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In vitro electron paramagnetic resonance characterization of free radicals: Relevance to exercise-induced lipid peroxidation and implications of ascorbate prophylaxis

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Abstract

This study tested the hypothesis that exercise-induced oxidative stress is caused by free radical-mediated damage to polyunsaturated fatty acids (PUFA) which can be prevented following ascorbate prophylaxis. Hyperfine coupling constants (HCC) of α-phenyl-*tert*-butylnitrone (PBN)-adducts were measured via room temperature electron paramagnetic resonance (EPR) spectroscopy in the venous blood of 12 subjects at rest and following maximal exercise during a randomized double-blind placebo-controlled trial and compared to those observed following room-air incubation (2 h at 37°C) of L-α-phosphatidycholine, linoleic acid, α-linolenic acid and arachidonic acid. All adducts exhibited similar HCC [a_N 13.6 Gauss (G) and $a\beta_H$ 1.8 G] with the exception of L-α-phosphatidycholine [$a_{N1} = 13.4$ G, $a\beta_{H1} = 1.6$ G (37%) and $a_{N2} = 14.9$ G, $a\beta_{H2} = 0.3$ G (63%)] consistent with the trapping of lipid-derived alkoxyl and oleate radicals, respectively. Ascorbate pre-treatment ablated radical formation in both systems. These findings identify circulating PUFA as a potential source of secondary radicals that are capable of initiating oxidative stress in the exercising human.

Keywords: *Free radicals, lipid peroxidation, electron paramagnetic resonance spectroscopy, spin-trapping, ascorbic acid*

Introduction

The origin of free radical species, particularly within the lipid peroxidation chain, has intrigued investigators for many years [1,2] and it is now known for example, that when a hydrogen atom is abstracted from a cell membrane polyunsaturated fatty acid (PUFA) side chain, a carbon-centred lipid radical is formed. This radical may undergo molecular rearrangement to form a conjugated diene and in the presence of molecular oxygen this reaction may proceed to form the aqueous oxygen-centred peroxyl radical [3,4]. Ascorbic acid is a known scavenger of the peroxyl radical [5,6] and, if present in required concentrations, the lipid peroxidation chain may terminate at this point. However, in the absence of sufficient ascorbic acid and/or other important chain breaking antioxidants, lipid hydroperoxides may accumulate and, via an iron dependent reaction, the oxygen-centred alkoxyl radical may be formed, which can subsequently form aldehydes such as malondialdehyde [7].

Free radicals have the potential to cause damage to lipids as a consequence of aerobic exercise. Exercise-induced oxidative stress is normally quantified by examining the by-products of lipid damage such as F_2-isoprostanes, malondialdehyde or lipid hydroperoxides. Recently, a number of studies examining the

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relationship between exercise and free radical generation have used Electron Paramagnetic Resonance (EPR) spectroscopy which is the most direct method available for the detection and subsequent identification of free radical species [8–11]. A number of free radical species such as peroxyl and/or alkoxyl radicals following exercise have been identified by EPR spectroscopy and it is postulated that these radicals originate from the phospholipid membrane [9,10].

However, no conclusive evidence has been provided to confirm this supposition and indeed there is a paucity of evidence demonstrating the possibility that free radicals detected *ex vivo* are as a consequence of lipid oxidation. Thus, our major objective was to determine the potential source and identity of free radicals generated during the course of lipid peroxidation in the exercising human. Using an EPR spin-trapping technique, our primary aims were to (1) compare the hyperfine coupling constants (HCC) of PBN-adducts generated *in vitro* during the auto-oxidation of individual PUFA/membrane phosopholipids with blood-borne species generated in the human circulation during exercise and (2) establish if ascorbic acid is capable of scavenging these lipid derived species and thus terminating the lipid peroxidation chain. We hypothesize that the blood-borne PBN-adducts generated *ex vivo* following exercise would have comparable HCC to those detected following the auto-oxidation of PUFA and membrane phospholipids. We further hypothesize that ascorbate prophylaxis would attenuate lipid peroxidation in both models.

Methodology

Human subjects and experimental design

Twelve apparently healthy male volunteers were recruited for the present study. All subjects were non-smokers and abstained from any dietary antioxidant supplementation for 6 weeks prior to experimental exercise. The Bro Taf Research Ethics Committee granted ethical approval and written informed consent was obtained from each volunteer prior to participation. On arrival at the laboratory subject body mass and stature was measured according to standard methods. Each volunteer was subsequently required to cycle to volitional exhaustion on a friction braked cycle ergometer (Monark 824ε, Stockholm, Sweden). The test was designed to be progressive and incremental in order to elicit maximal oxygen uptake (VO_{2max}). A cadence of 60 rpm was maintained while workload was increased by 0.5 kg every 3 min until volitional fatigue. Venous blood was collected from an antecubital forearm vein following 20 min supine rest and immediately post-exercise using the Vacutainer™ method (Becton-Dickinson, Oxford, UK).

