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DIRECT DETECTION OF RADICAL PRODUCTION IN THE ISCHAEMIC AND REPERFUSED MYOCARDIUM: CURRENT STATUS

MICHAEL J. DAVIES

Department of Chemistry, University of York, York YO1 5DD, U.K.

Electron spin resonance (e.s.r.) spectroscopy, which is a specific method for directly detecting free radicals in biological systems, has now been used in a number of studies to examine free radical generation in both ischaemic and reperfused myocardial tissue. This review critically assesses the information which has been obtained to date with particular reference to the elucidation of the nature and source of the radicals observed in these studies.

KEY WORDS: E.s.r., radicals, reperfusion, ischaemia, spin trapping, hydrogen peroxide, hydroxyl, superoxide.

INTRODUCTION

In the last few years evidence has been obtained which is consistent with the hypothesis that reactive oxygen-derived free radicals are generated in cells and tissues, and that such species are involved, as either primary or secondary agents, in a variety of pathological processes such as the reperfusion injury observed in the heart, lung, liver, kidney, brain and gastrointestinal tract.[1-5] This evidence has, in most cases, been obtained by indirect methods such as the detection of products arising from lipid peroxidation (thiobarbituric acid reactive material, lipid hydroperoxides, conjugated dienes and hydrocarbon production), the loss of cellular enzymes, and the decrease in heart arrhythmias and cellular damage observed on administration of protective enzymes, metal-ion chelators, and radical scavengers. To definitively link oxygen radical generation with such cellular injuries direct measurement of radical production is necessary; such experiments should also allow identification and quantification of the radicals formed, determination of their site and mechanism of formation, and the examination and evaluation of protective strategies.

Electron spin resonance (e.s.r.) spectroscopy, a technique that is specific for species with unpaired electrons (i.e., free radicals and some metal-ion complexes), would therefore appear to be the method of choice. The absorption lines (which are often recorded as their first derivatives) detected in an e.s.r. experiment arise from the promotion of the unpaired electron from a low energy state to a higher energy state; such transitions occur when the free radical is placed in a magnetic field and exposed to microwave energy of the appropriate frequency. The interaction of the unpaired electron with neighbouring magnetic nuclei results in a number of possible transitions; information obtained from the number, pattern and splittings between these lines (hyperfine coupling constants) together with the overall position of the absorptions in the magnetic field (g factor) gives valuable information as to both the nature and structure of the radical(s) present in the system.[6,7]

The lower limit for the direct detection of radicals by this technique is of the order of 10^{-8} M, though to adequately resolve spectral lines, and hence obtain information as to the structure of the radical, the limit is somewhat higher than this. Thus for direct detection of a radical it must be generated at a rapid rate and/or have a long lifetime. Many of the radicals suggested to be involved in both ischaemia- and reperfusion-induced cellular damage (for example oxygen-derived free radicals) do not, however, fall into this category because of their high reactivity (and hence short lifetimes), low rates of generation or the extremely broad nature of their absorption lines. To circumvent these problems, two different approaches have been used by groups attempting to study radical generation during ischaemia and reperfusion. The first of these is the use of rapid freezing, the second, the use of spin trapping. Both of these methods, though extremely useful, are prone to artifactual errors and misinterpretation; the rest of the article is concerned with a critical review of the direct evidence obtained for radical generation in both the ischaemic and reperfused myocardium.

RESULTS OBTAINED FROM RAPID FREEZING EXPERIMENTS

Methodology

Rapid freezing of tissue samples, usually to liquid nitrogen temperature, greatly reduces the rate of radical decay and hence provides a "snapshot" of some of the radicals present in the tissue at the time of freezing; very reactive radicals such as the hydroxyl radical, HO·, cannot be detected by this method as they decay during the time interval required to produce freezing.

