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The Copper Complex of Captopril is not a Superoxide Dismutase Mimic. Artefacts in DMPO Spin Trapping

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The effect of captopril and of its copper complex on several superoxide-dependent reactions used to detect and assay superoxide dismutase activity was studied, including pyrogallol and hematoxylin autoxidation and Nitro Blue Tetrazolium reduction. In none of these systems were superoxide dismutase-like properties of captopril/Cu apparent. Captopril/Cu decreased the yield of DMPO-OH adducts generated by KO_2 but this effect may be due to the acceleration of the decay of the adduct by captopril/Cu.

Key words: captopril, superoxide radical, copper, superoxide dismutase, SOD-mimics

Abbreviations: CAP, captopril; DMPO, 5,5,-dimethyl-l-pyrroline N-oxide; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetate; NBT, Nitro Blue Tetrazolium; SOD, superoxide dismutase

INTRODUCTION

Captopril ((S)-1-(3-mercapto-2-methyl-1-oxopropyl)-L-proline; CAP), inhibitor of the angiotensin-converting enzyme, is effective in the treatment of hypertension and congestive heart failure,[1] has antiinflammatory properties[2] and protects against reperfusion arrhythmias.[3] The antiinflammatory and cardioprotective action of CAP has been linked to its antioxidant action. However, the mechanism of the antioxidant activity of CAP has been the subject of controversy. Bagchi *et al.*[4] reported that CAP is a potent scavenger of $O_2^{-\bullet}$, $\bullet OH$, and OCl^-. Aruoma *et al.*[5], Kokreja *et al.*[6] and Jay *et al.*[7] did not find any significant superoxide scavenging by CAP, ascribing the antioxidant effects of CAP to scavenging of hypochlorite[5], nonspecific antioxidative activity[6] and chelation of metals, especially copper[7]. Recently it has been proposed that CAP-copper complex, in contrast to free CAP, reacts rapidly with $O_2^{-\bullet}$ by virtue of its superoxide dismutase (SOD)-like activity[8]. A similar function has also been ascribed to the CAP-iron complex[9]. In view of this controversy, relevant also to the mechanism of antioxidant action

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of naturally occurring thiols, we reexamined the behavior of CAP in the absence and in the presence of copper in several $O_2^{-\bullet}$-detecting systems. Our results do not support the hypothesis of the SOD-like activity of the CAP-copper complex.

MATERIALS AND METHODS

Potassium superoxide was obtained from ICN (Plainview, N.Y., USA). All other reagents were from Sigma (Deisenhofen, Germany). Water was purified in a Milli-QPlus system (Millipore) and had a resistivity of 18.2 MΩcm. CAP/Cu complexes were prepared according to Reguli and Mišík.[8]

Nitro Blue Tetrazolium (NBT) reduction by xanthine + xanthine oxidase was measured in a system of 100 μM NBT in 50 mM sodium phosphate buffer, pH 7.8, by monitoring the absorbance increase at 560 nm.[10] Urate production by the enzyme was estimated from the rate of absorbance increase at 292 nm, NBT being omitted from the system.

Cytochrome c reduction was quantified by measurement of absorbance changes at 550 nm in a system containing 0.9 mg/ml cytochrome c, 150 μM CAP and eventually 50 μg/ml Cu,ZnSOD in 50 mM sodium phosphate buffer, pH 7.4.[11]

Pyrogallol (0.2 mM) autoxidation was studied in 50 mM Tris-HCl buffer, pH 8.2, in the absence and in the presence of 1 mM ethylenediaminetetraacetate (EDTA). Absorbance increase was measured at 420 nm.[12]

Hematoxylin autoxidation was measured in 100 μM hematoxylin solution in 50 mM phosphate buffer, pH 7.4 and in 50 μM hematoxylin solution in 50 mM Tris/HCl, pH 9.0 by monitoring the absorbance increase at 560 nm[13]. Spectrophotometric measurements were made in a Cary 1 spectrophotometer (Varian, Australia).

