Reappraisal of the e.p.r. signals in (post)-ischaemic cardiac tissue

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The present study was designed to measure directly, using e.p.r. spectroscopy, oxygen-derived free radicals in (post)-ischaemic or (post)-anoxic rat hearts. Rat hearts were rapidly freeze-clamped at 77 K under normoxic, anoxic, ischaemic or reperfusion conditions. The samples were measured at three different temperatures (13, 77 and 115 K) and at several microwave power levels, and were compared with isolated rat heart mitochondria. Samples were prepared both by grinding and as tissue cuts. The two preparation techniques gave identical e.p.r. results, which excludes the occurrence of grinding artifacts. No free radical signals linked to reperfusion injury were detected. Several electron transfer centres known in the mitochondrial respiratory chain were measured. The signals previously assigned to post-ischaemic reperfusion injury were found to originate from electron transfer centres of the respiratory chain, predominantly the iron–sulphur cluster S-1 in succinate dehydrogenase. The differences in signal intensity between normoxic, ischaemic and reperfused hearts were found to result from the different redox stages of these centres under the various conditions tested. These findings do not necessarily imply that oxygen-derived free radicals are not formed in cardiac tissue during (post)-ischaemic reperfusion. The constitutive background of paramagnetism from the respiratory chain, however, seriously hampers the direct detection of comparatively low concentrations of free radicals in cardiac tissue. It is therefore expedient to focus future experiments in this field on the use of spin-trapping agents.

INTRODUCTION

The hypothesis that oxygen-derived free radicals (ODFR) are responsible for (post)-ischaemic myocardial injury has obtained much attention over the past few years [1,2]. Not only myocardial necrosis but also reperfusion-induced arrhythmias [3], ventricular fibrillation [4] and post-ischaemic coronary vasodilation [5] are now thought to be related to ODFR generation. Evidence in support of this free radical theory is mainly based on experiments in which protection of (post)-ischaemic cardiac tissue was reported with superoxide dismutase [6,7], catalase [8,9], free radical scavengers (e.g. mannitol and vitamin E [10,11]), and drugs that are able to block free radical generation (allopurinol, oxypurinol, deferoxamine, [12–15]). This evidence, however, is all indirect, since ODFR themselves were not measured in these studies. In order to obtain more direct information about ODFR involvement in (post)-ischaemic cardiac injury, e.p.r. spectroscopy has recently been introduced into this field [16,17].

In the present study we investigated freeze-clamped rat hearts which were perfused under normoxic, anoxic, ischaemic and reperfusion conditions. The study was especially designed to answer the following questions: (1) are the published e.p.r. signals [17–19] in (post)-ischaemic cardiac tissue reproducible; (2) do these signals originate from oxygen-derived free radicals; (3) to what extent are we able to measure electron transfer centres in cardiac tissue using e.p.r.; (4) do these signals interfere with the signals originating from ODFR; and (5) are there any signals artifactually induced in the myocardium as a result of the pulverization process under liquid N₂?

We report here that the originally published e.p.r. signals in (post)-ischaemic myocardial tissue [17–19] are reproducible and not artifactually induced; however, our interpretation of these signals is drastically different. The signals are found to originate from electron-transfer centres (mainly iron–sulphur clusters) which are located in respiratory chain complexes, specifically NADH dehydrogenase and succinate dehydrogenase. The published differences [17–19] in signal intensity between normoxic, ischaemic/anoxic and reperfused hearts are found to result from signal saturation and/or from different redox stages of these enzymes as a direct response to the presence or absence of oxygen.

