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THE PROOXIDANT PROPERTIES OF CAPTOPRIL

DOMENICO LAPENNA,*† SERGIO DE GIOIA,* ANDREA MEZZETTI,* GIULIANO CIOFANI,* CARMINE DI ILIO‡ and FRANCO CUCCURULLO*

*Istituto di Fisiopatologia Medica; and ‡Istituto di Scienze Biochimiche, Universita' degli Studi "G. D'Annunzio", Facolta' di Medicina e Chirurgia 66100, Chieti, Italy

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Abstract—The thiol drug captopril has been reported to possess reducing and transition metal-binding properties, which could result in specific changes in iron and copper prooxidant capacity. Thus, the effects of captopril on iron- and copper-induced oxidative injury were evaluated using deoxyribose as the oxidizable substrate in the presence of physiological phosphate concentrations but in the absence of the non-physiological chelator EDTA. In an iron(III)/H_2O_2/ascorbate oxidant system, captopril enhanced deoxyribose oxidation only when it was pre-mixed with iron, whereas it did not influence sugar degradation when not pre-mixed with the metal or when ascorbate was omitted. The physiological thiol GSH acted in a similar manner, whereas the SH-lacking angiotensin-converting enzyme inhibitor ramiprilat did not influence iron-induced deoxyribose oxidation, indicating that the thiol group is crucial in favouring enhanced iron reactivity due to 'malignant' chelation. Further specific experiments designed to evaluate possible thiol-dependent iron(III) reduction failed to demonstrate ferric to ferrous reduction by either captopril or reduced glutathione (GSH). When iron(III) was replaced by copper(II) to induce deoxyribose oxidation, captopril was prooxidant both in the presence and absence of ascorbate, and when pre-mixed or not with copper. On the other hand, GSH was prooxidant up to a 2:1 molar ratio with respect to copper but markedly inhibited copper-dependent sugar oxidation beginning at molar ratio of 4:1. Ramiprilat did not significantly influence copper-induced deoxyribose oxidation. Moreover, unlike the experiments performed with iron, captopril, as well as GSH, readily reduced copper(II) to copper(I). Hence, captopril can act as a prooxidant in the presence of iron or copper. In the former case, only 'malignant' iron chelation by the drug is involved in oxidant injury, whereas in the latter both copper chelation and reduction are operative, although specific chelating mechanisms are crucial in enhancing copper-induced oxidant injury. Captopril, therefore, cannot be considered simply as an 'antioxidant drug', and its catalytic transition metal-related prooxidant capacity should be taken into account in experimental and clinical investigations.

Key words: captopril; iron; copper; free radicals; oxidative stress; deoxyribose

CA§ is an ACE inhibitor largely used in the treatment of hypertension and congestive heart failure. Experimental studies have indicated that some beneficial effects of CA could also be due to antioxidant mechanisms, apparently related to the sulphydryl group in the drug molecule [1, 2].

Oxidative stress is dependent on the presence of catalytic transition metals such as iron and copper [3, 4]. Thus, the interaction of a drug with these metals must be relevant in conditioning its putative antioxidant capacity [3, 4]. The thiol group of CA, however, may be responsible for prooxidant more than antioxidant pharmacological properties in the presence of redox-active transition metals. Indeed, the sulphydryl group should give CA a reducing capacity, which may favour the reduction of catalytic transition metals, resulting in prooxidant generation via interconversion between metal redox states [3–5]. Moreover, the chemical structure of CA indicates that it may act as a bidentate ligand, with a potential transition metal binding capacity [6]. Accordingly, iron and copper chelation by CA has recently been reported [6–8], though it is not yet known if this phenomenon may condition specific changes in metal biological reactivity and oxidant capacity. In such a context, it is noteworthy that some chelators can exert a transition metal-related prooxidant action [3, 4, 9].

The present study was designed to investigate the effects of CA on catalytic transition metal-driven oxidative damage. The results show that CA does not act as an antioxidant against iron- and copper-dependent oxidant injury, which is even enhanced by the drug via peculiar interactions with such redox-active transition metals.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The water used in the study was deionized, glass-bidistilled and Chelex 100 resin-treated. Experiments were carried out in plastic or acid-washed glassware.

