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Detection of Hydroxyl and Carbon-Centred Radicals by EPR Spectroscopy after Ischaemia and Reperfusion of the Rat Kidney

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Recent studies suggest that oxygen-derived free radicals are involved in mediating renal reperfusion injury. EPR spectroscopy and spin trapping with the spin traps DMPO and PBN, were used to detect and quantitate the formation of hydroxyl radicals in rat kidney after ischaemia-reperfusion *in vivo* and *in vitro* in the isolated rat kidney perfused in the absence of leucocytes. EPR analysis of homogenised kidneys and of venous samples did not detect radical adducts with either spin trap. With PBN, radical adducts were not detected *in vitro*. When DMPO was used as the spin trap in kidneys perfused without albumin in the perfusate, EPR signals characteristic of hydroxyl and carbon-centred radical adducts were detected during early reperfusion following ischaemia. These studies confirm the generation of hydroxyl radicals during ischaemia-reperfusion in kidney. During reperfusion the total DMPO adduct concentration reached 4.35 ± 1.05 nmol/g kidney/3 min, $p < 0.05$. In control kidneys total adduct were present at lower concentration (2.55 ± 1.1 nmol/g kidney/3 min). Addition of 15 mM dimethylthiourea abolished formation of these adducts following ischaemia-reperfusion but did not prevent a reduction in glomerular filtration rate. These results indicate that significant levels of hydroxyl and carbon-centred radicals are formed in the absence of circulating neutrophils during early renal reperfusion following ischaemia.

Key words: hydroxyl radicals, ischaemia-reperfusion, electron paramagnetic resonance spectroscopy, 5,5-dimethyl-l-pyrroline N-oxide (DMPO), α-phenyl-N-*tert*-butyl nitrone (PBN), kidney, dimethylthiourea (DMTU)

INTRODUCTION

Oxygen-derived free radicals (OFR) are reported to play an important role in oxidative reperfusion injury in many tissues.[1-3] Hydroxyl radicals (•OH) are thought to be the most destructive of the radicals, which attack cellular components and cause damage.[4,5] Electron paramagnetic resonance (EPR) spectroscopy in conjunction with spin trapping has been widely used to identify these radicals.[6,7] This technique consists of using a

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spin trap (nitrone) to react with the initial highly reactive free radical to produce a more stable free radical (nitroxide adduct), which can then be observed by EPR spectroscopy.

Many studies in recent years have observed bursts of OFR formation during the initial minutes of post-ischaemic myocardial reperfusion.[8,9] In this regard spin trapping has been used as a valuable tool for the study of free radical processes in the heart.[10,11] In contrast, few studies have been reported on the use of this technique in kidneys and these have had controversial results. Using PBN (α-phenyl-N-*tert*-butyl nitrone) as a spin trap, Lai *et al.* observed that the amount of radicals produced during γ irradiation was greater in mice brain than heart, liver and spleen and no significant radical formation was observed from renal tissue extracts.[12] In 1990, Pincemail *et al.* had to pool the lipid residues of several rabbit renal venous plasma samples collected over 6 min to detect the paramagnetic species of PBN spin trapped radicals following ischaemia-reperfusion.[13] Using the spin trap 2-ethyl-2,5,5-trimethyloxazolidin-l-yloxy (OXANOH) in *in vivo* models of ischaemic-reperfused rabbit kidneys, Haraldsson *et al.* reported a 2-fold increase in the production of undefined OFR in comparison to pre-ischaemic values (3 μmol/min/10 g kidney).[14,15] However the type and amount of detected radicals differ among organs and different protocols. By using salicylate as a chemical trap we recently detected an increase in •OH formation as the product 2,5-dihydroxybenzoic acid (DHBA) in the isolated rat kidney perfused without leucocytes.[16] Since this product can also arise by renal salicylate metabolism, we repeated the study using DMPO (5,5-dimethyl-l-pyrroline, N-oxide) and PBN in order to identify whether ischaemia-reperfusion could produce a burst of ·OH radical formation in kidney perfused without leucocytes.

MATERIALS AND METHODS

All chemicals were obtained at the highest purity obtainable. PBN and DMSO were obtained from Sigma Chemical Co. (St. Louis, USA). DMTU and TEMPO were obtained from Aldrich Chemical Co. (Milwaukee, USA). DMPO (97% pure) was obtained from Sigma Chemical Co. and Aldrich Chemical Co. and further purified by double distillation under vacuum (dinitrogen atmosphere) and stored at −20°C in amber vials to prevent light-induced degradation. An EPR spectrum of purified DMPO (as is) was measured prior to performing the experiments. DMPO was repurified by the procedure described above when signals of DMPO radical adducts were observed.

