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ANGIOTENSIN CONVERTING ENZYME INHIBITORS AS OXYGEN FREE RADICAL SCAVENGERS*

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The authors have compared the ability of two non-SH-containing angiotensin converting enzyme (ACE) inhibitors (enalaprilat and lisinopril) with an -SH containing ACE inhibitor (captopril) to scavenge the hydroxyl radical (·OH). All three compounds were able to scavenge ·OH radicals generated in free solution at approximately diffusion-controled rates (10^{10} $M^{-1}s^{-1}$) as established by the deoxyribose assay in the presence of EDTA. The compounds also inhibited deoxyribose degradation in reaction mixtures which did not contain EDTA but not so effectively. This later finding also suggests that they have some degree of metal-binding capability. Chemiluminescence assays of oxidation of hypoxanthine by xanthine oxidase in the presence of luminol, confirm that the three ACE inhibitors are oxygen free radical scavengers. Our results indicate that the presence of a sulphydryl group in the chemical structure of ACE inhibitors is not relevant for their oxygen free radical scavenging ability.

KEY WORDS: angiotensin converting enzyme inhibitors, oxygen free radicals, hydroxyl radical, oxygen free radical scavengers, chemiluminescence

1. INTRODUCTION

Some angiotensin-converting enzyme (ACE) inhibitors have a number of properties which are independent from specific enzyme inhibition. They exhibit anti-inflammatory properties, and protect the ischemic myocardium against reperfusion injury. A pathogenic role for oxygen free radicals has been ascribed in both these situations. A possible explanation for alternative actions of ACE inhibitors may be attributed, at least in some degree, to the ability of ACE inhibitors to scavenge deleterious free radicals[1-6].

The superoxide anion radical (O_2^-) scavenging capability of some ACE inhibitors like captopril (an SH-containing ACE inhibitor) and enalaprilat (a non-SH-containing ACE inhibitor) has already been reported, although for captopril, contradictory results have appeared[1-7].

When assessing the antioxidant properties of such drugs, it is perhaps more important to evaluate if they scavenge, or prevent, the generation of more reactive oxygen species which, unlike O_2^-, are probably the direct agents of damage.

It is now well established that much of the damage done by O_2^- and H_2O_2 *in vivo* can be attributed to their conversion into highly-reactive oxidants, such as the hydroxyl radical (·OH)[8,9]. The hydroxyl radical reacts with almost all biological

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molecules in its vicinity; thus it will damage proteins, cause breakage of DNA strands, and initiate lipid peroxidation[10,11].

The generation of oxygen free radicals by the xanthine oxidase system has been proposed as one of the important mechanisms involved in reperfusion injury after myocardial ischemia[12,13], and electronparamagnetic resonance (EPR) studies have demonstrated that ·OH is an important mediator of such reperfusion injury[14-16].

The ability of captopril to scavenge the ·OH radical was already examined[2,7]. The free radical scavenging action of captopril is believed to be due to the presence of an -SH group in its structure. In this study, using the deoxyribose assay[17,18], two non-SH-containing ACE inhibitors, enalaprilat and lisinopril, were compared with captopril concerning the ability to effectively scavenge the ·OH radical.

The deoxyribose assay enables one to measure the second-order rate constants of these drugs for reaction with ·OH, as well as to evaluate their potential ability to chelate iron ions and interfere with "site-specific" generating of ·OH[9,18]. We have also examined the ability of ACE inhibitors to inhibit the luminol-enhanced luminescence, produced during the enzymatic reaction of xanthine oxidase on its substrate hypoxanthine[19].

2. MATERIAL AND METHODS

2.1. Chemicals and Enzymes

Reagents of the highest purity available were purchased and used without further purification. 2-Deoxy-D-ribose, L-ascorbic acid, N-ethylmaleimide, hypoxanthine, luminol, xanthine oxidase (Grade III from buttermilk), superoxide dismutase (bovine erythrocytes), catalase (bovine liver) and D-mannitol were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and ferric chloride from Fluka (Buchs, Switzerland). Enalaprilat and lisinopril were kindly supplied by Merck Sharp & Dohme (Hoddeston, Herts, UK) and captopril by Bristol-Myers Squibb (Moreton, Merseyside, UK). All the other reagents were obtained from Merck (Darmstadt, Germany).

