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EFFECT OF OXYGEN-DERIVED FREE RADICALS AND OXIDANTS ON THE DEGRADATION *IN VITRO* OF MEMBRANE PHOSPHOLIPIDS

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The abilities of chemically generated hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O^{\cdot -}$) and hydrogen peroxide (H_2O_2) to degrade rat myocardial membrane phospholipids previously lableed with [1-^{14}C]arachidonic acid were studied. $HO^{\cdot}$ and H_2O_2, but not $O_2^{\cdot -}$, caused the degradation of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). With $OH^{\cdot}$ and H_2O_2, the loss of radiolable in PC was accompanied by an increase in the radiolabel of lysophosphatidylcholine (LPC), but not in that of free fatty acid (FFA). These results suggest the hydrolysis of 1-oxygen ester bond of PC by $HO^{\cdot}$ and that H_2O_2 and that $HO^{\cdot}$ and H_2O_2, but not $O^{\cdot -}$, are detrimental to the structure and function of membrane phospholipids. However, since μM amounts of $HO^{\cdot}$ and mM amounts of H_2O_2 were necessary to affect the membrane phospholipids, it is likely that in the reprefused myocardium only $HO^{\cdot}$, but not H_2O_2, may directly cause the breakdown of membrane phospholipids.

KEY WORDS: Free radicals, phospholipids, membranes, myocardium, arachidonic acid, reperfusion injury.

INTRODUCTION

Myocardial reperfusion is associated with the generation of free radicals, degradation of phospholipids, massive influx of Ca^{2+}, and accumulation of free fatty acids (FFA) and their esters.[1-5] These biochemical events, either alone or in combination, have been implicated in the pathogenesis associated with ischemic reperfusion injury.

The free radical attack on lysosomal membrane-releasing phospholipases and the activation of Ca^{2+}-dependent phospholipases have been suggested as some of the mechanisms by which free radicals mediate the degradation of membrane phospholipids during ischemic-reperfusion[6]. However, free radicals can directly attack the polyunsaturated fatty acids of phospholipids, resulting in the peroxides of fatty acids in the phospholipids.[7] This initial attack by free radicals on phospholipids may stimulate the hydrolysis of phospholipids, or may result in the loss of functional phospholipids in the membrane. Therefore, in this paper we have examined the degradation of membrane phospholipids by chemically generated superoxide anion ($O^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2).

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MATERIALS AND METHODS

Preparation of Microsomal Membrane Containing [1-^{14}C]Arachidonate-Labled Phospholipids

Male Sprague-Dawley rats weighing 200–250 gm were properly anesthetized, and the hearts were removed. The isolated rat heart was perfused with [1-^{14}C]arachidomic acid essentially as described by Otani *et al.*[8] The hearts were then removed and homogenized in 0.25 M sucrose containing 10 mM tris-HCl buffer (pH 7.2). Differential centrifugation was carried out to isolate 120,000 g fraction containing microsomal membrane.[9] This membrane pellet was suspended in the same buffer at a protein concentration of 5 mg/ml and used for the studies. Protein was determined by the method of Lowry *et al.*[10]

Generation of O_2^- and $OH^{\cdot}$

$O_2^{\cdot -}$ was generated by the reaction of xanthine oxidase (XO) on xanthine (X)[11]. The reaction mixture contained 10 mM tris-HCl (pH 7.4), 8 mU XO, 100 μM X, and 10μM EDTA in a total volume of 1 ml. To generate $HO^{\cdot}$, 100 μM, $FeCl_3$ and 100 μM EDTA were added to the reaction mixture of $O_2^{\cdot -}$ generating system.[12] The generation of $O_2^{\cdot -}$ was confirmed by adding 100 μM cytochrome C to the $O_2^{\cdot -}$ generating system and following its reduction spectrophotometrically at 550 nm. To quantitate the generation of $OH^{\cdot}$, 2 mM salicylic acid was added to its generating system, and the adducts of salicylic acid and $HO^{\cdot}$ (2,5- and 2,3- dihydroxybenzoic acids) were separated using the Altex Ultrasphere 3 μ ODS (75 × 4.6 mm) equipped with a Waters Associated High Performance Liquid Chromatography (HPLC) unit. The dihydroxybenzoic acids were detected and quantitated by an electrochemical detector.

To study the effect of O_2 derived free radicals and oxidants on phospholipid degradation, the reaction mixture contained 150 μg [1-^{14}C]arachidonate labeled membrane (about 5,000 c.p.m.) and $O_2^{\cdot -}$ or $HO^{\cdot -}$ generating systems or $HO^{\cdot -}$ generating systems or 20 mM H_2O_2 in a total volume of 0.8 ml. The mixture was incubated for 30 min at 37°C with shaking, after which the reaction was terminated by the addition of 3.0 ml chloroform-methanol mixture (1:2 v/v containing 0.005% BHT). The lipids were extracted by the method of Bligh and Dyer.[13]

Individual phospholipids, except lysophosphatidylcholine (LPC), were separated on silica K̂ plates,[14] while LPC and FFA were separated from the other lipids on silica gel H and G plates, respectivaly.[15] All of the above solvent systems contained 0.05% BHT to prevent oxidation of polyunsaturated fatty acids. The lipids were visualized by exposure to I_2, and the radioactivities of individual lipids were determined.