Preparation of isolated perfused rat kidney (IPRK)

Male Wistar rats, weighing 250–350 g, with free access to standard rat food and water were used in all experiments. The rats were anaesthetized with 60 mg/kg intraperitoneal sodium pentobarbital and anticoagulated with 500 IU/kg intravenous sodium heparin. A midline abdominal incision was made and the superior mesentric artery and right renal artery were exposed. The right renal artery was cannulated through the superior mesenteric artery and perfusion pressure was continuously monitored within the renal artery using a Statham-type pressure gauge (model P23, Gould, UK) through a polyethylene line contained within the cannula perfusing the artery.[16,17] The perfusion medium consisted of 5 mM D-glucose and Krebs-Henseleit buffer [KHB; containing (in mM) 118 NaCl, 4.7 KCl, 24.6 $NaHCO_3$, 2.4 $MgSO_4$, 1.67 $CaCl_2$, dissolved in deionized water]. Albumin was not included due to its interference with formed spin adducts. The duration of experiments was limited to 46 min to avoid kidney swelling and minimise changes in renal function. The initial volume of medium in the system was 200 ml and the solution was gassed with 95% O_2/5% CO_2, bringing the pH to 7.4. Oxygen tension was measured in line by an oxygen electrode attached to a blood gas analyzer (PHM72 Radiometer, Copenhagen, Denmark). The mean arterial pressure was monitored and

held at 100 ± 20 mmHg using a process controller (model 2073, West Division, Gulton Industries Inc., Schiller Park, USA) that controlled the speed of the peristaltic pump (Watson and Marlow model 501U; Smith and Nephew, Watson-Marlow, UK). Flow rate was measured by wide beam ultrasound using a Transonic T206 flow meter with an in line 'cannulating' flow probe (SN22; Transonic Systems Inc., Ithaca, USA). Analog outputs from pressure, flow and oxygen meters were connected to separate channels of a chart recorder (Gould RS 3400, Gould Inc., Cleveland, USA). The right ureter was cannulated by a 2 cm length of 0.61 mm OD polyethylene tubing (ID 0.28 mm, Dural Plastics & Engineering, Auburn, Australia) connected to a 10 cm length of 0.96 mm polyethylene tubing (ID 0.58 mm). After 20 min equilibration, urine samples were obtained over 3 min intervals. Perfusate samples were taken at the mid-point of each urine collection period. The urinary clearances of ^{14}C-inulin, sodium and potassium were calculated from their respective urine and perfusate concentration ratios. Sodium and potassium were measured by flame photometry (FLM3, Radiometer, Copenhagen, Denmark). The radioactivity of ^{14}C-inulin was counted in an LKB 1217 Rackbeta scintillation counter. Only if the initial inulin clearance was greater than 0.5 ml/min/g kidney weight (calculated using the weight of the unperfused left kidney) were kidneys accepted for inclusion. Renal function was monitored throughout in all groups from ^{14}C-inulin clearance (equivalent to glomerular filtration rate, GFR) and fractional sodium excretion (FE_{Na}). Ischaemia was induced by clamping off one arm of a Y piece which stopped perfusion but allowed the perfusate to flow over the kidney maintaining temperature and hydration.

Experimental Protocol

Neither *in vivo* nor IPRK studies using PBN yielded interpretable EPR spectra of OFR and preliminary experiments with DMPO in the *in vivo* kidneys or in the albumin perfused IPRK were unsuccessful (see Results). Therefore the DMPO studies were only performed in IPRK in two groups as follows:

The kidneys were perfused for a total of 46 min. After an initial equilibration period of 20 min, renal function was assessed in each group (between 20–23 min). In 5 kidneys (Control) DMPO was infused to a final concentration of 5 mM and all effluent collected over 3 min. All kidneys were then made ischaemic for 20 min (between 23–43 min). At reperfusion DMPO was infused over 3 min (43–46 min), venous effluent was collected for analysis of both renal function and EPR spectroscopy. For assessment of renal function, the initial 20–23 min perfusion interval also served as the Control period for each group.

(a) Ischaemia-Reperfusion: After normoxic perfusion for 23 min, ischaemia was induced for 20 min. Following ischaemia and coinciding with normoxic reperfusion, DMPO was infused into the kidney and perfusate collected for 3 min.

(b) DMTU-Ischaemia-Reperfusion: 15 mM DMTU (dimethylthiourea) was added to the perfusate at 23 min immediately before the induction of 20 min ischaemia. Following ischaemia and coinciding with normoxic reperfusion, DMPO was infused into the kidney and perfusate collected for 3 min.