In order to determine the effect of ascorbic acid on blood oxidative stress, 12 healthy subjects also received an acute oral bolus dose of either 1000 mg (2×500 mg) of ascorbic acid (Nova Laboratories, Wigston, UK, batch SCP g1514) or placebo (Nova Laboratories, Wigston, UK, batch SCP g1515) (six placebo and six ascorbic acid), 2 h prior to the exercise challenge in a randomized, double-blind, placebo-controlled design. Blood was determined for PBN-adduct and malondialdehyde concentration at rest and immediately following termination of exercise.

Human exercise, PBN-adduct extraction and EPR spectroscopic analysis

An *ex vivo* EPR spin trapping technique using α-phenyl-*tert*-butylnitrone (PBN) was employed to investigate the formation of free radical species. Venous blood was drawn into a 6 ml glass serum separation vacutainer, containing 1.5 ml of 200 mm PBN previously mixed with physiological saline (50 mm final concentration). The vacutainer was inverted gently 10 times and placed in the dark to clot (PBN and blood incubation time was 10 min, therefore the free radical adducts trapped are described as *ex vivo* generated species that we assume reflect events that occur *in vivo*). Following centrifugation at 3000 rpm for 10 min at 4°C, 1 ml of HPLC grade toluene was added to 1 ml of the serum/spin adduct and vortex mixed for 30 s. The sample was centrifuged for 10 min to separate the organic layer; 200 µl of the toluene/organic layer was placed in a precision bore quartz EPR tube and vacuum degassed in a freeze/thaw procedure using a turbo molecular pump (West Technologies Ltd, Bristol, UK) to 10^{-3} Torr for three consecutive 8 min cycles. A Pirani 14 gauge detector was used to monitor pressure change (Edwards, UK). All samples were analysed under vacuum at room temperature on a Bruker EMX series X-band EPR spectrometer (Bruker, Karlsruhe, Germany). All EPR analyses were conducted on the experimental day and nuclear hyperfine coupling constants (HCC) were confirmed following computer simulation using SimEPR32 software [12]. The relative concentrations of PBN-adducts (expressed in arbitrary units (AU)) were determined by measuring the mean signal intensity of each spectral peak-to-trough line height.

Blood lipid peroxidation analysis

Plasma malondialdehyde was collected in di-potassium ethylene diamine tetra-acetic acid (EDTA) tubes and centrifuged at 3000 rpm at 4°C for 10 min. The serum was then placed into aliquots and stored at −80°C until analysis within 2 months. Malondialdehyde was estimated using the HPLC with fluorometric detection assay of Young and

Trimble [13]. This established technique increases measurement sensitivity and specificity, which may not be found when determining malondialdehyde concentrations using other assays. Intra- and inter-assay coefficient of variation (CV) at 0.56 μmol/L = 6.2% and 9.1%, respectively.

In vitro *experimental work*

The polyunsaturated fatty acids and total membrane phospholipid were chosen due to their double bond number and thus susceptibility to oxidation (linoleic, 18:2; α-linolenic, C18:3; arachidonic acid, C20:4; L-α-phosphatidycholine, total phospholipids). Linoleic acid, α-linolenic, arachidonic acid and L-α-phosphatidycholine were air auto-oxidized with the spin trap PBN and subsequently in the presence of ascorbic acid. All of the above substrates followed the same oxidizing protocol.

Lipid auto-oxidation

All substrates and chemicals were purchased from the Sigma-Aldrich chemical company (Poole, Dorset, UK). L-α-phosphatidycholine only was prepared by Sigma-Aldrich in 10 mg ml of chloroform; 50 μl of substrate was dissolved in 3 ml of toluene; 1.5 ml of PBN solution (dissolved in toluene, 100 mg/1.5 ml) was added to this lipid-aqueous mixture. This mixture was subsequently air auto-oxidized in a warm water bath (Clifton water baths, Nickel Electro, UK) at 37°C for 2 h. On completion of this incubation period, 200 μl of PBN-adduct was removed, transported into a new quartz EPR glass tube and degassed before EPR analysis was performed. The same Bruker EMX X-band EPR spectrometer that was used to measure free radicals in the human exercising study was used for all *in vitro* work presented. A sample from the auto-oxidation of α-linolenic acid only was drawn from the mixture at 10 min, 60 min and 90 min and measured in an attempt to determine the rate of lipid peroxidation. Control experiments involving blood without PBN, a blank sample, PBN, toluene and a PBN and toluene mix were also conducted.