Spin trapping was performed in 50 mM phosphate buffer, pH 7.4, containing 100 mM 5,5'-dimethyl-1-pyrroline N-oxide (DMPO) and eventually 2 mM CAP, 2 mM CAP/0.5 mM Cu^{2+} or 0.5 mM $CuSO_4$. Superoxide was generated by addition of supernatant of 0.1 M KO_2 solution in dimethylsulfoxide (DMSO) (10 μl per 35 μl of the final solution). When the effect of additives on the stability of the DMPO-OH• adduct was studied, 10 μl of the buffer containing DMPO was added with 10 μl of KO_2 in DMSO, followed by 5 μl of catalase (1 mg/ml) and, after 5-min incubation at room temperature, by 10 ul of water, CAP, CAP/Cu or $CuSO_4$ solution. ESR measurements were made in a Bruker ESP-300 spectrometer.

All the experiments were done at ambient temperature (21°C).

RESULTS AND DISCUSSION

The interesting hypothesis that CAP-copper complex may have a SOD-like activity was based on experiments in one superoxide-detecting system viz. spin trapping of $O_2^{-\bullet}$ with DMPO.[8] This idea might be valid also with respect to naturally occurring thiols, e.g. glutathione. In order to check the validity of this hypothesis, we examined the behavior of CAP in the absence and in the presence of Cu^{2+} in several other $O_2^{-\bullet}$-detecting systems used for determination of SOD activity.

CAP did not inhibit the NBT reduction by xanthine + xanthine oxidase. On the contrary, its presence increased the reduction rate of NBT. The reduction rate of NBT was diminished by Cu^{2+} and in the presence of CAP/Cu. However, these effects are apparently due not to scavenging of $O_2^{-\bullet}$ but to inhibition of xanthine oxidase by copper ions as demonstrated by the inhibition of copper on urate production by xanthine oxidase. CAP/Cu did not inhibit the enzyme (Table 1), apparently due to the higher affinity of cupric ions for CAP than for the enzyme. CAP reduced NBT in the absence of xanthine. The rate of this reaction increased with increasing pH and was much higher at pH 10.2 ($\Delta A_{560\ nm}$/min was 0.052 for 60 μM CAP). A contribution of CAP autoxidation to this reduction can be inferred from partial inhibition of this reaction by SOD (not shown).

TABLE 1 Effect of CAP on NBT reduction by xanthine + xanthine oxidase

System	$\Delta A_{560\,nm}/min \times 10^3$	$\Delta A_{292\,nm}/min \times 10^3$
Xanthine + XO	26.7	29.9
Xanthine + XO + 25 μM Cu^{2+}	8.1	5.6
Xanthine + XO + 100 μM CAP	37.3	35.4
Xanthine + XO + 100 μM CAP/25 μM Cu^{2+}	31.0	31.3
Xanthine	0	0
Xanthine + 100 μM CAP	2.1	0

Reaction medium contained 100 μM NBT + 700 μM xanthine + xanthine oxidase in 50 mM sodium phosphate buffer, pH 7.8 (measurements at 560 nm). In measurements of urate production (292 nm) NBT was omitted.

CAP reduced cytochrome c in the absence of xanthine oxidase. Again, the reduction was partly inhibited by SOD (not shown).

Neither CAP nor CAP/Cu affected the autoxidation rate of pyrogallol significantly in the presence of EDTA. In the absence of EDTA the rate of pyrogallol autoxidation was higher and was further augmented by Cu^{2+}. CAP alone stimulated the autoxidation slightly and the effect of CAP/Cu was intermediate between those of free Cu^{2+} and free CAP (Table 2).

Hematoxylin autoxidation rate at pH 7.4 (which is inhibitable by SOD) was not affected by CAP. The addition of Cu^{2+} augmented the autoxidation rate, in agreement with the findings of Martin *et al.*[13] The CAP/Cu complex behaved in the same manner as free Cu^{2+}, augmenting the autoxidation rate of hematoxylin (Table 3). Like in the case of pyrogallol, the stimulation of hematoxylin autoxidation by transition metal ions is abolished by EDTA[13]. Apparently, complexation of Cu^{2+} by CAP is not sufficient for an EDTA-like effect which can be expected taking into account the relatively low affinity of CAP for Cu^{2+} (dissociation constant of the CAP/Cu^{2+} complex was estimated to be about 3×10^{-6} M).[7]

Hematoxylin autoxidation rate at alkaline pH is known to be augmented by SOD.[13] At pH 9.0 this rate was slightly diminished by CAP and increased by Cu^{2+}. CAP/Cu augmented the autoxidation rate of hematoxylin but to a smaller extent than free Cu^{2+}.

