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SPIN TRAPPING OF FREE RADICALS FORMED DURING PEROXIDATION OF SARCOLEMMAL MEMBRANES (SLM)

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The generation of free radicals in a superoxide ($O_2^{\cdot -}$) driven Fe^{+3} catalysed reactions with isolated myocytic sarcolemma using electron spin resonance was investigated. Incubation of highly purified canine myocytic sarcolemma in the presence of the spin trap, 2-methyl-2-nitrosopropane (MNP), followed by the addition of dihydroxyfurmarate (DHF) and Fe^{+3}-ADP resulted in the generation and detection of radical adducts of this spin trap. Spin trapping of the alkyl radicals with 2-methyl-2-nitrosopropane led to the identification of methyl radical adduct following exposure to DHF/Fe^{+3}-ADP. With sarcolemma and the alkyl nitroso compound, the only radical product trapped was the methyl radical formed by β-scission of alkoxyl radical. The participation of hydroperoxide-derived radicals in this system verified that the decomposition of unsaturated hydroperoxy fatty acid does proceed via a free radical mechanism.

KEY WORDS: Sarcolemmal membranes, lipid peroxidation, alkyl radical, hydroxyl radical, superoxide anion, spin trapping.

ABBREVIATIONS: SLM, sarcolemmal membranes; MNP, 2-methyl-2-nitrosopropane; DHF, dihydroxyfumarate; ADP, adenosine-5′diphosphate; ESR, electron spin resonance; SOD, superoxide dismutase; DMPO, 5,5′-dimethyl-1-pyrroline-N-oxide; DTBN, di-tert-butyl-nitroxide; PUFA, polyunsaturated fatty acid.

INTRODUCTION

In many pathological states such as ischemia and reperfusion, there is an increased production of reduced-oxygen intermediates, which are capable of extensive tissue damage.[1,2] These reduced-oxygen intermediates include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), which are highly reactive, short-lived species. The myocytic sarcolemma have been proposed to be the principal target organelles of free radical attack in the myocardial injury.[3] In addition, oxygen free radicals have been shown to disrupt calcium transport by cardiac muscle sarcoplasmic reticulum.[4] Since an oxygen radical mediated mechanism is now thought to be of pathogenic relevance in the genesis of myocardial membrane injuries, but has not yet been demonstrated directly, the aim of this study was to use the spin-trapping

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ESR technique to monitor suggested radicals. The role of free radicals in stimulating lipid peroxidation of myocardial sarcolemma was studied by adding a free radical generating system [dihydroxyfumarate + Fe^{+3}-ADP] to purified myocytic sarcolemmal membranes (SLM).

MATERIALS AND METHODS

Dihydroxyfumarate (DHF), adenosine-5′-diphosphate (ADP), ferric chloride ($FeCl_3$–$6H_2O$), mannitol, superoxide dismutase (SOD, specific activity 3080 units/mg), and catalase (specific activity 24,000 units/mg) were from Sigma Chemical Co. (St. Louis, MO). All chemicals were of the highest purity commercially available. 2-Methyl-2-nitrosopropane (MNP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Aqueous solutions of MNP were prepared in water by adding 25 mg of the spin-trap to 10 ml of H_2O and stirring in the dark at 45 °C for 2 hours.[5] Sarcolemmal membranes were prepared from adult canine myocytes as described previously.[6] The sarcolemmal fractions were enriched at least 50-fold in the activity of the marker enzyme Na, K-ATPase. The free radicals were generated during the autooxidation of DHF (0.33 mM) in the presence of $FeCl_3$ (6.25 uM) chelated by ADP (62.5 uM).[7,8] Unless otherwise stated, SLM (100 ug protein/ml) were suspended in a buffer containing 120 mM KCl, 50 mM sucrose and 10 mM potassium phosphate, pH 7.2, before addition of the spin trap. After 10 min. of incubation at 37 °C in the presence of the spin trap agent, the lipid peroxidation was initiated by the final additions of DHF and Fe^{+3}-ADP. For the MNP experiments, incubations were carried out at 37 °C in a total volume of 0.5 ml. Mixtures contained the incubation buffer, pH 7.2, 100 ug/ml of membranes, 0.017 M MNP, 0.33 mM DHF and 6.25 uM Fe^{+3}-ADP. After addition of DHF/Fe^{+3}-ADP, the system was rapidly transferred to an aqueous flat cell for ESR examination at 25 °C. Intervention studies were carried out by adding different concentrations of mannitol (10 mM), catalase (15 ug/ml), SOD (15 ug/ml) or SOD plus catalase (15 ug/ml) to the system. ESR observations were made at room temperature, 22 °C, with a Bruker ER-100 spectrometer equipped with a TM_{110} cavity and a 100 KHz field modulation. The spin concentration was determined by double integration of the ESR signal and comparison with the double integral of the signal from an aqueous solution of 3-carbamoyl-2,2,5,5-tetramethylpyrrolin-1-yloxy (from Aldrich). All hyperfine splitting constants were obtained from spectra simulated on an IBM/PC/XT computer system using ESRSIM software.