2.2. Deoxyribose Degradation by Fe^{3+} (±EDTA), Ascorbic Acid and H_2O_2

The experimental procedure was essentially as described by Halliwell *et al.*[17] with a few modifications as described. Each assay contained, in a final volume of 1 ml, the following reagents at the final concentrations stated: KH_2PO_4-KOH buffer, pH 7.4 (20 mM), deoxyribose (2.8 mM), $FeCl_3$ (100 μM), EDTA when added (104 μM), H_2O_2 (300 μM), one of drugs (enalaprilat, lisinopril or captopril) at concentrations shown in appropriate figures or tables and ascorbic acid (100 μM).

$FeCl_3$ and EDTA (if added) were pre-mixed just before addition to the reaction mixture. Solutions of $FeCl_3$ and ascorbate were made-up immediatelly before use. Ascorbic acid was added to start the reaction. The final concentration of the drug in the reaction mixtures ranged from 1 to 10 mM. Reaction mixtures were incubated at 37° for 1 h. Colour was developed by adding 1 ml of 2-thiobarbituric acid (TBA) 1% (w/v) in 0.05 M NaOH and 1 ml of trichloroacetic acid (TCA) 2.8% (w/v). The mixture was heated at 100° for 15 min. After cooling, the pink chromogen, which was formed, was measured at 532 nm.

For all concentrations of enalaprilat and lisinopril assayed, controls in which

deoxyribose is omitted from the reaction mixture, were performed. The three drugs released TBA-reactive material when attacked by ·OH, and the absorbance values (A_{532}) obtained for these controls were subtracted from the values of complete assays plus the drugs. The control TBA values ranged between 5–15% of the absorbance measured for the complete system, without EDTA, and in the presence of EDTA, the control values ranged between 15–25%. It was also found that the three drugs did not interfere with subsequent measurement of deoxyribose degradation products, since they had no effect, when they were added to the reaction mixture at the end of the incubation, just before addition of TCA and TBA.

2.3. Luminol-Enhanced Chemiluminescence Method to Detect Oxygen Radical Scavengers

This study was carried out as described by Betts[19] with minor modifications as described below. Experimental solutions contained, in a final volume of 1 ml, EDTA (100 μM), hypoxanthine (600 μM), luminol (20 μM), $FeCl_3$ when added (5 μM), test drug at concentrations shown in appropriate figures or tables, xanthine oxidase (3×10^{-2} U/ml) in phosphate buffer, pH 7.4 (50 mM). The addition of EDTA was essential in order to get reproducible results. However, it was confirmed that the yield of chemiluminescence was dependent upon EDTA concentration. Above 100 μM EDTA there was an increasing inhibition, and for the 10–100 μM range there was no significant change in the chemiluminescence results (data not shown). All reactions were incubated at 37°. The xanthine oxidase was added during vortex mixing, and the samples were quickly introduced into the luminometer (LKB 1250) for counting. The luminometer was set to integrate for 20 intervals of 10 s each. The results were expressed as light intensity (LI) in mV and represent the output accumulated after each integration period of 10 s. For each assay, three to five replicates were done. The chemiluminescence profiles were selected according to the median of maximal light intensity (LI_{max}) values obtained in each experiment. It was carefully checked that none of the drugs studied inhibited xanthine oxidase, and did not act as light quenchers in the luminol ($A_{425\ nm}$) assay.

3. RESULTS

In the deoxyribose method ·OH radicals are generated by a mixture of ascorbic acid, H_2O_2 and $FeCl_3$. It was observed that ·OH generation is greatly stimulated if iron is complexed with EDTA, in a equimolar ratio. This is because the Fe^{3+}-EDTA chelate reacts promptly with O_2^- [20,21] and with ascorbate[22].

Figure 1 shows the ability of enalaprilat, lisinopril and captopril to inhibit deoxyribose degradation in reaction mixtures, with or without EDTA. From the slope of the competition plots which were obtained in the presence of EDTA, the second-order rate constants were calculated (Table 1).

