Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 223-232
Reprints available directly from the publisher
Photocopying permitted by license only

STUDIES ON THE EFFECTS OF ANTIOXIDANTS AND INHIBITORS OF RADICAL GENERATION ON FREE RADICAL PRODUCTION IN THE REPERFUSED RAT HEART USING ELECTRON SPIN RESONANCE SPECTROSCOPY

S.L. SHUTER, M.J. DAVIES,[†] P.B. GARLICK,[‡] D.J. HEARSE[§] and T.F. SLATER

Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH, [†]Department of Chemistry, University of York, Heslington, York, YO1 5DD, [‡]Department of Radiological Sciences, 18th Floor Guy's Tower, Guy's Hospital, London, SE1 9RT, and [§]Cardiovascular Research, The Rayne Institute, St. Thomas' Hospital, London SE1 7EH, UK

Reperfusion of the heart after a period of ischaemia can precipitate ventricular arrhythmias and lead to an exacerbation of tissue injury. Direct evidence to suggest the involvement of free radicals has been obtained using electron spin resonance (esr) spectroscopy and the spin trap N-*tert.* butyl-α-phenyl nitrone (PBN). In the present study, we have used esr spectroscopy and PBN to examine the individual effects of superoxide dismutase (SOD), catalase, allopurinol or desferal on radical production in the isolated, reperfused rat heart. A burst of radical production was observed in the control group during the first 5 minutes of reperfusion; the peak occurred during the first minute, when signal intensity had increased by almost 300%, but returned to the baseline by 15 minutes of reperfusion. The esr signals were consistent with the trapping of either alkoxyl or carbon-centered radicals (a_N = 13.6 and a_H = 1.56 G). In the desferal-treated group, a burst of radical production was observed during the first five minutes of reperfusion; this was maximal during the second minute, when signal intensity had increased by almost 200%, but had returned to the baseline value by 30 minutes of reperfusion. In the SOD-treated group, a burst of radical production was observed during the first 10 minutes of reperfusion; signal intensity was maximal during the tenth minute of reperfusion, when signal intensity had increased by almost 200%, but had returned to the baseline value by 30 minutes of reperfusion. In the allopurinol- and catalase-treated groups, no significant burst of radical production could be detected. These data further support the concept that cytotoxic, oxygen-derived species are formed upon reperfusion and that hydrogen peroxide and/or hydroxyl radicals, are likely to be involved.

KEY WORDS: *Esr spectroscopy, free radicals, spin trapping, reperfusion injury, antioxidant.*

INTRODUCTION

It has been proposed that oxygen-derived free radicals, generated in the reperfused myocardium, can act as a trigger in the initiation of arrhythmias.[1-5] There is circumstantial evidence that supports this hypothesis; several indirect studies have suggested that a number of enzyme inhibitors, antioxidant enzymes, free radical scavengers and iron chelators are able to protect against reperfusion injury in a number of species.

Address for correspondence: S.L. Shuter, Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH.

For example, superoxide dismutase (SOD) and catalase have both been shown individually to protect the myocardium against reperfusion-induced injury.[3,4,6,7] Pretreatment with allopurinol, an inhibitor of xanthine oxidase, has also been shown to be protective.[9–11] Desferal, a chelator of iron that inhibits the iron-catalysed Haber-Weiss reaction[12] which can lead to the generation of the highly reactive HO· radical, has also been shown to protect the heart against reperfusion-induced arrhythmias.[3]

Recent experiments using electron spin resonance (esr) spectroscopy, the only method currently available for the direct detection of free radicals in biological systems, have provided further evidence in support of the hypothesis that free radicals may act as triggers of reperfusion injury; it has been demonstrated that a burst of free radicals is generated upon aerobic reperfusion of the myocardium in a number of species.[13–19] This evidence has recently been reviewed.[20]

In the present study we have used the technique of esr spectroscopy to examine directly the individual effects of two antioxidant enzymes (SOD and catalase), an enzyme inhibitor (allopurinol) and an iron chelator (desferal), on the generation of free radicals in the isolated, reperfused rat myocardium.

METHODS

Animals

Male Wistar rats (280–300 g body weight) were used in all studies.