MATERIALS AND METHODS

Perfusion protocol

A total of 22 male Wistar rats of body weight 200–250 g were used in the present study. After a short anaesthesia with diethyl ether, the heart was quickly removed from the body and put into ice-cold Tyrode's buffer. Within 1 min of this procedure, the hearts were cannulated in the aorta and perfused retrogradely according to Langendorff [20]. The perfusions were carried out at 37 °C and pH 7.4, using a modified Tyrode's buffer containing 128 mM-NaCl, 4.7 mM-KCl, 1.3 mM-CaCl₂, 20.2 mM-NaHCO₃, 0.4 mM-NaH₂PO₄, 1 mM-MgCl₂ and 11 mM-glucose and saturated with O₂/CO₂ (19:1). Perfusions were performed with a constant pressure of 80 cm of water. At the end of an initial equilibration period of 15 min, in which the hearts were stabilized, total ischaemia, anoxia or control perfusions were started for 15, 30 or 45 min (13 rat hearts in all; see Table 1 for the

Abbreviation used: ODFR, oxygen-derived free radicals.

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exact conditions used). Tissue temperature during total ischaemia was maintained by immersing the hearts in Tyrode's buffer at 37 °C, saturated with N_2/CO_2 (19:1). Anoxia was induced by perfusing the hearts under the same conditions with Tyrode's buffer saturated with N_2/CO_2 (19:1). Nine separate hearts were reperfused for 20 s after a period of 15, 30 or 45 min of ischaemia or anoxia (see Table 1).

Sample preparation

At the end of each perfusion experiment, the hearts were rapidly frozen using Wollenberger tongs precooled in liquid N_2 (77 K [21]). Directly after this procedure, samples were prepared under liquid N_2 using two different techniques. The first method involved grinding of the hearts, at 77 K, by means of a mortar (filled with liquid N_2) and a pestle of porcelain. The second procedure was performed by cutting the hearts under liquid N_2 into pieces with the same mortar and a razor-sharp 1 cm chisel. After these procedures the samples were transferred to e.p.r. tubes under liquid N_2 . Heart mitochondria were prepared according to [22].

E.p.r. spectroscopy

E.p.r. spectroscopy was done with a Bruker (ED 200 D) e.p.r. spectrometer. The microwave frequency was measured with a Systron Donner frequency counter (model 1292 A). The d.c. magnetic field was measured with an AEG Kernresonanz Magnetfeldmesser (type GA-EPR 11/21-02). The field was modulated with a frequency of 100 kHz and an amplitude of 0.63 mT. Heart samples were measured at N_2 flow temperatures (115 K), using a Bruker (ER 4111 VT) variable temperature unit, and at helium flow temperatures (13–77 K) using a home build cryostat, in which the temperature was measured by means of a calibrated 5 k Ω Allen-Bradley carbon resistor.

All data were collected as 1024-point spectra using an Olivetti M24 PC with software written in Asyst. This program allowed us to perform signal averaging, baseline correction, frequency alignment and signal integrations.

RESULTS

E.p.r. spectroscopy at 13 K

Fig. 1 shows an e.p.r. spectrum at 13 K of a pulverized, freeze-clamped rat heart after 30 min of ischaemia and 20 s of reperfusion. Under these conditions three types of signals can be observed: (1) metal-related signals, (2) radical signals, and (3) a doublet signal.

(1) Metal-related signals. The concentration of the respiratory chain in this sample is so high that the obtainable e.p.r. spectrum is almost the same in quality as those of purified enzymes from the respiratory chain. The e.p.r. properties of these enzymes have been documented in great detail in the literature [23–35]. With this information it is straightforward to identify in Fig. 1 the electron transfer centres (iron–sulphur clusters) N-1, N-2, N-3 and N-4 known in NADH dehydrogenase [23–26], S-1 and S-3 in succinate dehydrogenase [27–30], E in electron-transferring flavoprotein dehydrogenase [31], R in the Rieske protein [32,33], and copper in cytochrome *c* oxidase (Cu_A [34,35]). Centre S-2 from succinate dehydrogenase is the only non-detectable iron–sulphur cluster in Fig. 1 because of its extreme spectral width [30].