RESULTS

When myocytic sarcolemmal membranes in the presence of the spin trap MNP were exposed to the oxygen radical generating system, DHF/Fe^{+3}-ADP, the ESR spectrum in Figure 1A was obtained. Initial analysis of this ESR spectrum shows that it consists of at least three different MNP spin adducts, in addition to the large triplet (labeled S, a_N = 17.2 G) corresponding to di-tertiary-butyl-nitroxide (DTBN), an impurity frequently formed in experiments using MNP.[5] The most clearly observed MNP adduct in Figure 1A consists of a triplet of quartets arranged in a 1:3:3:1 pattern. This indicates that the unpaired nitroxide electron is interacting equally with three beta hydrogens suggesting that the ESR spectrum corresponds to the methyl radical

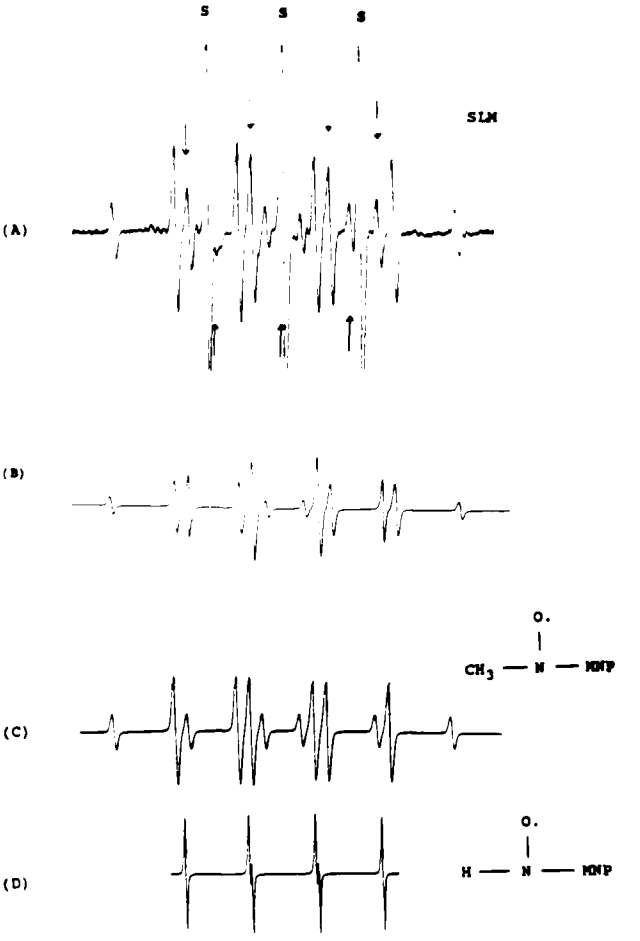

FIGURE 1 The ESR spectra of MNP radical adducts formed during peroxidation of sarcolemmal membranes (SLM). The composition and conditions of incubation of the system as well as the manner in which the ESR observations were made are described under "Materials and Methods." (A) ESR spectrum of MNP–SLM 26 min after starting incubation; SLM (100 ug protein/ml) were suspended in a buffer containing 120 mM KCl, 50 mM sucrose and 100 mM potassium phosphate, pH 7.2. Mixture contained the incubation buffer, 100 ug/ml of membrane, 0.017 M MNP, 0.33 mM DHF and 6.25 uM Fe^{+3}-ADP in a total volume of 0.5 mL. (B) computer simulations of spectra C–D; (C) simulated spectrum of MNP/CH_3 adduct; (D) simulated spectrum of MNP/H adduct; Instrumental conditions were: power = 10 milliwatts, modulation amplitude = 1.00 G, time constant = 0.5 s, scan time = 16 min, scan range 100 G, gain 1.25×10^5.