Perfusion Techniques and Perfusion Media

Rats were anaesthetised with diethyl ether and, after the administration of heparin (200 IU), hearts were excised and perfused,[21] at a constant pressure of 65 cm H_2O at 37°C. The perfusion medium was bicarbonate buffer containing (in mM), NaCl (118.5), $NaHCO_3$ (25.0), KCl (3.1), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5) and glucose (11.1), pH 7.4 after gassing with 95% O_2 and 5% CO_2. In order to remove any particulate matter, the buffer was passed through a 0.22 μm filter prior to use.

Experimental Time Course

Hearts (n = 6/group; five groups) were perfused aerobically for 35 minutes, followed by 15 minutes of global ischaemia (zero flow) which was induced by clamping the aortic input line. The hearts were then reperfused for 30 minutes.

In order to trap reactive radicals generated in the myocardium,[13,22] all hearts were perfused with buffer containing the spin trap N-*tert.* butyl-α-phenyl nitrone (PBN; Sigma, UK) for the last 5 minutes of the aerobic perfusion period and throughout reperfusion. The spin trap was used at a concentration of 3 mM since it has been shown[23] that this does not produce cardiotoxic effects. In order to avoid the photolytic degradation of PBN, the perfusion apparatus was kept in darkness throughout the experimental period.

In 3 of the treatment groups, the perfusion fluid during the last 5 minutes of aerobic perfusion and for the whole of reperfusion contained human recombinant superoxide dismutase (8×10^4 U/l, a generous gift from Pharmacia, AB Uppsala, Sweden; specific activity 3325 IU/mg protein), or catalase (5×10^5 U/l. Sigma, UK; specific activity 3310 IU/mg protein) or desferal (deferoxamine mesylate; 0.15 mM. Sigma,

UK). In the fourth treatment group, allopurinol (4 mg/ml. Sigma, UK) was administered orally (0.5 ml/100 g body weight; 20 mg/kg) as an aqueous suspension 48, 24 and 1 hour before the study.[5]

Sample Processing and Esr Spectroscopy

Coronary effluent fractions were collected after 34 minutes of aerobic perfusion and at 1, 2, 3, 4, 5, 10, 15 and 30 minutes of reperfusion and the coronary flow was recorded. Due to the large volumes of coronary effluent (Table 2), the radical adducts were present at a relatively low concentration. To minimise this effect, 5 ml aliquots of effluent were added to 0.75 ml toluene (at 4°C) and vortex-mixed for 30 sec. After centrifugation (800 g, 5 minutes), the toluene layer was aspirated and its esr spectrum recorded using a Bruker ER 200 D spectrometer equipped with 100 KHz modulation and an ER 411 variable temperature unit. Although the major radical adduct observed was relatively stable at room temperature, esr spectra were recorded at 213 K in order to facilitate the observation of other, less stable adducts, which may have been present. Hyperfine coupling constants and signal intensities were measured directly from the field scan. It has been established by control experiments that the peak height of the PBN adducts correlates closely with the double-integral of the signal; thus, peak height may be used as a measure of free radical content.

Statistical Analysis of Results

Results were expressed as the mean ± SEM. A one-way analysis of variance was carried out to test for differences between the mean values of each group. Once a difference had been established, each of the treated groups was compared to the control using the unpaired t-test and p-values of less than 0.05 were considered to be significant.

RESULTS

Control

Typical esr spectra obtained from coronary effluent collected from hearts perfused with PBN only are shown in Figure 1. Signal intensity was greatest during the first 5 minutes of reperfusion (Figure 1C) and was virtually undetectable in fresh buffer and in coronary effluent collected either before ischaemia or after 15 minutes of reperfusion (Figures 1A, B and D).

The hyperfine splitting constants (a_N = 13.6 G and a_H = 1.56 G) of the spectra obtained during reperfusion were typical of the PBN adducts of carbon-centred or alkoxyl radicals.[24] These species may have been formed via secondary reactions of oxygen radicals with membrane lipids. A time-course of spin adduct appearance in the coronary effluent was constructed from the spectra (see Figure 2A). Upon reperfusion with PBN alone, a burst of spin adduct formation was observed which was maximal during the first minute (when signal intensity increased to 279 ± 87% of the baseline value), and subsequently decreased to the baseline value by 15 minutes of reperfusion.

