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Comparison of the Free Radical-scavenging Ability of Captopril and Ascorbic Acid in an In-vitro Model of Lipid Oxidation. Implications for Reperfusion Injury and ACE Inhibitor Therapy

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Abstract—The free radical-scavenging activity of captopril and ascorbic acid was determined by assessing their ability to inhibit the peroxidation of linoleic acid in sodium dodecyl sulphate (SDS) micelles at 37°C. The extent of peroxidation was monitored polarographically using an oxygen electrode to determine oxygen consumption rates. Ascorbic acid was observed to inhibit the peroxidation in a concentration-dependent fashion with an IC50 value of 17 μM. In contrast, the addition of captopril at concentrations up to 500 μM did not result in any detectable inhibition. Unlike ascorbic acid, no synergistic effect, as evidenced by the duration of inhibition, was observed with captopril/α-tocopherol mixtures. Measurements of the partitioning of captopril between SDS micelles and the aqueous phase show that 37% of the bulk captopril concentration is localized within the micellar phase; this indicates that phase separation of captopril from the lipid peroxyl radicals is not the cause of the observed lack of inhibitory activity.

Reperfusion injury refers to the myocardial damage that occurs upon the restoration of blood flow following the breakup of a coronary artery blockade. Due to the increasingly widespread use of thrombolytic therapy following myocardial infarction, the pathogenesis and treatment of reperfusion injury is of considerable clinical importance. There is now a considerable body of evidence implicating oxygen-centred free radicals as causative agents in ischaemic-reperfused myocardial injury (Kloner et al 1989); this has led to the suggestion that antioxidants may have a direct therapeutic effect due to their ability to scavenge free radicals (Bolli et al 1989). In particular, Westlin & Mullane (1988) have shown that the angiotensin-converting enzyme (ACE) inhibitor captopril can attenuate post-ischaemic reperfusion injury in dogs by a mechanism that is independent of ACE inhibition. It was suggested that the scavenging of oxygen-derived free radicals by the sulphydryl group of captopril was responsible for the effects observed in-vivo.

Subsequent to the report of Westlin & Mullane (1988), several investigations regarding the in-vitro radical-scavenging ability of captopril have appeared. The reaction of captopril with superoxide ion has been assessed in the hypoxanthane/xanthine oxidase test system (Bagchi et al 1989a), the dianisidine photooxidation assay (Chopra et al 1990), and by the direct electrolytic generation of superoxide (Pi & Chen 1989). The results of these investigations have been contradictory; although initial results indicated that captopril is a potent scavenger of superoxide, subsequent studies revealed little or no scavenging activity (Kukreja et al 1990; Mehta et al 1990).

The ability of captopril to scavenge the hydroxyl radical has been indirectly demonstrated by Bagchi et al (1989b) through the decreased production of salicylate oxidation products, and by Mak et al (1990) using spin-trapping techniques. However, the relevance of these findings to in-vivo radical scavenging is questionable since the hydroxyl radical is an extremely powerful oxidant and will undergo reaction with virtually all molecules (Howard 1972). Recently, Chen et al (1991) have shown that several ACE inhibitors are capable of trapping hydroxyl radicals regardless of whether a sulphydryl group is present in the scavenging molecule.

The utility of ascorbic acid derivatives in ameliorating reperfusion-induced myocardial damage has also been studied. Following the demonstration that 2-*O*-alkylascorbic acid derivatives were able to suppress arrhythmias in a rat heart ischaemia-reperfusion model (Kato et al 1988), an extensive study of the free radical-scavenging activity of alkylascorbic acid derivatives has been reported. Of particular interest is the demonstration that these derivatives are capable of inhibiting the peroxidation of lipids in a variety of in-vitro models (Nihro et al 1991a,b). Unfortunately, the radical-scavenging efficiencies were generally determined only by qualitative or semiquantitative methods using peroxidation systems that are not well characterized in terms of the elementary chemical reactions taking place.