Results

This technique has been used by Zweier *et al.*[8] to study radical production during total global ischaemia and subsequent reperfusion in isolated, Langendorff-perfused, rabbit hearts. Hearts were freeze clamped during control perfusion, after 10 min of normothermic global ischaemia, and after a similar length of ischaemia followed by various periods of reflow with oxygenated perfusate. The tissue from each heart was ground to a fine powder under liquid nitrogen then transferred to e.s.r. tubes. The spectra of these hearts exhibited three different signals with different power saturation characteristics and thermal stabilities; temperature annealing and spectral subtraction allowed the spectra from each radical to be identified. The major species observed in control hearts consisted of an isotropic signal with g 2.004; this signal has been assigned to a semiquinone radical. The second signal, which was anisotropic with g_{11} 2.033 and $g_{\perp}$ 2.005 (assigned to an alkyl peroxyl or superoxide free radical), and the third signal, which consists of an isotropic triplet with g 2.000 and a hyperfine splitting a_N 24 Gauss (assigned to a nitrogen-centered free radical) were observed predominantly in the reperfused tissue with the maximum intensities observed 10 sec after initiation of reflow. Hypoxic reperfusion did not increase the intensity of any of the radical signals above the levels observed during ischaemia. Later studies by this same group[9] demonstrated that recombinant human superoxide dismutase (r-h-SOD) decreased the intensity of both the alkyl peroxyl and nitrogen-centered free radical signals by 49% and 38% respectively at 10 sec after initiation of reflow. In contrast the intensity of the semiquinone signal was increased by 51% at this time. Peroxide

inactivated r-h-SOD did not show these effects. Similar experiments using rabbit hearts treated with the potent iron chelator desferal (0.5 mM) either during ischaemia or at the start of reflow, resulted in a decrease in the alkyl peroxyl radical signal to $\simeq 40\%$ of controls.[10] The results obtained in these studies led the authors to conclude that (i) oxygen-centered free radicals were generated during reperfusion of previously ischaemic tissue, (ii) superoxide-derived free radicals were produced and (iii) the radicals observed on reperfusion were greatly reduced by the iron chelator desferal.

Recent studies carried out by other groups[11–13] have cast doubt on the significance of some of these findings and in particular on the validity of data obtained from heart tissue that has been subjected to severe mechanical stress (such as grinding). In the first of these studies Baker, Kalyanaraman and co-workers[11] compared e.s.r. spectra obtained at -196°C from aerobically perfused rat and rabbit hearts which had been either *pulverized* to a fine powder or *chopped* into small fragments. The spectra obtained from the *pulverized* tissue consisted of three components: a semiquinone radical (g 2.004), a lipid peroxyl radical ($g_{\parallel}$ 2.04, $g_{\perp}$ 2.006), and a carbon-centered radical which was tentatively identified as a lipid species (g_{iso} 2.002, a_H 50 Gauss). In contrast *chopped* tissue gave only the spectrum of the semiquinone radical, which has been tentatively assigned to the ubisemiquinone radical. The intensity of this signal was observed to vary in the rat heart between aerobically perfused tissue, ischaemic tissue (10 min, global, normothermic) and reperfused tissue in the order reperfused > aerobic > ischaemic with the maximum signal intensity observed at 10–15 seconds of reflow. Anaerobic reperfusion did not give this increase in signal intensity.

Very similar results have been obtained by Nakazawa and co-workers[12] using isolated buffer-perfused rat and rabbit hearts and open-chest canine hearts. In each system e.s.r. spectra were obtained from heart tissue which had either been carefully chopped or pulverized to a fine powder, at liquid nitrogen temperature. In all cases the pulverized samples showed two additional signals which were not present in the chopped sample. The intensities of these additional signals were found to depend on both the speed and length of pulverization and the type of tissue examined, with ischaemic and reperfused tissue more susceptible to the generation of these signals. It was concluded that these additional species were artifactually generated. In agreement with Baker *et al.*[11] the concentration of the non-artifactual semiquinone radical was observed to be maximal in reperfused tissue and at a minimum in ischaemic samples.

