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APPLICATION OF METHIONINE AS A DETECTOR MOLECULE FOR THE ASSESSMENT OF OXYGEN RADICAL GENERATION BY HUMAN NEUTROPHILS AND ENDOTHELIAL CELLS

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Diverse cell types can generate reactive oxygen species (ROS) which are implicated in many disease processes and are ascribed both beneficial and deleterious roles. *In vitro* studies of this phenomenon indicate that properties of the microenvironment in culture influence the cells' behaviour with regard to ROS generation *in vivo*. To date, however, the assessment of cellular ROS generation has been limited to techniques which are invasive of the culture environment, or require cells to be in suspension. This study describes the application of NMR spectroscopy to the detection of ROS generation, a technique which is non-invasive of the cell culturing environment.

KEY WORDS: NMR spectroscopy, neutrophil free radical generation, endothelial cell free radical generation, methionine, cell culture

INTRODUCTION

The assessment of reactive oxygen species (ROS) generation by living cells is an important requirement in the study of the physiological and pathological roles of radical generating mechanisms *in vivo*. For example, the microbicidal action of ROS produced by activated neutrophils is a well known example of a beneficial mechanism. In fact, patients whose neutrophils lack the capability of generating ROS (cf. chronic granulomatous disease) have a very poor prognosis. On the other hand, inappropriate ROS production by a variety of known cell types has now been implicated in the pathogenesis of many inflammatory conditions.[1] There are many known sources of ROS *in vivo* including mitochondrial electron transport chain components, the endoplasmic reticulum and nuclear membrane electron transport systems. The oxidants produced by these systems could play a rôle in tissue damage associated with ischaemia and inflammation,[2] but the most popular candidates are the enzymes xanthine oxidoreductase (XOD) of capillary endothelial cells and the NADPH oxidase of the human neutrophil. The former source has received particular attention as the ROS-generating system in ischaemia/reperfusion injury, as has been described in the heart, gut and joint.[3] Since the primary location of XOD is the capillary

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endothelial cell[4,5] it is ideally situated to be driven by ischaemic episodes followed by reperfusion. O_2^-, H_2O_2 and ·OH liberated locally will no doubt have a considerable damaging effect on the cell of origin. It follows that the delicately balanced haemostatic functions of the endothelium will be locally compromised, resulting in the triggering of, or perpetuation of an inflammatory process. In situations where particular organs are prone to ischaemia it would be of benefit to therapeutically intervene in this process. To this end, a sensible approach would be to halt or reduce ROS generation at the level of the endothelial cell. A variety of strategies are available and are currently under investigation in endothelial cell cultures. However, to date the assessment of endothelial cell ROS generation has largely been limited to techniques which are invasive of the culturing environment, or require the cells to be in suspension. Here we describe a new technique for detecting ROS using endogenous culture medium methionine, wherein the relative amounts of methionine and its ROS-induced oxidation product, methionine sulphoxide,[6] are measured using proton Hahn spin-echo nuclear magnetic resonance spectroscopy (NMR). We have previously reported that NMR spectroscopy may be employed to detect low-molecular-mass products derived from ROS-mediated oxidative damage to a range of bimolecules present in human body fluids.[7]

MATERIALS AND METHODS

Neutrophil preparation

Human peripheral blood neutrophils were isolated from fresh heparinised blood by sedimentation on dextran (M wt 485,000 daltons), density gradient centrifugation on Histopaque 1.077 followed by hypotonic lysis of contaminating erythrocytes. Neutrophile were suspended in E-199 (Gibco—the amino acid content of E199 is given in Table I), counted and diluted to a concentration of 3×10^6 cells/ml. Cells were incubated for 2 hours at 37°C with 1 mM PMA to stimulate O_2^- release. Superoxide dismutase (300 U/ml) was added to a control incubation. Other controls included PMA-free E-199 medium, incubated under the same conditions as above, with and without neutrophils. After the incubation period the cells were pelleted by centrifugation and the supernatant stored at −70°C until ready for assay.