To clarify the importance of -SH-containing ACE inhibitors and ascorbic acid derivatives as free radical scavengers, I investigated the reaction of captopril and ascorbic acid with the peroxyl radical derived from linoleic acid. Lipid-derived peroxyl radicals are produced during the oxidation of membrane phospholipids and are believed to play a role in several disease states. The radical-initiated peroxidation of linoleic acid in sodium dodecyl sulphate micelles has been extensively studied, and the basic reactions and associated rate constants for the overall oxidation have been determined (Barclay et al 1987; Pryor et al 1988). In this paper, the antioxidant efficiency of captopril and ascorbic acid in this in-vitro model of phospholipid peroxidation is described.

Materials and Methods

Chemicals

Captopril (99%), linoleic acid (99%), (−)-ascorbic acid

(98·8%), α-tocopherol (95%), and EDTA (98%) were obtained from Sigma (St Louis, MO) and used as received. Sodium dodecyl sulphate (SDS) was obtained from Bethesda Research Laboratories (Bethesda, MD) and was Ultrapure grade. The free-radical initiator 2,2′-azobis(2-amadinopropane) dihydrochloride (ABP) was a gift from Wako Chemicals USA, Inc (Dallas, TX). Buffer salts were obtained from Fisher Chemicals (Pittsburg, PA) and were HPLC grade. Deionized water was obtained from a Millipore Milli-Q purification system.

Oxidation studies

The free-radical oxidation of linoleic acid in SDS micelles was monitored polarographically by measuring the decrease in dissolved oxygen concentration using a YSI Model 5300 Biological Oxygen Monitor. A solution of linoleic acid (0·041 M) solubilized in 0·5 M SDS in a pH 7·4 phosphate buffer at 37°C was saturated with air. The solution was made up to 2 μM in EDTA to inhibit adventitious metal-catalysed reactions. The oxidation was initiated by the addition of the thermally labile free-radical initiator ABP (0·010 M), and the decrease in oxygen concentration was monitored as a function of time. Known concentrations of captopril and ascorbic acid were then added and the new rate of oxidation was monitored with time. In practice, it was most convenient to sample the oxygen electrode output at defined time intervals so that the digitized data could be transferred directly to a computer for analysis.

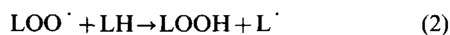
Determination of micelle-water distribution coefficients

The partitioning of captopril and ascorbic acid between the micellar and bulk water phase was measured at 37°C by the diffusion coefficient technique of Burkey et al (1984). Phosphate buffer (or SDS/buffer) was pumped through a 4 m × 0·0153 cm i.d. stainless steel tube thermostated to 37°C at a flow rate of 0·1 mL min^{-1}. The dispersion of the sample was monitored by UV detection and recorded on a Spectra-Physics SP4270 integrator. To minimize precolumn sample dispersion, samples were introduced using a Perkin Elmer ISS 100 autosampler with an injection volume of 10 μL. Pyrenecarboxaldehyde was used as the standard compound for which complete incorporation into the micelle is assumed.

Results

The oxidation of linoleic acid in SDS micelles occurs by a free radical chain mechanism involving the propagation steps shown in equations 1 and 2:

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (1)$$



$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (2)$$

The chain reaction is initiated by the addition of a thermally labile azo compound. Azo compounds are chemically different from the oxygen-centred radicals believed to be the primary initiators of in-vivo lipid peroxidation. However, the azo initiator is well suited to quantitative kinetic studies because its known decomposition rate produces linoleoyl radicals $L^{\cdot}$ at a constant rate. Of the two propagation steps, the addition of oxygen to the linoleoyl radical (eqn 1) is several orders of magnitude faster than the hydrogen abstraction (eqn 2); consequently, the vast majority of lipid-derived radicals exist as peroxyl radicals $LOO^{\cdot}$. Antioxidants exert their effect by scavenging these peroxyl radicals, thereby inhibiting the second propagation step and breaking the chain reaction. Experimentally, this is reflected in a decrease in the rate of oxygen consumed in the first propagation step.