Summary

The results presented in these two reports, together with data from our own laboratories[13] which are in broad agreement with the above findings, and data from previous studies[14–21] in other tissues which show that a wide variety of types of mechanical stress can result in artifactual radical production, suggest that some of the radical signals observed by Zweier *et al.*[8–10] *may* have been produced during the grinding process. Unfortunately some form of tissue manipulation is often necessary as the physical size of commonly used e.s.r. cavities and low temperature inserts, limits the diameter of the sample tubes that can be used to a maximum external diameter of approximately 4.5 mm. Few, if any, artifactual radical signals have been observed in tissue which has been manipulated in the unfrozen state either because of reduced bond cleavage or because of rapid decay of the artifactual species. Thus it has been reported that sampling *in vivo* by needle biopsy, drill biopsy, guillotine devices and freeze clamping methods do not generate new, observable, radical species.[12] It is therefore suggested

that such devices, or special grooved freeze clamps[13] (developed in our laboratories) which produce frozen cylinders of tissue suitable for direct insertion into e.s.r. sample tubes with a minimum of tissue disruption, may be of some use in such e.s.r. studies, provided that appropriate control experiments are always performed.

The observation of the semiquinone signal in tissue which has not been pulverized or ground[11,12] suggests that this species is present in the tissue at the time of freezing and it would appear that the concentration of this radical varies with the status of the tissue; both groups[11,12] report larger signals in reperfused tissue than in control perfused tissue with the lowest relative concentrations being observed in ischaemic samples. Absolute concentrations of this species can, however, only be determined by double integration of the signal due to significant changes in the line width as well as the intensity (line height) of the signal.

RESULTS OBTAINED FROM SPIN TRAPPING EXPERIMENTS

Methodology

In e.s.r. spin trapping experiments as organic compound (the spin trap), usually containing a nitroso or nitrone function, is added to the system under study to convert reactive radicals, which may be otherwise undetectable, into more stable, detectable, radical adducts (reactions 1 and 2). High concentrations of the spin trap (usually $\gg$ mM) are required in order to compete with reaction of the radicals with naturally occurring material; such concentrations may, however, cause significant alterations in the system under study (vide infra). A wide variety of spin traps are commercially available in relatively pure form; the correct choice of the spin trap is of great importance since different spin traps have different specificities with respect to the radicals with which they will react rapidly to form stable adducts. For further details the reader is referred to previous review articles.[22–26]

$$\underset{\text{Nitroso spin trap}}{R - N = O} + X^{\cdot} \rightarrow \underset{\text{Nitroxide spin adduct}}{R - \underset{\displaystyle X}{\underset{|}{N}} - O^{\cdot}} \quad (1)$$

$$\underset{\text{Nitrone Spin trap}}{R' - CH = \overset{+}{\underset{\displaystyle O^{-}}{\underset{|}{N}}} - R'' + X^{\cdot}} \rightarrow \underset{\text{Nitroxide spin adduct}}{R' - \underset{\displaystyle X}{\underset{|}{CH}} - \underset{\displaystyle O^{\cdot}}{\underset{|}{N}} - R''} \quad (2)$$

RESULTS

Radical Production During Ischaemia

Blasig and co-workers[27] using 5,5-dimethyl-l-pyrroline-N-oxide (DMPO, 100 mM) as the spin trap have detected e.s.r. signals from two spin adducts in coronary effluent samples obtained from isolated rat hearts perfused in the Langendorff mode during 90 min of low flow ischaemia after a 30 min equilibration period. These adducts,

which were not observed during the equilibration period, have e.s.r. hyperfine coupling constants of a_N 15.6 Gauss and a_H 23.0 Gauss for the first (major) species and $a_N = a_H = 14.8$ Gauss for the second (minor) species, and are assigned to a carbon-centered lipid radical adduct and the hydroxyl radical adduct respectively. No spin adducts were observed in heart *tissue* samples from the same experiments.