TABLE I
Amino acid content of medium E-199

Amino acid	mg/dm³	Amino acid	mg/dm³
Alanine	50.00	Leucine	120.00
Arginine	70.00	Lysine	70.00
Aspartic acid	60.00	Methionine	30.00
Cysteine	0.11	Phenylalamine	50.00
Glutamic acid	150.00	Proline	40.00
Glutamine	100.00	Serine	50.00
Glycine	50.00	Threonine	60.00
Histidine	21.88	Tyrosine	20.00
Hydroxyproline	10.00	Valine	50.00
Isoleucine	40.00	$Fe^{III}(NO_3)_3 \cdot 9H_2O$	0.72

Isolation, growth and subculture of human umbilical vein endothelial cells (HUVEC)

Endothelial cells were obtained from human umbilical veins as described by Jaffe *et al.*[8] with minor modifications. A 2 mg/ml solution (w/v in serum-free medium M199) of collagenase (Type IV, Sigma Chemical Co, UK) was filter sterilised through 0.22 μm filters (Sartorius). The vein was incubated with the collagenase solution at 37°C in 95% air and 5% CO_2 for 15 min, in order to enzymically detach the endothelial cells lining the vessel from the basement membrane. The collagenase solution was subsequently removed and the vein flushed with 10 ml PBS, which was centrifuged together with the collagenase solution for 10 min at 1300 rpm. The pellet of cells was resuspended in 10 ml of serum-containing (foetal calf serum, 20% v/v) medium E199 with ECGF (30 μg/ml) and heparin (10 IU/ml), then seeded onto a 75 cm^2 plastic culture flask (Costar® T75). This primary culture was maintained by the replacement of the medium every 48 hours. At confluency, the cells were "passaged", i.e. subcultured (at 1:3 in Costar® T75 tissue culture flasks) by trypsinisation and replating.

Simulation of ischaemia/reperfusion cycles on HUVEC

HUVEC at the second passage were seeded onto T75 Costar culture flasks and allowed to grow to confluence. A hole was made with a hot needle in the top of the flask at the beginning of the experimental period. The modified lid incorporating a pasteur pipette was connected to a 95% N_2/5% CO_2 gas supply via a rubber tube. The flask was flushed with this gas mixture at high pressure for 1 minute then perfused with a gentle stream of the gas (1 l/min) for 40 minutes. After this time the gas supply was switched to 95% air/5% CO_2 and similarly perfused for 40 minutes. This cycle was repeated twice to simulate intermittent ischaemic episodes, after which time samples of the medium (1 ml) were taken and stored at −70°C until assessed by NMR.

NMR measurement on neutrophil and endothelial cell supernatant media prior and subsequent to treatment

Proton NMR measurements were performed on Bruker WH 400 (University of London Intercollegiate Research Service (ULIRS), Queen Mary and Westfield College, London, UK) and JEOL JNM-GSX 500 (ULIRS, Biomedical NMR Centre, Birkbeck College, London, UK) spectrometers operating at 400 and 500 MHz respectively for ^1H. The Bruker WH 400 spectrometer was equipped with a Bruker Aspect 3000 data system. All spectra were recorded at ambient probe temperature (23 ± 1°C). Typically, 0.60 ml of cell culture medium was placed in a 5 mm diameter NMR tube, and 0.07 ml of ^2H_2O was added to provide a field frequency lock. The broad protein resonances and the intense water signal were suppressed by a combination of the Hahn spin-echo sequence and the application of continuous secondary irradiation at the water frequency respectively. The Hahn spin-echo sequence D[90°x-τ-180°y-τ-collect] was repeated 128 times with τ = 68 ms for the Bruker WH 400 and 60 ms for the JEOL JNM-GSX 500 spectrometers. Chemical shifts were referenced to external sodium-3-(trimethylsilyl)-1-propanesulphonate (TSP, δ = 0.00 ppm). The methyl group resonances of valine (1.050 ppm), lactate (1.330 ppm) and alanine (1.487 ppm) served as secondary internal references.

Resonance assignments were made by a consideration of chemical shift values, spin-spin coupling patterns and coupling constants. Where appropriate, assignments were confirmed by making standard additions of components to the media.

