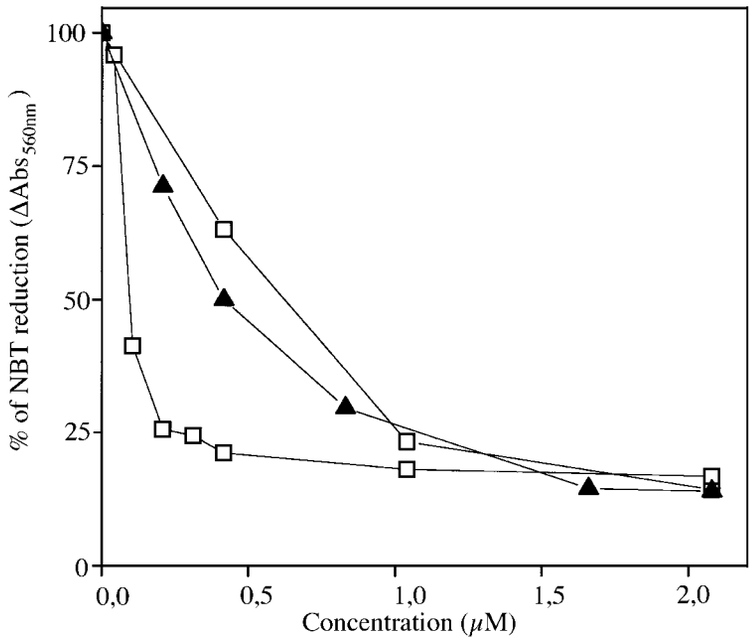

Fig. 4. Effects of increasing concentrations of L-Cu(II) (□), AcL-Cu(II) (■), and $CuSO_4$ (▲) on the rate of NBT reduction by superoxide in the X/XO system. Mean values ± SD (from four separate assays) were expressed as the percentages of control (no additions) and calculated over a period of 5 min at 25°C.

Cu(II). The acetylated form of L-Cu(II) was also tested in the assay showing a remarkable inhibition of NBT reduction. Values of IC_{50} for the L-Cu(II) and its acetylated derivative were calculated to be 0.67 and 0.083 μM, respectively. The non-linear kinetic yielded by $CuSO_4$ did not allow reliable IC_{50} calculations. Maximum inhibition of NBT reduction by the three copper compounds was steadily reached at approx. 1.5 μM. To monitor the effects of L-Cu(II), AcL-Cu(II) and $CuSO_4$ on the kinetics of urate production these compounds were incubated in the X/XO assay mixture at the fixed concentration of

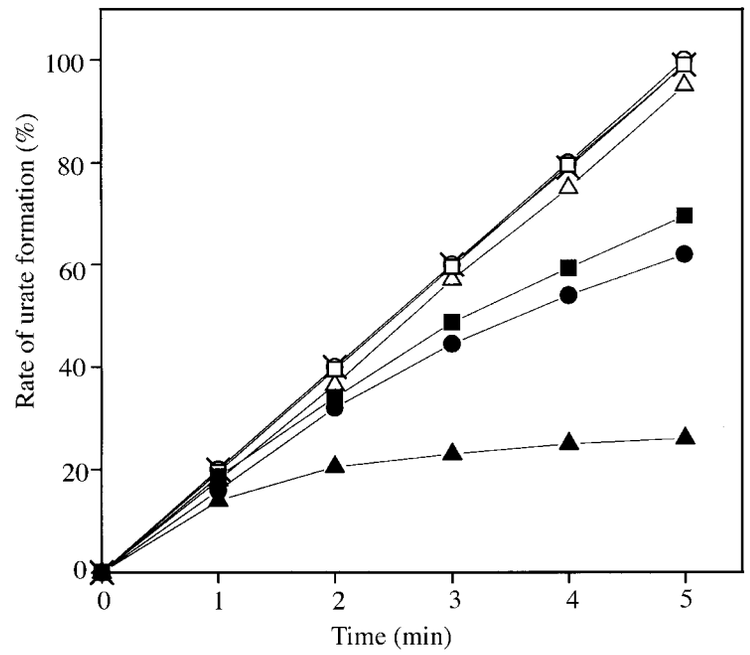

Fig. 5. Kinetics of urate production by the X/XO system in the absence and in the presence of a fixed amount (1.575 μM, final concentration in cuvette) of Cu(II) compounds. ○, control; X, $CuSO_4$ + SOD + CAT; ▲, $CuSO_4$; △, $CuSO_4$ + SOD; ●, $CuSO_4$ + CAT; □, L-Cu(II); ■, AcL-Cu(II). Values were expressed as the percentages of control rates (no additions). CuZn-SOD (0.1 μg ml^{-1}) and catalase (10 μg ml^{-1}), when included, were delivered to cuvette prior to start the reaction by xanthine addition. Assays were carried out at 25°C for 5 min.

1.575 μM and the reaction was followed over a period of 5 min. As shown in Fig. 5, L-Cu(II) caused no appreciable variation over control kinetics; in turn, $CuSO_4$ exerted a striking inhibition of the rate of urate production. Of note, the inhibition appeared to be irreversible (data not shown) but it could be fully prevented by the addition of CuZn-SOD prior to starting the reaction. The protective effect was exerted also by the combined addition of CuZn-SOD and catalase, while catalase alone allowed only a partial relief of inhibition. Eventually, the rate of urate production in the presence of AcL-Cu(II) showed it to be partially and progressively inhibited yielding a kinetic in between those of L-Cu(II) and Cu(II). The inhibitory effect of AcL-Cu(II) on urate production, however, was far from being comparable to its exceedingly high efficacy on the extent of NBT reduction seen in Fig. 4.