EPR Studies

DMPO experiments

DMPO (50 mg) was dissolved in 1 ml of KHB immediately before use and covered to prevent light-induced degradation.[18] The spin trapping studies were performed by infusion of DMPO through a side arm located just proximal to the renal arterial cannula, so that DMPO was infused directly into the kidney. The effluent was collected from the kidney during control perfusion (between 20–23 min) before induction of ischaemia and again at the beginning of the reperfusion

(between 43–46 min). The collected effluents were immediately frozen in liquid nitrogen to prevent spin adduct decay and further freeze-dried to concentrate the samples. Since the quantity of •OH radical formation was low, we were unable to establish the time course for DMPO-radical adduct production. The freeze-dried samples were dissolved in deionized water immediately before EPR measurements.

Hydroxyl radicals were generated chemically in a Fenton type reaction [$FeCl_3$ (2 mM), adenosine diphosphate (ADP, 10 mM), $FeSO_4$ (2 mM) and H_2O_2][19] and after reaction with DMPO detected as the DMPO-OH adduct.[20–22] 50 µl dimethylsulfoxide (DMSO) or 50 µl ethanol were added to this system to produce typical DMPO-alkyl adducts.[23–25]

PBN experiments

For IPRK experiments, PBN was dissolved in a mixture of 80% normal saline and 20% water and then added to the circuit to a final concentration of 3 mM, 5 min before induction of 20 min ischaemia. Additional 15 mM PBN (2.65 mg/1 ml) was infused directly into the kidney through a side arm located just proximal to the renal arterial cannula at the beginning of reperfusion and in non-ischaemic time matched controls. After 3–5 min, kidneys were either minced or freeze-clamped and then homogenized, extracted with toluene,[26] centrifuged and the toluene layer evaporated to dryness by a rotary evaporator or dried in a stream of dinitrogen gas. The venous effluent from control and ischaemic kidneys was also collected, an aliquot taken and frozen for direct EPR measurements and the rest immediately extracted in toluene, centrifuged and the toluene layer evaporated to dryness.

In *in vivo* studies, 3–15 mM PBN was injected through a cannula in the renal artery after induction of ischaemia for 20 min and at the time of reperfusion. The venous blood was collected, serum separated by centrifugation and analysed by EPR directly or after solvent extraction into toluene. A secondary radical trapping technique[27] was also used by dissolving 120 mg PBN in 0.5 ml of DMSO. After induction of anaesthesia, 0.5 ml of the PBN/DMSO solution was injected intraperitoneally. Ten to 15 min later ischaemia was induced for 20 min by placement of an atraumatic clamp on the right renal artery. At the beginning of reperfusion, blood was collected from the renal vein in a heparinized tube. Plasma was separated by centrifugation and transferred to an amber vial and frozen or extracted with toluene and dried as described above.

EPR spectroscopy

EPR spectra of the DMPO and PBN radical adducts were measured on a Bruker ESP300E EPR spectrometer operating at X-band microwave frequencies. An aqueous flat cell (Wilmad WG-812-Q) was employed in conjunction with a rectangular TE_{102} cavity to minimize the dielectric loss. Spectra were recorded at room temperature under aerobic conditions and a field modulation of 100 kHz frequency was employed. The microwave frequency and magnetic field were calibrated with an EIP 548B microwave frequency counter and a Bruker ER035M gaussmeter which enabled an accurate determination of the isotropic g- and A- values.

Computer simulation was performed using the FORTRAN computer programme epr50fit.f[28] running on a SUN SPARCstation 10/30. The quality of the simulations was determined using the least squares error (LSE) parameter defined by Martinelli *et al.*[29] Quantitation of radical adduct formation was performed using the comparison method in conjunction with a dual TE_{104} rectangular cavity in which the reference sample was 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO). An important point which should be noted when using a dual cavity is that the individual cavities may have different modulation amplitude calibrations and microwave magnetic field strengths. The former was simply solved by calibrating each cavity, while the latter was

overcome by first measuring the standard and reference samples in the front and back cavities and then swapping the samples and repeating the measurement. The concentration of the radical adducts was calculated after Pilbrow and Hanson.[30]

STATISTICAL ANALYSIS

Values are reported as mean ± S.D. throughout the study. t-test comparisons with Bonferroni corrections were used to identify significant differences. The significant level was set at 95%.

RESULTS

Chemical System

Addition of DMPO to an •OH chemical generator system resulted in formation of a 1:2:2:1 quartet EPR spectrum attributable to a DMPO-OH adduct (g, 2.0056; A_N, 14.15×10^{-4} cm^{-1}; A_H, 14.38×10^{-4} cm^{-1}; Figure 1a). Addition of 50 µl of DMSO or 50 µl ethanol to this system reduced the intensity of DMPO-OH and produced a spectrum consisting of six equally intense resonances (g, 2.0056; A_N, 14.99×10^{-4} cm^{-1}; A_H, 22.22×10^{-4} cm^{-1}) which is typical of DMPO-alkyl adducts[7,31] (Figure 1b, resonance labelled '*'). When these experiments were performed in the presence of 6.7% albumin, the EPR spectra from DMPO-OH and DMPO-alkyl adducts were no longer observed. Presumably this was due to the scavenging properties of albumin[32] or the reduction of the radicals to diamagnetic compounds.