In Figure 1 it can be seen that the three ACE inhibitors have a very similar behaviour. They inhibit deoxyribose degradation in the system without EDTA, but more efficiently when it is present. This suggests that the three drugs are interfering with "site-specific" ·OH generation, i.e., they probably remove iron ions from deoxyribose and form complexes less reactive in generating ·OH[9]. However, all three drugs appear to be better scavengers of ·OH radicals, when ·OH is generated in

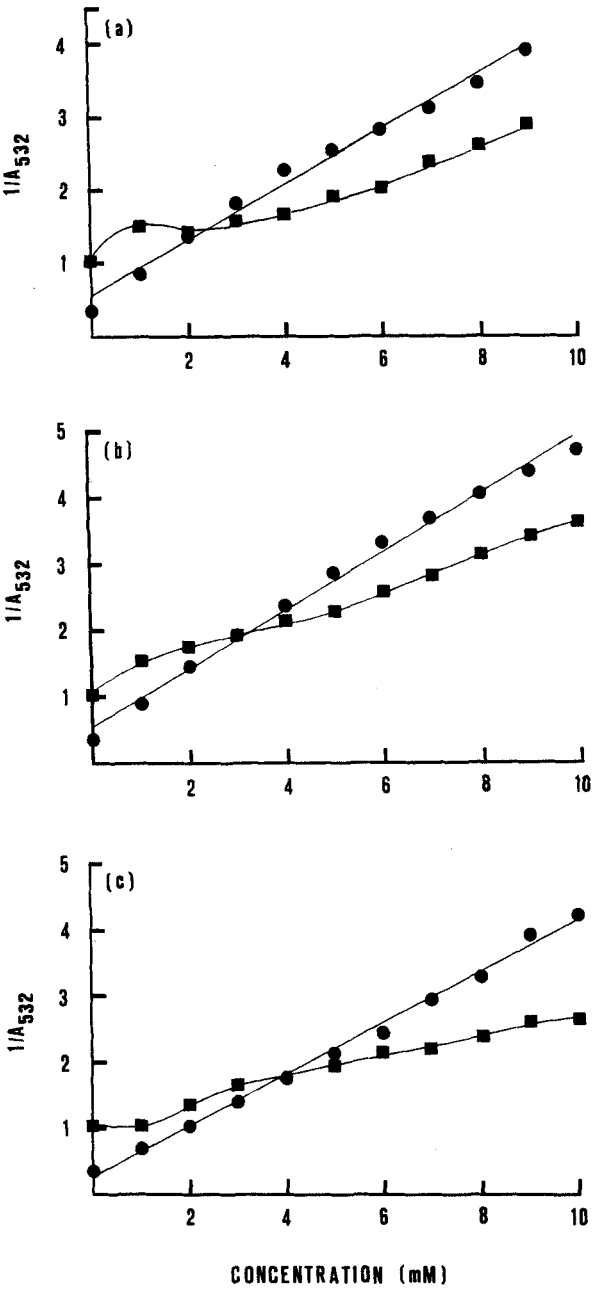

FIGURE 1 Inhibition of deoxyribose degradation by enalaprilat, lisinopril and captopril. Each assay contained, in a final volume of 1 ml, the following reagents: 20 mM KH_2PO_4-KOH buffer (pH 7.4), 2.8 mM deoxyribose, 100 μM $FeCl_3$ and 104 μM EDTA (when added), 300 μM H_2O_2, drug and 100 μM ascorbic acid. The reaction mixtures were incubated at 37° for 1 h and the colour was developed by the TBA method[17]. ● with EDTA; ■ without EDTA. (a) Enalaprilat, (b) lisinopril, (c) captopril. Data represent the means of at least four experiments done in triplicate that differed by no more than 10%.

free solution (i.e., in reaction mixtures containing EDTA). These results are in agreement with those obtained with captopril by Aruoma *et al.*[7].