Therefore, in the colorimetric tests for detection and estimation of SOD activity used by us, CAP/Cu complexes did not show any SOD-like activity.

A common feature of the effect of CAP in the autoxidation assays is the appearance of the lag period, apparently due to interaction with initiating free radical species (the same effect was seen with adrenalin autoxidation; data not shown). The occurrence of the lag period may lead to an erroneous conclusion that a thiol compound reduces

TABLE 2 Effect of CAP and Cu^{2+} on pyrogallol autoxidation

System	$\Delta A_{420\,nm}/min \times 10^3$	
	With EDTA	No EDTA
Control	30.2	42.2
25 μM Cu^{2+}	30.9	306.5
100 μM CAP	30.2	49.2
100 μM CAP/25 μM Cu^{2+}	29.1	87.6

Reaction medium contained 0.2 mM pyrogallol in 50 mM Tris-HCl buffer, pH 8.2, supplemented or not with 1 mM EDTA.

TABLE 3 Effect of CAP and Cu^{2+} on hematoxylin autoxidation at pH 7.4

System	$\Delta A_{560\,nm}/min \times 10^3$	Induction time [min]
Control	31	0
10 μM CAP	31	11.2
2 μM Cu^{2+}	59	0
10 μM CAP/2 μM Cu^{2+}	60	2.73
5 μM Cu^{2+}	74	0
10 μM CAP/5 μM Cu^{2+}	72	2.00
10 μM Cu^{2+}	111	0
10 μM CAP/10 μM Cu^{2+}	111	1.11

