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SUPEROXIDE SCAVENGING BY THIOL/COPPER COMPLEX OF CAPTOPRIL - AN EPR SPECTROSCOPY STUDY

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Scavenging of superoxide radical by angiotensin converting enzyme (ACE) inhibitor captopril (CAP), a thiol compound, was studied by several investigators and the results were contradictory; while some reported a high superoxide scavenging activity of CAP others found that CAP removed superoxide inefficiently. In this work we show that in the presence of copper ions the apparent rate of superoxide removal by CAP (molar ratio CAP:$CuSO_4$ = 4:1) was two orders of magnitude higher ($\approx 1.5 \times 10^5$ $M^{-1}s^{-1}$ at pH 7.4) than the literature value for superoxide scavenging by CAP alone ($< 10^3$ $M^{-1}s^{-1}$ at pH 7.4). We presume that in the presence of copper ions a CAP/copper complex with a SOD-mimicking activity is being formed. Similar results were also obtained with another thiol glutathione (GSH). The possible role of the CAP/copper complexes in the anti-inflammatory effect of CAP is discussed.

KEY WORDS: Superoxide radical, captopril, glutathione, thiol/copper complexes, SOD-mimics, EPR spin trapping.

Abbreviations: CAP - captopril; GSH - reduced glutathione; RSH - thiol, RS· - thiyl radical, DMPO - 5,5-dimethyl-1-pyrroline N-oxide; SOD - superoxide dismutase.

INTRODUCTION

Captopril (CAP), an angiotensin converting enzyme (ACE) inhibitor, is effective in treating hypertension and congestive heart failure.[1,2] Captopril also possesses anti-inflammatory activity,[3] and was reported to provide protection against ischemia/reperfusion - induced arrhythmias, a property not shared by other ACE inhibitors.[4] Since free radical-mediated damage may be an important mechanism in ischemia/reperfusion injury,[5] free radical scavenging by CAP has been studied; scavenging of hydroxyl radicals,[6-9] hypohalite radicals (OCl·) and hypochlorous acid (HOCl),[6,8] has been reported. The thiol group of CAP was demonstrated to be involved in the scavenging of hydroxyl radicals.[9] Scavenging of superoxide by CAP has been studied by several investigators, however, the results were conflicting: while some[6,10] reported that captopril was an effective scavenger of superoxide others[8,11] found that the reaction with superoxide is very slow ($< 10^3$ $M^{-1}s^{-1}$ at pH 7.4).[8]

Recently Jay *et al.*[12] suggested that the antioxidative mechanism of CAP may be

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related to a formation of inactive complexes of CAP with Cu(II) ions, and possibly with other transition metals, rather than to a direct radical scavenging.

The aim of this work was therefore, to analyze superoxide scavenging by CAP, using a technique of EPR spin trapping, and study the role of copper ions in the scavenging mechanism.

MATERIALS AND METHODS

Materials

Captopril [CAP; (S)-1-(3-Mercapto-2- methyl-1-oxopropyl)-L-proline], glutathione (GSH), crown-ether (18-crown-6), cupric sulphate ($CuSO_4.5H_2O$), and ascorbic acid were obtained from Sigma (St. Louis, MO), spin trap 5,5-Dimethyl-1-pyrroline N-oxide (DMPO, Sigma) was distilled prior use. Potassium superoxide (KO_2) and DMSO were from Fluka, Chemie AG (Germany).

Preparation of Thiol Copper Complexes

The thiol/Cu(I) complexes of CAP and GSH were prepared as described earlier.[13] Water solutions of 4 mM CAP or GSH were mixed with 1 mM $CuSO_4$.. Premixed solutions of thiols with copper were kept anaerobic and used within 1–2 hours. In some experiments ascorbate was used instead of the thiols to reduce Cu(II) ions.