NMR measurements on chemical model systems

0.60 ml Aliquots of control and gamma-irradiated 0.10 mol·dm^{-3} aqueous solutions of L-methionine and DL-methionine sulphoxide were placed in a 5 mm diameter NMR tube, and 0.07 ml of ^2H_2O was added to provide a field frequency lock. Aliquots of medium which had been treated for 2 hours with 1) 40 U/ml xanthine oxidase (XO-Boehringer) and with 2) 880 μM H_2O_2 were likewise prepared. Single-pulse spectra of these solutions were obtained on a Bruker WH 400 spectrometer using a pulse angle of 30–40° and a total delay between pulses of 3 s to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated. These spectra were also recorded at ambient probe temperature and referenced to TSP. Single-pulse spectra of aqueous solutions of individual culture medium reference components were similarly obtained.

Gamma-irradiation treatment of cell culture media and aqueous solutions of L-methionine and DL-methionine sulphoxide

Cell culture media E-199 and aqueous solutions of L-methionine and DL-methionine sulphoxide were subjected to gamma-radiolysis in the presence of atmospheric oxygen at a temperature of 25°C, using a ^{60}Co source (Department of Immunology, The London Hospital Medical College). The total dose employed was 5.00 kGy (dose rate 4.76 Gy/min). Under these experimental conditions, the major primary radiolytic products of water are ·OH (G = 2.7), $e^-_{(aq.)}$ (G = 2.7) and H· (G = 0.5) (equation 1). In the presence of atmospheric O_2, the aquated electrons ($e^-_{(aq.)}$) generated are predominantly converted to superoxide anion (equation 2).

$$H_2O \rightarrow \cdot OH,\ e^-_{(aq.)},\ H\cdot,\ H_2,\ H_2O_2,\ H_3O^+ \quad (1)$$

$$e^-_{(aq.)} + O_2 \rightarrow O_2^{\cdot -} \quad (2)$$

RESULTS

Figure 1(a) exhibits the high-field (low-frequency) regions of 400 MHz proton Hahn spin-echo NMR spectra of fresh E-199 medium which was incubated at 37°C for 2 hr. The spectrum contains a variety of resonances attributable to a wide range of low-molecular-mass constituents of the medium. The corresponding spectrum of E-199 which was incubated under the same conditions with neutrophils (3×10^6 cells/ml) is shown in Figure 1(b). Notable metabolic changes include reductions in the concentrations of glucose and methionine, together with the production of threonine.

Stimulation of neutrophils with PMA gave rise to modifications in the chemical composition of E-199 culture medium as illustrated in Figure 1(c). A further depletion of the glucose content was accompanied by a total loss of NMR detectable methionine. In addition, a multiplet signal centred at ca. 2.7 ppm is markedly diminished in intensity after stimulation. However, the most striking modification observed was