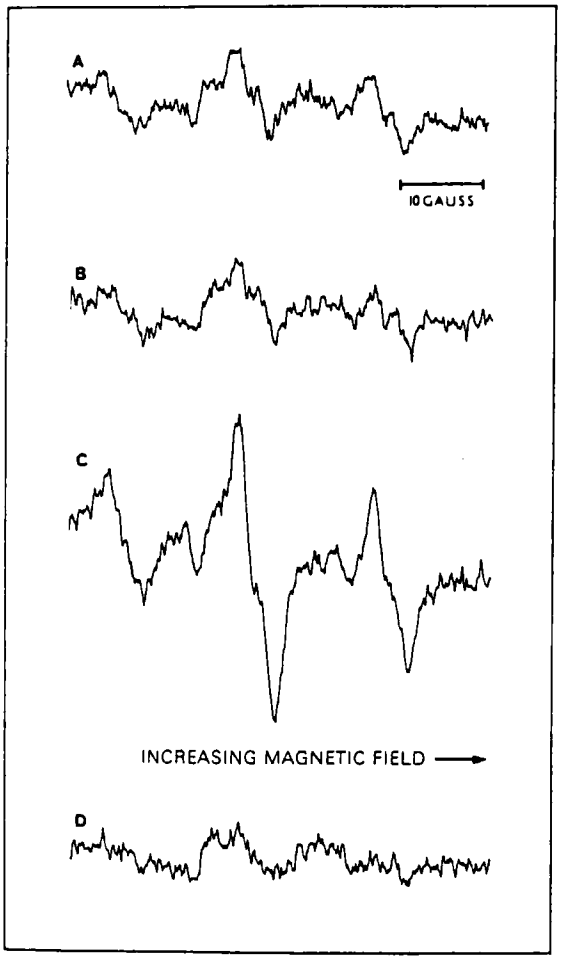

FIGURE 1 Electron spin resonance spectra from: A, Oxygenated buffer prior to perfusion. B, Coronary effluent collected after 34 minutes of aerobic perfusion. C, Coronary effluent collected after 2 minutes of reperfusion. D, Coronary effluent collected after 15 minutes of reperfusion. Spectra B–D are recorded from control hearts.

Reperfusion in the Presence of Desferal

The esr spectra obtained from coronary effluent collected from hearts perfused with desferal and PBN were qualitatively similar to those obtained from the PBN controls. In the presence of 150 μM desferal (Figure 2B), a burst of spin adduct formation was observed which was maximal after 2 minutes of reperfusion (when signal intensity increased to 193 $\pm$ 29% of the baseline value), and subsequently decreased to the baseline value by 30 minutes or reperfusion. There were no statistically significant differences between the desferal-treated and the PBN control group at any of the measured time-points. The coronary flow rate was unaffected by desferal (Table 1).