Iron- and copper-dependent deoxyribose oxidation. It is known that iron and copper can oxidatively damage the sugar deoxyribose [3, 4, 10–12]. When transition metals such as iron are chelated with EDTA, the generation of prooxidants (i.e. hydroxyl

† Corresponding author. Tel. 0871 355 266; FAX 0871 345501 or 0871 355267.

§ Abbreviations: CA, captopril; ACE, angiotensin-converting enzyme; GSH, reduced glutathione; DTPA, diethylenetriaminepentaacetic acid; TBAR, thiobarbituric acid reactant.

radical (OH·)) happens freely in solution, so that OH· is equally accessible to deoxyribose and any other substance present in the reaction mixture; indeed, the 'deoxyribose test' is largely used to evaluate the hydroxyl radical scavenging properties of various drugs [3, 4, 11, 12]. Using this test, a direct OH· scavenging action by CA has been reported [12]. However, it must be noted that the deoxyribose test performed with EDTA is rather artificial when potentially extrapolated to the *in vivo* setting, since EDTA is not present *in vivo*. Indeed, phosphates (which are present in the intracellular and extracellular fluids) are physiological transition metal chelators [13]. Thus, we have specifically evaluated the effects of CA on transition metal-dependent deoxyribose oxidation in the absence of EDTA but in the presence of phosphates; CA was pre-mixed or not with iron and copper to investigate the effects of transition metal–drug complexes and of drug-reducing capacity towards such metals on oxidant injury.

Reaction mixtures contained, in a final volume of 1.0 mL, the following reagents at the final concentrations stated: 10 mM potassium phosphate buffer, pH 7.4, 50 μM $FeCl_3$ (or 50 μM $CuCl_2$), pre-mixed or not with 25, 50, 100, 200 and 400 μM CA, 2.8 mM deoxyribose, 2 mM H_2O_2 and 0.1 mM ascorbate (the latter was omitted in other experiments). When transition metals were not pre-mixed with CA, they were added to reaction mixtures before drug addition to favour their preferential binding by phosphates and not by CA [14]. Reaction mixtures were incubated at 37° for 30 min. Then, glacial acetic acid (1.0 mL) and 0.6% aqueous solution of TBA (1.0 mL) were added to each millilitre of reaction mixture, followed by 30 min heating at 95°. After cooling, absorbance values at 532 nm were recorded spectrophotometrically against appropriate blanks. Results were expressed as nmol TBA reactants (TBAR)/mL, with a molar extinction coefficient of $1.54 \times 10^5\ M^{-1}\ cm^{-1}$ used for calculations.

In other specific experiments, the effects of the physiological thiol GSH and the SH-lacking ACE inhibitor ramiprilat (both used at the same concentrations of CA) on deoxyribose oxidation induced by iron or copper were also evaluated.

Iron(III) and copper(II) reduction. Transition metal reduction is crucial to generating oxidant species capable of inducing biomolecule oxidant damage [3–5, 15]. The potential reduction of 10 μM $FeCl_3$ or $CuCl_2$, induced by 5, 10, 20, 30 and 40 μM CA (i.e. at the same CA/metal molar ratios used in the deoxyribose experiments) as well as by the same concentrations of GSH and ramiprilat was evaluated in physiological saline and not in phosphate buffer, in order to avoid phosphate-induced iron(II) autoxidation [15] potentially causing the amount of metal reduced to be underestimated. However, the use of another phosphate-independent medium (i.e. 10 mM sodium acetate medium, pH 7.4) led to results very similar to those observed with physiological saline. Incubation of $FeCl_3$ or $CuCl_2$ with various drug concentrations was for 30 min at 37°; ferene S and neocuproine (both at 30 μM final concentration) were used specifically to detect iron(II) and copper(I) produced from iron(III) and copper(II) reduction, using a molar extinction coefficient for the ferene S–iron(II) and the neocuproine–copper(I) complexes of 3.55×10^4 and $7.5 \times 10^3\ M^{-1}\ cm^{-1}$ at 593 and 454 nm, respectively [16–18].