The stability of the DMPO adducts was evaluated by incubating DMPO and a constant volume of the •OH chemical generator system in the presence of renal tissues. Incubation with 10 mg of homogenised kidney reduced the intensity of the spectrum (Figure 1c) and incubation with 100 mg eliminated the EPR signals from the DMPO adducts (Figure 1d), presumably because of the presence of endogenous antioxidants[33] or the binding of •OH radicals to proteins and other macromolecules.

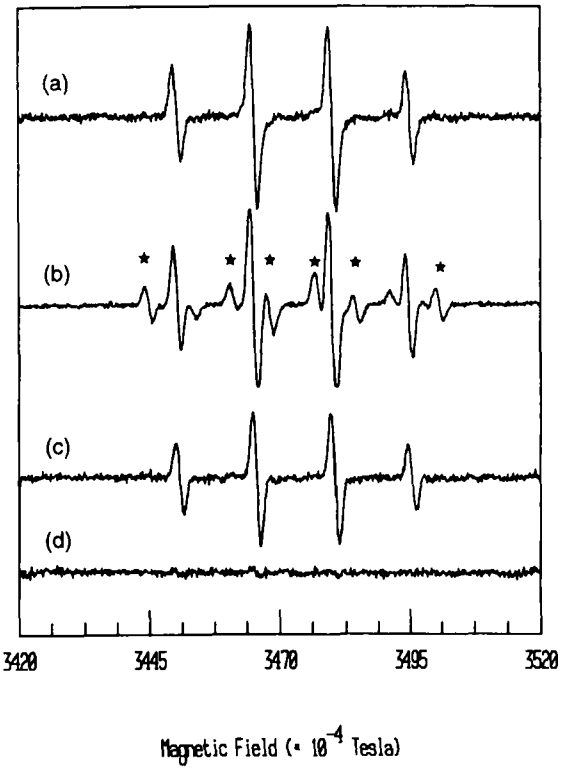

FIGURE 1 EPR spectra of the DMPO-OH radical adduct, v = 9.7845 GHz. (a) DMPO plus $FeCl_3$ (2 mM), ADP (10 mM), $FeSO_4$ (2 mM) and H_2O_2, (b) as for (a) with the addition of DMSO (50 µl), (c) as for (a) with the addition of homogenized renal tissue (10 mg), (d) as for (a) with the addition of homogenized renal tissue (100 mg).

DMPO Experiments

Preliminary EPR experiments involving the detection of •OH in renal tissue, or venous effluent were performed in the *in vivo* kidney and in the IPRK with albumin-containing perfusate. These did not detect any DMPO-OH or DMPO-alkyl adducts. In light of the results obtained from the OH chemical generating system, the remaining experiments were conducted in IPRK using albumin-free medium. DMPO was not recirculated and perfusate samples were immediately freeze-dried and then redissolved in water for EPR measurement. An EPR spectrum of the renal

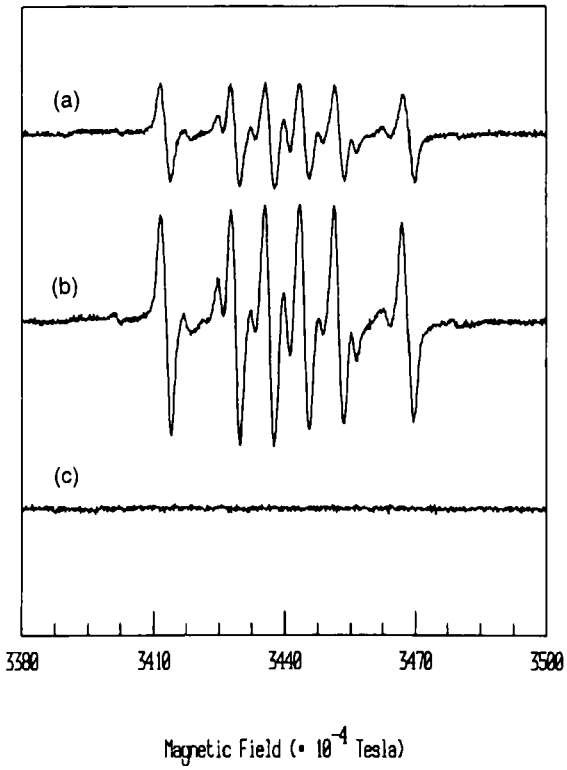

FIGURE 2 Typical EPR spectra of the radical adducts from (a) a control kidney, $v = 9.6578$ GHz, (b) an ischaemic kidney, $v = 9.6578$ GHz, (c) an ischaemic kidney plus 15 mM DMTU, $v = 9.7256$ GHz.