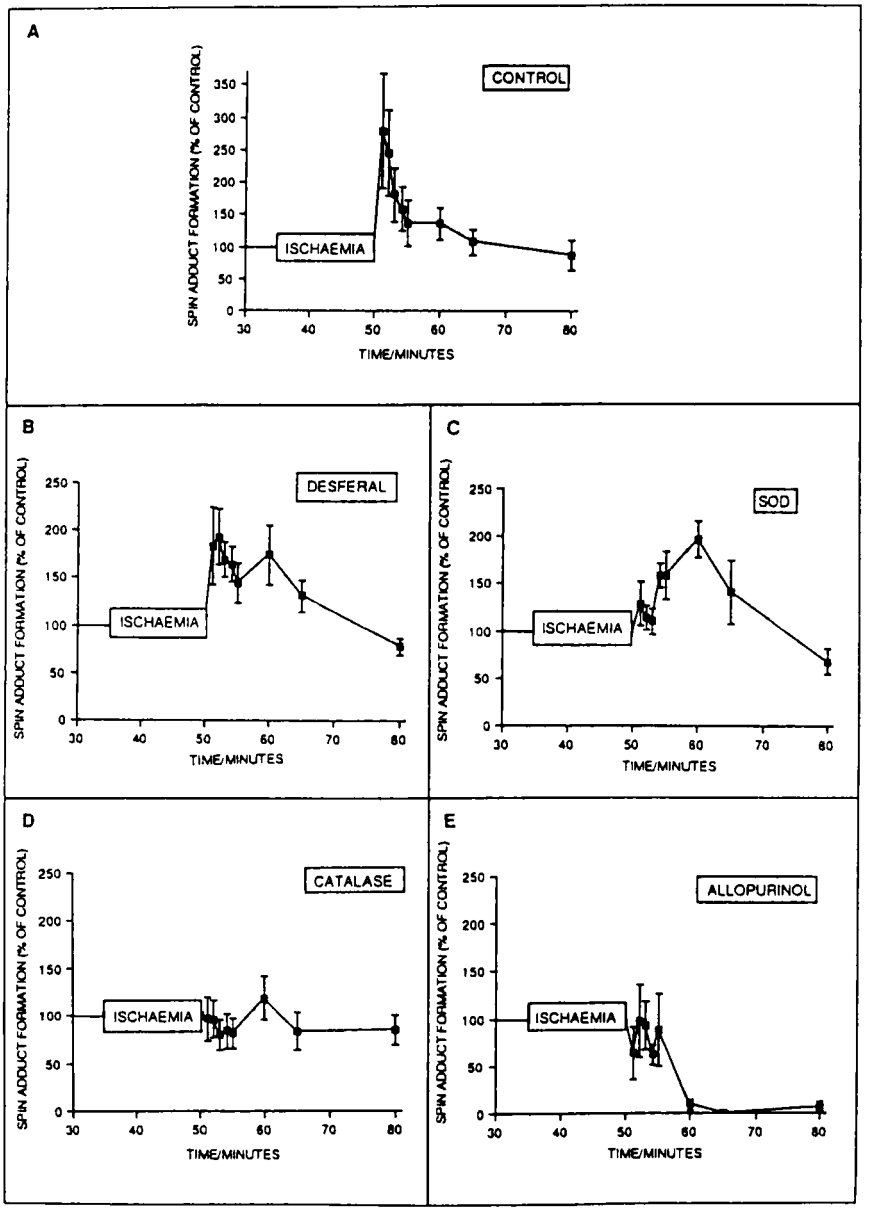

FIGURE 2 Graphs showing the time course of spin adduct formation in the reperfused rat heart in the presence of: A, Control, 3 mM PBN only; B, 150 μM desferal; C, 8 $\times$ 10^4 U/l superoxide dismutase (SOD); D, 5 $\times$ 10^5 U/l catalase; E, After pre-treatment of animals with allopurinol (20 mg/kg body weight, 48, 24 and 1 hour before experiment). n = 6/group. Results expressed as mean $\pm$ SEM. *p < 0.05 compared with zero time control (t = 34) for each group.

TABLE I

Coronary flow rates at various times throughout the experiment for: control hearts; SOD-perfused hearts; catalase-perfused hearts; desferal-perfused hearts and hearts from animals pre-treated with allopurinol. All results are expressed as mean ± SEM

Time (Minutes)	Coronary flow (Ml/Minute)				
	Control	SOD	Catalase	Desferal	Allopurinol
34	8.3 ± 0.9	7.8 ± 0.7	8.7 ± 0.5	7.7 ± 0.4	9.1 ± 0.8
51	3.2 ± 1.1	4.0 ± 0.4	*6.5 ± 0.7	5.3 ± 0.4	5.3 ± 0.8
52	5.7 ± 1.1	5.3 ± 0.3	7.3 ± 0.3	6.4 ± 0.3	6.9 ± 0.9
53	6.3 ± 0.8	6.1 ± 0.2	7.6 ± 0.3	7.0 ± 0.3	7.1 ± 1.0
54	7.3 ± 0.7	6.1 ± 0.4	7.8 ± 0.4	7.1 ± 0.2	7.2 ± 1.0
55	7.7 ± 0.5	6.6 ± 0.4	8.0 ± 0.5	7.5 ± 0.2	7.4 ± 0.9
60	8.7 ± 0.7	7.4 ± 0.3	8.8 ± 0.6	8.2 ± 0.2	8.1 ± 0.6
80	8.1 ± 0.9	6.4 ± 0.3	9.0 ± 0.7	7.8 ± 0.3	7.7 ± 0.8

* $p < 0.05$ compared with the control.