Table 1. Effect of CA on iron-dependent deoxyribose oxidation

	Ascorbate	No ascorbate
Control	8.0 ± 0.45	5.6 ± 0.4
$CA–FeCl_3$ pre-mixed		
25 μM CA	9.3 ± 0.3*	5.75 ± 0.45
50 μM CA	9.6 ± 0.5*	5.8 ± 0.6
100 μM CA	9.9 ± 0.35*†	5.7 ± 0.55
200 μM CA	10.1 ± 0.45*†	6.0 ± 0.65
400 μM CA	10 ± 0.4*†	5.85 ± 0.5
$FeCl_3$ and CA not pre-mixed		
25 μM CA	7.9 ± 0.35‡	5.7 ± 0.4
50 μM CA	8.1 ± 0.4‡	5.65 ± 0.5
100 μM CA	8.2 ± 0.5‡	5.8 ± 0.6
200 μM CA	8.05 ± 0.45‡	5.75 ± 0.65
400 μM CA	8.15 ± 0.55‡	5.5 ± 0.45

Reaction mixtures contained 10 mM KH_2PO_4–KOH buffer (pH 7.4), 50 μM $FeCl_3$, pre-mixed or not with stated drug concentrations, 2.8 mM deoxyribose and 2 mM H_2O_2, with or without 0.1 mM ascorbic acid. Iron-dependent deoxyribose oxidation was assessed via the TBAR assay, as fully explained in the Materials and Methods section. Results express means ± SD of six different experiments, and are given as nmol TBAR/mL.

* $P < 0.05$ versus control values (i.e. obtained in the absence of CA; one-way analysis of variance, followed by the Student–Newman–Keuls test).

† $P < 0.05$ versus 25 μM CA (one-way analysis of variance, followed by the Student–Newman–Keuls test).

‡ $P < 0.0001$ versus CA-$FeCl_3$ pre-mixed (unpaired Student's *t*-test).

Statistics. Data were calculated as means ± SD of six different experiments, unless otherwise indicated. Dose-dependent drug effects were studied by the one-way analysis of variance, followed by the Student–Newman–Keuls test [19]. Differences between TBAR values obtained from pre-mixing the drug or not with transition metals were analysed by the Student's *t*-test for unpaired data [19]. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of CA on iron-dependent deoxyribose oxidation

When pre-mixed with iron, CA induced a significant increase in deoxyribose oxidation with respect to iron/H_2O_2/ascorbate (Table 1). CA, therefore, can interact with iron and bind it, but cannot inactivate the metal. The iron-binding capacity of CA is quite different from that of other chelators, such as desferrioxamine (which inhibits iron-dependent oxidative reactions) and DTPA, which bind and inactivate iron at approx. 2:1 molar excess over the metal [9]. The iron-binding capacity of CA resembles that of the thiol compound penicillamine, which can stimulate iron-driven

biomolecule oxidant damage even at 8:1 molar excess over iron [9]. When CA was added to the reaction mixtures without being pre-mixed with iron, it did not influence iron-dependent deoxyribose oxidation (Table 1).

When ascorbate was omitted in reaction mixtures, control TBAR values were approx. 30% lower than those detected in the presence of ascorbate. This essentially agrees with previous studies which showed a prooxidant effect of iron(III)/H_2O_2 on deoxyribose, which was significantly lower, however, than that of iron(III)/H_2O_2/ascorbate [20]. Under these experimental conditions, CA, pre-mixed or not with iron, did not significantly influence sugar oxidative degradation (Table 1). This indicates that the CA–iron complex requires the presence of ascorbate to trigger oxidative injury, perhaps because this complex could be recycled by ascorbate to produce oxidizing species. The aforementioned results also suggest that CA is incapable of directly reducing ferric iron.