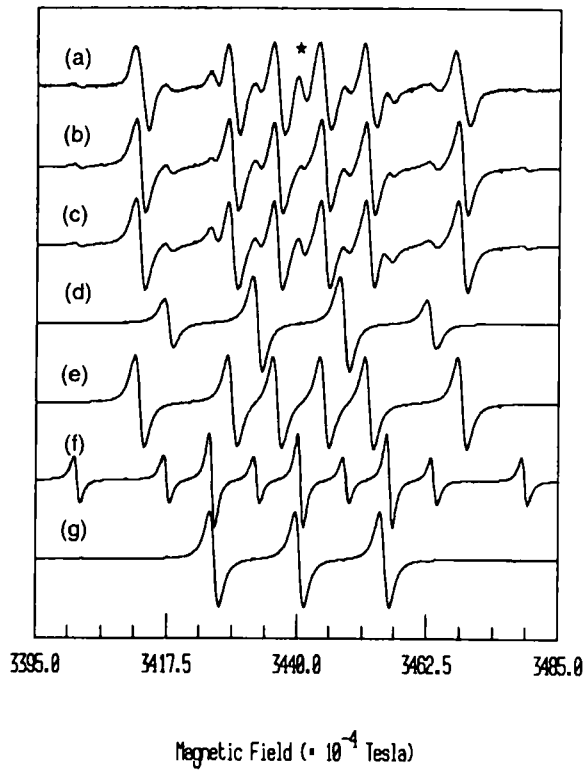

FIGURE 3 EPR spectra of the DMPO radical adducts in the ischaemic kidney, $v = 9.6578$ GHz. (a) experimental spectrum, (b) computer simulation of (a) obtained by adding spectra ($d \times 0.12 + e \times 0.81 + f \times 0.07$), LSE = 1.043×10^{-2}, (c) computer simulation of (a) obtained by adding spectra ($d \times 0.10 + e \times 0.70 + f \times 0.14 + g \times 0.06$), LSE = 9.143×10^{-3} (d) computer simulation of DMPO-OH radical adduct, (e) computer simulation of DMPO-R radical adduct, (f) computer simulation of DMPO-H radical adduct and (g) computer simulation of DMPO-oxidized nitroxide radical adduct. Spin Hamiltonian parameters for these radicals are given in Table 1.

perfusate sample from the Ischaemia-Reperfusion group (Figure 2b and reproduced in Figure 3a) clearly contains multiple DMPO-radical adducts. Results from computer simulation studies are shown in Table 1 and Figure 3. The computer simulated spectrum shown in Figure 3b was obtained by adding the spectra from the radical adducts; DMPO-OH, DMPO-R and DMPO-H in the ratio of 0.12: 0.81: 0.07 respectively. A comparison of the simulated (Figure 3b) and experimental (Figure 3a) spectra yields an LSE value of 1.043×10^{-2}. Although the simulated spectrum in Figure 3b reproduces the experimental resonant field positions accurately, the intensity of the resonance at $g = 2.0056$ (labelled '*' in Figure 3a) is not the same. Inclusion of the spectrum from oxidized DMPO yields the spectrum shown in Figure 3c, where the ratio of the various adducts is as follows: DMPO-OH: DMPO-R: DMPO-H: oxidized DMPO; 0.10: 0.70: 0.14: 0.06. The relative intensities of the four radical adducts were obtained by comparison of the intensity of these resonances (341.8 mT for DMPO-OH, 341.2 mT for DMPO-R, 340.2 mT for DMPO-H and 344.0 mT for oxidized DMPO) in the experimental spectrum with those in the simulated spectrum. Inclusion of all four radical adducts yielded a slightly better fit, LSE = 9.143×10^{-3}. While the EPR spectrum from ischaemic-reperfused rat kidneys (Figure 3a) and rabbit heart[7] contain the same oxidized-DMPO nitroxide radical adduct, albeit in different

TABLE 1 EPR parameters for DMPO-radical adducts.

DMPO-adduct	g	A_N[a]	A_H[a]	Intensity ratios	Figure no.
DMPO-OH	2.0056	14.15	14.38	1:2:2:1	3d
DMPO-R	2.0056	14.99	22.22	1:1:1:1:1:1	3e
DMPO-H	2.0056	14.15	14.38[b]	1:1:2:1:2:1:2:1:1	3f
Oxidized-DMPO	2.0056	14.15	14.38	1:1:1	3g

[a]Units for hyperfine coupling constants are 10^{-4} cm^{-1}.
[b]The two protons are magnetically equivalent.

proportions, the spectra generated chemically (Figure 1) do not show the presence of the DMPO-H and oxidized DMPO. DMPO-R radicals can be formed from the reaction of •OH radicals with any macromolecule or aromatic ring which can be secondarily trapped by DMPO to yield DMPO-R radicals.[7,34,52] Similarly under our experimental conditions the •OH radicals formed in the kidney can potentially react with biological molecules to form secondary alkyl (•R) or alkoxyl (•OR) radicals, which can be subsequently trapped by DMPO. Formation of these adducts indicates generation of •OH radicals during reperfusion to a total DMPO adduct concentration of 4.35 ± 1.05 nmol/g kidney/3 min, n = 6, Figure 4. In control kidneys, the adducts were present at a lower concentration (2.55 ± 1.1 nmol/g kidney/3 min, n = 5, $p < 0.05$, Figure 4).

