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EFFECTS OF FREE RADICALS ON THE FLUIDITY OF MYOCARDIAL MEMBRANES

M. BAGCHI[1], M.R. PRASAD[1], R.M. ENGELMAN[2] and D.K. DAS[1]

Cardiovascular Division, Department of Surgery, University of Connecticut School of Medicine, Farmington, Connecticut 06032 and Department of Surgery[2], Baystate Medical Center, Springfield, Massachusetts 01107

Free radicals, including superoxide anions ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and hypohalite radical ($OCl^{\cdot}$), as well as oxidants such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), have been indicated in the pathogenesis of myocardial ischemic and reperfusion injury. In this report, we compared the integrity of the myocardial membrane when exposed to these free radicals/oxidants. Isolated rat heart membrane preparations were exposed to chemically generated free radicals with or without their respective scavengers. Membrane fluidity was monitored by fluorescence polarization using the diphenylhexatriene probe, as well as by electron spin resonance (ESR) spectroscopy using 2,2,6,6-tetramethyl piperidine-n-oxyl as the spin labeling agent. $HO^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ + HOCl increased the fluorescence polarization (FP) and microviscosity significantly by 1.7-fold, 1.8-fold, and 1.7-fold, respectively, as compared to an only 1.2-fold increase in FP by $O_2^{\cdot -}$. $O_2^{\cdot -}$ did not alter the fatty acid profiles of the membrane phospholipids. However, $HO^{\cdot}$ and H_2O_2 reduced the arachidonic acid contents in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). These radicals also stimulated the lipid peroxidation by several-fold, while that by $O_2^{\cdot -}$ was only insignificant. These results suggest that $HO^{\cdot}$ and H_2O_2 decreased the membrane fluidity and induced lipid peroxidation by releasing the arachidonic acid from PC, PE, and PI.

KEY WORDS: Membrane fluidity, heart, free radicals, microviscosity, phospholipids, arachidonic acid.

INTRODUCTION

Oxygen-derived free radicals have been implicated in the pathogenesis of myocardial ischemic-reperfusion injury leading to the membrane perturbation.[1,2] The phospholipids that constitute the myocardial membrane, especially those lipids containing unsaturated double bonds, are susceptible to free radical attack leading to the formation of lipid peroxides and aldehydes.[3,4] Under a normal physiological state, the lipids in membranes are arranged in the form of a bilayer where the fluidity of lipids is operationally confined to the hydrocarbon region and where the asymmetric features are less pronounced than those around the lipid head groups. It is, therefore, conceivable that if unsaturated fatty acids are released from the membrane phospholipids or undergoes lipid peroxidation, the fluidity will be modified and will influence the dynamics of functional units (protein) which are embedded in the lipid matrix. Although it has been conclusively demonstrated that breakdown of membrane phospholipids and lipid peroxidation occur during the reperfusion of ischemic myocardium[5] and that this breakdown process is probably mediated by free radicals,[1,5] nothing is known regarding the changes in myocardial membrane fluidity

Mail correspondence to: Dipak K. Das, Ph.D., Director, Cardiovascular Division, Department of Surgery, University of Connecticut School of Medicine, Farmington, Connecticut 06032, (203) 679-3687.

during ischemia and reperfusion and how it is related to free radical injury. The present study examines the effects of the potential free radicals as oxidants that might be generated in the reperfused heart on the fluidity of the myocardial membrane.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 200 gm were anesthetized with intraperitoneal pentobarbital. Hearts were quickly removed and kept in ice-cold Tris-sucrose buffer (pH 7.4). Hearts were then homogenized in the same buffer, using a Polytron Homogenizer (Brinkman, NY). Subcellular fractionations were achieved by centrifuging the tissue homogenates, and membranes were isolated between 12,500 g and 105,000 g. Isolated membranes were further purified by repeated washings and recentrifugation.

Free radicals were generated by the chemical reactions. Superoxide anions ($O_2^{\cdot -}$) were generated by the action of xanthine oxidase (XO) (8 mU/ml) on hypoxanthine (100 μM), whereas hydroxyl radicals ($HO^{\cdot}$) were formed by further adding $FeCl_3$ (100 μM) and EDTA (100 μM) to the $O_2^{\cdot -}$ generating system. Hypohalite radicals ($OCl^{\cdot}$) and hypochlorous acids (HOCl) were generated by the action of a weak acid on 1 mM sodium hypochlorite.