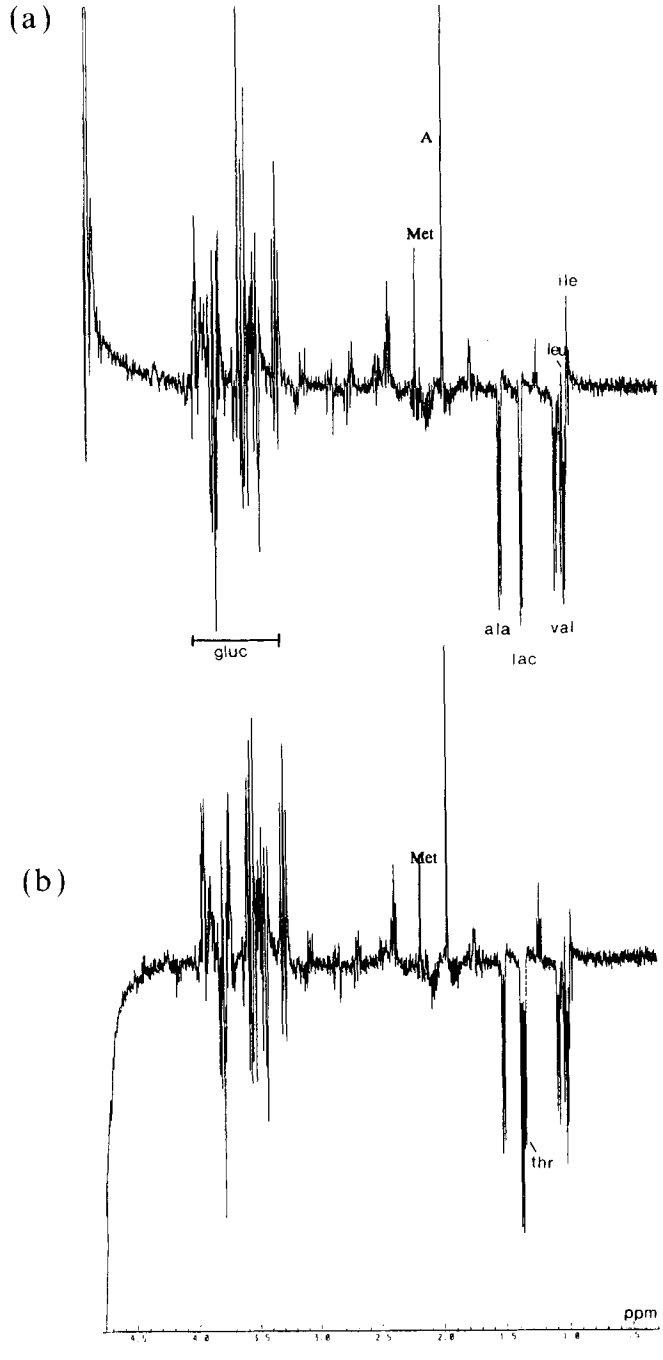

FIGURE 1 Low frequency (high-field) regions of 400 MHz ^{1}H Hahn spin-echo NMR spectra of (a) E-199 culture medium, (b) as (a) but after a 2 hr incubation with neutrophils.

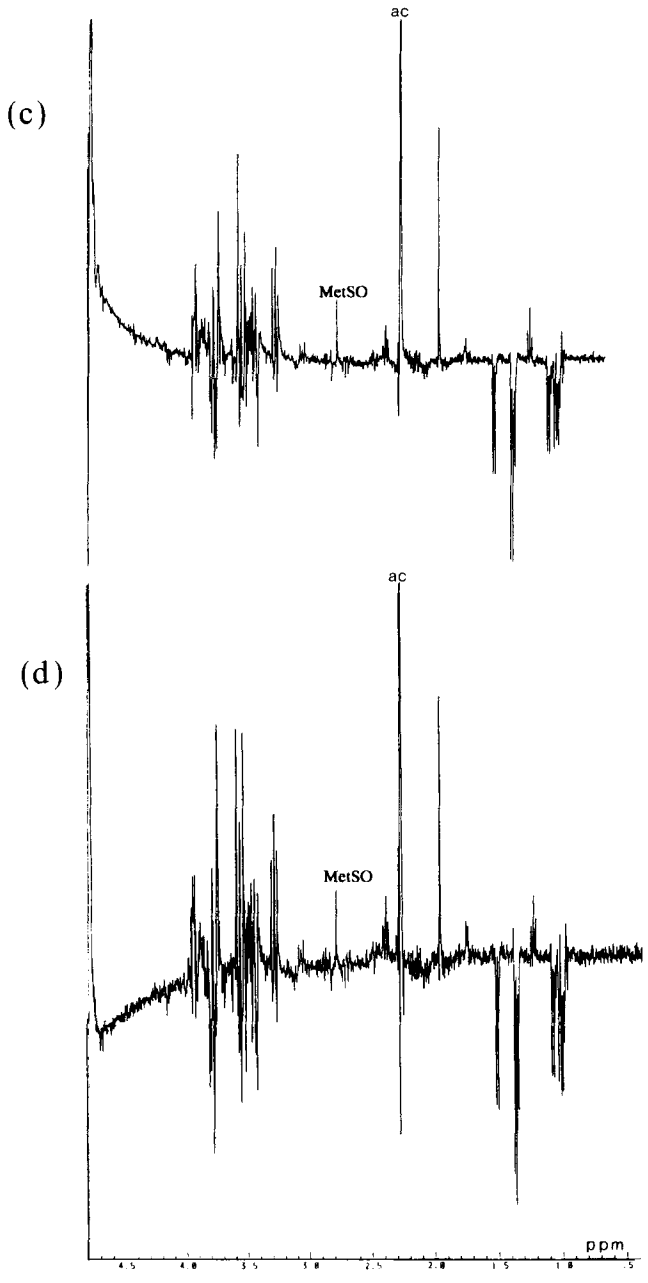

FIGURE 1 *continued.* (c) as (b) but incubated in the presence of 1 mM PMA and (d) as (c) but with inclusion of 300 U/ml superoxide dismutase. Abbreviations: A, acetate-CH_3; ac, acetone-CH_3; ala, alanine-CH_3; gluc, glucose proton resonances; ile, isoleucine-CH_3; lac, lactate-CH_3; leu, leucine-CH_3; Met, methionine-CH_3; MetSO, methionine sulphoxide-SO-CH_3; thr, threonine-CH_3; val, valine-CH_3. The acetone detected in spectra (c) and (d) is the solvent in which PMA was solubilised.