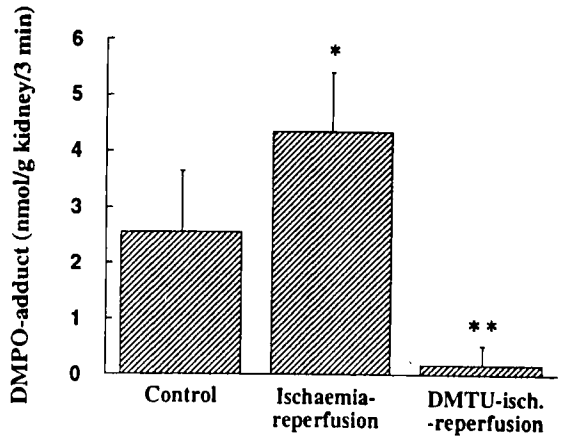

FIGURE 4 Modulation of renal DMPO-adduct concentration (mean ± S.D.) by ischaemia-reperfusion. Basal DMPO-adduct formation was 2.55 ± 1.1 nmol/g kidney/3 min between 20–23 min of perfusion (Control). Following ischaemia-reperfusion there was a 70% increase in the DMPO-adduct concentration (* $p < 0.05$). The addition of 15 mM DMTU to the perfusate inhibited both basal DMPO-adduct production (** $p < 0.01$) and the increase after ischaemia-reperfusion ($p < 0.001$).

Addition of 15 mM DMTU abolished formation of both DMPO adducts (Figure 2c & Figure 4). These results indicate that significant •OH radicals formed in the absence of circulating neutrophils during early renal reperfusion following ischaemia. The data support previous studies where •OH radicals were detected by HPLC *in vitro*[16] and where non-specific OFR were detected by EPR spectroscopy and the spin trap OXANOH in an *in vivo* model of ischaemia-reperfusion.[14]

Renal physiological function

Renal function was assessed from the inulin clearance and the fractional sodium excretion (FE_{Na}). Initial renal function was not significantly different in either group and the data was averaged for both groups as shown (Figure 5, Control). Reperfusion following 20 min ischaemia markedly reduced renal function, as shown by the decrease in GFR ($p < 0.005$) and by the increase in FE_{Na} ($p < 0.005$) compared with the Control group (Figure 5). Functional parameters in the DMTU-Ischaemia-Reperfusion group were not significantly different from the Ischaemia-Reperfusion group.

PBN experiments

Initial EPR studies of direct infusion of different concentrations of PBN into the *in vivo* post-ischaemic rat kidney and into IPRK (both with and without albumin in the perfusate medium) did not detect radical adducts in either homogenised

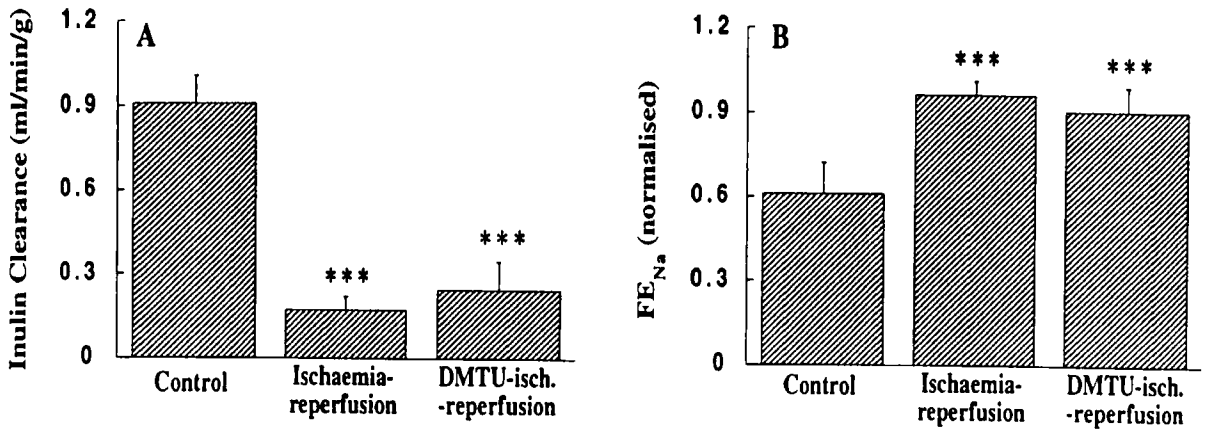

FIGURE 5 Renal function before and after ischaemia. (A) Inulin clearance (GFR); (B) Fractional excretion of sodium (FE_{Na}) in perfused rat kidney. The initial inulin clearance (ml/min/g) for both groups has been averaged and is shown as Control. Following ischaemia-reperfusion, inulin clearance decreased both in the absence and presence of DMTU. Values represent the mean ± S.D. for each group. Significance level: *** $p < 0.005$.