Generation of $O_2^{\cdot -}$ was monitored from the extent of cytochrome C reduction, as described previously.[6] The presence of $HO^{\cdot}$ was confirmed by assaying salicylate-$HO^{\cdot}$ spin adducts using High Pressure Liquid Chromatography.[7] The generation of all the free radicals, including $O_2^{\cdot -}$, $HO^{\cdot}$, and $OCl^{\cdot}$, was confirmed by the luminol-chemiluminescence assay using a luminometer.[8]

The myocardial membranes were then exposed to various free radical generating systems and oxidants in the presence and absence of their corresponding scavengers. The reaction mixture was incubated at 37°C for 30 min. The reaction was then terminated by layering the reaction mixture on 0.25 M sucrose solution, followed by sedimentation of the membranes by ultracentrifugation. 1,6-diphenyl-1,3,5-hexatriene (DPH, 0.5 M) was added to the membrane suspension, which was incubated for 2 hr at 25°C, and then left overnight at 4°C. Fluorescence polarization, a measure of membrane fluidity, was determined at 25°C with a Perkin Elmer spectrofluorimeter equipped with perpendicular and parallel polarizers, using an excitation wavelength of 365 nm and an emission of 430 nm.[9] Fluorescence polarization was calculated as

$$P = \frac{I_{II} - I_I}{I_{II} + I_I} \quad (1)$$

where I_{II} and I_I are the fluorescence emission intensities measured parallel and perpendicular to the polarization axis of the polarizer, respectively. Apparent microviscosity was calculated as

$$(\eta) = \frac{2P}{0.46 - P} \quad (2)$$

where P is polarization calculated from above.

The fluidity of membranes was further examined by electron paramagnetic resonance (EPR) spectroscopy using 7-doxyl spin-labeled stearic acid as the spin-labeling probe, which is known to partition between aqueous and hydrophobic

regions of the biologic membranes.[10] Tempo (5×10^{-3} M) was attached to the isolated myocardial membrane by incubating the membrane in the same way as DPH. Order parameters 'S' and correlation times 'T_c' were calculated from the following equation:

$$S = \frac{A_{II} - A_I - C}{A_{II} + 2A + 2C} \times 1.66 \quad (3)$$

where A_{II} and A_I are apparent parallel and perpendicular hyperfine splitting parameters of the EPR spectrometer. The constant $C = 1.4 - 0.053\,(A_{II} - A_I)$ is an empirical correction for the difference between the true and apparent values of A_I, and the factor 1.66 is a solvent polarity correction factor.[10]

RESULTS AND DISCUSSION

In this study, we used fluorescence depolarization techniques as well as spin-label techniques to investigate the dynamics of lipid bilayers of the isolated myocardial membrane during the free radical exposure. It is customary to express the fluidity of the membrane in terms of microviscosity (η), which simulates the anisotropic lipid core with an equivalent isotropic fluid and is expressed in macroscopic units.[11] We have determined the microviscosity within lipid bilayers utilizing steady state fluor-