Fig. 1. Overview of e.p.r. detectable redox centres in whole-heart tissue

The heart was subjected to 30 min of global ischaemia followed by 20 s of aerobic reperfusion. After freeze-clamping and pulverization at 77 K, a 4-times-averaged e.p.r. spectrum was taken at 13 K with a microwave power of 3.2 mW and a microwave frequency of 9.33 GHz. The centres N-1 to N-4 are iron–sulphur clusters in NADH dehydrogenase; S-1 and S-3 are iron–sulphur clusters in succinate dehydrogenase; E is the iron–sulphur cluster in ETF dehydrogenase; R is the iron–sulphur cluster in the Rieske protein; and Cu_A is one of the copper ions in cytochrome *c* oxidase [23–35]. Selected *g* values are also given. The solid bar indicates the limited field range used in previous e.p.r. studies on perfused hearts [17–19].

(2) Radical signals. A clear isotropic signal with a *g*-value close to 2.00 can be detected in Fig. 1. This signal is thought to originate from coenzyme Q_{10} and the flavins FMN in NADH dehydrogenase and FAD in succinate dehydrogenase [23–35]. The semiquinone states (half-reduced or single-electron reduced states) of these compounds are all paramagnetic. FAD in succinate dehydrogenase in its semiquinone form gives rise to a well-developed radical signal at *g* = 2.005 with a 'peak-to-peak' width of the signal of 1.2 mT [36]. The situation for coenzyme Q_{10} is more complicated as there appear to be two different forms of coenzyme Q_{10} that give rise to two slightly different isotropic resonances. One is located at *g* = 2.005 and the other at *g* = 2.006, with 'peak to peak' widths of 1.0 and 0.83 mT respectively [37]. A radical from FMN in NADH dehydrogenase has thus far never been positively identified. The semiquinone e.p.r. signals from the respiratory chain account for the isotropic signal observed here and in previous studies [16–19] at *g* ~ 2.00. The slight broadening of this signal observed in ischaemic samples (see e.g. Fig. 5c) indicates that the spectrum comes mainly from the FAD radical (1.2 mT width) whereas the spectrum from normoxic heart is dominated by coenzyme Q_{10} (1.0 and 0.83 mT width). This conclusion is in agreement with earlier reports of Baker *et al.* [18].

(3) The doublet signal. As shown in Fig. 1, the isotropic signal at *g* ~ 2.00 is flanked by two sharp unidentified

Fig. 2. Low-temperature (13 K) e.p.r. spectra as a function of perfusion condition

Trace A, isolated mitochondria in air; trace B, heart subjected to 30 min of aerobic perfusion; trace C, heart subjected to 30 min of global ischaemia; trace D, heart subjected to 30 min of global ischaemia followed by 20 s of aerobic reperfusion. The six arrows and g values indicate the redox centres quantified in Table 1. E.p.r. conditions: microwave power, 3.2 mW; microwave frequency, 9.35 GHz.

signals (at $g \sim 2.01$ and at $g = 1.985$). We further label these signals 'centre C' after Zweier *et al.* [17] and Nakazawa *et al.* [19]. Zweier *et al.* ascribed centre C to an N_2 -centred free radical associated with reperfusion injury. We will discuss this centre below.

E.p.r. signals in ischaemic/anoxic and reperfused hearts at 13 K

E.p.r. detectable responses of heart tissue under normoxic, ischaemic and reperfusion conditions are presented in Fig. 2. For comparison, a spectrum of isolated resting mitochondria is also shown. This latter spectrum is dominated by a nearly isotropic signal of the oxidized centre S-3 from succinate dehydrogenase ($g = 2.015$). The respiratory chain in these mitochondria is almost completely oxidized, as only a trace of reduced Fe-S clusters is detectable around $g = 1.93$. Note that the line at $g = 1.985$ of the unidentified centre C [17,19] is also detectable in these mitochondria. The normoxic or control heart sample represents an intermediate redox situation in which oxidized S-3 is still easy to detect. In

this control spectrum, an enhanced reduction of Fe-S clusters from NADH dehydrogenase and succinate dehydrogenase can be seen at around $g = 1.93$. The six arrows and g -values indicate the Fe-S clusters and centre C as they were quantified in Table 1 (see below). The ischaemic heart sample is characterized by a further reduced respiratory chain which is especially evident by a decrease of the S-3 signal ($g = 2.015$). Reperfusion for 20 s appears to induce only minor changes in the reduction state.