the production of an intense methionine sulphoxide $-SO-CH_3$ group singlet resonance located at 2.752 ppm. The production of this species is consistent with ROS-mediated oxidative damage to methionine which explains the observed loss of this amino acid from the medium. Co-incubation of neutrophils with superoxide dismutase and PMA (Figure 1(d)) did not prevent the generation of methionine sulphoxide as shown in Figure 1(c). Single-pulse proton NMR (400 MHz) spectra of control and gamma-irradiated 0.1 $mol \cdot dm^{-3}$ aqueous solutions of L-methionine are shown in Figures 2(a) and (b) respectively. Subsequent to gamma radiolysis (5.0 kGy) in the presence of atmospheric oxygen, the methionine $-S-CH_3$ group singlet and $\gamma-CH_2-$ group triplet ($\delta = 2.13$ and 2.635 ppm respectively) show a marked decrease in intensity.

Concurrently, the 2.752 ppm $-SO-CH_3$ singlet and 3.021 ppm γ-CH_2-multiplet resonances of methionine sulphoxide have become clearly visible in the spectrum, demonstrating the conversion of methionine to its corresponding sulphoxide by radiolytically-generated $\cdot OH$. A corresponding spectrum of an aqueous solution containing an authentic sample of DL-methionine sulphoxide is exhibited in Figure 2(c).

Figures 2(d) and (e) show 400 MHz proton Hahn spin-echo NMR spectra of both control and gamma-irradiated (5.00 kGy) E-199 culture medium. The major differences observed between these spectra were (1) a marked radiolytically-dependent reduction in the intensities of the methionine $-S-CH_3$ group singlet and γ-CH_2-triplet resonances located at 2.13 and 2.635 ppm respectively, consistent with radiolytic depletion of this amino acid, and (2) the corresponding production of a methionine sulphoxide $-SO-CH_3$ group singlet resonance located at 2.752 ppm. These data demonstrate the oxidation of methionine endogenous in E-199 to its sulphoxide by radiolytically-generated $\cdot OH$.

Treatment of E-199 medium with the enzymic xanthine/XO $O_2^{\cdot}$-generating system also gave rise to the production of a relatively intense methionine sulphoxide $-SO-CH_3$ group resonance with a corresponding total loss of NMR-detectable methionine (Figure 3(a)). This oxidative modification of methionine was similarly observed following incubation of the medium with H_2O_2 (Figure 3(b)).

Figure 4 shows 400 MHz proton Hahn spin-echo NMR spectra of E-199 culture medium after endothelial cells had been subjected to simulated cycles of ischaemia/reperfusion (4b) and a normally oxygenated control (4a). The major differences observed between these spectra were a marked reduction in the intensity of the methionine-$S-CH_3$ group singlet resonance located at 2.13 compared to the control cells. This is consistent with oxidative depletion of this amino acid and corresponds with the production of a small methionine sulphoxide $-SO-CH_3$ group singlet resonance located at 2.752 ppm.

DISCUSSION

Endothelial cells in culture and peripheral blood neutrophils can easily be stimulated to produce ROS but detection is often difficult with currently available systems and can be impaired by many inherent complicating factors. These include, in the case of endothelial cells, ROS interactions with endogenous scavengers and with those present in the culture media. The purpose of this study was to develop a system in which ROS generation can be controllably stimulated and reliably measured without