renal tissue or venous plasma by direct analysis of aqueous samples or toluene extracts. One possible explanation is that superoxide can reduce PBN nitroxide to diamagnetic species *in vivo*.[35,36]

Therefore in a second approach *in vivo*, we used the secondary radical trapping technique described by Burkitt *et al.*[37] With this technique, •OH radicals react with DMSO to form methyl radicals, which are subsequently detected by use of PBN as a spin trap.[38] In PBN spin trapping studies, different reports demonstrated PBN adducts with different spectral characteristics. A broad three line spectrum (g, 2.0063; A_N, 14.2×10^{-4} cm^{-1}) was observed in all our studies but was difficult to assign. Because of these uncertainties no further experiments with PBN were conducted.

DISCUSSION

OFR have been implicated as possible mediators of reperfusion injury following ischaemia in a variety of tissues including kidney.[1-4] However direct identification of OFR in biological systems is difficult, because of their low concentration and the rapidity with which these radicals react with cellular components. For example •OH radicals which are the most reactive species known, react with themselves or attack whatever they are close to.[39] Consequently, they never reach a concentration high enough to be detected directly.[40] This has left uncertain the exact role of these radicals in renal reperfusion injury. To overcome these problems, in recent years PBN and DMPO have been widely used to stabilize these short-lived radicals and make them visible by use of EPR spectroscopy.[41] However in our preliminary studies, no radical adducts were detected using PBN by direct EPR analysis of renal tissue or renal venous plasma, when PBN was used *in vivo* or *in vitro*. Since superoxide has been reported to reduce PBN nitroxide to a diamagnetic species, we tried to implement the secondary radical trapping technique of Burkitt *et al.*[37] which uses PBN to detect methyl radicals formed by interaction of DMSO with •OH radicals. We obtained a broad three line spectrum which was difficult to interpret. Janzen has suggested that nitroxides with unresolved hyperfine coupling constants may be formed as a result of abstraction of a β-hydrogen of a radical adduct by a nontrapped radical.[42] A similar signal has been reported for a PBN-dependent adduct during ischaemia-reperfusion in the gerbil brain[43] and has been attributed to a nitroxyl adduct resulting from the spin trapping of oxygen and

carbon-centred radicals by PBN. This spectrum may be the result of the complex PBN chemistry[27,37] or oxidative degradation of PBN in the oxygenated buffer[44] which because of its large line width can potentially hide other radical adducts. Since the DMPO studies were able to detect •OH radicals in IPRK perfused without albumin, we did not pursue the use of PBN further.

Amongst the various available spin traps, DMPO is widely used for trapping •OH radicals in many biological systems because of its low toxicity,[26] large reaction rate constant with •OH[40,45] and its ability to penetrate cell membranes[26] and react with radicals produced intracellularly.[26,45] EPR studies with DMPO as the spin trap in isolated heart and other organs have demonstrated the formation of OFR.[7,45] In our preliminary experiments, we were not able to demonstrate radical formation when 6.7% albumin was present in the perfusion medium. Subsequent experiments were conducted by using an albumin-free perfusate. This may be explained by the scavenging properties of albumin,[32] the low concentration of radicals produced, or by the detection limits of EPR spectroscopy. Thus radical species may be undetected even if formed. To distinguish between failure of production of hydroxyl radicals in albumin perfused ischaemic kidneys and immediate conversion of radicals to EPR silent spectra, we added albumin to the hydroxyl radical chemical generating system. We found that in the presence of albumin there were no spin adducts formed suggesting that any formed radical adducts were immediately converted to an EPR silent species.[46] However we were successful in detecting the formation of •OH and carbon-centred radicals when albumin was absent from the perfusion medium (Figure 2).

Not only are the free radicals themselves short-lived and difficult to detect, their spin adducts are also subject to metabolism and degradation. The rate constant for the reaction of •OH with DMPO is high (3.4×10^9 M^{-1} s^{-1}) and the DMPO-OH adduct is reported to be relatively stable[21,26] for several hours at room temperature in aqueous solutions. In the presence of cells DMPO-OH adducts are rapidly metabolised.[26] It has been suggested that several natural antioxidant defence systems in whole blood including ascorbic acid, superoxide dismutase (SOD), catalase and glutathione may react with OFR and their DMPO adducts to yield EPR silent species.[33] For example, ascorbate which is an important antioxidant defence against OFR has a high concentration in the kidney. Incubation of an oxygen radical generating system with human tumor cells,[47] swine plasma,[33] red blood cells, hamster V76 cells[48] and a microsomal system[21] are reported to result in rapid metabolism or conversion of DMPO-superoxide or -•OH radical adducts. However Rojanasakul *et al.*[49] have shown that the magnitude of the DMPO-OH adduct from the radical generating system was only slightly quenched in the presence of alveolar macrophages.