As already stated, the line at $g = 1.985$ of the unidentified centre C is easily detectable. The relative quantification (% amplitude) of this signal, however, is difficult as it is always on the ascending slope of the stronger isotropic signal. Because of its possible importance, being previously reported to represent an N_2 -centred free radical associated with reperfusion injury [17], the amplitude of this signal was semi-quantitatively measured on a scale of four gradations from 0 to + + +. The gradation of + + + corresponds to a well-developed resonance as in traces B and C of Fig. 2. The weaker signals of traces A and D would be labeled + +. The symbol + is used when the signal is just barely detectable as an inflection on the ascending slope.

The results of these quantifications together with the amplitudes of the electron transfer centres in all 22 hearts at 13 K are presented in Table 1. The columns are arranged according to an increasing resonance field corresponding to the arrows in Fig. 2 from left to right. The amplitudes are proportional to the concentration of the centres as indicated on top of each column (see also Fig. 1). All amplitudes have been normalized by making the average amplitude at each g -value in the three control samples equal to 1.0. According to Table 1, centre C is present in normoxic, anoxic, ischaemic and reperfused hearts. For this reason we conclude, in contrast to Zweier *et al.* [17], that this signal is not associated with reperfusion injury.

From the Fe-S clusters listed in Table 1, only S-3 is paramagnetic in its oxidized state. All other centres are paramagnetic in their reduced state. The amplitudes in Table 1 at $g = 2.05$ (centre N-2), $g = 1.925$ (sum of amplitudes from centres N-2 and N-4) and $g = 1.88$ (centres N-3 and E) are remarkably invariant to changes in the perfusion condition. Apparently the steady-state reduction level of the NADH dehydrogenase and the ETF dehydrogenase are rather insensitive to the oxygenation history of the heart. Although the reduction level of centre N-1 in NADH dehydrogenase cannot be measured separately in these experiments, it is reasonable to assume that this centre behaves in concert with the other three centres (N-2, N-3 and N-4) in the enzyme. Therefore the changes at $g = 1.935$ (N-1/S-1) are mainly due to a change of centre S-1 from succinate dehydrogenase.

When the heart is made ischaemic or anoxic, the S-1 signal ($g = 1.935$) responds with an extensive increase in amplitude, i.e. reduction. This response is paralleled by a decrease, of comparable extent, in the signal at $g = 2.015$ from centre S-3 (i.e. also a reduction of this iron-sulphur cluster). Both centres appear to only slightly reoxidize over a period of 20 s of aerobic reperfusion. The extent of reduction appears to be dependent on the duration of ischaemia. This is especially evident in the signal from S-3, which is the strongest and, therefore, the most accurately measurable signal. This time dependency in

Table 1. Relative e.p.r. amplitudes at a temperature of 13 K for the redox centres in each of the 22 whole-heart preparations at the various experimental conditions

Amplitudes were normalized by putting the average amplitude of the control samples equal to 1.00. Values are given for iron-sulphur clusters in NADH dehydrogenase (N-1 to N-4), succinate dehydrogenase (S-1 to S-3) and in ETF dehydrogenase (E). C is 'centre C' (see text). The *g*-values at which the amplitude was measured are given in parentheses for each centre. The reperfusion time was 20 s.