Overall, the present data indicate that CA does not act as an antioxidant against iron-mediated oxidative damage but rather as a prooxidant, essentially via 'malignant' iron chelation and not iron reduction. This chelation appears to be thiol-dependent; indeed, GSH showed an action very similar to that of CA on iron-induced deoxyribose oxidation when pre-mixed or not with the metal and with or without ascorbate, whereas the SH-lacking ACE inhibitor ramiprilat was ineffective (data not shown).

Table 3. Effect of GSH on copper-dependent deoxyribose oxidation

	Ascorbate	No ascorbate
Control	11.3 ± 0.5	4.5 ± 0.3
GSH–$CuCl_2$ pre-mixed		
25 μM GSH	13.8 ± 0.7*	6.5 ± 0.6*
50 μM GSH	14.5 ± 0.6*†	7.8 ± 0.4*†
100 μM GSH	11.9 ± 0.45*†	8.1 ± 0.5*
200 μM GSH	3.6 ± 0.3*†	3.4 ± 0.35*†
400 μM GSH	2.95 ± 0.25*†	2.85 ± 0.3*†
$CuCl_2$ and GSH not pre-mixed		
25 μM GSH	13.7 ± 0.45*	6.6 ± 0.45*
50 μM GSH	14.2 ± 0.4*†	7.7 ± 0.5*†
100 μM GSH	11.8 ± 0.5*†	7.95 ± 0.55*
200 μM GSH	3.7 ± 0.25*†	3.25 ± 0.3*†
400 μM GSH	2.9 ± 0.2*†	2.8 ± 0.25*†

Experimental conditions were similar to those described in the legend to Table 2. Results express means ± SD of six different experiments, and are given as nmol TBAR/mL.

* $P < 0.05$ versus control values (one-way analysis of variance followed by the Student–Newman–Keuls test).

† $P < 0.05$ versus the values that precede (one-way analysis of variance, followed by the Student–Newman–Keuls test). Significant differences between the values of pre-mixing and not pre-mixing experiments were not detected (unpaired Student's *t*-test).

Table 2. Effect of CA on copper-dependent deoxyribose oxidation

	Ascorbate	No ascorbate
Control	11.3 ± 0.5	4.5 ± 0.3
CA–$CuCl_2$ pre-mixed		
25 μM CA	13.9 ± 0.55*	6.8 ± 0.35*
50 μM CA	15.6 ± 0.6*†	7.9 ± 0.5*†
100 μM CA	15.7 ± 0.7*	7.7 ± 0.45*
200 μM CA	15.5 ± 0.55*	8.1 ± 0.4*
400 μM CA	15.1 ± 0.8*	7.6 ± 0.6*
$CuCl_2$ and CA not pre-mixed		
25 μM CA	14.05 ± 0.6*	6.6 ± 0.5*
50 μM CA	15.5 ± 0.8*†	7.7 ± 0.4*†
100 μM CA	15.6 ± 0.6*	8.0 ± 0.55*
200 μM CA	15.3 ± 0.65*	8.2 ± 0.6*
400 μM CA	15.0 ± 0.7*	7.8 ± 0.65*

Reaction mixtures contained 10 mM KH_2PO_4–KOH buffer (pH 7.4), 50 μM $CuCl_2$, premixed or not with stated drug concentrations, 2.8 mM deoxyribose and 2 mM H_2O_2, with or without 0.1 mM ascorbic acid. Copper-dependent deoxyribose oxidation was assessed via the TBAR assay, as fully explained in the Materials and Methods section. Results express means ± SD of six different experiments, and are given as nmol TBAR/mL.

* $P < 0.05$ versus control values (i.e. obtained in the absence of CA; one-way analysis of variance, followed by the Student–Newman–Keuls test).

† $P < 0.05$ versus the values that precede (one-way analysis of variance, followed by the Student–Newman–Keuls test). Significant differences between the values of pre-mixing and not pre-mixing experiments were not detected (unpaired Student's *t*-test).