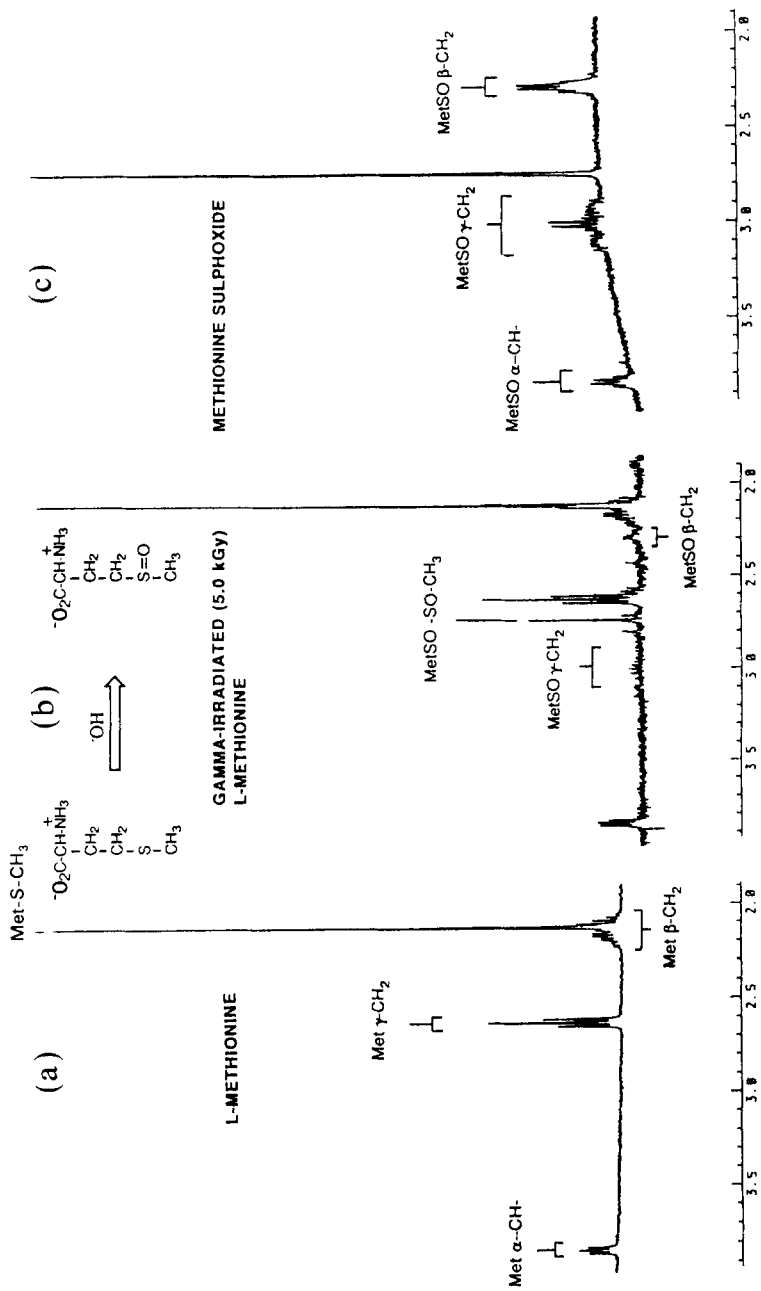

FIGURE 2 1.9–3.9 ppm regions of the single pulse ^{1}H NMR spectrum of L-methionine, (b) as (1), but following gamma-radiolysis (5.00 kGy) in the presence of atmospheric oxygen, and (c) DL-methionine sulphoxide.