Conditions	Centre					
	N-2 (2.05)	S-3 (2.015)	C (1.985)	N-1/S-1 (1.935)	N-2/N-4 (1.925)	N-3/E (1.88)
Control:						
15 min	0.89	1.03	+	1.39	0.87	0.90
30 min	1.19	0.97	+	0.96	1.28	0.99
45 min	0.92	1.00	+	0.65	0.85	1.12
Ischaemia:						
15 min	1.02	0.48	0	4.13	1.59	1.43
30 min	1.02	0.30	0	3.78	1.62	1.34
30 min	0.66	0.23	+	3.61	1.23	1.11
30 min	0.90	0.27	+	4.13	1.23	1.06
45 min	1.02	0.16	+	4.96	1.37	1.06
Anoxia:						
15 min	0.89	0.23	+	3.09	1.15	0.91
30 min	0.97	0.22	+	4.22	1.17	1.09
30 min	0.75	0.24	+	5.30	1.09	0.95
30 min	1.00	0.20	+	4.35	1.21	1.01
45 min	0.80	0.20	+	3.74	0.93	0.94
Ischaemia:						
15 min + reperfusion	0.93	0.55	+	1.78	1.10	0.80
30 min + reperfusion	0.93	0.39	+	1.52	0.98	0.70
30 min + reperfusion	0.95	0.27	+	3.83	1.20	0.99
30 min + reperfusion	1.02	0.42	+	2.74	1.29	1.18
45 min + reperfusion	1.01	0.20	+	3.62	1.44	1.18
Anoxia:						
15 min + reperfusion	0.97	0.30	+	2.65	0.93	0.93
30 min + reperfusion	0.96	0.24	+	2.09	1.08	0.85
30 min + reperfusion	1.09	0.20	+	4.26	1.14	1.06
45 min + reperfusion	1.01	0.16	+	3.35	0.79	0.60

ischaemic samples is reflected in its reoxidation response to reperfusion. The S-3 signals in the anoxic samples were found to be somewhat smaller compared with the ischaemic signals and not time-dependent. This difference might be related to the fact that rat hearts during anoxic perfusion are still beating, in contrast to ischaemic hearts [14]. This can imply that during anoxic perfusion a higher degree of reduction is reached.

In summary, the reduction level of iron-sulphur clusters in the enzyme succinate dehydrogenase is strongly correlated with the perfusion history of the heart. The amplitude of the signal from centre C is not correlated with the perfusion condition. It is clear that the signal intensity of centre C in reperfusion samples is not higher than those in control samples (Table 1).

Independence of the e.p.r. spectroscopy of sample preparation technique

The relevance of the original observations by Zweier *et al.* [17] was recently discredited by Baker *et al.* [18] and Nakazawa *et al.* [19] as due to an artifact of sample preparation. The latter two groups claimed that two out of the three e.p.r. signals were absent when the freeze-clamped heart was not pulverized in a mortar, but rather

cut into pieces with a spatula [18] or a chisel [19]. We have prepared several heart samples by grinding one half and chiselling the other half at 77 K. Between these pair samples we have not found significant differences in their e.p.r. spectra either at 13 K or at 77 K. In Fig. 3, an example is shown of a low-temperature e.p.r. spectrum from pair samples prepared from a reperfused heart after 30 min of ischaemia. Note especially that the signal amplitudes at $g = 2.03$ – 2.04 and at $g = 1.985$, previously assigned to artifacts of grinding [18,19], are not significantly different for the two samples.

E.p.r. spectroscopy at 77 K

Fig. 4 shows data of a warming-up experiment of normoxic samples measured at two microwave powers (0.5 and 13 mW). The experiment confirms previous observations [17,19] that the unidentified centre C (i.e. the two satellite lines flanking the central radical line at $g = 2.005$ in the control spectrum) is unstable towards warming-up for 1 min to -80°C ; also a peak at $g = 2.03$ is unstable towards 30 min of warming at -20°C . However, this experiment shows that both the isotropic radical at $g = 2.005$ and the centre C saturate much more readily than the other observable signals.

Fig. 3. Comparison of low-temperature e.p.r. data from samples prepared by different techniques

A heart was subjected to 30 min of global ischaemia followed by 20 s of aerobic reperfusion. Trace A was from the one half of the freeze-clamped heart cut with a chisel into 1 mm pieces; Trace B was from the other half of the heart which was ground to a fine powder in a mortar. E.p.r. conditions were as for Fig. 1.