The ability of captopril to scavenge ·OH may be due to its -SH group. To confirm this possibility, experiments were carried out with the -SH group of captopril blocked by N-ethylmaleimide (NEM). Under these conditions captopril loses its ability to inhibit deoxyribose degradation in reaction mixtures which do not contain EDTA. However, in assays with EDTA, captopril still inhibits deoxyribose degradation (Table 2).

Chemiluminescence generated during the oxidation of hypoxanthine by xanthine oxidase, in the presence of luminol, was used to study the scavenging action of the three drugs.

Figure 2 shows the chemiluminescence profiles in the presence of increasing amounts of enalaprilat, lisinopril or captopril. The concentrations required to cause an IC_{50} are 700 μM for enalaprilat and lisinopril and 190 μM for captopril.

In order to confirm the oxygen free radical scavenging actions of the three ACE inhibitors, the effect of known free radical scavengers such as superoxide dismutase (SOD), catalase, dimethyl sulfoxide (DMSO), thiourea and mannitol were determined. These scavengers produced a similar set of chemiluminescence profiles. The chemiluminescence assay was highly sensitive to SOD, with an IC_{50} of 0.25 U/ml. The IC_{50} for catalase, DMSO, thiourea and mannitol were 13 U/ml, 725 μM, 3.1, and 3 mM, respectively.

TABLE 1
Rate constants for reactions of drugs with ·OH obtained by the deoxyribose method

Drugs	Second-order rate constant for reaction with ·OH ($M^{-1}s^{-1}$)
Enalaprilat	$(7.8 - 8.8) \times 10^9$
Lisinopril	$(9.8 - 10.2) \times 10^9$
Captopril	$(8.1 - 9.2) \times 10^9$

The rate constants were determined from the slopes of the lines obtained in the presence of EDTA (K = slope $\times K_{DR} \times [DR] \times A^\circ_{532}$, where $K_{DR} = 3.1 \times 10^9\ M^{-1}s^{-1}$, [DR] = 2.8 mM and A°_{532} is the absorbance measured in the absence of drug[17]). Values given are the range from at least four separate experiments done in triplicate that differed by no more than 10%.

TABLE 2
Effect of N-ethylmaleimide on the capacity of captopril to inhibit the deoxyribose degradation

Additions to the reaction mixtures	TBA reactivity A_{532} nm			
	Assay with Fe^{3+}		Fe^{3+}-EDTA	
None	0.964	(-)	2.591	(-)
Captopril (4 mM)	0.611	(37)	0.565	(78)
Captopril - NEM (4 mM)	0.986	(0)	0.599	(77)
Captopril (8 mM)	0.470	(51)	0.333	(87)
Captopril - NEM (8 mM)	0.834	(13)	0.357	(86)

Assays were performed essentially as described in the legend of Figure 1. In the assays with NEM, this alkylating reagent is pre-mixed and stays in contact with captopril, for at least 15 min, before being added to the reaction mixture. The values in parenthesis are the percentage inhibition of deoxyribose degradation. The results shown are those from a representative of three to five experiments done in triplicate that differed by no more than 10%.

The effect of iron on chemiluminescence was also studied, using a constant concentration of EDTA (100 μM) and concentrations of ferric chloride from 1 to 10 μM. Chemiluminescence was maximal with 5 μM ferric chloride. At this concentration of $FeCl_3$ the drug concentrations required to inhibit chemiluminescence by 50% were 510 μM for enalaprilat and lisinopril and 150 μM for captopril (Table 3). This assay was less sensitive to SOD and catalase but more sensitive to ·OH radical scavengers (Table 3).