Oxygen uptake (O_2) was measured every 15 min period using a Clarke type oxygen electrode (Jenway, DO_2 meter, USA) during linolenic acid oxidation only.

Lipid auto-oxidation and ascorbic acid

For the ascorbic acid experimental component, lipids were prepared as above. Pure (99%) white crystalline ascorbic acid (Sigma-Aldrich, Poole, Dorset, UK) was dissolved in 3 ml de-ionized water to a concentration of 100 μmol/L. After 2 h of substrate auto-oxidation, 1.5 ml of the ascorbic acid solution was added, thus yielding a final concentration of 50 μmol/L. This antioxidant and pro-oxidant mixture was further oxidized for 2 h. On completion of this incubation period, 200 μl of PBN-adduct was removed and transported into a new quartz ESR glass tube and vacuum degassed before EPR analysis was performed.

In order to determine if ascorbic acid interfered with PBN-adduct stability or acted as an authentic radical scavenger, a further experiment was performed using human serum ($n = 1$); 2 ml of PBN adduct was added to 2 ml of toluene and centrifuged (as above); 1 ml supernatant was recovered and 400 μl was vacuum degassed as standard and subsequently analysed using EPR (control run). For the experimental run, 2 ml of PBN adduct was added to 2 ml of toluene and centrifuged; 1 ml supernatant was added to a 2 ml eppendorf containing white crystalline ascorbic acid (Sigma-Aldrich, Poole, Dorset, UK) to yield a final concentration of 100 μmol/L; 400 μl was vacuum degassed and subsequently analysed using EPR.

Human blood and in vitro *EPR operating conditions*

All experiments were performed in a darkened room with minimal exposure to daylight in order to minimize the risk of sample photolytic degradation. Identical room temperature EPR operating conditions were used for all experiments and were: modulation frequency, 100 kHz; microwave frequency, 9.688 GHz; incident microwave power, 20 mW; scan width, 50 gauss; modulation amplitude, 0.50 gauss; magnetic field centre, 3455 gauss; receiver gain 1.00×10^5; time constant, 83.9 ms; sweep time, 83.8 s for 10 scans. Intra-assay CV at 1795 arbitrary units = 5.2%.

Statistical analysis

Statistical analysis was performed using the SPSS package (Version 15.0, Surrey, UK). The implications of exercise for PBN-adduct formation was examined using a paired samples *t*-test following mathematical confirmation of a normal distribution by repeated Shapiro-Wilk *W*-tests. The efficacy of ascorbate prophylaxis against oxidative stress was determined using a two-way repeated measures ANOVA that incorporated one within (state: rest vs exercise) and one between (group: ascorbic acid vs placebo) subjects factors. Following a significant interaction effect (state × group), within subjects factors were analysed using Bonferroni-corrected paired samples *t*-tests. Between subjects differences were analysed using a one-way ANOVA with *a posteriori* Tukey Honestly Significant Difference (HSD) test. The relationship between selected dependent variables was examined using a Pearson's Product Moment Correlation. The alpha was established at $p < 0.05$ and values reported as mean ± SD.

Results

Exercise, PBN-adduct and malondialdehyde data

Typical EPR spectra at rest and post-exercise in serum are illustrated in Figures 1(A and B), exhibiting the characteristic triplet of doublet from nitroxide PBN spin trapping. The HCC were a_N 13.6 G and $a\beta_H = 1.8$ G, as confirmed by computer simulation (Figure 1C) and are consistent with the trapping of secondary oxygen-centred lipid-derived free radicals. Ascorbic acid supplementation selectively attenuated the exercise-induced increase in PBN-adduct and malondialdehyde concentration (Figures 2A and B) ($p < 0.05$).

Lipid auto-oxidation and PBN-adduct data

Typical EPR spectra of PBN-adducts extracted *in vitro* following auto-oxidation of linoleic, α-linolenic and arachadonic acid are shown in Figures 3(A–C), respectively. The HCC were a_N 13.6 G and $a\beta_H =$ 1.8 G for all adducts detected. Mean peak amplitude height, indicating PBN-adduct/free radical concentration was 121 201 AU, 230 072 AU and 70 801 AU for linoleic acid, α-linolenic acid and arachidonic acid oxidation, respectively. Typical EPR spectra of PBN-adducts extracted *in vitro* following auto-oxidation of *L*-α-phosphatidycholine is shown in Figure 4A. Computer simulation (Figure 4B) identified two primary species with nuclear hyperfine splittings of $a_{N1} = 13.4$ G, $a\beta_{H1} = 1.6$ G (37%) and $a_{N2} = 14.9$ G, $a\beta_{H2} = 0.3$ G (63%). Mean peak amplitude height = 49 620 AU. The rate of α-linolenic oxidation and subsequent consumption of oxygen increased linearly over time, as illustrated in Figures 5(A and B).