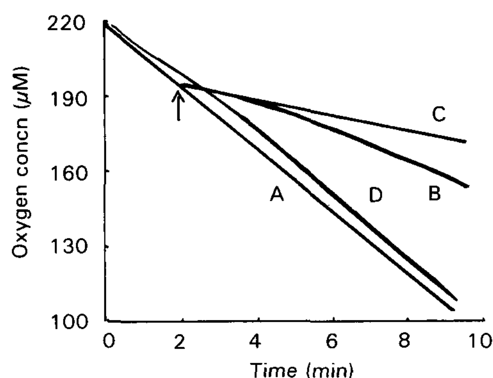


FIG. 1. Oxygen concn vs time curves for the peroxidation of linoleic acid in SDS micelles at 37°C. A uninhibited oxidation; B α-tocopherol, 1·7 μM; C ascorbic acid, 50 μM; D captopril, 500 μM. In panels B, C, and D the arrow indicates where the inhibitor was added to the reaction.

Table 1. Oxygen consumption rates for the free radical peroxidation of linoleic acid in SDS micelles at 37°C in the presence of test inhibitors captopril and ascorbic acid.

Inhibitor concn (μM)	$-d[O_2]/dt$ (μM s^{-1})	
	Captopril	Ascorbic Acid
0	0·223±0·009 (n=6)	
25	n.d.	0·0783±0·0020
50	0·228±0·010	0·0502±0·0022
100	0·248±0·005	0·0278±0·0014
250	0·227±0·002	0·0132±0·0003
500	0·229±0·004	n.d.

Values are shown ± the range based on two measurements. n.d. = not determined.

Table 2. Diffusion coefficients (cm^2 s^{-1} × 10^6) in phosphate buffer (0·05 M, pH 7·4) and buffer containing 0·5 M SDS at 37°C.

Compound	Buffer	SDS/buffer	f^a
Ascorbic acid	8·78±1·19	7·20±0·28	0·21
Captopril	8·16±0·89	5·56±0·08	0·37
1-Pyrenecarboxaldehyde	n.d.	1.17±0·09	(1·00)[b]

Errors are standard deviations based on triplicate determinations. [a] Fraction of molecules associated with the micelle. [b] Assumed value, see text.

Fig. 1 shows oxygen concentration vs time curves for the uninhibited oxidation reaction, and for the same reaction in the presence of tocopherol, ascorbic acid, and captopril. As previously reported (Barclay et al 1987; Pryor et al 1988), tocopherol is a highly efficient antioxidant in this micellar test system, and causes a rapid and substantial decrease in the

rate of oxygen consumption even at micromolar levels. Similarly, inhibition by ascorbic acid is readily apparent in Fig. 1, but much higher concentrations are required. Oxygen consumption rates are virtually identical in the presence and absence of captopril.

Oxygen consumption rates as a function of the concentration of captopril and ascorbic acid are shown in Table 1. Inhibition by ascorbate is observed even at the lowest level tested, and increases in a concentration-dependent manner. A plot of the oxygen consumption rate against the reciprocal of the ascorbate concentration (Howard 1972) gave a satisfactory linear fit from which an IC50 (concentration needed to decrease the oxidation rate to half its uninhibited value) of 17 μM was calculated. In contrast, captopril was found to be completely without effect up to the highest concentration studied (500 μM). Since even a 10% decrease in the oxidation rate would be detectable with the apparatus used here, the IC50 value for captopril must be no less than 1000 μM, and may be substantially greater.

Ascorbic acid exhibits synergism with tocopherol in inhibiting lipid oxidation in-vitro (Barclay et al 1983). To determine whether captopril is also capable of this synergism, the duration of inhibition by tocopherol in the presence and absence of captopril was determined. The duration of inhibition was defined as the time required for the oxidation rate to return to its uninhibited value due to complete depletion of the antioxidants. The duration of inhibition (476 ± 7 s) was unaffected by added captopril (474 ± 6 for 10·1 μM; 473 ± 11 for 50·7 μM).