adduct of MNP (MNP–CH_3). The additional MNP adducts are marked in Figure 1A with arrows. Although it is hard to exactly identify these adducts, the spin adduct indicated by arrow pointing down (↓) is observed as a quartet but is possibly a triplet of doublets. This would be the case if the unpaired nitroxide electron is interacting with a single hydrogen nucleus suggesting that the triplet of doublets corresponds to the hydrogen adduct of MNP (MNP–H). Because of the large intensity of the DTBN

triplet in Figure 1A, not much can be said about the MNP adduct indicated by arrows pointing upward, except that it consists of a triplet. Figure 1B is the addition of the computer generated ESR spectra shown in Figure 1C an d D. Figure 1C is the computer simulation that best matches the experimental triplet of quartets (Figure 1A). This ESR spectrum was generated using hyperfine coupling constants, a_N = 17.4 G and a_H^β = 14.37 G. These hyperfine coupling constants are consistent with those previously reported for the MNP–CH_3 spin adduct.[9] Figure 1D is the computer simulation that best matches the apparent quartet (↑) in Figure 1A. The hyperfine coupling constants, a_N = 14.70 G and a^α = 14.0 G, used to generate the ESR spectrum in Figure 1D are consistent with those reported for the MNP–H adduct.[9]

In order to determine the nature of the MNP adduct indicated in Figure 1A by the arrows pointing upward, the sample was extracted with petroleum ether to eliminate the DTBN impurity. Figure 2A shows the ESR spectrum obtained following this extraction. Although it is possible that the MNP–H adduct may have been lost during the extraction, the MNP–CH_3 adduct in addition to a well defined triplet is still present. Figure 2B is the addition of the computer generated ESR spectra in Figure 1C (MNP–CH_3) and Figure 2C. This spectrum shows a good match to the experimentally obtained ESR spectrum in Figure 2A. The computer simulated ESR spec-