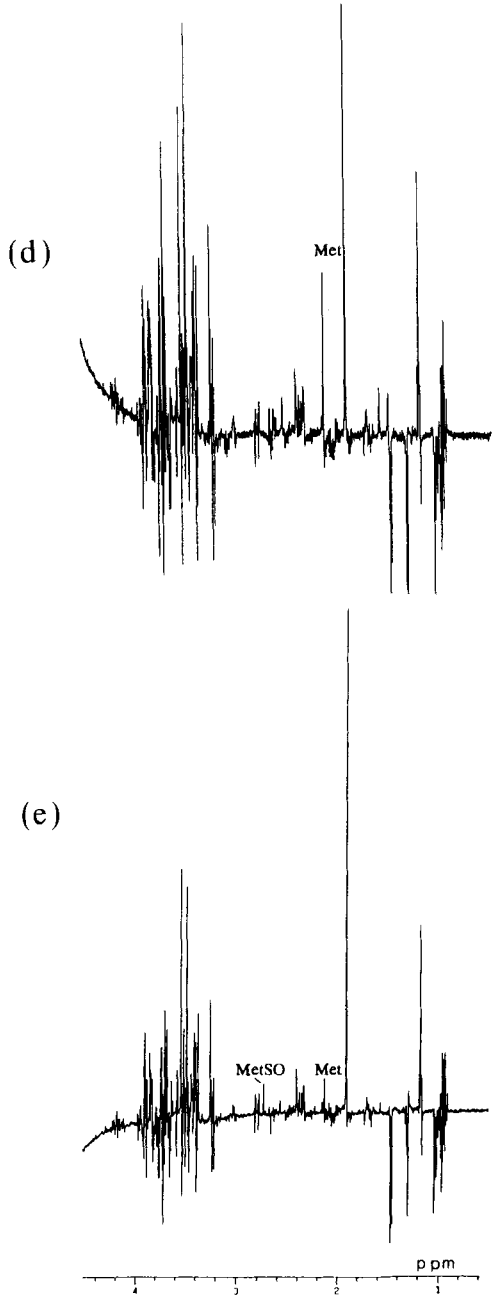

FIGURE 2 *continued*. (d) and (e) High field regions of the 500 MHz ^{1}H NMR spectra of control and gamma-irradiated (5.00kGy) E-199 culture medium respectively. Abbreviations: Met α-CH, β-CH_2–, γ-CH_2–, –S–CH_3 and MetSO α-CH, β-CH_2–, γ-CH_2–, –SO–CH_3 represent the characteristic resonances of methionine and methionine sulphoxide respectively.

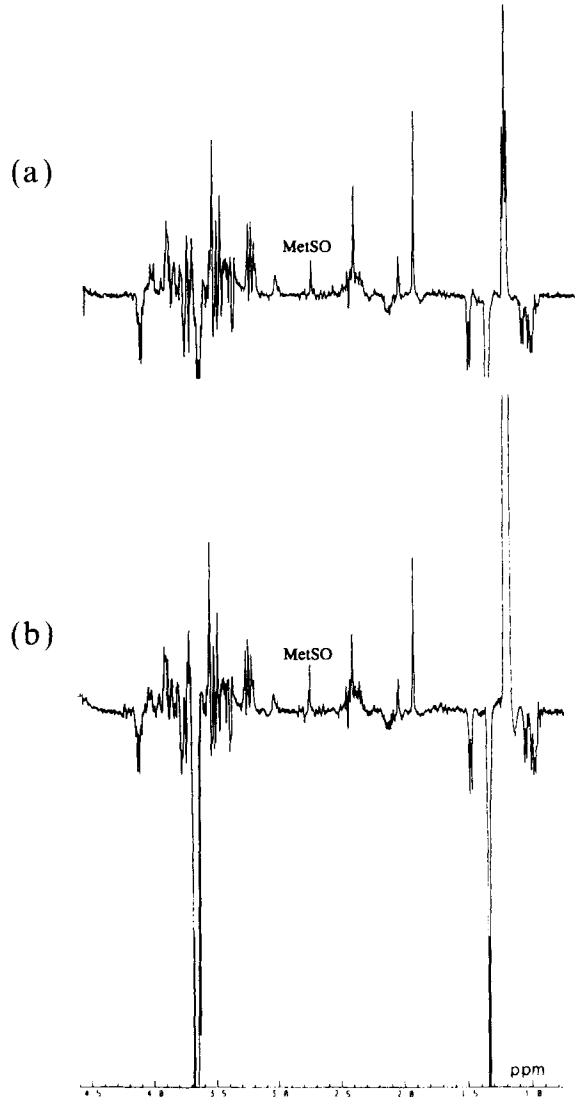

FIGURE 3 Low frequency (high field) regions of ^{1}H NMR spectra of (a) E-199 culture medium treated with 40 U/ml XO (operating frequency 400 MHz), (b) E-199 medium treated with 882 μM H_2O_2 (400 MHz).

being invasive of the optimal physiological environment, and ultimately to employ such a system to investigate the rôle of endothelial cell-derived ROS in pathological processes.