Reperfusion in the Presence of SOD

The esr spectra obtained from coronary effluent collected from hearts perfused with SOD and PBN were qualitatively similar to those obtained from the PBN controls. In the presence of 8×10^4 U/l SOD (Figure 2C), a burst of spin adduct formation was observed which was maximal after 10 minutes of reperfusion (when signal intensity increased to 197 ± 19% of the baseline value), and subsequently decreased to the baseline value by 30 minutes of reperfusion. There was a statistically significant difference between the SOD-treated and the PBN control group after 10 minutes of reperfusion. The coronary flow rate was unaffected by SOD (Table 1).

Reperfusion in the Presence of Catalase

The esr spectra obtained from coronary effluent collected from hearts perfused with catalase and PBN were qualitatively similar to the controls. Figure 2D shows the time course of spin adduct formation in the presence of 5×10^5 U/l catalase. There was no evidence for a burst of radical production at any time during reperfusion; statistically significant differences between the catalase-treated and the PBN control group were found at all measured time-points during the first four minutes ($p < 0.05$). The pre-ischaemic coronary flow rate was not significantly different from the control value. The flow rate was significantly different from the control at the first minute of reperfusion (6.5 ± 0.7 ml/minute in the presence of catalase, compared with 3.2 ± 1.1 ml/minute in the control group; $p < 0.05$) (Table 1).

Effects of Pre-treatment with Allopurinol

Figure 2E shows the time course of spin adduct formation following pre-treatment of the animals with allopurinol. As with catalase, there was no evidence for a burst of radical production at any time during reperfusion; statistically significant differences between the allopurinol-treated and the PBN control group were found at all time points during the first four minutes of reperfusion ($p < 0.05$). The coronary flow rate was unaffected by pre-treatment with allopurinol (Table 1).

DISCUSSION

Using esr spectroscopy, a number of investigators[13–19] have provided direct evidence for a burst of free radical production during the early minutes of reperfusion of the previously ischaemic heart. The primary free radicals produced are believed to be oxygen-derived species that are generated as a consequence of the readmission of oxygen that occurs upon reperfusion. This burst may overwhelm endogenous defence mechanisms and initiate free radical chain reactions such as lipid peroxidation, which would be associated with secondary free radical production. The membrane damage that occurs as a consequence of lipid peroxidation may lead to the ionic imbalances which trigger the ventricular arrhythmias that are observed upon reperfusion.[1] Alternatively, the radicals may alter the redox state of membrane channels and carrier proteins[25] leading to ionic imbalance.[20]

Using the spin trap PBN, we have observed carbon-centred or alkoxyl radical adducts in the effluent collected from the reperfused rat myocardium. This is in agreement with previous findings.[13] These radicals may have been formed via reactions of cytotoxic oxygen-derived species with membrane lipids. In the present study, we have used known inhibitors of radical production or antioxidant enzymes in an attempt to attenuate the burst of free radical production that is detectable in the effluent of the reperfused myocardium.

In the presence of catalase, there was no evidence for a burst of free radical production at any time during reperfusion. There was also no evidence for a burst of free radical production in hearts from allopurinol-treated animals. SOD did not significantly decrease the burst of free radical production normally observed upon reperfusion of the isolated rat heart. However, free radical production was delayed; the peak occurring after 10 minutes of reperfusion in the SOD group in comparison with a peak at the first minute of reperfusion in the PBN group. Since exogenous SOD was probably confined to the vascular space, it could only have scavenged $O_2^{\cdot -}$ originating in, or close to, that tissue compartment. Thus, it may have been that $O_2^{\cdot -}$ formed during the early moments of reperfusion, for example via xanthine oxidase, was scavenged by SOD. At later times, however, it is possible that $O_2^{\cdot -}$ may have initiated damage in areas of tissue inaccessible to SOD. The superoxide anion radical is, however, a relatively unreactive radical; for example, the rate constant for the reaction of $O_2^{\cdot -}$ with linoleic acid at pH 7.0 is small,[26] whereas the rate constant for the reaction of HO· with linoleic acid under similar conditions is $9.0 \times 10^9\ M^{-1} s^{-1}$.[27] It is possible therefore, that $O_2^{\cdot -}$ was indirectly toxic in this system; $O_2^{\cdot -}$ could have led to the formation of HO· via reaction with H_2O_2 in the iron-catalysed Haber-Weiss reaction.[28] Both catalase (by direct removal) and allopurinol (by decreasing the overall rate of $O_2^{\cdot -}$ and H_2O_2 production) would be expected to decrease the concentration of H_2O_2 in the heart. Conversely, SOD would be expected to increase the amount of H_2O_2, possibly leading to increased HO· generation. Our study provides indirect evidence in support of the hypothesis that H_2O_2, possibly via iron-catalysed HO· generation, is an important species mediating reperfusion-induced myocardial injury.