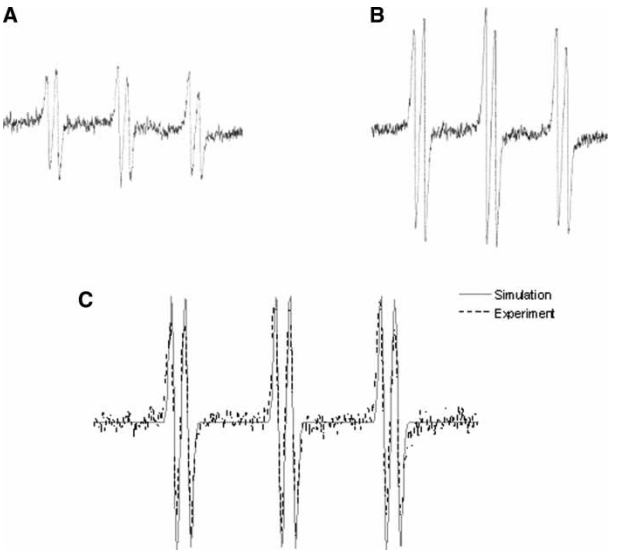

Figure 1. Typical EPR spectra of PBN-adducts extracted *ex vivo* from human blood in one volunteer at rest (A) and following maximal exercise (B). Computer simulation (C) identified a single species with nuclear hyperfine splittings of $a_N = 13.6$ G and $a\beta_H =$ 1.8 G. All spectra were filtered and scaled identically.

The observation that PBN-adduct concentration did not change when ascorbic acid (100 μmol final concentration) was added following toluene extraction of PBN-adduct from human serum, indicates that ascorbic acid did not diminish trap-radical adduct stability and provides tentative evidence for the authentic scavenging of lipid-derived radicals (see Figures 6A and B).

Discussion

The primary purpose of these *in vitro* experiments was to establish whether free radical species detected *ex vivo* in a human exercising model originate from polyunsaturated fatty acids. The HCC recorded at baseline and following exercise in human subjects are similar to those observed following *in vitro* oxidation of fatty acid substrates and thus suggests that the free

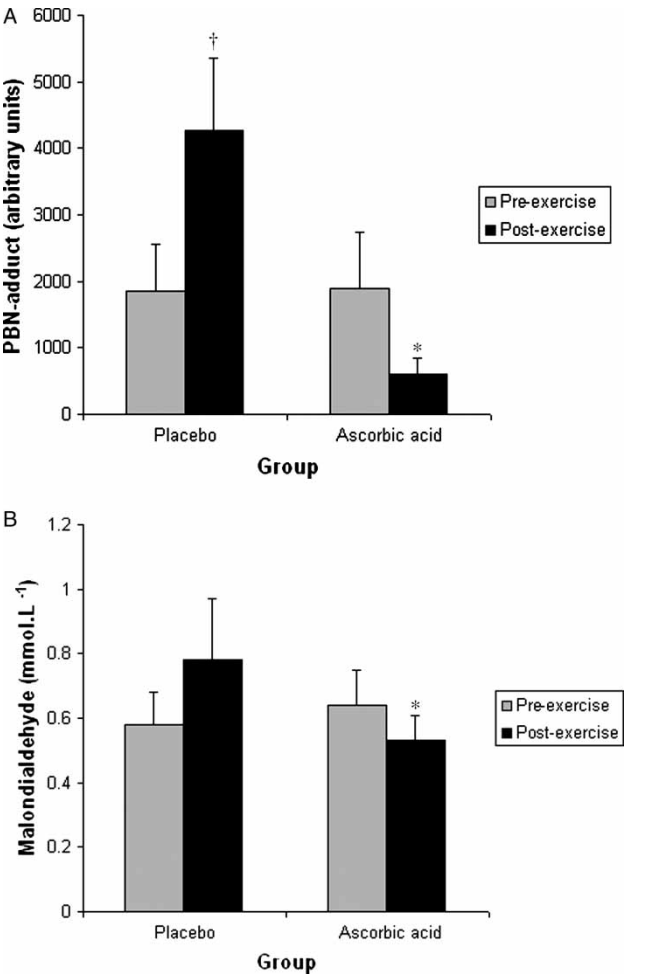

Figure 2. Effects of oral ascorbate prophylaxis against exercise-induced PBN-adduct (A) and malondialdehyde formation in venous blood. (B) † denotes a difference within groups ($p < 0.05$), while * denotes a difference between groups as a function of state ($p < 0.05$). A main effect for treatment was also observed for PBN-adduct and malondialdehyde concentration (pooled data, ascorbic acid vs placebo, $p < 0.05$).