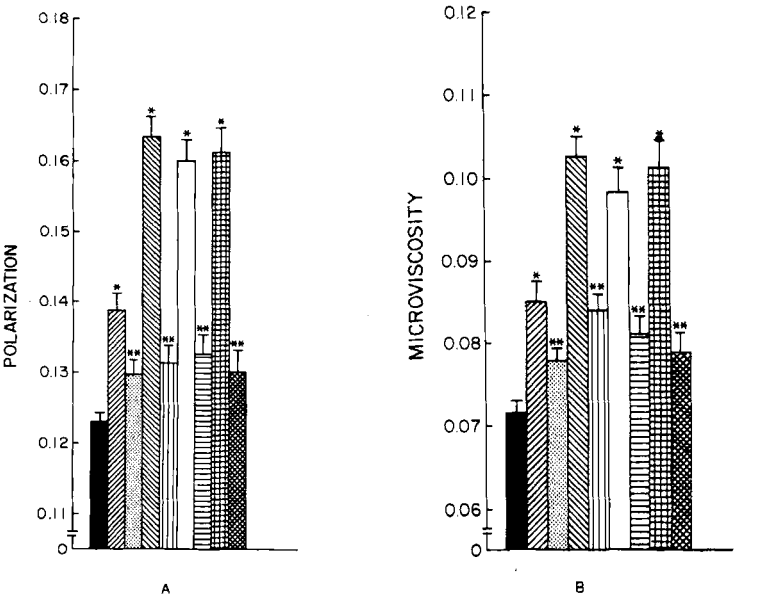

FIGURE 1 Membrane polarization (A) and microviscosity (B) as functions of free radical/oxidant exposure.
■ – control; ▨ – $O_2^{\cdot-}$; ▩ – $O_2^{\cdot-}$ + SOD + CAT; ▧ – $HO^{\cdot}$; ▥ – $HO^{\cdot}$ + DMTU; □ – H_2O_2; ■ – H_2O_2 + catalase; ▪ – $OCl^{\cdot}$ + HOCl; ▪ – $OCL^{\cdot}$ + HOCl + allopurinol.
*$p < 0.05$ compared to control.
**$p < 0.05$ compared to corresponding free radical.

TABLE I
Effects of free radicals and oxidants and their scavengers on the order parameters ('S'), calculated from the EPR spectra

Free Radical Generating System	Free Radical/ Oxidant	S
Control	—	0.618 ± 0.002
Hypoxanthine + XO	$O_2^{\cdot -}$	0.620 ± 0.004
Hypoxanthine + XO + SOD	—	0.619 ± 0.003
Hypoxanthine + XO + $FeCl_3$ + EDTA	$HO^{\cdot}$	0.571 ± 0.004[a]
Hypoxanthine + XO + $FeCl_3$ + EDTA + DMTU	—	0.615 ± 0.003[b]
H_2O_2	H_2O_2	0.588 ± 0.004[a]
H_2O_2 + catalase	—	0.617 ± 0.002[b]
NaOCl	$OCl^{\cdot}$ + HOCl	0.580 ± 0.005[a]
NaOCl + allopurinol	—	0.620 ± 0.004[b]

[a] $p < 0.05$ compared to control.
[b] $p < 0.05$ compared to corresponding free radical.

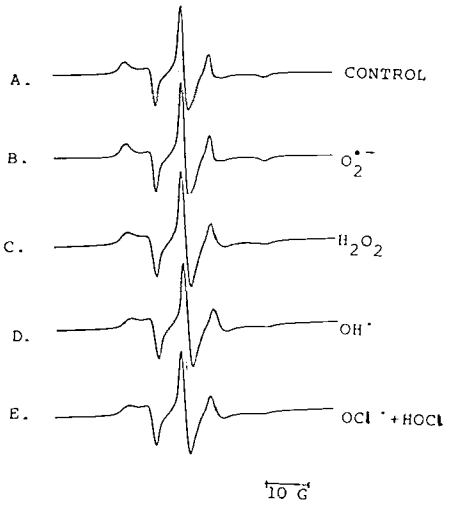

FIGURE 2 Efects of free radicals/oxidants on the ESR spectra for 7-doxyl stearic acid intercalated into myocardial membrane. (A) control, S = 0.618; (B) $O_2^{\cdot -}$, S = 0.620; (C) H_2O_2, S = 0.588; (D) $HO^{\cdot}$, S = 0.571; (E) $OCl^{\cdot}$ + HOCl, S = 0.580.

escence polarization (FP). All the free radicals and oxidants used in our study enhanced the FP (Figure 1A). $O_2^{\cdot -}$ exerted a minimal effect compared with $HO^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ + HOCl. The corresponding scavengers were able to restore the FPs to their corresponding control values. Figure 1B shows the results for the microviscosity calculated from FPs. Since microviscosity is inversely related to the lipid fluidity, the results of these studies suggest that these free radicals and oxidants decreased the fluidity of the membrane. The results of the FPs were further confirmed from the spin-label study using 7-doxyl spin-labeled stearic acid. EDR spectra as shown in Figure 2 are characteristic of the anisotropic motion anticipated in the acyl chain of

TABLE II
Effects of free radicals and oxidants on the arachidonic acid compositions of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and photphatidylinositol (PI)

Free Radicals/ Oxidants	PC	PE (mol %)	PI
Control	8.98 ± 0.30	10.23 ± 0.42	9.67 ± 0.90
$O_2^{\cdot -}$	9.27 ± 0.33	10.07 ± 0.40	9.89 ± 0.82
$HO^{\cdot}$	6.07 ± 0.46[a]	7.05 ± 0.90[a]	4.02 ± 0.40[a]
H_2O_2	7.61 ± 0.92[b]	8.50 ± 1.52[b]	7.20 ± 0.69[a]

[a] $p < 0.01$ compared to control.
[b] $p < 0.05$ compared to control.