In a slightly different system, though again using buffer-perfused isolated rat hearts and 100 mM DMPO, Arroyo *et al.*[28] have detected two spin adducts in the coronary effluent collected during 40 min of low coronary flow ischaemia after a 20 minute stabilisation period. In this study the major species detected was the hydroxyl radical adduct together with a carbon-centered radical adduct with hyperfine coupling constants a_N 15.5 Gauss and a_H 22.6 Gauss. Both of these signals were inhibited when SOD (93 μg/ml) was included in the perfusate demonstrating that both species have the superoxide radical as precursor. After long periods of ischaemia, in the presence of SOD, a third signal was observed which was assigned, on the basis of its coupling constant, to the ascorbyl radical. Further work by this same group[29] on open-chest, buffer-perfused, canine hearts with other spin traps has produced further evidence for radical generation. In hearts subjected to 20, 45 or 90 min of regional (LAD) ischaemia after a 30 min loading period in which the spin traps α-phenyl-N-t-butyl nitrone (PBN, 50 mM) or 2-methyl-2-nitrosopropane (MNP, 50 mM) were introduced, e.s.r. spin adduct signals were detected in both tissue samples and subsequent lipid extracts obtained from the ischaemic region but *not* from the non-ischaemic areas. With PBN as the spin trap, two adducts were observed in the ischaemic tissue samples; on the basis of their coupling constants, these signals were assigned to a carbon-centered radical adduct and an alkoxyl radical adduct. The former signal was also observed in the lipid extract, suggesting that this radical was derived from membrane lipids, whereas the latter adduct was not. In experiments where MNP was the spin trap, a single adduct was observed in both the tissue samples and the lipid extracts; this adduct has been assigned as a lipid-derived carbon-centred species.

Radical Production During Reperfusion

The detection of radical generation in the reperfused myocardium has received considerably more attention than that during the ischaemic period. In studies carried out on isolated, buffer-perfused, rat hearts Blasig and co-workers[30] have detected, using DMPO (100 mM) as the spin trap, two radical adducts in the coronary effluent during reperfusion after 30 min of control perfusion and 90 min of ischaemia. These two adducts have been identified as a carbon-centered radical adduct and the hydroxyl radical adduct.

In similar studies carried out by Weglicki and co-workers[31] using isolated, buffer-perfused, rat hearts subjected to reperfusion after varying periods of ischaemia, the hydroxyl radical adduct to DMPO was also detected. The intensity of the observed signal was increased with increasing duration of ischaemia in the range 20–40 minutes, though the time course of adduct detection remained relatively constant with maximal concentrations being observed 3 minutes after initiation of reperfusion. Inclusion of SOD (93 μg/ml) abolished this burst of radical production, implicating the superoxide radical as a precursor of the observed hydroxyl radical adduct. This hypothesis was confirmed by later work[28] from the same group where the superoxide radical adduct itself was detected in coronary effluent collected 20 sec after the initiation of reper-

fusion in the presence of 100 mm DMPO. This signal was shown to be rapidly replaced by that of the hydroxyl radical adduct at longer periods of reperfusion.

Similar observations have been reported by Zweier in studies on isolated, buffer-perfused rabbit hearts.[32] In this study both hydroxyl and carbon-centered radical adducts to DMPO (40 mM) were detected in coronary effluent collected during reperfusion of rabbit hearts after 25 mins of stabilisation and 30 min of global ischaemia. The peak concentrations of these adducts were observed in the first 30 sec of reflow. These radical adducts were shown to arise via superoxide radical production by inclusion of recombinant human superoxide dismutase (60,000 U) in the reperfusion medium; this resulted in a marked reduction in the signal intensity of both adducts. Peroxide-inactivated recombinant human superoxide dismutase had no effect. In experiments where recovery of contractile function was measured in parallel with free radical generation a direct relationship was observed, suggesting that the observed radicals were mediators of cellular dysfunction.