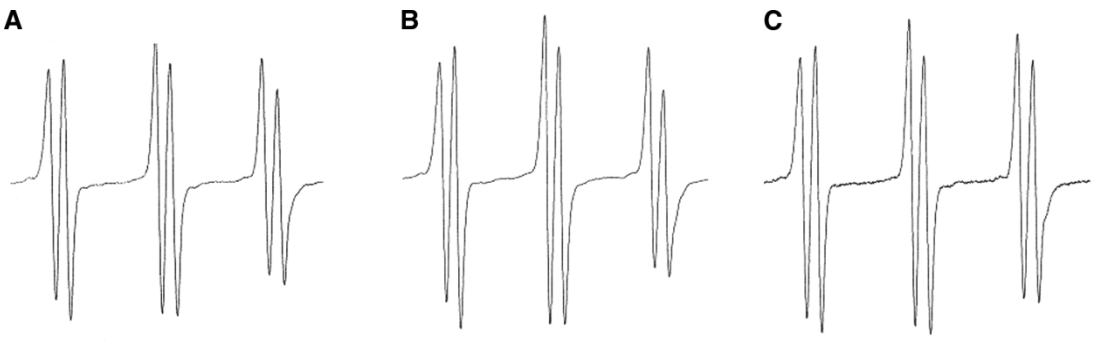

Figure 3. Typical EPR spectra of PBN-adducts extracted *in vitro* following auto-oxidation of linoleic (A), α-linolenic (B) and arachadonic acid (C) in room air. Nuclear hyperfine splittings were $a_N = 13.6$ G and $a\beta_H$ 1.8 G for all adducts detected. All spectra were filtered and scaled identically.

radicals detected following human exercise are possibly derived from circulating PUFA.

Identity, source and mechanisms of free radical formation

In studies examining the relationship between systemic free radical accumulation and exercise, a similar source to the above involving lipid peroxidation and subsequent radical generation has been suggested. For example, Davison et al. [9,10] postulates that the HCC for all *ex vivo* PBN trapped radicals detected from human blood following exercise are oxygen-centred lipid alkoxyl free radicals, possibly derived from oxidative damage to cellular membranes. In addition, it is thought that these species are likely to be secondary or even tertiary radicals. These studies also observed an increase in lipid hydroperoxides (LOOH), a major reactant of lipid oxidation, and further claim that the increased detection of lipid-derived free radicals and LOOH concentration strongly supports the presence of increased oxidative damage to lipid membranes. In further support, Bailey et al. [11] have recently shown an increase in PBN-adducts from rest to exercise in