Effect of CA on copper-dependent deoxyribose oxidation

CA significantly increased Cu(II)/H_2O_2/ascorbate-dependent deoxyribose oxidation both when it was pre-mixed and not with the transition metal (Table 2). A similar trend was also observed when ascorbate was omitted in reaction mixtures; this procedure, however, resulted in TBAR values approx. 60% lower than those detected with ascorbic acid, emphasizing the relevance of ascorbate as the reducing agent in conditioning the entity of copper-driven oxidant damage (Table 2). Thus, CA enhances copper-dependent oxidative injury, this action being apparently due to mechanisms involving both metal chelation and reduction. Specific copper binding by CA, however, appears crucial in drug prooxidant activity; indeed, GSH (which showed a reductive capacity on copper(II) similar to that of CA, see below) was markedly inhibitory on copper-induced deoxyribose degradation beginning at a molar ratio of 4:1 with respect to the metal (Table 3). Copper chelation by CA also requires the thiol group but is unrelated to ACE inhibition, since ramiprilat did not influence copper-driven sugar oxidative degradation when pre-mixed or not with the metal and with or without ascorbate (data not shown).

Effect of CA on iron(III) and copper(II) reduction

In agreement with the data from the deoxyribose test, CA, as well as ramiprilat, could not reduce iron(III) to iron(II) under the experimental

conditions used. GSH was also incapable of favouring such a reduction; this agrees with a previous report by Rowley and Halliwell, indicating that GSH itself cannot reduce trivalent iron [21].

CA, however, was capable of reducing copper(II) to copper(I) with a high efficiency; indeed, at 5, 10, 20, 30 and 40 μM concentration, CA resulted in 6.5 ± 0.2, 8.4 ± 0.6, 8.6 ± 0.35, 8.7 ± 0.25 and 8.8 ± 0.4 μM of copper(I), respectively (only 5 versus 10, 20, 30 and 40 μM CA, $P < 0.05$; for the other values, P = NS; N = 5). GSH gave very similar results; indeed, 6.45 ± 0.25, 8.3 ± 0.7, 8.7 ± 0.35, 8.65 ± 0.4 and 8.75 ± 0.45 μM of copper(I) were detected in the presence of 5, 10, 20, 30 and 40 μM of GSH, respectively (only 5 versus 10, 20, 30 and 40 μM GSH, $P < 0.05$; for the other values, P = NS; N = 5).

Finally, ramiprilat had no reducing capacity on either iron(III) or copper(II).

DISCUSSION

The present study shows that CA is a prooxidant when it interacts with iron and, especially, with copper.

In particular, the interaction of CA with iron enhances metal oxidative capacity through 'malignant' chelation mechanisms, whereas iron reduction is apparently not involved. This specific prooxidant chelation is essentially thiol-dependent; indeed, GSH, as with CA, favours iron-induced deoxyribose oxidation only when pre-mixed with the metal and in the presence of ascorbate. In such a context, it is known that the complexation of iron with some chelating agents facilitates prooxidant reactions, since these specific iron–chelator complexes represent a highly soluble iron redox-active form [3, 4, 9]. Moreover, it is known that iron-driven radical generation requires the availability of at least one metal coordination site that is open or occupied by a readily dissociable ligand, such as water [22]. The interaction of CA with iron, therefore, could favour the formation of soluble active iron complexes which may be recycled by ascorbate and/or the availability of the metal coordination site with the generation of iron oxidizing species, such as OH· or iron(IV) [3, 4, 14, 22].