Desferal is an iron-chelator well known for its ability to inhibit the iron-dependent HO· generation that occurs via reactions such as the Haber-Weiss reaction and possibly therefore, lipid peroxidation.[12,29] In this study, desferal did not significantly decrease the burst of free radical production observed upon reperfusion. It is known that desferal penetrates only slowly into endothelial cells *in vitro*;[30] thus, during the

course of our experiment, desferal is likely to have been restricted to the vascular space. This would suggest that extracellular iron is not involved in the genesis of the radicals observed in this system. Alternatively, iron may have been released in a form which desferal was unable to chelate. The identity of the iron-catalyst that is involved in reperfusion-induced generation of HO· is, however, unknown, although oxymyoglobin, which is in abundance in the reperfused myocardium, is a potential source of iron.[31] Hydrogen peroxide is known to react with myoglobin to generate radicals, possibly by inducing iron release from the haem group of the myoglobin molecule,[29] or by elevating the iron to a higher oxidation state.[32] Free radical-induced myocardial injury may thus be dependent upon H_2O_2 production in close proximity to myoglobin. It has been shown[29] that desferal is able to stabilise myoglobin by preventing haem breakdown, thus inhibiting iron-stimulated radical generation. Desferal may, however, have been in the wrong location in our system to facilitate protection.

In the present study, PBN-radical adducts were detected in the coronary effluent in the first minute of reperfusion. Since adducts were observed so rapidly, we suggest that, in the reperfused myocardium, free radical generation begins at the endothelium. For example, $O_2^{\cdot-}$ and H_2O_2, generated via xanthine oxidase, could react with trace metal ions to form HO· that, in turn, may initiate free radical chain reactions such as lipid peroxidation which may then lead to damage elsewhere in the cell and ultimately, in the surrounding tissues. Lipid peroxidation produces a complex variety of substances, some of which are biologically active and yet can diffuse over distances that are relatively large in relation to cellular dimensions.[33] In consequence, lipid peroxidation that is initiated by a free radical reaction in the endothelium may cause significant effects at a distance due to the production of substances such as lipid hydroperoxides, and 4-hydroxyalkenals that can act as intercellular signals.[34] The free radicals that were spin trapped in this study, may thus have been lipid radicals, produced as a result of lipid peroxidation, and which originated in the endothelial cell membrane.

It is improbable that exogenous SOD, catalase or desferal would have been able to cross cell membranes during the time course of these experiments. Therefore, all three agents will have been confined to, and must have exerted their effects in, the vascular space or on the endothelial surface. Similarly, since xanthine oxidase is located primarily in endothelial cells[35,36] allopurinol would have exerted its effects here, thus providing further support for the hypothesis that most of the radicals scavenged in this study are likely to have been either endothelial or extracellular in origin.

In conclusion, oxygen-derived free radicals are formed upon reperfusion; the burst of radical production can be attenuated by allopurinol and by catalase, but not by SOD or desferal. It is possible, therefore, that hydrogen peroxide and/or hydroxyl radicals may be the cytotoxic species that mediate myocardial injury.

Acknowledgements

This work was supported in part by grants from the British Heart Foundation (BHF 86/87), STRUTH, the National Heart Lung and Blood Institute (RO1 – HL 372780), and The Nuffield Foundation. We thank Ms Sue Maddix for her excellent technical assistance.