Hematoxylin was 100 μM in 50 mM sodium phosphate, pH 7.4

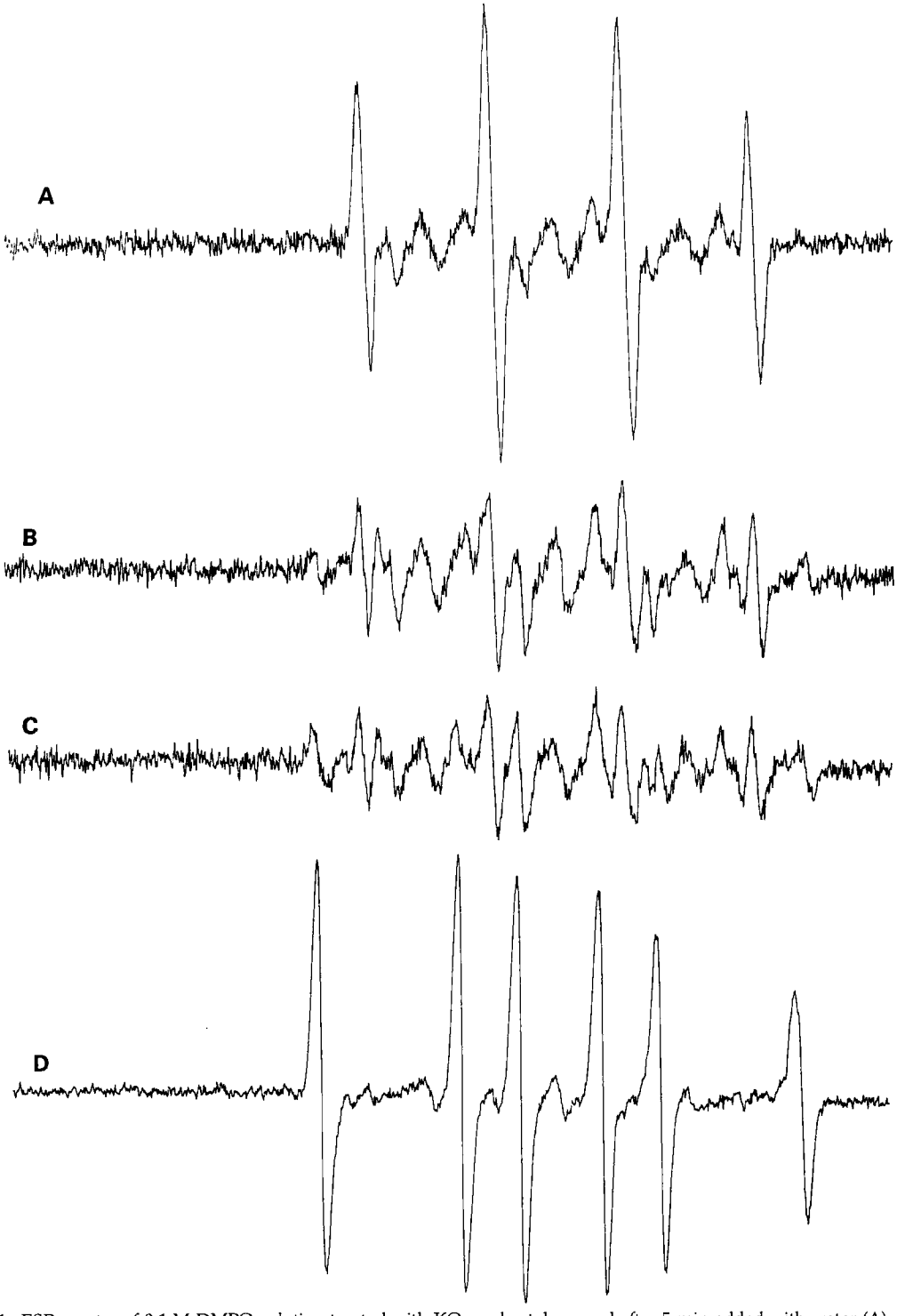

FIGURE 1 ESR spectra of 0.1 M DMPO solution treated with KO_2 and catalase, and after 5 min added with water (A), CAP (final: 2 mM; B), 2 mM CAP/0.5 mM Cu (C) or 0.5 mM $CuSO_4$ (D). Measurement started 60 s after introduction of the additives. Measurement conditions: microwave power 6.39 mW, modulation frequency 100 kHz, sweep width 100 G, modulation amplitude 0.5 G, receiver gain 2×10^4, time constant 10.24 ms.

TABLE 4 Effect of CAP and Cu^{2+} on hematoxylin autoxidation at pH 9.0

System	$\Delta A_{560\,nm}/min \times 10^3$	Induction time [min]
Control	241	0
10 μM CAP	220	2.83
2 μM Cu^{2+}	310	0
10 μM CAP/2 μM Cu^{2+}	297	1.0
4 μM Cu^{2+}	360	0
10 μM CAP/4 μM Cu^{2+}	310	0.83
10 μM Cu^{2+}	340	0
10 μM CAP/10 μM Cu^{2+}	315	0.40

Hematoxylin was 50 μM in Tris-HCl buffer, pH 9.0

the autoxidation rate to zero when the reaction is not monitored for a prolonged time period. However, as shown in Table 3, the effect of CAP effect may be confined to introduction of a lag time without any effect on the maximal autoxidation rate.

We observed a significant diminution of the formation of ESR spectra of the •OH-DMPO adduct generated by addition of KO_2 solution in DMSO in the presence of the CAP/Cu complex in agreement with the finding of Reguli and Misik[8] (not shown). However, when the adduct was first generated by addition of KO_2 followed by addition of catalase to remove the hydrogen peroxide formed, and after 5 min the aliquots were added with CAP, Cu^{2+} or CAP/Cu, there was a significant acceleration of the decay of the adduct by CAP and a much more pronounced acceleration by the CAP/Cu complex. The decay was apparently monoexponential with half-times of 19.7 min for control, 11.5 min in the presence of CAP and 7.7 min in the presence of CAP/Cu. Addition of Cu^{2+} alone further accelerated the decay of the adduct but augmented the sextet signal, attributable to the adduct of $•CH_3$ formed from DMSO (Figure 1). It seems therefore that the apparent SOD-like action of the CAP/Cu complex detectable in the spin trapping assay may be due to the acceleration of the decay of the DMPO adduct formed, rather than to the prevention of its formation, by the complex.

Halliwell discussed methodological pitfalls in the determination of 'antioxidant activity', including spin trapping.[14] One of the prerequisites for a valid measurement is that the supposed antioxidant should not interfere with the product measurement i.e. should be inactive when added to the reaction mixture at the end of the incubation. Apparently, both captopril and its copper complex does not fulfill this condition in the case of the spin trapping assay.[8]

In conclusion, we see no reason to accept the view of SOD-like activity of the CAP/copper complex.

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