4. DISCUSSION

This study shows that the ACE inhibitors, enalaprilat, lisinopril and captopril, are effective scavengers of ·OH when the radical is generated in free solution. Rate constants of around 10^{10} $M^{-1}s^{-1}$ were established by using the deoxyribose assay in

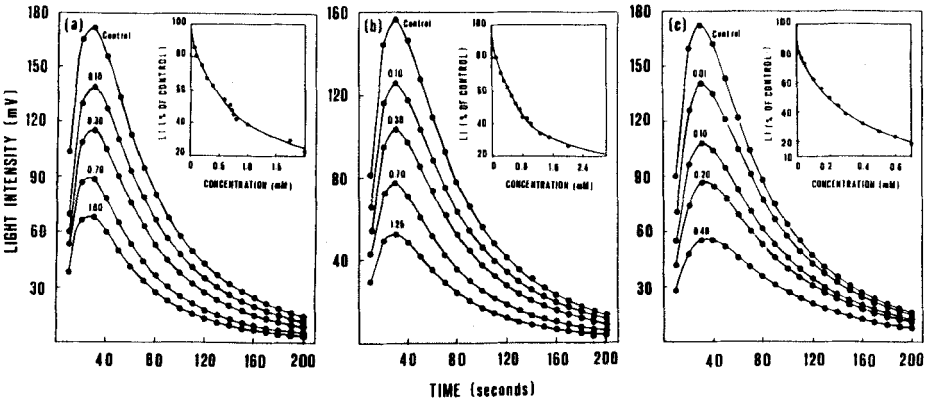

FIGURE 2 Inhibition of chemiluminescence by enalaprilat, lisinopril and captopril. Each assay contained 100 μM EDTA, 600 μM hypoxanthine, 20 μM luminol, and the drugs in the concentration (mM) as indicated in the Figure, 3×10^{-2} U/ml xanthine oxidase in 50 mM phosphate buffer (pH 7.4). Inserts show inhibition curves of LI_{max} obtained for each drug. (a) Enalaprilat, (b) lisinopril, (c) captopril. The values shown are from a representative of four experiments.

TABLE 3
Concentrations of compounds required to cause 50% inhibition (IC_{50}) in the chemiluminescence assays

	IC_{50}	
Compounds	Assay with EDTA	Assay with Fe-EDTA
Enalaprilat	700 μM	510 μM
Lisinopril	700 μM	510 μM
Captopril	190 μm	150 μM
Catalase	13 U/ml	21 U/ml
SOD	0.25 U/ml	0.58 U/ml
DMSO	725 μM	650 μM
Thiourea	3.1 mM	2.0 mM
Mannitol	3.0 mM	2.2 mM

The assays were carried out as described under Materials and Methods. The values shown are the median of at least four experiments.

the presence of EDTA. High concentrations of drugs, which are unlike to be achieved *in vivo* during normal therapeutic regimens, were used to calculate these constants. A further consideration is the high yield of free radicals, generated by the *in vitro* system which is certainly much higher than would be generated in biological systems. Nevertheless, even with the lowest concentration assayed (1 mM) of the ACE inhibitors, a clear inhibitory effect was observed (50 to 65%).

The three drugs studied also appeared to be able to chelate iron ions. This effect is relevant because the antioxidant ability of a compound can also be examined by studying its capacity to chelate iron ions in such a way that it interferes with the metal ion dependent "site-specific" generation of ·OH[23].

When assaying the three ACE inhibitors we also wanted to know if the presence of an -SH group in captopril conferred greater antioxidant capacity to it once the other two drugs do not possess such a group.

Enalaprilat and lisinopril have similar molecular structures (Figure 3) and they both possess a phenyl and an acid group, the 2-pyrrolidinecarboxilate. This group also exists in the captopril molecule but in addition it possesses a thiol group. Since the carboxylate and -SH groups can act as ligands of iron ions[24] we suggest that the three compounds can chelate iron through their carboxylate groups. In addition, captopril may also chelate iron ions through its thiol group.

Clearly, all three drugs possess groups which can react with ·OH[25,26], and may effect scavenging of ·OH through a decarboxylation reaction of the carboxyl group. Moreover, enalaprilat and lisinopril may both scavenge ·OH through an addition reaction on the aromatic ring and captopril may scavenge ·OH by the oxidation of the -SH group. The captopril molecule therefore possesses two groups which may both chelate iron and scavenge ·OH radicals. In the deoxyribose assays without EDTA present, and when the -SH groups of captopril are blocked (with NEM) the carboxyl groups remain to chelate iron, leaving no free groups to react with ·OH. This suggestion could explain why captopril does not inhibit deoxyribose degradation in assays without EDTA. In the assay system in which all the iron is chelated to EDTA, captopril continues to inhibit deoxyribose degradation even when its -SH groups are blocked, suggesting this occurs by its carboxyl groups, remaining free.