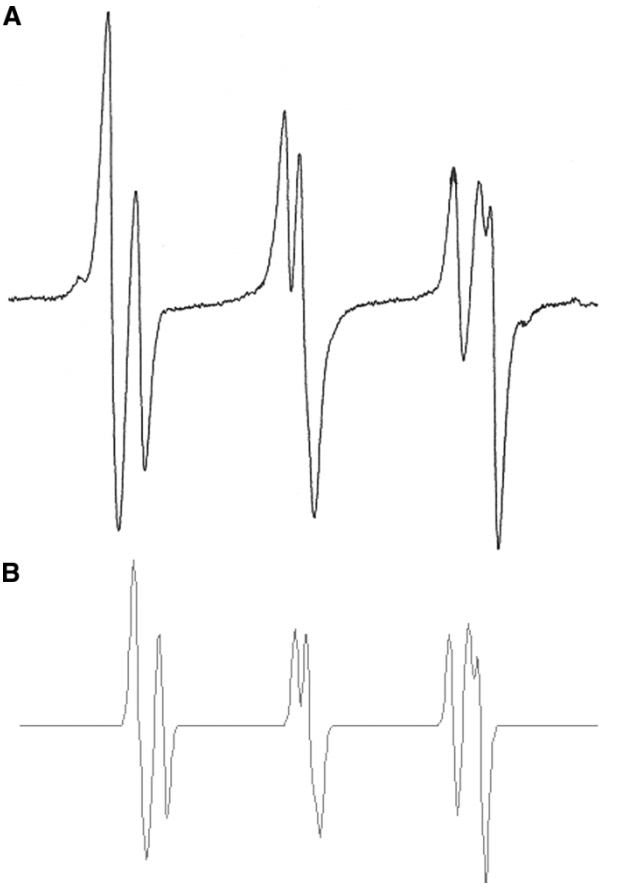

Figure 4. Typical EPR spectra of PBN-adducts extracted *in vitro* following auto-oxidation of L-α-phosphatidycholine in room-air (A). Computer simulation (B) identified two primary species with nuclear hyperfine splittings of a_N 13.6 G and $a\beta_H$ 1.8 G and for L-α-phosphatidycholine $a_{N1} =$ 13.4 G, $a\beta_{H1} = 1.6$ G (37%) and $a_{N2} = 14.9$ G, $a\beta_{H2} = 0.3$ G (63%). All spectra were filtered and scaled identically.

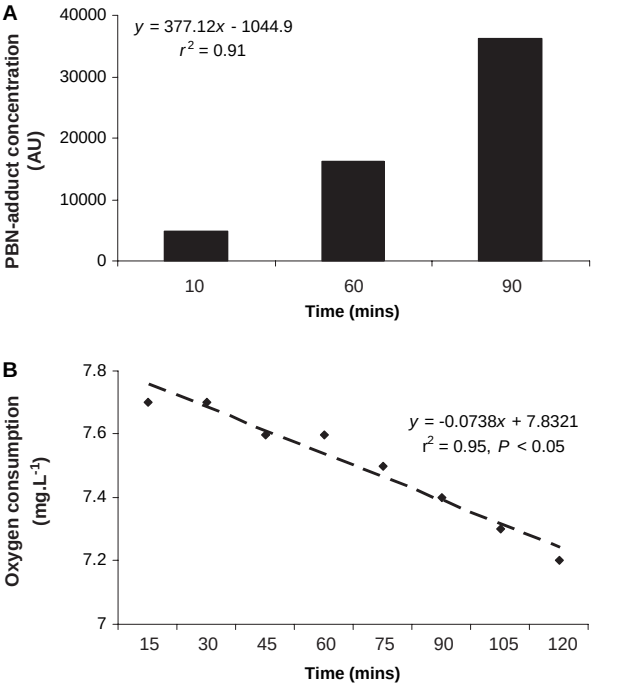

Figure 5. Rate kinetics of lipid peroxidation (A) and oxygen consumption (B) during auto-oxidation of α-linolenic acid in room air. Rate of PBN-adduct formation and oxygen consumption was calculated as ≈394 arbitrary units (AU)/min and ≈4.8 μg/L/min, respectively.

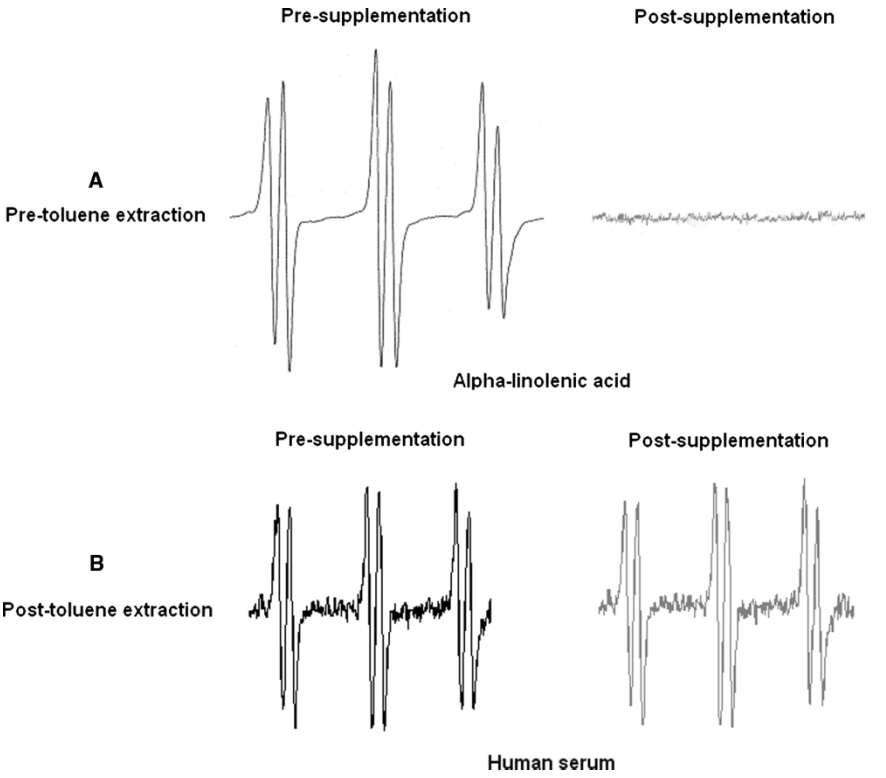

Figure 6. Scavenging of PBN-adducts by ascorbate (final concentration 100 μmol) during the auto-oxidation of α-linolenic acid prior to toluene extraction (A). PBN-adduct concentration did not change when ascorbate (100 μmol final concentration) was added following toluene extraction of PBN-adducts from human serum and immediately prior to degassing (B). The latter indicated that ascorbate did not diminish trap-radical adduct stability.