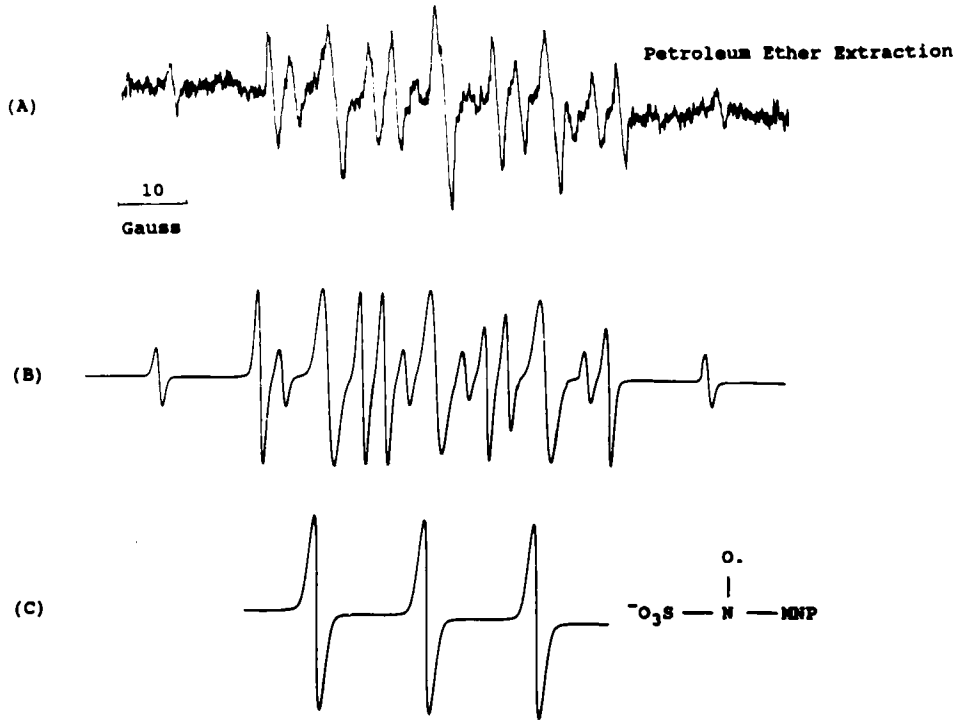

FIGURE 2 The ESR spectra of MNP adducts formed after petroleum ether extration of SLM + MNP/DHF/Fe^{+3}-ADP under the same experimental conditions, pH 7.2, as for Figure 1A., A. B, computer simulations of spectra of MNP–CH_3 adduct and C, simulated spectrum of MNP/SO_3^- adduct. Instrumental condition as for Figure 1.

trum in Figure 2C matches the triplet in Figure 2A. This ESR spectrum was generated using a hyperfine coupling constant a_N = 14.75 G. This value is consistent with the reported hyperfine coupling constant for the sulfite radical adduct of MNP (MNP-SO_3^-).[9]

The results obtained in Figures 1 and 2 have the formation of methyl, hydrogen and sulfite adducts of MNP when sarcolemmal membranes incubated with MNP are exposed to DHF/Fe^{+3}-ADP.

DISCUSSION

Several studies were done in order to determine the origin of MNP adducts observed in Figures 1 and 2. In the absence of DHF/Fe^{+3}ADP from the sarcolemmal membrane system containing MNP, no spin adduct spectra were observed. To determine whether the methyl adduct may originate from the DHF itself, a control experiment using MNP in the presence of DHF/Fe^{+3}-ADP and in the absence of sarcolemmal membranes was carried out. An identical experiment using the spin trap DMPO instead of MNP was also carried out. The reagent concentrations in these experiments were the same as those used in the sarcolemmal membrane experiments. No ESR spectrum was obtained in the experiment with MNP (Figure 3A). This is not unusual