Measurement of Superoxide Scavenging

A strongly buffered system was used to ensure that pH variations will not affect the rate of a non-catalyzed dismutation of superoxide or change the rate of superoxide spin-trapping by DMPO.[14] To 50 μl of 0.1 M sodium phosphate buffer (pH 7.4) 10 μl of water (control), 1 mM $CuSO_4$, or the premixed complexes were added, followed by 25 μl of 1 M DMPO. Superoxide was introduced in the system in 15 μl of DMSO (0.1 M KO_2 was solubilized by complexation of K^+ with 0.19 M crown-ether).[15] Note that at the pH used in this study most of the superoxide ($\sim$99.7%) was in the non-protonated form $O_2^{\cdot -}$ ($pK_a = 4.88$). The samples were drawn in 50 μl glass capillaries and transferred in the EPR cavity. EPR spectra were measured 2 minutes after mixing all components using a Bruker ER 200D-SRC spectrometer. Isotropic spectral simulation was performed using an ASPECT computer equipped with a Bruker software.

RESULTS

When KO_2 was added to a buffer containing DMPO – a spin trap, the spectrum with splitting constants $a_N = 14.8$ G, $a_H = 14.7$ G was detected [Figure 1A] and was identified as the spectrum of DMPO/$\cdot$OH adduct. These splittings are slightly different from the typical values reported in aqueous solutions ($a_N = a_H = 14.9$ G).[26] We verified that the difference is due to the presence of DMSO in our system; a spectrum with identical splittings with those of the spectrum shown in Figure 1A was obtained when the DMPO/$\cdot$OH adducts were initially formed in aqueous solution by UV-light decomposition of 1% H_2O_2 and subsequently 15% (volume) DMSO

was added to the samples and the EPR spectrum was measured. In the presence of 1.7 M ethanol the DMPO/·OH signal was not replaced by DMPO/·CH(OH)CH_3 [Figure 1B] which would have been formed if hydroxyl radicals were generated in the system; given the relative concentrations of ethanol/DMPO = 6.8 and using the rate constants of hydroxyl radical scavenging by ethanol (1.9×10^9 $M^{-1}s^{-1}$; Reference 27) and by DMPO (3.4×10^9 $M^{-1}s^{-1}$; Reference 14), from a simple kinetic analysis it follows that the intensity of the DMPO/·OH signal would have to decrease by 75% if it were formed by trapping of ·OH radicals. This experiment verified that the observed DMPO/·OH signal did not originate from trapping of hydroxyl radicals but rather from rearrangement of DMPO/·OOH adducts.[16] This system was, therefore, suitable for measurement of superoxide scavenging.

In the presence of 0.4 mM CAP the DMPO/·OH signal decreased by about 80% and was overlapped by a similarly looking quadruplet signal with splitting constants distinct from a DMPO/·OH adduct (a_N = 15.1 G, a_H = 15.6 G) [Figure 1C]. A spectrum with identical splittings was obtained when DMPO/·CAP-thiyl adducts were formed in aqueous solutions by hydrogen atom abstraction from CAP by ·OH radicals produced by 265 nm light photolysis of hydrogen peroxide,[9] followed by the addition of 15% DMSO to the samples after irradiation. Thus, the spectrum shown in Figure 1C was identified as DMPO/·CAP -thiyl adduct. Assignment of the splitting constants and determination of the portion of the remaining DMPO/·OH in the mixed spectrum was determined using computer simulation (Figure 1D). Since only negligible amounts of hydroxyl radicals were detected in our system the trapped thiyl radical must have been formed by the reaction with superoxide:

$$RSH + O_2\cdot^- + H^+ \longrightarrow RS\cdot + H_2O_2 \qquad (1)$$

A simple estimate of the rate constant of CAP with superoxide, calculated from a competition kinetics between DMPO and CAP, gives a value $\approx 7.5 \times 10^4$ $M^{-1}s^{-1}$ (see Footnote 1 for details).

When the same experiment was carried out in the presence of 0.4 mM CAP premixed with 0.1 mM $CuSO_4$ (see Methods), the DMPO/·OH signal became hardly detectable [Figure 1E, Table I]. This could be a result of superoxide scavenging by CAP/Cu but could also result from reduction of the nitroxide radical of the DMPO/·OH by Cu(I) which is formed during reduction of Cu(II) by CAP.[9] To exclude the latter possibility CAP was replaced by ascorbate which also reduces Cu(II), however, the intesity of the DMPO/·OH signal increased rather than decreased [Figure 1F, Table I]. The same result was also obtained when only Cu(II) was present in the reaction mixture in the absence of any reductant [Figure 1G, Table I]. The increase of the DMPO/·OH signal was probably a consequence of generation of hydroxyl radicals via reactions 2–4. The additional six line signal (a_N = 15.75 G, a_H = 22.55 G) [Figures 1F and 1G] has characteristics of a DMPO/carbon-centered radical adduct, which may have been formed by trapping of methyl radicals