human muscle (a_N 13.7 G and $a\beta_H$ 1.9 G) and suggested that the trapped species are likely derived from tissue membrane phospholipids. It is important to emphasize that whereas EPR is considered the most direct, specific and sensitive analytic technique for the molecular detection of free radical species [14], the exercise related spin trapping approach employed in the present study still relies on *ex vivo* detection of resonance-stabilized reactants formed clearly downstream of the primary production pathway that we assume reflects events initiated *in vivo*.

Multiple factors including the rate of superoxide ($O_2^{\bullet -}$) production and the activity/concentration of the antioxidants in blood dictate the extent of lipid radical accumulation following exercise. One potential mechanism involving the production of $O_2^{\bullet -}$ is the oxidative burst. Activated vascular polymorphonuclear leukocyte cells can increase membrane-bound NADPH oxidase activity, which subsequently may reduce molecular oxygen to $O_2^{\bullet -}$ [15]. However, using a comparable *ex vivo* spin trapping technique using 5,5-dimethylpyrroline-*N*-oxide, we have not been able to detect any 'upstream' initiators of lipid peroxidation namely blood-borne $O_2^{\bullet -}$ and the hydroxyl radical ($^{\bullet}OH$) (Bailey et al., unpublished observations).

It is conceivable, however, that transition metal ions (specifically Fe^{2+}) released from metalloproteins during the exercise phase, which in addition to $O_2^{\bullet -}$ release from activated polymorphonuclear leukocytes, may have catalysed the reductive decomposition of existing LOOH to form secondary and EPR detected lipid free radicals. Although a definitive assignment is unrealistic in light of the complex nature of human blood, the fact that comparable spectral characteristics were observed following extraction of the aqueous reaction mixture containing Fe^{2+} and Cu-OOH *in vitro* further suggests that these secondary species may have evolved during the metal-catalysed reductive decomposition of LOOH *ex vivo* subsequent to primary radical (recently identified as mitochondrial $UQ^{\bullet -}$)-mediated damage to tissue membrane phospholipids initiated *in vivo* [11]. Furthermore, we can't rule out the possibility of primary free radicals being generated as a result of the oxidative burst during the incubation phase of the PBN/blood mixture, thereby increasing the production of lipid radicals *ex vivo*.

Although the circulating concentrations of antioxidants were not measured in this study since we chose specifically to focus on free radicals as our primary outcome variable, they have clear implications for exercise-induced oxidative stress. Davison et al. [9] observed a decrease in the venous concentration of α-tocopherol, α-carotene and retinol in the presence of raised levels of LOOH and identical PBN-adducts following exhaustive exercise, while Ashton et al. [16] demonstrated an attenuation of the exercise-induced

increase in PBN-adducts following ascorbic acid supplementation. The above clearly suggests that exercise-induced lipid free radical generation may be due in combination to an over-production of primary stimulated free radical species and an inadequate antioxidant defence system. Alternatively ascorbic acid prophylaxis may have the potential to promote iron-induced lipid-derived alkoxyl and alkyl free radical release via a Fenton-type reaction initiated during ischemia [17].

The EPR spectra observed following auto-oxidation of α-linolenic acid exhibits identical HCC to those of human blood which partially confirms that the free radical species detected *ex vivo* are lipid-derived in origin. A number of *in vitro* studies reported similar HCC to those observed in the present experiments. Using ozone to induce PUFA oxidation, Pryor et al. [1] detected PBN-adducts from methyl linoleate and suggested that the HCC (a_N 13.7 G and $a\beta_H$ 1.8 G) were indicative of a lipid-derived radical. In a further study, Dikalov and Mason [18] using the spin trapping technique detected lipid free radicals from the oxidation of linoleic, arachidonic and linolenic acid. Iwahashi et al. [19] also reported HCC of $a_N = 15.8$ G and $a\beta_H = 2.2$ G from the *in vitro* oxidation of arachidonic acid and suggest they resemble the trapping of PBN lipid-derived free radicals. The fact that the HCC in the latter study are slightly higher than those reported in the present work is likely attributable to line broadening since samples were not subject to vacuum degassing. Aside from PBN, Witting et al. [20] detected lipid radicals from the oxidation of L-α-phosphatidylcholine using DMPO and, while using the spin trap PBN, we detected two different lipid-derived free radical species following the *in vitro* auto-oxidation of L-α-phosphatidylcholine. Computer simulation confirmed the presence of two primary species ($a_{N1} = 13.4$ G and $a\beta_{H1} = 1.6$ G, 37%, and $a_{N2} = 14.9$ G and $a\beta_{H2} = 0.3$ G, 63%) that were consistent with the trapping of the alkoxyl and oleate radical, respectively [21].