Experiments with NEM suggest that the -SH group of captopril contributes to its antioxidant capacity. However, in relation to ·OH scavenging, the three ACE inhibitors show much the same reactivity (rate constants of about 10^{10} $M^{-1}s^{-1}$). This is perhaps to be expected from their chemical structures since enalaprilat and lisinopril, although they have no -SH group, possess an aromatic ring.

The chemiluminescence assays allowed us to use much lower drug concentrations, and confirmed that the three ACE inhibitors are free radical scavengers. The advantages of sensitivity, simplicity and quickness make the chemiluminescence technique

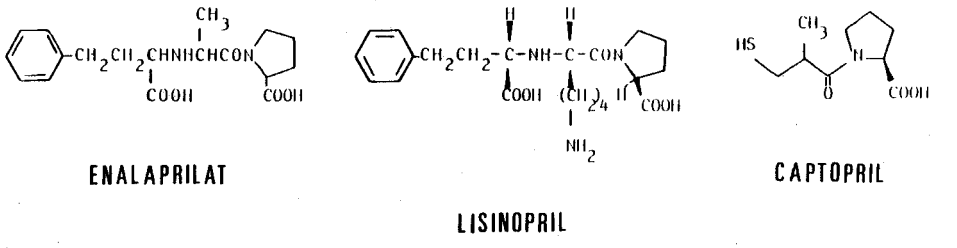

FIGURE 3 Chemical structures of ACE inhibitors, enalaprilat, lisinopril and captopril.

a very useful way to study the continuous production of radicals. However, a major disadvantage of this assay is the difficulty in knowing which radicals are responsible for the luminescence. It has been suggested that luminol reacts with $O_2^{\cdot-}$, hydrogen peroxide anion (HO_2^-), ·OH and singlet oxygen ($^1O_2$)[27]. However, the use of specific oxygen radical scavengers allows the identification of some of the oxygen radicals involved. Xanthine oxidase has been widely used as a $O_2^{\cdot-}$ and H_2O_2 generating system but it should also be realised that since iron traces are present ·OH radical will also be generated. The chemiluminescence which was observed to be increased by the addition of $FeCl_3$, must reflect ·OH generation via an iron-catalysed Haber-Weiss reaction. Under these conditions we get lower IC_{50} values for DMSO, thiourea and mannitol (three known ·OH radical scavengers) and also for the three ACE inhibitors which were studied, supporting that these are effective ·OH radical scavengers.

Our results indicate that the presence of a sulphydryl group in the chemical structure of ACE inhibitors is not a determinant condition for the compounds being able to scavenge oxygen free radicals as well as to bind metal ions.

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References

1. W. Westlin and K. Mullane (1988) Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation*, **77** (Suppl I), 30–39.
2. D. Bagchi, R. Prasad and D.K. Das (1989) Direct scavenging of free radicals by captopril, an angiotensin converting enzyme inhibitor. *Biochemical and Biophysical Research Communications*, **158**, 52–57.
3. R.C. Kukreja, H.A. Kontos and M.L. Hess (1990) Captopril and enalaprilat do not scavenge the superoxide anion. *American Journal of Cardiology*, **65**, 241–271.
4. X.-J. Pi and X. Chen (1989) Captopril and ramiprilat protect against free radical injury in isolated working rat hearts. *Journal of Molecular and Cellular Cardiology*, **21**, 1261–1271.
5. J. McMurray and M. Chopra (1991) Influence of ACE inhibitors on free radicals and reperfusion injury: pharmacological curiosity of therapeutic hope? *British Journal of Clinical Pharmacology*, **31**, 373–379.
6. D. Jay, A. Cuéllar, R. Zamorano, E. Muñoz and R. Gleason (1991) Captopril does not scavenge superoxide: captopril prevents $O_2^{\cdot-}$ production by chelating copper. *Archives of Biochemistry and Biophysics*, **290**, 463–467.
7. O.I. Aruoma, D. Akanmu, R. Cecchini and B. Halliwell (1991) Evaluation of the ability of the angiotensin-converting enzyme inhibitor captopril to scavenge reactive oxygen species. *Chemico-Biological Interactions*, **77**, 303–314.
8. B. Halliwell and J.M.C. Gutteridge (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Archives of Biochemistry and Biophysics*, **246**, 501–514.
9. B. Halliwell (1990) How to characterize a biological antioxidant. *Free Radical Research Communications*, **9**, 1–32.
10. B.A. Freeman, J.D. Crapo (1982) Biology of Disease. Free radicals and tissue injury. *Laboratory Investigation*, **47**, 412–426.
11. P.A. Southorn and G. Powis (1988) Free Radicals in Medicine. I. Chemical nature and biologic reactions. *Mayo Clinic Proceedings*, **63**, 381–389.
12. J.M. McCord (1985) Oxygen-derived free radicals in postischemic tissue injury. *The New England Journal of Medicine*, **312**, 159–163.
13. P.A. Southorn and G. Powis (1988) Free Radicals in Medicine. II. Involvement in human disease. *Mayo Clinic Proceedings*, **63**, 390–408.