To aid in the interpretation of the antioxidant data in this biphasic micellar system, the equilibrium partitioning of captopril and ascorbate between the micellar and bulk aqueous phases was measured by the diffusion coefficient technique. In this method, diffusion coefficients are measured by capillary flow band broadening in the presence and absence of SDS micelles. The diffusion coefficient of a highly hydrophobic molecule which is assumed to partition completely into the micelle is also measured; this measurement is actually the diffusion coefficient of the host micelle and is independent of the identity of the guest molecule. The observed diffusion coefficient D_o for an incompletely partitioned molecule is then a linear function of the aqueous and micelle diffusion coefficients (D_a and D_m, respectively) and the fraction f of molecules in the micelle is as follows:

$$D_o = f D_m + (1 - f) D_a \qquad (3)$$

Table 2 presents the experimental diffusion coefficients and calculated partitioning factors.

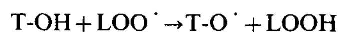
Discussion

The principal aim of this work was to evaluate the antioxidant activity of captopril and ascorbic acid towards lipid-derived peroxyl radicals. To accomplish this, the linoleic acid/SDS test system was used because the elementary reactions and rate constants pertaining to lipid oxidation in this system have been well characterized. The data shown in Fig. 1 and Table 1 indicate that captopril is completely ineffective at inhibiting the oxidation reaction even when used at concentrations well in excess of achievable therapeutic levels. As a peroxyl radical scavenger, captopril is at least 60 times less effective than ascorbate in this test system.

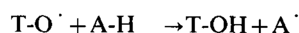
A possible reason for the observed results is that captopril and the lipid peroxyl radical may reside in different phases of the micellar solution. This reasoning, however, is inconsistent with the observed radical scavenging activity of the hydrophilic ascorbate ion. Moreover, direct measurement of the equilibrium partitioning indicates that 37% of the bulk captopril concentration is localized within the micellar phase. It is concluded that captopril is of intrinsically low reactivity towards the linoleoyl peroxyl radical.

In apparent contrast to these results, Mak et al (1990) have recently reported that captopril is able to inhibit the free-radical lipid peroxidation of cultured endothelial cells in a concentration-dependent manner. However, those authors attributed the observed inhibition to scavenging of the hydroxyl radicals used to initiate the reaction, rather than the scavenging of chain-carrying lipid peroxyl radicals.

Previous research has shown that tocopherol and ascorbate can act as synergistic inhibitors of lipid peroxidation in-vitro (Barclay et al 1983). The mechanism of this synergism is thought to involve rapid peroxyl radical scavenging by tocopherol (T-OH) to produce the tocopheryl radical (T-O$^{\cdot}$), followed by regeneration of intact tocopherol upon reaction with ascorbate (AH):



$$\text{T-OH} + \text{LOO}^{\cdot} \rightarrow \text{T-O}^{\cdot} + \text{LOOH}$$



$$\text{T-O}^{\cdot} + \text{A-H} \rightarrow \text{T-OH} + \text{A}^{\cdot}$$

Note that all the peroxyl radical scavenging is ascribed to the highly efficient tocopherol molecule, and that ascorbate is used solely to prevent the depletion of tocopherol. The net effect is that ascorbate prolongs the duration of action of tocopherol. Due to the presence of a free sulphhydryl group, captopril may also exhibit a synergistic effect by this mechanism. However, the results show that the duration of action of tocopherol was not affected by the presence of captopril. Apparently, the tocopheryl radical is capable of trapping another linoleoyl peroxyl radical at a rate that is sufficiently rapid such that the reaction with captopril is not significant.

The results with ascorbic acid are consistent with the studies of Nihro et al (1991a,b) who have demonstrated inhibitory activity for a variety of 2-*O*- and 3-*O*-alkylascorbic acid derivatives in several in-vitro models of lipid peroxidation. Unfortunately, these workers depended mostly on semiquantitative measures of lipid peroxidation such as the thiobarbituric colour assay, whereas the present results are based on quantitative kinetic measurements.

In summary, the results presented in this paper indicate that ascorbic acid, but not captopril, is capable of scavenging lipid-derived peroxyl radicals and thereby inhibiting lipid peroxidation in this micellar model. Although it is difficult to extrapolate the quantitative aspects of the micellar data to more complex intact cell systems, the complete lack of reactivity of captopril with lipid peroxyl radicals in-vitro suggests that it is unlikely to be an important reaction in-vivo.

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