Early studies[33,34] carried out by the Brunel University and St Thomas' Hospital groups on isolated, buffer-perfused, rat hearts with PBN (3 mM) as the spin trap, demonstrated that a burst of radical production occurs in the myocardium on reperfusion after 15 min of total global ischaemia. The concentration of spin trap and period of ischaemia were chosen such that direct comparison of data could be made with previous studies where, under identical conditions, PBN provided significant protection of the myocardium against reperfusion-induced arrhythmias. This burst of radical production was observed by monitoring the concentration of spin adducts in the coronary effluent; the observed spin adduct has been assigned to either a carbon-centered or alkoxyl radical adduct, with the latter more likely. The time course of radical production was determined by collection of sequential aliquots of coronary effluent; the maximum radical concentration was observed at 4 minutes, with levels decreasing to control values by 30 min of reperfusion. This burst of radical production was observed at similar times to the previously determined maximum vulnerability of the myocardium to reperfusion-induced arrhythmias, suggesting that these two phenomena may be linked. The roles of flow and oxygen in this phenomenon were investigated by reperfusing the ischaemic myocardium with anoxic buffer; no increase in radical production was observed on restarting the flow, reintroduction of oxygen, however, resulted in an immediate burst of radical production similar in magnitude to that seen in wholly aerobic reperfusion experiments. Further experiments by this group[35] have demonstrated that allopurinol (a xanthine oxidase inhibitor) and catalase cause major inhibition of this burst of radical production whereas the iron chelator desferal and recombinant human SOD gave only marginal protection. These data are consistent with extracellular H_2O_2 generation and intracellular $HO^{\cdot}$ radical production.

A similar type of radical signal, assigned to a carbon-centered radical adduct, has been detected, using the spin trap trimethoxy phenyl-t-butylnitrone, in lipid extracts of cardiac tissue after 15 min of reperfusion.[36] Inclusion of the spin trap afforded slight protection against loss of mechanical function and reduced the release of creatine phosphokinase during the reperfusion period.

A recent, elegant, study by Bolli and co-workers[37] has demonstrated that a burst of radical production, similar to that described above, can be detected, by use of PBN as the spin trap, in open chest, blood-perfused, dog hearts. In this study hearts were subjected to 15 min coronary artery occlusion and subsequent reperfusion, with PBN infused into the myocardium via a selected coronary artery; e.s.r. signals of alkoxyl

and/or carbon-centered radical adducts to PBN were detected in the venous blood draining from the reperfused vascular bed. A slight release of PBN adducts was observed during the coronary occlusion but this increased dramatically during the first few minutes of reperfusion. Wash out of the radical adducts abated after this initial burst, but was still observable up to 3 hours after initiation of reflow. Simultaneous measurement of coronary function showed that there was a direct relationship between the magnitude of adduct production and the degree of ischaemic flow reduction; recovery of contractile function after reperfusion was also shown to be significantly greater in dogs treated with PBN than in controls.

SUMMARY

In each of the above studies spin traps have been successfully employed to detect the production of reactive free radicals during either the ischaemic or reperfusion phases. Evidence has been obtained, by the use of a number of different spin traps, in two buffer-perfused model systems (the isolated rat heart[27,28] and the *in vivo* canine heart[29]) for the generation, *during ischaemia*, of hydroxyl, alkoxyl and carbon-centered radicals. Little information has been obtained, to date, as to the site of generation or mechanism of production of these species, but it has been postulated, on the basis of the effects of SOD and the fact that some of the observed adducts are present in lipid extracts, that the superoxide radical is the initially generated species and that subsequent reactions of the hydroxyl radical (produced from H_2O_2 which arises from superoxide dismutation) with membrane lipids gives rise to the observed carbon-centered species. How oxygen-derived species such as $O_2^{\cdot -}$ and HO· are generated in *ischaemic* tissue remains to be determined.

A considerable body of evidence has also been obtained for the generation of radical species such as $O_2^{\cdot -}$, HO·, RO·, and R· in the reperfused myocardium of a number of species. Spin trapping studies using DMPO have produced evidence for the production of $O_2^{\cdot -}$ and HO· radicals in both the isolated, buffer-perfused, rat[30,31] and rabbit[32] hearts.