DISCUSSION

The antioxidative and anti-inflammatory properties of the L-Cu(II) complex, under *in vivo*, *ex vivo*, and *in vitro* conditions, have confirmed and extended the previously reported scavenger activities of this compound [19] and, conceivably, of other histidine-containing Cu(II)-complexes [17]. However, it was also shown here that, in most cases, quite similar SOD-mimetic effects could be reproduced by Cu(II)-ions whose biological effects were often overlooked in the literature. Indeed, both the $CuSO_4$ and L-Cu(II) showed almost overlapping activities in protecting rat cerebral homogenates from induced lipid peroxidation *in vitro* and *ex vivo* and also in reducing the formation of carrageenan-induced paw edema *in vivo*. These findings might be explained by admitting that Cu(II), whether arising from the complex or from the free aquoion, reacted with tissue components to yield unknown compounds which served subsequently as the real scavengers. The lability of the Cu(II)-tetrapetide complexes made feasible the release of Cu(II) in the presence of tissue components; therefore, the delivery of analogous metal–oligopeptide complexes to animals did not seem to offer special pharmacological advantages over the simple injection of inorganic Cu(II) solutions. Several endogenous molecules such as ceruloplasmin, albumin and aminoacids could complex and transport copper [30, 31]. Moreover, these newly-formed active Cu(II) compounds seemed to possess a high degree of bioavailability since they were transported away from the site of injection to reach peripheral tissues in the body and, eventually, cross the blood–brain barrier with their effects lasting up to 1 h from administration.

However, while *in vivo* biological effects of $CuSO_4$ and L-Cu(II) were comparable, their biochemical activities *in vitro* differed strikingly. $CuSO_4$ decreased both superoxide and urate production to suggest a progressive inactivation of XO; L-Cu(II), instead, was able to inhibit superoxide-driven NBT reduction without interfering at all with XO activity and the steady production of superoxide and urate as we might expect for a true SOD-like compound. To explain these discrepancies it must be mentioned that in the presence of 10 mM Na-phosphate buffer (as used for the X/XO reaction) most of the $CuSO_4$ was promptly converted into the $Cu(HPO_4)$ complex [32]. We may assume that Cu(II) in the $Cu(PO_4)$ complex reacted with superoxide yielding the strong oxidant species Cu(III), according to the following equation proposed by Goldstein *et al.* [17]:

$$Cu(II) + O_2^- + 2H^+ \leftrightarrow Cu(III) + H_2O_2$$

The interaction between XO, notably sensitive to oxidation [33], and Cu(III) may cause the irreversible enzyme inactivation which, however, was fully prevented by the addition of SOD prior to starting the reaction, to imply that superoxide anions were especially involved in the termination of the reaction by $CuSO_4$. Another chance of XO inactivation might potentially be represented by the presence of hydroxyl radical generated from a Fenton-type reaction of Cu(I) with hydrogen peroxide and this would explain the partial protective effect of catalase on XO activity. On the other hand, L-Cu(II) was likely to remain practically unaltered, even at low ligand-to-metal ratio (1:1, in this case) and act probably in a SOD-like fashion through the turnover Cu(II)/Cu(I) [34]. It was also worth mentioning that L-Cu(II) reacts only weekly with H_2O_2, formed during the O_2-reduction, to yield hydroxyl radical [19].

We have no data on the redox potentials of L-Cu(II) and AcL-Cu(II) to explain their notably different biochemical reactivities *in vitro*. However, the acetylation of the NH_2 end group, that was known to be involved in the complexation [21], may hamper the formation of the amino–copper bond and change the structure of the complex thus releasing some of the $[Cu(HPO_4)]$ species in the solution to generate Cu(III) species and inactivate XO. The moderate decrease in urate production by AcL-Cu did not match with its exceedingly high inhibition of superoxide-mediated NBT reduction. This fact would suggest that some metal was released from the organic complex to partially reproduce the effect of Cu(II) ions. The acetylation of L-Cu(II) might have decreased the stability of the complex but, in turn, greatly enhanced its true SOD-like activity.

Eventually, the inhibition of macrophage oxidative burst by either Cu(II) and L-Cu(II) could be addressed to the fact that the metal was trapped into biomolecules released from macrophages to produce scavenging effects similar to those observed *in vivo* and *ex vivo*. However, on the basis of results of the

X/XO assay, it was also possible that the metal, whether deriving from Cu(II) ions or displaced from the L-Cu(II), might complex with highly concentrated phosphate anions in the Hank's solution to enter the above reported equation and generate Cu(III). In this case, the inhibition of cytochrome *c* reduction rather than being ascribed to a superoxide scavenger activity might rely on the inactivation of NADPH-oxidase as also pointed out by Pellettier *et al.* [35].

On the whole, our results may have reshuffled the pharmacological potential of Cu(II)-oligopeptides, but, in turn, they provided new information on the role of Cu(II) ions and their complexes in biological systems pointing to the differences in chemical reactivity and stability between inorganic and organic Cu(II)-chelates. A great deal of low molecular weight copper complexes have been developed during the past 20 years for the treatment of inflammatory diseases, but, at present, it is not known how much and which kind of biologically active Cu compounds actually reach the site of inflammation. We believe that this work might be of help as a guideline to develop and evaluate SOD-mimetic activities of copper complexes in the view of their potential pharmacological use.

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