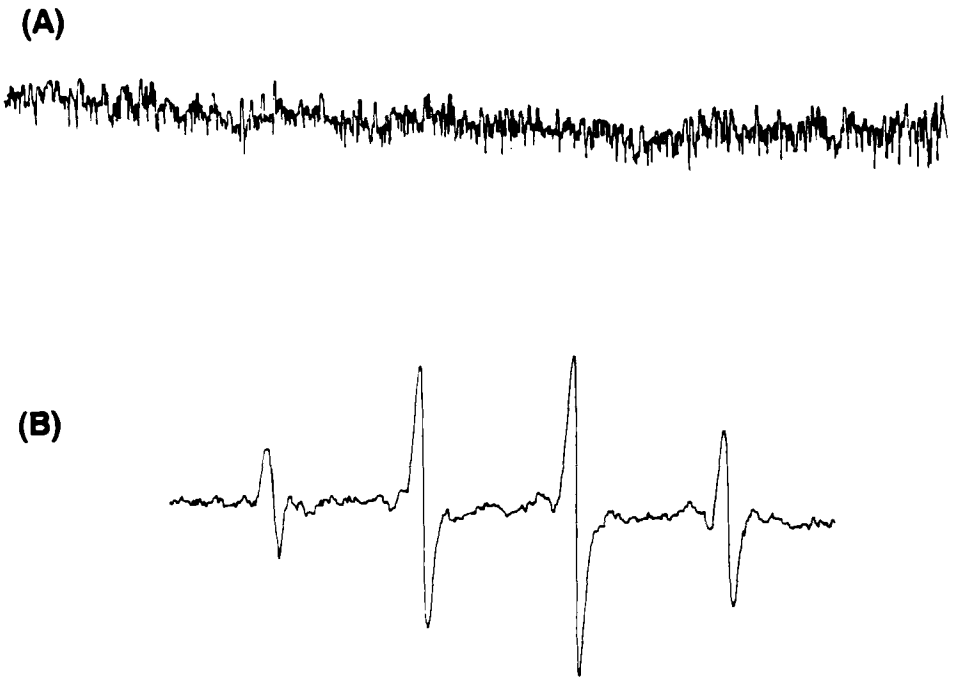

FIGURE 3 ESR spectra of the free radicals generated system (DHF/Fe^{+3}-ADP): (A) Mixture containing 0.33 mM DHF in the presence of $FeCl_3$ (6.25 uM) chelated by ADP (62.5 uM) and 0.6 mg/ml of MNP in KSP buffer, to give a final pH of 7.2. (B) Same conditions as A, except in this case the spin trap 5,5′-dimethyl-1-pyrroline-N-oxide, DMPO, 90 mM in KSP was used.

because the autooxidation of DHF produces $O_2^{\cdot-}$ which in the presence of Fe^{+3}-ADP leads to the formation of ·OH (Figure 3B). The MNP adducts of these oxygen-centered radicals are unstable and immediately disappear.

However, in the experiment containing DMPO an ESR spectrum was obtained consisting of a 1:2:2:1 quartet with hyperfine coupling constants, $a_N = a_H^\beta = 14.9$ G. This is the DMPO-OH adduct.[10] These results suggest that the MNP adducts observed in Figures 1 and 2, obtained in experiments containing the sarcolemmal membranes, are from secondary radicals originating from reactions involving either $O_2^{\cdot-}$ or ·OH.

To further substantiate these observations, experiments in which various sarcolemmal membrane preparations were incubated at 37 °C with MNP for 10 min and further incubated for an additional 5 min, prior to addition of DHF/Fe^{+3}-ADP, each with one of the following: superoxide dismutase (SOD, 15 ug/ml), catalase (15 ug/ml), SOD plus catalase (15 ug/ml each) and mannitol (10 mM). No MNP spin adduct spectra were obtained in the presence of SOD, catalase, or SOD plus catalase. The results obtained from the SOD and catalase experiments suggest that $O_2^{\cdot-}$ produced by the autooxidation of DHF is the initiating species. However, in the absence of SOD and catalase but in the presence of the hydroxyl radical scavenger, mannitol, the MNP spin adduct spectra in Figures 1 and 2 were also quenched. This result suggests that the $O_2^{\cdot-}$ formed did not directly react with sarcolemmal membrane constituents but disproportionated forming H_2O_2 which in the presence of trace amounts of ferrous ions forms ·OH which are the primary reactive species leading to the formation of the radicals trapped by MNP (Figures 1 and 2). The results suggest that the spin adducts observed in Figures 1 and 2 originate from hydroxyl radical initiated lipid peroxidation of the sarcolemmal membrane. In support of lipid peroxidation occurring in sarcolemmal membranes exposed to DHF/Fe^{+3}-ADP is the stimulation of large increases in malondialdehyde (MDA) formation.[11] Initiation of lipid peroxidation in a membrane is due to the attack of any species that has sufficient reactivity to abstract a hydrogen atom (eq. 1).

$$\text{Lipid-H} + \cdot\text{OH} \rightarrow \text{Lipid}\cdot + \text{H}_2\text{O} \quad (1)$$

The carbon radical in a polyunsaturated fatty acid tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with O_2 to give a hydroperoxyl radical (eq. 2)

$$\text{Lipid}\cdot + \text{O}_2 \rightarrow \text{Lipid} - \text{O}_2^{\cdot} \quad (2)$$

(after molecular rearrangement)
Hydroperoxyl radicals abstract hydrogen atoms from other lipid molecules and so continue the chain reaction of lipid peroxidation (eq. 3).