References

1. Manning, A.S. Reperfusion-induced arrhythmias: Do free radicals play a critical role? *Free Rad. Biol. Med.*, **4**, 305–316, (1988).

2. McCord, J.M. Free radicals and myocardial ischaemia: Overview and outlook. *Free Rad. Biol. Med.*, **4**, 9–14, (1988).
3. Bernier, M., Hearse, D.J. and Manning, A.S. Reperfusion-induced arrhythmias and oxygen free radicals. *Circ. Res.*, **58**, 331–340, (1986).
4. Woodward, B. and Zakaria, M.V.M. Effect of some free radical scavengers on reperfusion-induced arrhythmias in the isolated rat heart. *J. Mol. Cell. Cardiol.*, **17**, 485–493, (1985).
5. Manning, A.S., Coltart, D.J. and Hearse, D.J. Reperfusion-induced arrhythmias: Mechanisms and prevention. *Circ. Res.*, **55**, 545–548, (1984).
6. Kim, M.S. and Akera, T. Oxygen free radicals: Cause of ischemia-reperfusion injury to cardiac Na^+-K^+/ATPase. *Am. J. Physiol.*, **252**, H252–H257, (1987).
7. Riva, E., Manning, A.S. and Hearse, D.J. Superoxide dismutase and the reduction of reperfusion-induced arrhythmias: *in vivo* dose-response studies in the rat. *Cardiovasc. Drugs Ther.*, **1**, 133–139, (1987).
8. Elion, G.B., Kovensky, A., Hitchings, G.H., Metz, E. and Rundless, R.W. Metabolic studies of allopurinol, an inhibitor of xanthine oxidase. *Biochem. Pharmacol.*, **15**, 863–880, (1966).
9. Chambers, D.E., Parks, D.A., Patterson, G., Roy, R., McCord, J.M., Yoshida, S., Parmley, L.F. and Downey, J.M. Xanthine oxidase as a source of free radical damage in myocardial ischaemia. *J. Mol. Cell. Cardiol.*, **17**, 145–152, (1985).
10. Manning, A.S., Coltart, D.J. and Hearse, D.J. Ischaemia and reperfusion-induced arrhythmias in the rat. *Circ. Res.*, **55**, 545–548, (1984).
11. Wearns, S.W., Shea, M.J., Driscoll, E.M., Mitsos, S.E., Fantone, J.C., Pitt, B. and Lucchesi, B.R. Effect of xanthine oxidase inhibition on canine myocardial ischaemia. *Circ. Res.*, **56**, 895–898, (1985).
12. Gutteridge, J.M.C., Richmond, R. and Halliwell, B. Inhibition of iron-catalysed formation of hydroxyl radicals and superoxide and lipid peroxidation by desferal. *Biochem. J.*, **184**, 469–472, (1979).
13. Garlick, P.B., Davies, M.J., Hearse, D.J. and Slater, T.F. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ. Res.*, **61**, 757–760, (1987).
14. Arroyo, C.M., Kramer, J.H., Dickens, B.F. and Wegliki, W.B. Identification of free radicals in myocardial ischaemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Letters*, **221**, 101–104, (1987).
15. Baker, J.E., Felix, C.C., Olinger, G.N. and Kalynaraman, B. Myocardial ischaemia and reperfusion: Direct evidence for free radical generation by esr spectroscopy. *Proc. Nat. Acad. Sci. USA*, **85**, 2786–2789, (1988).
16. Blasig, I.E., Ebert, E. and Lowe, H. Identification of free radicals trapped during myocardial ischaemia trapped *in vitro* by esr. *Studia Biophysica.*, **116**, 35–42, (1986).
17. Bolli, R., Patel, B.S., Jeroudi, M.O., Lai, E.K. and McCay, P.B. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap α-phenyl N-*tert.*-butyl nitrone. *J. Clin. Invest.*, **82**, 476–485, (1988).
18. Nakazawa, H., Ichimori, K., Shinozaki, Y., Okino, H. and Hori, S. Is superoxide demonstration by electron-spin resonance spectroscopy really superoxide? *Am. J. Physiol.*, **255**, H213–215, (1988).
19. Zweier, J.L., Flaherty, J.T. and Weisfeldt, M.L. Direct measurement of free radical generation following reperfusion of the ischaemic myocardium. *Proc. Nat. Acad. Sci. USA.*, **84**, 1404–1407, (1987).
20. Hearse, D.J. Free radicals and myocardial injury during ischaemia and reperfusion: a short-lived phenomenon? In: *Lethal Arrhythmias Resulting from Myocardial Ischaemia and Reperfusion* (ed. M.R. Rosen and Y. Palti), Boston: Kluwer Academic Publishers, pp 105–115, (1989).
21. Langendorff, O. Untersuchungen uber lebender saugertier herzen. *Pflugers Arch. ges. Physiol.*, **61**, 291–332, (1897).
22. Janzen, E.G. A critical review of spin trapping in biological systems. In: *Free Radicals in Biology* (ed. W.A. Pryor), New York: Academic Press, pp 115–154, (1980).
23. Hearse, D.J. and Tosaki, A. Free radicals and reperfusion-induced arrhythmias. Protection by PBN in the rat heart. *Circ. Res.*, **60**, 375–383, (1987).
24. Buettner, G.R. Spin trapping: ESR parameters of spin adducts. *Free Rad. Biol. Med.*, **3**, 259–303, (1987).
25. Reeves, J.P., Bailey, C.A. and Hale, C.C. Redox modification of sodium-calcium exchange activity in cardiac sarcolemmal vesicles. *J. Biol. Chem.*, **261**, 4948–4955, (1986).
26. Bielski, B.H., Arundi, R.H. and Sutherland, M.W. A study of the reactivity of HO_2/O_2^- with unsaturated fatty acids. *J. Biol. Chem.*, **258**, 4759–4761, (1983).
27. Buxton, G.V., Greenstock, C.L., Helman, W.P. and Ross, A.B. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (.OH/.O^-) in aqueous solution. *J. Phys. Chem. Ref. Data.*, **17**, 513–886, (1988).