In fact, at powers where the line at $g = 2.03$ is readily detectable (upper two spectra at high power), the isotropic radical and centre C are partially saturated (smaller compared with the low power situation). For this reason we conclude that difference spectra previously used to prove ODFR generation in post-ischaemic cardiac tissue are difficult to interpret [17,19]. For the same reason we criticize the previous conclusions that centre C is either a triplet spectrum with 2.5 mT splitting [17,19] or a doublet spectrum with 5 mT splitting ([18], see discussion).

It is also clear from Fig. 4, when comparing the low and high power spectra and the post-annealing spectra, that the peak at $g = 2.03$ is not linked to the strong line at $g = 2.005$ as claimed by Zweier *et al.* [17] and Nakazawa *et al.* [19]. It can be seen, however, from Fig. 4 that the peak at $g = 2.03$ goes together with the signals at $g = 1.94$ and $g = 1.91$. This conclusion destroys the basis for the previous assignment that the signal at $g = 2.03$ is part of an axial spectrum from an oxygen-centred radical [17,19]. We found that the signal at $g = 2.03$ is part of the spectrum from the iron-sulphur cluster S-1 in succinate dehydrogenase. This cluster is known to generate an e.p.r. signal with three g -values at 2.03, 1.94 and 1.91 [28]. In the high power spectra of Fig. 4 we also see a weak, very broad peak at $g \sim 2.17$ (0.3085 T) and a corresponding broad derivative-shaped feature around $g \sim 2.03$ characteristic for the Cu_A centre in cytochrome *c* oxidase [34,35].

E.p.r. spectroscopy and saturation characteristics at 115 K

All three previous studies [17–19] carried out at 77 K report an increase of the isotropic signal at $g = 2.005$ in

reperfused hearts compared with control hearts. We have confirmed these observations at 77 K (results not shown). In view of the above-noted problem of partial power saturation of this signal at 77 K, we have measured the samples under definitely non-saturating conditions (115 K and microwave power of 0.05 mW).

Fig. 5(a) shows that detection of the signals in control hearts is still possible at this 'high' temperature of 115 K using a microwave power of 80 mW. The signal from centre S-1 can be readily detected ($g = 2.03$, 1.94 and 1.91) in these control hearts. The isotropic signal at $g = 2.005$ and the signal from centre C are extensively reduced in amplitude by microwave power saturation. In Fig. 5(b) the peak-to-peak amplitude of the isotropic signal is plotted as a function of the microwave power. It is clear from this graph that even at the elevated temperature of 115 K only power levels below 0.2 mW will not induce saturation. Fig. 5(c) shows e.p.r. spectra at the definitely non-saturation condition of 115 K and 0.05 mW. Evidently, the previously found apparent increase of the isotropic radical signal [17–19] in reperfused hearts at 77 K was not encountered under the present conditions. We therefore conclude that the earlier published reperfusion-dependent increase of the isotropic signal [17–19] is a result of microwave power saturation.

More data concerning the various perfusion conditions measured with non-saturating power are shown in Table 2. The amplitudes of the isotropic radical signal at $g = 2.005$ in 10 hearts were again normalized with respect to control levels. Table 2 clearly shows that the signal intensity is halved upon deprivation of oxygen. Reperfusion induces only a small recovery of the signal. The line width in the control sample is approx. 0.9 mT and increases maximally to 0.2–0.3 mT upon ischaemia/anoxia or reperfusion (Fig. 5). Even corrected for this broadening, the intensities (i.e. concentrations) never significantly exceeded those of the normoxic samples, which contrasts with the findings published in [17–19].