[1] This value was estimated from the competition kinetics between DMPO and CAP based on the known reaction rate of DMPO with superoxide ($30\ M^{-1}s^{-1}$ at pH 7.4)[14]:

$$4 \times k_{DMPO}[DMPO][O_2\cdot^-] \approx k_{CAP}[CAP][O_2\cdot^-]$$

The value of 4 comes from the fact that the DMPO/·OH signal decreased by about 80% in the presence of CAP, consequently, 4 times more superoxide was scavenged by 0.4 mM CAP than by 250 mM DMPO. Thus, the estimate of the rate constant of CAP with superoxide at pH 7.4 is $\approx 7.5 \times 10^4$ $M^{-1}s^{-1}$.

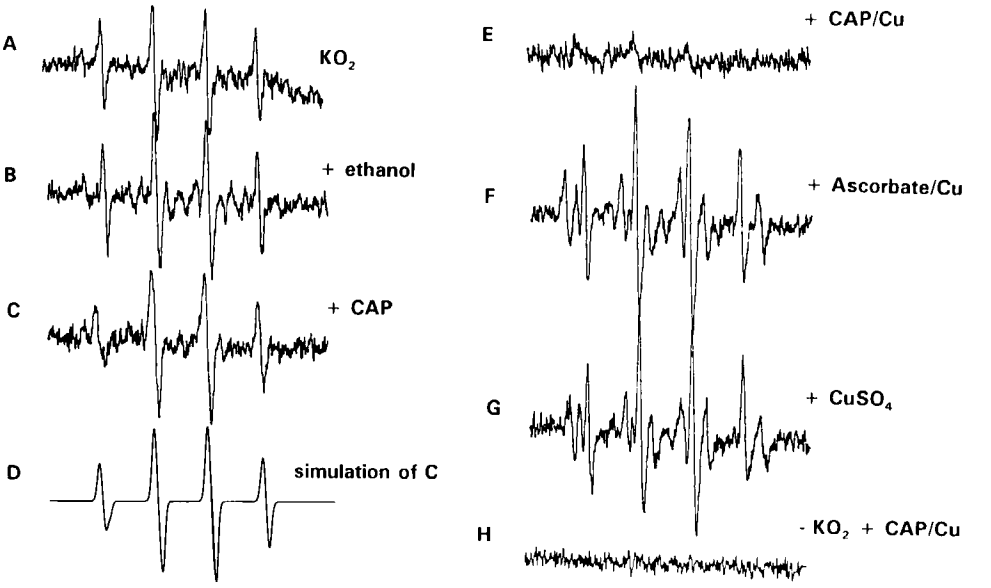

FIGURE 1 EPR spectra of DMPO adducts generated by the addition of KO_2 to the system containing 250 mM DMPO: A - control, B - 1.7 M ethanol, C - 0.4 mM captopril, D - simulation of the spectrum B using a_N = 15.1 G, a_H = 15.6 G and 20% of the simulated spetrum A (a_N = 14.8 G, a_H = 14.7 G) with 0.0005 units smaller g-factor; E - 0.4 mM captopril and 0.1 mM $CuSO_4$, F - 0.4 mM ascorbate and 0.1 mM $CuSO_4$, G - 0.1 mM $CuSO_4$. No KO_2 was added and only 0.4 mM captopril and 0.1 mM $CuSO_4$ were added (H). Experimental settings: microwave power 20 mW, modulation amplitude 1 G, time constant 100 ms, scan rate 1.6 G/sec, gain 5×10^5, spectra width 80 G.