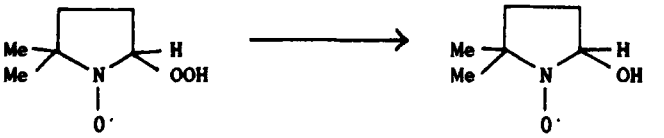
Carbon-centered and alkoxyl radicals have been detected in the coronary effluent from isolated, buffer-perfused, rat[30,33,35] and rabbit[32] hearts and the *in vivo*, blood-perfused, canine heart.[37] In some of these studies the effects of protective enzymes and inhibitors such as catalase, allopurinol and the iron-chelator deferoxamine (desferal) have yielded evidence as to the mechanism of generation of these species; in some of the reported cases superoxide dismutase has also proved to be an effective inhibitor of the observed signals suggesting that the superoxide radical is a key species. This radical itself is unlikely to be the major initiator of cellular damage due to its low reactivity.[38]

The protection offered by catalase and the observation of HO· adducts (though see later) suggests that H_2O_2 is the source of the damaging radical(s) which are assumed to be HO· radicals. Subsequent reaction(s) of HO· (with membrane components ?) yields the observed carbon-centered and alkoxyl radicals.

In each of these studies where the time course of radical production was investigated it has been shown that there is a rapid burst of radical production which peaks within the first few minutes of reperfusion. The similar nature of the observed time courses between different groups using very different systems suggests that this burst of radical production may be a common feature. The correlation observed in several

of these studies between levels of radical production and markers of biological damage is encouraging; it has been demonstrated that spin traps not only decrease the incidence of ventricular arrhythmias in isolated rat hearts,[39,40] but also improve recovery of contractile function in both isolated rabbit hearts[32] and *in vivo* canine hearts.[37]

The evidence reported in some of these reports should however be interpreted with caution. In particular the detection of the hydroxyl radical adduct to DMPO[27-32] should not be taken as compelling evidence for the production of hydroxyl radicals, partly because this species is extremely reactive and will hence be very difficult to scavenge with a spin trap, and partly because several other routes which result in the production of this adduct which do *not* involve the generation of hydroxyl radicals are known.[25] Not least of these is the often reported decomposition of the superoxide radical adduct (reaction 3) – in some of the reported studies the observed hydroxyl radical adducts to DMPO might be arising via this pathway.



(3)

The second cautionary note arises from the use of extremely high concentrations of spin traps in some of these studies (up to 100 mM).[27-32] The presence of these compounds at high concentrations may have very serious toxicological or hemodynamic effects; this is certainly true in other organs where enzyme inhibition and GSH and ATP depletion have been observed,[26] and there is some evidence that similar effects occur in heart tissue.[39,40] It is therefore of great importance that appropriate control experiments are performed – in some of the above studies this does not appear to have been done. This is *not* true however of all of these studies, in some cases the spin trap concentrations used were deliberately chosen in order to minimise any such pre-determined effects.[33-35,37,39] At low concentrations PBN does not appear to have toxic effects.[41]

CONCLUSIONS

Data obtained from e.s.r. experiments have considerably enhanced our knowledge of the role of free radicals in both ischaemia and reperfusion injury in the myocardium, and has provided some tentative causative links between radical production and physical damage. Two major types of experiment have been used to detect radicals – those which involve rapid freezing and those which use spin traps. It has been shown that the former technique is prone to severe problems of artifactual radical generation during tissue manipulation and much of the early data obtained by use of this technique should be interpreted with caution. Methods which minimise tissue disruption have given some useful information concerning the levels of semiquinone radicals during ischaemia and reperfusion.

Spin trapping experiments have provided a considerable body of evidence for the generation of a variety of radicals during both the ischaemic and reperfusion phases. The similarity of the data obtained by a number of groups using different model systems suggests that the observed short, sharp, burst of radical generation is a real

effect and common to a number of species. Some evidence has been obtained which links this radical production to observable damage, though considerable effort still needs to be made in *proving* that free radicals are the causative agents in cellular dysfunction. Little is known about the site or mechanism of radical production though a recent study[42] on the spin trapping of radicals in isolated endothelial cells provides some important clues; further work in this area is clearly called for.

An attempt to circumvent the problems associated with the use of either the rapid freezing or spin trapping techniques has been made by examining hearts *directly* within the cavity of the e.s.r. spectrometer. This has had limited success due to poor spectrometer sensitivity.[43] Whether such a technique will be of use in detecting radical production during ischaemia and reperfusion remains to be determined.

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