DISCUSSION

The proposal that ODFR are involved in (post)-ischaemic myocardial injury [38], combined with the notion that e.p.r. spectroscopy can be used to detect these radicals, has in recent years encouraged several groups to use e.p.r. spectroscopy in attempts to gain insight into the molecular mechanisms underlying ischaemic injury. Zweier *et al.* [17] were the first to claim the direct measurement of three different radical species, among which was an oxygen-centred one, generated upon reperfusion. Subsequently, Baker *et al.* [18] and Nakazawa *et al.* [19] claimed that two out of the three signals reported by Zweier *et al.* [17], including the one attributed to an oxygen-centred radical, are artifacts due to sample preparation. Baker *et al.* [18], however, repeated the claim that reperfused myocardium contained an increased amount of radicals. In a recent letter by Baker & Kalyanaramun, this increase in radical concentration is no longer mentioned [39]. In the same paper an e.p.r. signal in ischaemic hearts is ascribed to reduction of an iron-sulphur centre in a mitochondrial dehydrogenase, and this leads the authors to conjecture that the respiratory chain of intact mitochondria might contribute to 'oxy-radical production during reperfusion'.

In the present study we have repeated the experiments of Zweier *et al.* [17], Baker *et al.* [18] and Nakazawa *et al.*

Fig. 4. Warming-up experiment to abolish stepwise e.p.r. signals in perfused heart

The e.p.r. was monitored at a temperature of 77 K and at two different microwave power levels. The heart was subjected to 30 min of normoxic perfusion, freeze-clamped and pulverized before the signals were measured (traces A). After this the sample was warmed up to -80°C for 1 min, and re-cooled to 77 K (trace B). Finally the sample was warmed up to -20°C for 30 min and re-cooled to 77 K (trace C). E.p.r. conditions: all traces are an average of four scans; microwave frequency, 9.37 GHz; microwave power, 0.5 mW (a) or 13 mW (b)

Fig. 5. E.p.r. spectroscopy of whole-heart samples at a temperature of 115 K

(a) High-power (80 mW) spectrum showing, in addition to the radical line at $g = 2.005$, the complete spectrum (i.e. $g = 2.03$, 1.94 and 1.91) of iron-sulphur cluster S-1 in a control heart (30 min of normoxic perfusion). The spectrum is an average of four scans corrected for an average of four baseline scans. (b) Power plot of the radical line at $g = 2.005$ (see text). (c) Low-power (0.05 mW) spectra of the radical line at $g = 2.005$ in a control heart (trace A; 30 min of normoxic perfusion), a heart subjected to 30 min of global ischaemia (trace B), and a heart subjected to 20 s of aerobic reperfusion after 30 min of ischaemia (trace C). For all spectra, the microwave frequency was 9.50 GHz.

Table 2. Peak-to-peak amplitude of the isotropic radical signal at $g = 2.005$ as a function of perfusion conditions in 10 hearts

The temperature was 115 K and the microwave power was 0.05 mW. Amplitudes were normalized by putting the average amplitude of the control samples equal to 1.00.

Condition	Amplitude
Normoxia (30 min)	0.89
Normoxia (30 min)	1.11
Ischaemia (30 min)	0.46
Ischaemia (30 min)	0.43
Anoxia (30 min)	0.49
Anoxia (30 min)	0.56
Ischaemia (30 min)+reperfusion (20 s)	0.35
Ischaemia (30 min)+reperfusion (20 s)	0.54
Anoxia (30 min)+reperfusion (20 s)	0.56
Anoxia (30 min)+reperfusion (20 s)	0.75

[19] at 77 K. We have extended our measurements to lower (13 K) and higher (115 K) temperatures, we have studied a wide magnetic-field scan range and have varied the microwave power extensively. All the signals we measure, however, in normoxic, ischaemic, anoxic and reperfused hearts can be ascribed to components intrinsic to the mitochondrial respiratory chain and not to potentially dangerous radicals.