14. J.L. Zweier, J.T. Flaherty and M.L. Weisfeldt (1988) Measurement of free radical generation in the post-ischemic heart. In *Oxy-radicals in Molecular Biology and Pathology* (eds. P. Cerutti, I. Fridovich and J.M. McCord), Alain R. Liss, New York, pp. 365–383.
15. J.L. Zweier, P. Kuppusamy and G.A. Lutty (1988) Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 4046–4050.
16. M. Culcasi, S. Pietri and P.J. Cozzone (1989) Use of 3,3,5,5-tetramethyl-1-pyrroline-1-oxide spin trap for the continuous flow ESR monitoring of hydroxyl radical generation in the ischemic and reperfused myocardium. *Biochemical and Biophysical Research Communications*, **164**, 1274–1280.
17. B. Halliwell, J.M.C. Gutteridge and O.I. Aruoma (1987) The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry*, **65**, 215–219.
18. O.I. Aruoma and B. Halliwell (1988) The iron-binding and hydroxyl radical scavenging action of anti-inflammatory drugs. *Xenobiotica*, **18**, 459–470.
19. W.H. Betts (1985) Detecting oxy radicals by chemiluminescence. In *CRC Handbook of Methods for Oxygen Radical Research* (ed. R.A. Greenwald), CRC Press, Boca Raton, pp. 197–201.
20. J. Butler and B. Halliwell (1982) Reaction of iron-EDTA chelates with the superoxide radical. *Archives of Biochemistry and Biophysics*, **218**, 174–178.
21. G.R. Buettner, T.P. Doherty and L.K. Patterson (1983) The kinetics of the reaction of superoxide radical with Fe(III) complexes of EDTA, DETAPAC and HEDTA. *FEBS Letters*, **158**, 143–198.
22. C.C. Winterbourn (1981) Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate and superoxide. *Biochemical Journal*, **198**, 125–131.
23. J.M.C. Gutteridge (1987) Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl radical scavengers in the presence of EDTA. *Biochemical Journal* **243**, 709–714.
24. M.N. Hughes (1981) *The Inorganic Chemistry of Biological Processes*. John Wiley & Sons, Chicester.
25. G. Czapski (1984) Reaction of ·OH. In *Oxygen Radicals in Biological Systems* (ed. L. Packer), Academic Press, Orlando, pp. 209–215.
26. A.I. Cederbaum and G. Cohen (1985) Microsomal oxidation of hydroxyl radical scavenging agents. In CRC *Handbook of Methods for Oxygen Radical Research* (ed. R.A. Greenwald), CRC Press, Boca Raton, pp. 81–87.
27. A.K. Campbell, M.E. Holt and A. Patel (1985) Chemiluminescence in medical biochemistry. In *Recent Advances in Clinical Biochemistry* (eds. C.P. Prince and K.G.M.M. Alberti), Churchill Livingston, Edinburgh, pp. 1–30.

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