Oxygen uptake was shown to increase over the duration of α-linolenic acid oxidation. This finding is in agreement with the work of Mason et al. [22], who showed an increase in oxygen consumption with increasing arachidonic acid free radical formation. It is proposed that oxygen is required in increased concentrations during increased rates of lipid peroxidation. Oxygen is fundamental to the progression of lipid peroxidation, as it binds to lipid-derived radicals ($L^{\cdot}$) to generate the peroxyl radical [3] as illustrated in equation (1):

$$\mathrm{PUFA} \xrightarrow{H^+} L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \longrightarrow \mathrm{LOOH} \longrightarrow LO^{\cdot} \quad (1)$$

where: 1: *PUFA, polyunsaturated fatty acids*; H^+, *hydrogen ion*; $L^{\cdot}$, *lipid radical*; O_2, *oxygen*; $LOO^{\cdot}$, *peroxyl radical*; *LOOH, lipid hydroperoxides*; $LO^{\cdot}$, *alkoxyl radical*. Our data suggests that oxygen is fundamental for both peroxyl and alkoxyl free radical formation.

It is proposed that this binding/formation would inevitably continue the lipid peroxidation cascade to generate lipid radicals [7] as demonstrated by EPR detection in the present experiment.

A time course experiment of α-linolenic was performed and monitored by EPR. There was a three-fold increase in free radical concentration throughout the period of α-linolenic oxidation. This data shows an accumulation of lipid-derived free radicals over time and suggests a proportionate increase in oxidative damage. The rate of PBN-adduct formation and oxygen consumption was calculated at ≈394 arbitrary units (AU)/min and ≈4.8 μg/L/min, respectively.

Scavenging of lipid-derived free radicals by ascorbic acid

All *in vitro* EPR signals were undetectable following addition of the ascorbic acid to the incubation lipid-aqueous mixtures during the course of adduct formation (Figure 6A). An important control experiment identified that ascorbate addition to a sample following adduct extraction did not alter EPR signal amplitude (Figure 6B), indicating that ascorbate did not simply destroy the trap-radical adduct by reducing the nitroxide to an 'EPR-silent' hydroxylamine. Thus, we are confident that the near ablation of the PBN-adduct signal observed following ascorbate prophylaxis in both *in vivo* and *in vitro* systems can indeed be attributed to its authentic ability to scavenge peroxyl radicals and terminate lipid peroxidation by inhibiting formation of the alkoxyl radical. Furthermore, ascorbate prophylaxis was also shown to attenuate the exercise-induced increase in malondialdehyde in the human circulation, thus adding further support to this contention. Previous work has shown a decrease in peroxyl radical detection in the presence of ascorbic acid during methyl linoleate oxidation [23]. Frei et al. [24] have also shown that ascorbic acid can protect lipids against oxidative damage induced by peroxyl radicals. Scarpa et al. [25] have demonstrated a decrease in the peroxidation rate of soybean L-α-phosphatidylcholine liposomes in the presence of ascorbic acid and α-tocopherol. These authors suggested that the α-tocopherol radical generated in the phosphatidylcholine bilayer is regenerated by ascorbic acid and the scavenging effect of α-tocopherol on lipid peroxidation is maintained only in the presence of ascorbic acid. This is supported by Leung et al. [26], who provides evidence for the synergistic effect of ascorbic acid and α-tocopherol as inhibitors of lipid peroxidation in model systems.

Conclusion

These experiments demonstrate that auto-oxidation of substrate lipids can generate free radical species. It is also postulated that the PBN-trapped free radicals measured via EPR spectroscopy following human exercise are likely derived from the oxidation of polyunsaturated fatty acid membranes. The species are identified as downstream lipid-derived oxygen-centred free radicals. What is not determined however from the present research is the contribution of individual membrane lipid oxidation to the overall free radical concentration detected following exercise. Furthermore, clear *ex vivo* and *in vitro* evidence suggests that ascorbic acid is an effective antioxidant when required to terminate lipid peroxidation and inhibit the generation of oxygen-centred alkoxyl radicals.

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