generated by hydroxyl radical attack to DMSO present in the samples, but could also result from a copper catalyzed oxidation of DMPO.[16]

$$Cu(I) + O_2\cdot^- + 2H^+ \longrightarrow Cu(II) + H_2O_2 \quad (2)$$

$$Cu(II) + O_2\cdot^- \longrightarrow Cu(I) + O_2 \quad (3)$$

$$Cu(I) + H_2O_2 \longrightarrow Cu(II) + \cdot OH + {}^-OH \quad (4)$$

TABLE I
Scavenging of superoxide - effect of thiols and copper ions. Results were determined from the intensity of the second peak of the DMPO/·OH adduct which is proportional to superoxide spin trapping. Concentration of DMPO was 250 mM

	Detected superoxide (rel.u.)
control	6.2 ± 0.7*
1.7 M ethanol	6.7 ± 0.6
0.4 mM CAP	1.2 ± 0.2**
0.4 mM CAP + 0.1 mM $CuSO_4$	0.6 ± 0.15
0.4 mM GSH	4.9 ± 0.55***
0.4 mM GSH + 0.1 mM $CuSO_4$	0.5 ± 0.1
0.4 mM ascorbate + 0.1 mM $CuSO_4$	9.9 ± 1.2
0.1 mM $CuSO_4$	8.55 ± 0.9

* Results are expressed as means ± SD (n = 3).
** CAP-thiyl radicals were produced (see text).
*** Glutathione-thiyl radicals were not detected.

Thus, our data show that CAP is an effective scavenger of superoxide if copper ions are present. The rate constant of superoxide scavenging by CAP/Cu was estimated $\approx 1.5 \times 10^5\ M^{-1}s^{-1}$ at pH 7.4 (see Footnote 2 for details).

No DMPO/·OH signals from CAP/Cu complexes could be detected when no KO_2 was added (Figure 1H). This demonstrates that the CAP/Cu complexes did not autooxidize to give rise to superoxide or hydroxyl radicals.

The importance of the thiol group in the formation of the CAP/Cu product with superoxide scavenging activity was documented using another thiol - glutathione (GSH). When the same experiment was performed in the presence of 0.4 mM GSH pre-mixed with 0.1 mM $CuSO_4$ the signal of the DMPO/·OH was removed almost completely, while GSH alone had only a minor effect without concomitant production of glutathione-thiyl radicals [Table I]. This indicates that in the absence of copper ions the reaction of superoxide with GSH is slower than with CAP.

DISCUSSION

Our results demonstrate that CAP reacts with superoxide to produce thiyl radicals [Figure 1C]. The estimated rate constant of this reaction ($\approx 7.5 \times 10^4\ M^{-1}s^{-1}$) is in the range reported for thiol cysteine [$5 \times 10^4\ M^{-1}s^{-1}$ (Reference 17); $1.8 \times 10^4\ M^{-1}s^{-1}$ (Reference 18)]. However a considerably smaller rate constant was reported for the reaction of CAP with superoxide ($< 1000\ M^{-1}s^{-1}$).[8] This difference could most likely be attributed to the difference in the systems used to detect the superoxide: In our system the spin trap DMPO removed CAP-thiyl radicals from the reaction system. If, however, spin trap is not present, it is possible that CAP-thiyl radicals enter further reactions (Equations 5 and 6), in analogy with cysteine thiyl radicals which results in formation of superoxide:[19]

$$RS\cdot + RSH(RS^-) \longrightarrow RS\cdot SR^- + H^+ \quad (5)$$

$$RS\cdot SR^- + O_2 \longrightarrow RSSR + O_2\cdot^- \quad (6)$$

In this case the reaction of CAP with superoxide would not cause a net loss of superoxide and should not be considered as superoxide scavenging. It may be, therefore, more appropriate to use the value of $< 1000\ M^{-1}s^{-1}$, determined using ferricytochrome c and nitro-blue tetrazolium reduction methods,[8] which do not interfere with the thiyl radicals, as ability of CAP to remove superoxide in biological systems. However, this value should not be referred to as a reaction rate of CAP with superoxide (which is considerably higher), but as a rate of superoxide scavenging or removal by CAP. In addition to the above reactions, thiyl radicals can react rapidly with biologically important macromolecules[20] and may be, therefore, even more damaging to the biological system than superoxide. Therefore, it is clear that, in the