$$\text{Lipid} - \text{O}_2^{\cdot} + \text{Lipid} - \text{H} \rightarrow \text{Lipid} - \text{O}_2\text{H} + \text{Lipid}\cdot \quad (3)$$

Reduced iron compounds react with lipid hydroperoxides (Lipid–O_2H) to give alkoxyl (lipid–O·) radicals.

$$\text{Lipid} - \text{O}_2\text{H} + \text{Fe}^{+2} - \text{complex} \rightarrow \text{Fe}^{+3} - \text{complex} + \text{OH}^- + \text{Lipid-O}\cdot \quad (4)$$

Hydroperoxides of PUFA generate alkoxyl radicals on reduction by Fe(II). The alkoxyl radicals may undergo either β-scission, as in Figure 4, or epoxidation. β-scission in fatty acids may give a variety of products. Our results are consistent with the known chemistry of the radical decomposition of a tertiary organic hydroperoxide

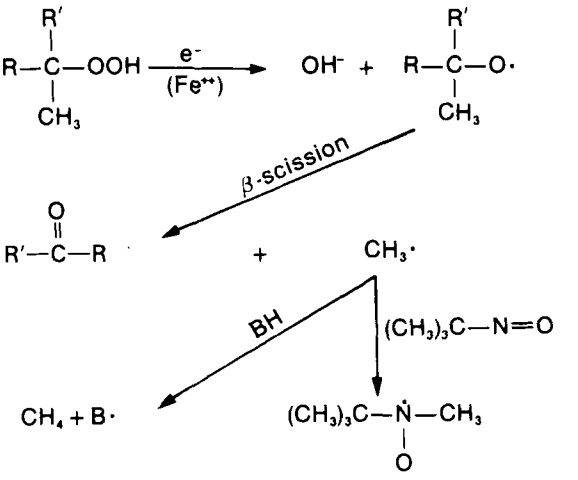

FIGURE 4 Proposed mechanism for the initiated radical decomposition of a lipid hydroperoxide, including the trapping of MNP. BH is an electron donor.

which undergoes one-electron reduction, then β-scission to yield a methyl radical and a diamagnetic specie.[12,13] The reaction scheme shown in Figure 4 provides a possible explanation of the experimental results obtained by the use of one nitroso spin trap as a probe of the reaction of DHF/Fe^{+3}-ADP with sarcolemmal membranes. Support for this proposal includes: 1) spin trapping studies of the chemical decomposition of fatty acid hydroperoxides[14] and (2) radical decomposition of other organic hydroperoxides catalyzed by hemeproteins.[13] In this work the methyl adduct of MNP was identified as the principal radical trapped in the myocytic sarcolemmal membrane system. The hydrogen atom adduct is unexpected and probably arises from the reduction of MNP.[15] A species such as the hydroxymethyl radical is a strong reducing agent.[16] This species could be formed as one product of the reaction of oxygen with the methyl radical. In any case, the hydroxymethyl radical has been proposed to reduce MNP to its hydrogen atom adduct.[17] The exact origin of the sulfite radical is unclear. Since this radical is a natural product of cysteine degradation, it may have originated from protein constituents in the membrane or from the culture media used in the isolation of sarcolemmal membranes.

In the present study, we have provided direct *in vitro* evidence that the exogenous generation of oxygen free radicals induces lipid peroxidation in myoctic sarcolemma. Postulated steps of the lipid peroxidation reaction are given in eqs. 1–4. Long chain polyunsaturated fatty acids are oxidized, particularly by the action of oxygen free radicals, and degraded, via a chain reaction, ultimately to short chain fragments. When such oxidative catabolism takes place in the membrane lipid bilayer, its consequences are grave, as free radical damage results in fatty acid oxidation, aldehyde formation, amino acid oxidation, cross-linking of proteins and lipids, protein strand scission, etc., resulting in impairments of the permeability, substrate and ion transports, and receptor function of membranes.[18,19]

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