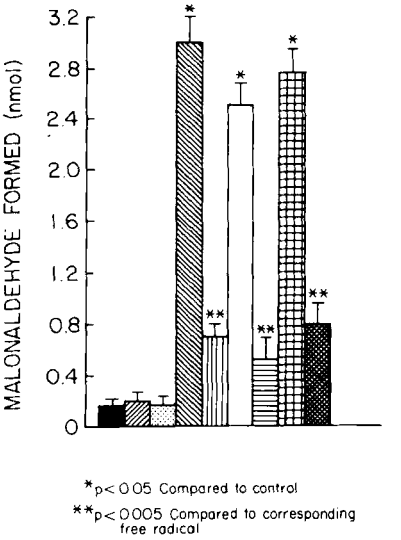

FIGURE 3 Malonaldehyde formation as a function of free radical/oxidant exposure.
■ – control; ▨ – $O_2^{\cdot -}$; ▩ – $O_2^{\cdot -}$ + SOD + CAT; ▧ – $HO^{\cdot}$; ▥ – $HO^{\cdot}$ + DMTU; □ – H_2O_2; ▤ – H_2O_2 + catalase; ⊞ – $OCl^{\cdot}$ + HOCl; ⊠ – $OCl^{\cdot}$ + HOCl + allopurinol.
* $p < 0.05$ compared to control.
** < 0.05 compared to corresponding free radical.

the phospholipid membrane, enabling calculation of an order parameter S via equation 3. All the free radicals and oxidants, except $O_2^{\cdot -}$, increased the values of S significantly, which were restored in the presence of corresponding free radical scavengers (Table I). This suggests that $HO^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ + HOCl all modify the acyl chain composition of the phospholipids and, thus, the membrane fluidity.

In order to examine which of the major phospholipid classes is particularly affected by the free radical attack, we determined the fatty acid profiles of three major phospholipid classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). Among the fatty acids examined (16:0, 18:0, 18:1, 18:2, and 20:4), only arachidonic acid (20:4) contents of PC, PE, and PI suffered significant loss during exposure to either $HO^{\cdot}$ or H_2O_2 (Table II). These results would tend to

indicate that $O_2^{\cdot -}$ itself may not cause any breakdown of membrane phospholipids unless it is being converted to more cytotoxic $OH^{\cdot}$.

Since the end products of phospholipid free radical interaction is malonaldehyde,[1,2] we also assayed the malonaldehyde formation during the free radical exposure. $HO^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ + HOCl all cause some degree of lipid peroxidation, leading to the formation of malonaldehyde (Figure 3).

The results of our study indicate that free radicals, including $O_2^{\cdot -}$, $HO^{\cdot}$, and $OCl^{\cdot}$, and oxidants such as H_2O_2 and HOCl can all decrease myocardial membrane fluidity. However, $O_2^{\cdot -}$ has only a minimal effect. Furthermore, $O_2^{\cdot -}$ can neither affect the acyl chain composition of membrane phospholipids nor can it induce any lipid peroxidation. All other free radicals and oxidants seem to be detrimental to the myocardial membrane. The formation of oxygen-derived free radicals has been indicated during reperfusion of ischemic myocardium,[1,2] with simultaneous breakdown of membrane phospholipids[3,4] and lipid peroxide formation.[1,4] $O_2^{\cdot -}$, if formed in the ischemic-reperfused myocardium, may undergo a metal-catalyzed Fenton-type reaction forming $HO^{\cdot}$ radicals.[12] Furthermore, $OCl^{\cdot}$ and HOCl may also be formed from the polymorphonuclear leukocytes (PMN) present in the circulating blood and catalyzed by the myeloperoxide present in PMN.[12] These results demonstrate that these free radicals and oxidants can release arachidonic acid from PC, PE, and PI, leading to the enhancement of microviscosity as a result of modification in acyl chain composition.

Acknowledgements

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