[2] For this estimation a similar reasoning was used as in case of CAP alone (see Footnote 1). This value was estimated from the competition kinetics between DMPO and CAP/Cu mixture (molar ratio 4/1) based on the known raction rate of DMPO with superoxide ($30\ M^{-1}s^{-1}$ at pH 7.4):[14]

$$9 \times k_{DMPO}[DMPO]\ [O_2\cdot^-] < k_{CAP/Cu}[CAP/Cu][O_2\cdot^-]$$

The value of 9 comes from the fact that the DMPO/·OH signal decreased by about 90% in the presence of CAP/Cu (4/1), consequently, 9 times more superoxide was scavenged by 0.4 mM CAP + 0.1 mM Cu than by 250 mM DMPO. Thus the estimate of the apparent rate constant of the reaction of captopril mixed with Cu with superoxide at pH 7.4 is $\approx 1.5 \times 10^5\ M^{-1}s^{-1}$.

absence of copper ions, CAP is not a suitable scavenger of superoxide in biological systems.

Our results show that in the presence of copper ions CAP scavenges superoxide efficiently (for CAP/Cu = 4/1 the estimate is $\approx 1.5 \times 10^5$ $M^{-1}s^{-1}$ at pH 7.4) without concomitant formation of the potentialy harmful thiyl radicals. Thus, the rate of superoxide scavenging by CAP in the presence of copper ions is two orders of magnitude higher than the reported value for CAP alone (< 1000 $M^{-1}s^{-1}$).[8]

Thiols like captopril,[9] GSH,[12,21] and penicillamine[22] were shown to form Cu(I)-type complexes in reactions with Cu(II) with various thiol:Cu(I) stoichiometry. Formation of such complexes is shown schematically in Equation 7.

$$kRSH + Cu(II) \longrightarrow (RS)_m - Cu(I) \quad (7)$$

Note, that the stoichiometry of the thiol:Cu(I) complex (m) may be different from the initial RSH/Cu(II) ratio (k). Equation 7 is a schematic representation of a multi-step reaction, where in the first step Cu(II) is reduced by RSH (a 1:1 stoichiometry of this reaction was demonstrated with CAP).[9] We presume that the resulting thiol/Cu(I) complexes act as SOD-mimics [Equations 8 and 9].

$$(RS)_m - Cu(I) + O_2\cdot^- + 2H^+ \longrightarrow (RS)_m - Cu(II) + H_2O_2 \quad (8)$$

$$(RS)_m - Cu(II) + O_2\cdot^- \longrightarrow (RS)_m - Cu(I) + O_2 \quad (9)$$

Unlike free copper, which may also catalyze reactions 8 and 9 but is also capable of decomposing hydrogen peroxide to hydroxyl radicals (Equation 4), the thiol/copper complexes are catalyticaly inactive in the latter reactions (also compare Figures 1F and 1G with Figure 1E) and may, therefore, qualify for successfull SOD-mimics.

Our results suggest that contamination with trace amounts of adventitious copper ions in the experimental systems[23] could contribute to the discrepancy between results showing that CAP is a poor scavenger of superoxide,[8,11] with those claiming high superoxide scavenging ability of CAP.[6,10] Recently Jay *et al.*[12] suggested that the conflicting results in the literature regarding superoxide scavenging could be attributed to the inactivation of catalytically active copper ions via formation of redox-inert CAP/Cu complexes. Our data also show the importance of the interaction of CAP with copper ions, however, the mechanism of superoxide inhibition is not a mere sequestration of the catalytically active copper as suggested by these authors,[12] but is rather linked to formation of catalytically active CAP/Cu complex with SOD-mimicking activity.

The importance of superoxide scavenging by CAP/copper complexes under normal physiological conditions remains in question in light of the high reactivity of GSH/copper complexes [Table I], and the high GSH/CAP plasma ratio. However, the significance of this reaction may increase when GSH levels are depleted under oxidative stress, such as inflammation. We believe that the SOD-mimicking activity of CAP/copper complexes may play a role in the mechanism of reported anti-inflammatory activity of CAP,[3] in analogy with the action of aspirin/copper complexes.[24] In this respect it is noteworthy that treatment with CAP increased plasma copper levels in spontaneously hypertensive rats.[25]

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