The results demonstrate that methionine sulphoxide is a major product generated by the attack of neutrophil-derived ROS on methionine. In addition, it appears that in the case of endothelial cell ROS generation, a similar but less pronounced oxidation of methionine occurs on cyclical hypoxia and reperfusion epixodes. In these

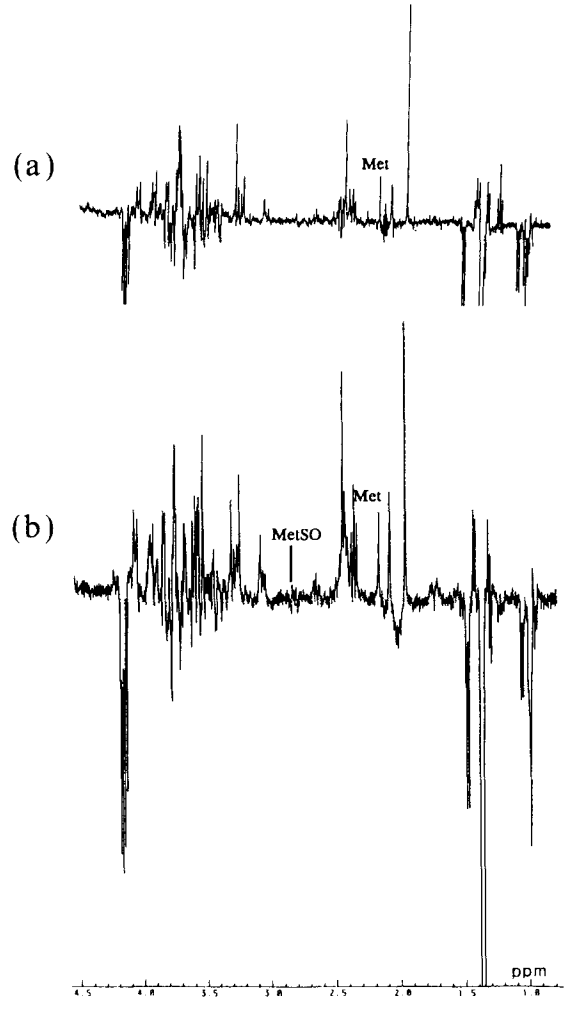

FIGURE 4 400 MHz proton Hahn spin-echo NMR spectra of E-199 culture medium after endothelial cells had been subjected to simulated cycles of ischaemia/reperfusion (4a) and a normally oxygenated control (4b).

experiments, however, the concentration attainable of endothelial cells is 5 times less than the concentration of neutrophils used.

It is well known that a variety of endothelial cell types have the capability of generating ROS. This observation has implicated a rôle for ROS in hypoxic reperfusion injury, inflammation and the control of vascular tone. Microvascular endothelial cells are a rich source of XO,[4,5] facilitating a mechanism for ROS generation via the enzymatic conversion of hypoxanthine to xanthine.[2] Superoxide is the primary ROS generated which dismutates to H_2O_2. Since the oxidative damage to methionine observed in the neutrophil study was uninhibitable by superoxide dismutase, it would appear that oxidation was mediated by hydroxyl ($^{\cdot}OH$).

However, it should be noted that alkoxyl (RO·) and peroxyl (ROO·) radicals, together with hypochlorous acid (HOCl) derived from the myeloperoxidase/H_2O_2/Cl^- system also have the ability to convert methionine to its sulphoxide and hence the involvement of these species as oxidants in this system cannot be ruled out. Hydroxyl radical generation is dependent on the interaction of H_2O_2 with iron(II) present in the culture medium (equation 3).[9]

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^- \quad (3)$$

The iron(II) is likely to be produced from the direct reduction of iron(III) by electron donors such as ascorbate and cysteine (present in E-199 at levels of 50 and 110 $\mu g/dm^3$ respectively). The observations made in this study indicate that the superoxide generated by these cellular systems does not act as a precursor to ·OH radical formation via the iron catalysed Haber-Weiss reaction.

Although it has been previously demonstrated that ethylene gas results from the attack of ·OH radical on the methionine molecule,[10] the detection of this product by ^1H NMR is, of course, precluded.

In conclusion, these data show that stimulation of neutrophils and endothelial cells to produce ROS can be detected by high field ^1H NMR analysis of the culture medium in which they thrive, thereby avoiding variables arising from changes in the culture environment. The procedure is currently being refined to imitate *in vivo* ROS generating systems in which potential antioxidant compounds can be evaluated.

Acknowledgements

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