Our results also show that when CA interacts with copper, both metal chelation and reduction by the drug are involved in copper–CA-dependent oxidative damage. However, specific copper binding by CA (followed by metal reduction by ascorbate and/or CA itself) appears crucial in drug prooxidant activity, since GSH, which is characterized by a copper(II)-reducing capacity similar to that of CA, is markedly inhibitory on copper-dependent oxidative injury beginning at a 4:1 molar ratio with respect to the metal. This basically agrees with the study by Hanna and Mason, which showed an inhibitory action of GSH excess over copper on metal-driven radical generation at physiological pH values in a phosphate buffer [23]. In light of their results, these authors have proposed that GSH could play a major role in moderating the toxicological effects of copper *in vivo* [23]. On the other hand, the pharmacological thiol compound CA is peculiar in favouring copper-induced oxidant damage, even at high drug to copper molar ratios. This action, which requires copper complexation conveivably at the level of drug SH, COOH and C=O groups with a specific steric configuration [8], is independent of ACE inhibition, since ramiprilat does not influence copper-driven deoxyribose oxidation. The recognized copper chelation by CA [8] is, therefore, prooxidant, and different from that by other chelators with or without the sulphydryl group, such as diethyldithiocarbamate or histidine, which antagonize copper-mediated oxidative damage [10, 24]. Moreover, it is noteworthy that iron- and copper-binding capacity by CA is essentially prooxidant, whereas that by other thiols (e.g. penicillamine) is prooxidant on iron but antioxidant on copper [9, 10].

It is intriguing to attempt to reconcile our findings with previous studies on CA antioxidant activity. However, it is possible that the antioxidant properties of CA observed in cell systems, such as in the heart, may be 'indirect' and related to enhanced CA-mediated prostacyclin production [25, 26]. Accordingly, recent studies have shown that CA affords protection towards free radical-induced endothelial cell injury and reperfusion arrhythmias (which are radical-mediated [27]) specifically through prostacyclin-mediated mechanisms [28, 29]. In this regard, there is evidence that prostacyclin reduces tissue-free radical generation [30] and the overflow of catecholamines (which are recognized radical-generating molecules [3]) in the ischaemic myocardium [29]. Furthermore, prostacyclin antagonizes inflammatory mediator release from mast cells [31] and oxidant generation by neutrophils [32], which may contribute to a decrease in oxidative burden *in vivo*. Some 'indirect' antioxidant effects of CA could also be due to its recognized ACE inhibitory capacity, with reduced angiotensin levels. In this context, angiotensin increases cell calcium [33, 34], which may favour oxidative stress [35]. Consistent with this fact is the evidence that the 'stunned myocardium' (a free radical-related phenomenon [27]) is favourably influenced not only by CA, but also by enalapril, which lacks the SH group [2]. The 'indirect' antioxidant effects of CA could sometimes be prevalent over its prooxidant properties *in vivo*, especially when high CA concentrations are used (often the case in experimental protocols). Under these rather artificial conditions, radical scavenging by CA, especially against hypochlorous acid [12], could also be operative, though this appears very unlikely in the clinical setting because of insufficiently high CA therapeutical concentrations [12, 36].

In conclusion, CA acts as a prooxidant via specific interactions with iron and copper. 'Free' iron concentrations seem to be approx. 2 μM *in vivo* [37]; although little is known concerning the levels of 'free' copper *in vivo*, it has been reported that loosely bound catalytic copper concentrations are approx. 0.5 μM in synovial fluid from rheumatoid patients and in cerebrospinal fluid [38]. Considering mean CA therapeutical concentrations of approx. 1 μM [36], drug prooxidant effects could be feasible under some conditions *in vivo* as a result of CA–transition metal interaction. Conversely, the physiological thiol GSH should be effectively

antioxidant on copper-dependent oxidative stress, since it is present in the millimolar range in the cell environment [39]. Moreover, GSH exerts antioxidant effects *in vivo* because it is an integral component of powerful antioxidant enzymes, such as glutathione peroxidases [39]. On the other hand, CA is a poor thiol donor [40], which should not favour cell thiol protection and the activity of GSH-related antioxidant defences. Under a pharmacological profile, although caution is needed before extrapolating our findings to the *in vivo* setting, it could be hypothesized that some side effects of CA, especially on kidney function, may be a consequence of its prooxidant properties. Indeed, free radicals and oxidative stress have been emphasized in the pathophysiology of glomerular dysfunction and kidney diseases [41]. In any event, our study shows that CA cannot be considered simply as an 'antioxidant drug', and that its catalytic transition metal-related prooxidant capacity should be taken into account in future experimental and clinical investigations.

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