Zweier *et al.* [17] reported two signals to be related to aerobic reperfusion. First, they observed an axial signal (i.e. two g -values, at $g = 2.033$ and $g = 2.005$) which the authors assigned to superoxide or an alkyl peroxide radical. They claim that resistance to microwave power saturation and instability above 100 K are properties of these oxygen-centred radicals. However, here they misread the literature [40,41], since Copeland [40] reports that alkyl peroxide radicals can withstand annealing for 10 min at 193 K and no power saturation data whatsoever were reported in this study. In addition, Knowles *et al.* [41] report that superoxide is stable at 123 K and that it can be recognized by its ease of saturation. On the basis of our multi-temperature and multi-microwave power study we can now reassign this 'oxygen-radical' axial signal ($g = 2.033$ and $g = 2.005$, [17]) to originate partly from centre S-1 from succinate dehydrogenase (three g -values at $g = 2.03$, $g = 1.94$ and $g = 1.91$, [28]) and partly from the saturated isotropic signal at $g = 2.005$ at 77 K.

The second signal reported by Zweier *et al.* [17], 'centre C', was interpreted as an isotropic triplet from an N_2 -centred free radical of unknown identity. Baker *et al.* [18] re-read the signal as an isotropic doublet from a carbon-centred radical, possibly a lipid radical. In the present study we have made several observations that may help in identifying this elusive centre. The middle part of the signal from centre C cannot be defined because it is overshadowed by the isotropic signal from semiquinone and the strong signal of S-3 at low temperatures. The two outermost features of the signal ($g \sim 2.01$ and $g = 1.985$) have a positive absorption shape and a negative absorption shape respectively, which is characteristic for a transition metal. The amplitude of centre C in our hands definitely does not peak in reperfused heart samples, but was shown to be present under

a variety of conditions. This means that the signal is not related to reperfusion injury. The apparent g -values together with the observed slow relaxation is reminiscent of molybdenum [42,43]. Since the high field line of this centre, at $g = 1.985$, was also encountered in this study in mitochondria, we surveyed the literature for more data. We found that a similar signal was detected as early as 1971 in membrane fragments from mitochondria [23]. This group of workers later suggested that this signal originates from the QH_2 :ferrocytochrome c oxidoreductase complex [44]; however, it is also observable in published spectra from the NADH:Q oxidoreductase complex [24]. A similar 'triplet-pattern' has recently been observed in the bacterium *Paracoccus denitrificans* [45] which has a respiratory chain that is quite similar to the mammalian respiratory chain.

In summary, centre C appears to be an intrinsic component of the mitochondrial respiratory chain. Its chemical identity remains at present obscure. It may be the transition metal molybdenum or a radical or a biradical related to coenzyme Q_{10} . With respect to the latter possibility we note, however, that the signal of centre C differs in its effective g -values and its temperature-dependence from a signal in beef heart mitochondria that was ascribed to a Q-Q interaction signal [46].

Finally, e.p.r. spectroscopy in cardiac tissue detects a variety of compounds from the mitochondrial respiratory chain, which respond to the varying perfusion conditions (ischaemia, anoxia, reperfusion). Free radicals linked to reperfusion injury were not detected. All of the signals previously assigned to post-ischaemic reperfusion injury can be explained to originate from electron-transfer centres in the mitochondrial respiratory chain and/or from signal saturation. This conclusion does not necessarily imply that ODFR are not formed during post-ischaemic reperfusion in cardiac tissue. There is still much indirect evidence indicating free radical involvement [1–15]. Moreover, ODFR have recently been detected with e.p.r. spectroscopy in perfusates of reperfused rat hearts as adducts of the spin-traps DMPO (5,5'-dimethyl-1-pyrroline- N -oxide [16,47]) or PBN (N -tert-butyl- α -phenylnitrone [48]). Recently we have confirmed these observations using DMPO (A. M. M. van der Kraaij, J. F. Koster & W. R. Hagen, unpublished work). Since we show in this study that the constitutive background of paramagnetism from the respiratory chain seriously hampers the detection of relatively low concentrations of free radicals in cardiac tissue, it is expedient to focus future experiments in this field on the use of spin-trapping agents.

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