In view of the reported instability of OFR adducts in cellular systems,[26] it is not surprising that OFR studies in kidney have been difficult to detect. Hence *in vivo* OFR formation in kidney needs more investigation. Due to the instability of radical adducts in the presence of cell components we attempted to slow the rate of disappearance of the radicals by rapidly freezing the samples. EPR spectra observed after ischaemia-reperfusion consisted of a 1:2:2:1 quartet and a 1:1:1:1:1:1 six line spectrum. The •OH radicals induce lipid peroxidation by abstraction of a methylene hydrogen atom from an unsaturated fatty acid to form a lipid alkyl radical[50] which can be subsequently trapped by DMPO. Thus the DMPO-R spectra can be formed by •OH radical attack on any aromatic ring or macromolecule in the incubation medium.[51] It is suggested that the R• radicals are derived only from generated hydroxyl radicals.[7,40] The prominent DMPO-R signal (1:1:1:1:1:1 six line spectrum, Figure 2) may be an indication of formation of hydroxyl radicals, because if only superoxide were formed no DMPO-R signal would be observed.[7,23,52] More study may be necessary to determine the source of carbon-centred radicals in biological systems.

In this study in IPRK perfused in the absence of albumin (Figure 2) as well as in our earlier study using salicylate as a chemical trap in the presence of albumin[16] there was spontaneous •OH generation (Figure 4). These observations are consistent with that of Nilsson *et al.* which demonstrated significant formation of OFR even before induction of *in vivo* renal ischaemia-reperfusion. In addition the results demonstrate that •OH are generated in the ischaemic-reperfused kidney in the absence of circulating neutrophils or erythrocytes. While activated circulating neutrophils may contribute to renal reperfusion injury *in vivo*, our studies confirm that even in the absence of neutrophils, renal parenchymal cells in the intact kidney generated significant quantities of •OH spontaneously, and that this generation was increased significantly by ischaemia-reperfusion. Cytoplasmic, mitochondrial, vascular and tubular compartments are possible sites for hydroxyl radical generation. Alternative extracellular sites for hydroxyl radical formation in this model include the vascular endothelium and the luminal brush border. We did not attempt to differentiate between these sites, but studies in cultured renal proximal tubular epithelial cells confirm that these cells are capable of generating •OH.[53]

The functional parameters in ischaemic-reperfused group were not significantly different from the ischaemia-reperfusion group in our previous study,[16] which we used as a retrospective group for comparison. For example in the absence of DMPO, after ischaemia-reperfusion GFR was reduced by 87.6 ± 2.5%, while in the present study with DMPO, GFR was reduced by 80.8 ± 5.9% ($p = 0.11$. ns). This suggests no effect of DMPO or PBN on ischaemia-reperfusion-induced injury in this model. These results are consistent with the conclusion that scavenging •OH radicals with DMTU or trapping them with DMPO or PBN did not improve renal function following ischaemia-reperfusion injury.

The role of renal •OH generation in renal damage following ischaemia-reperfusion remains uncertain. In this study, ischaemia-reperfusion caused a marked reduction in renal function, as monitored by the reduced GFR and increased FE_{Na}. Although, the free radical scavenger (DMTU) reduced hydroxyl radical formation (Figure 2c), it did not protect against early loss of renal physiological function. This is similar to the observation of protection by DMTU and DMSO against increases in total renal sodium (monitored by ^{23}Na NMR) in the absence of protection of renal physiological function.[17] Ischaemia and hypoxia induce extensive histological and functional damage in this model, whereas the changes protected against by •OH scavengers appear to be relatively subtle. As our studies were confined to the first minutes of reperfusion, these results do not exclude the possibility that long term recovery of function may be enhanced by DMTU during prolonged reperfusion, such as the 60 min period used by Linas *et al.*[2] *in vitro* and 24 hours duration by Paller *et al.*[3] following *in vivo* ischaemia.

We conclude that significant hydroxyl radicals are generated by the intact isolated rat kidney following ischaemia plus reperfusion in the absence of circulating neutrophils. The fact that 15 mM DMTU inhibits •OH formation but does *not* prevent functional injury during limited reperfusion after 20 min ischaemia in rat kidneys suggests that •OH radical formation does not play a major role in ischaemia-reperfusion injury in this model.

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