28. McCord, J.M. and Day, E.D. Superoxide-dependent production of hydroxyl radical catalysed by iron-EDTA complex. *FEBS Letters*, **86**, 139–142, (1978).
29. Puppo, A. and Halliwell, B. Formation of hydroxyl radicals in biological systems: Does myoglobin stimulate hydroxyl radicals from hydrogen peroxide? *Free Rad. Res. Commun.*, **4**, 415–422, (1988).
30. Gannon, D.E., Varani, J., Phan, S.H., Ward, J.H., Kaplan, J., Till, G.O., Simon, R.H., Ryan, U.S. and Ward, P.A. Source of iron in neutrophil-mediated killing of endothelial cells. *Lab. Invest.*, **57**, 37–44, (1987).
31. McCord, J.M. Oxygen-derived free radicals in post-ischaemic tissue injury. *N. Engl. J. Med.*, **312**, 159–163, (1985).
32. Harel, S. and Kanner, J. Generation of ferryl/HO· radicals during interaction of haemoproteins with hydrogen peroxide. *Free Rad. Res. Commun.*, **5**, 21–33, (1988).
33. Slater, T.F. Biochemical pathology in microtime. In: *Recent Advances in Biochemical Pathology: Toxic Liver Injury*, (ed M.U. Dianzani, G. Ugazio and L.M. Sena), Torino: Minerva Medica, pp 99–108, (1976).
34. Slater, T.F. Lipid peroxidation and intercellular messengers in relation to cell injury. *Agents and Actions*, **22**, 3–4, (1987).
35. Bindoli, A., Cavallini, L., Rigobello, M.P., Coassin, M. and DiLisa, F. Modification of xanthine-converting enzyme of perfused rat heart during ischaemia. *Free Rad. Biol. Med.*, **4**, 163–167, (1988).
36. Bailey, I.A., Blackwell, C.P. and Woodward, B. Chemiluminescence measurements of xanthine oxidase and xanthine dehydrogenase in four types of cardiovascular cell. *Basic Res. Cardiol.*, **83**, 392–400, (1988).

Accepted by Prof. E.G. Janzen