RESULTS AND DISCUSSION

Generation and Characterization of Oxygen-Derived Free Radicals

The $O_2^{\cdot -}$ generating system produced as much as 2 nmol of $O_2^{\cdot -}$/min/ml of reaction mixture. Hydroxyl radicals were produced by adding $FeCl_3$ and EDTA to the superoxide anion generating system. The hydroxylated products of salicylate were analyzed on HPLC (Figure 1). At zero xanthine concentration, the peak eluting at

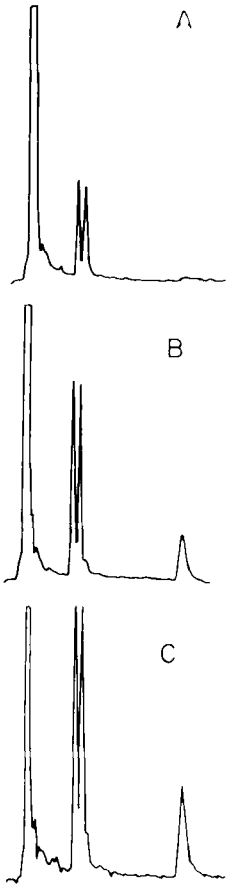

FIGURE 1 HPLC identification of the hydroxylated products of salicylic acid. The incubation mixture contained a total volume of 1.8 ml, 10 mM tris-HCl, 2 mM salicylic acid, 8 mU XO, 100 μM $feCl_3$, 100 μM EDTA, and 0 μM (A), 40 μM (B), and 100 (C) xanthine. After incubating at 25°C for 10 min, the samples were processed and subjected to HPLC as described under Methods.

7.5 min was almost absent, while at 40 and 100 μM the response of this peak increased with the increase in xanthine concentration. The addition of 20 and 40 μM DMTU, a known inhibitor of $HO^{\cdot}$, progressively decreased the response of this peak. These results suggest that this peak is the adduct of $HO^{\cdot}$ and salicylic acid; indeed, 2,3- and 2,5-dihydroxybenzoic acid coeluted wth the peak.

Effects of $O_2^{\cdot -}$, $HO^{\cdot}$ and H_2O_2 on the Membrane Phospholipids

Phospholipids of the purified microsomal fraction prelabeled with [1-^{14}C]arachidonic acid were exposed to $HO^{\cdot}$, $O_2^{\cdot -}$, and H_2O_2. Addition of $FeCl_3$ and EDTA alone to the membranes caused a significant decrease in the radioactivity of PC (Table I). The decrease of radioactivity in PC was accompanied by an increase in the accumulation

TABLE 1
Effect of $O_2^{\cdot}$, $HO^{\cdot}$, and H_2O_2 on the Degradation of Microsomal Membrane Phospholipids Previously Labeled with [1-^{14}C]Archidonic Acid

Additions	PHOSPHOLIPIDS					FFA
	LPC	PC	PI	PS	PE	
			(c.p.m.)			
None	232 ± 27	2650 ± 176	402 ± 39	195 ± 24	354 ± 46	304 ± 82
$FeCl_3$ + EDTA	460 ± 18	2412 ± 200	379 ± 40	175 ± 29	323 ± 50	375 ± 120
Xanthine (X) + Xanthine Oxidase (XO) [$O_2^{\cdot -}$]	206 ± 23	2499 ± 165	361 ± 23	179 ± 17	320 ± 13	577 ± 176
X + XO + $FeCl_3$ + EDTA	936 ± 80[a]	1899 ± 157[b]	286 ± 20[a]	141 ± 17	207 ± 14[a]	416 ± 33
[$HO^{\cdot}$]	581 ± 60[c]	2242 ± 80[c]	336 ± 21	176 ± 14	251 ± 25[c]	320 ± 30
H_2O_2						

The values are Means ± S.E.M. of five separate experiments. Three different batches of membranes were used.

a = $p < 0.001$; b = $p < 0.01$; c = $p < p < 0.05$ compared ot control.

tion of radioactive LPC, but not in the increased accumulation of radioactive FFA. However, there was not any significant decrease in the radioactivity of either phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylinositol (PI). When the membranes were exposed to $O_2^{\cdot -}$, there was neither a significant decrease in the radioactivites of any phospholipids, nor an increase in the radioactivity of LPC; however, there was a slight increase in the radioactivity of FFA. On the other hand, when membranes were exposed ot $OH^{\cdot}$, the radioactivities of PC, PE, and PI were significantly lowered as compared ot the control ($FeCl_3$ + EDTA) or $O_2^{\cdot -}$ generating system alone. Surprisingly, this decrease in PC radioactivity was accompaned by an increase in the accumulation of radioactive LPC, but not in that of FFA. When the membrane was exposed to H_2O_2 alone, significant decreases in the isotopic label of PC, PE, and PI were observed. With H_2O_2 also, the decrease in radiolabeled PC was again accompanied by an increase in LPC radiolabel, but not in the radiolabel of FFA.

When generation of $O_2^{\cdot -}$ was increased by increasing the xanthine concentration, there was neither a loss of radiolabel in PC nor an increase in radiolabels in LPC or FFA (Figure 2). When xanthine concentrations were increased in the presence of XO, $FeCl_3$, and EDTA, an increase of $HO^{\cdot}$ was observed (Figure 3). Generation of $O_2^{\cdot -}$ (when $FeCl_3$ and EDTA were omitted) and $HO^{\cdot}$ in the presence of $FeCl_3$ and EDTA increased up to 60 μM xanthine, after which the amount of generated free radicals leveled off. The loss of radiolabel in PC increased in concert with the increase in generated $HO^{\cdot}$ (Figure 3), and a maximum of 10% of the radiolabel was lost from membrane PC. The progressive decrease of radiolabel in PC was accompanied by an increase of radiolabel in LPC. The degradation [^{14}C)PC increased with increase in H_2O_2, and at 20mM concentration, about 6% of the radiolabel was lost from membrane PC. Again, this decrease of radiolabel in PC was accompanied by an increase in the radiolabel of LPC (Figure 4).

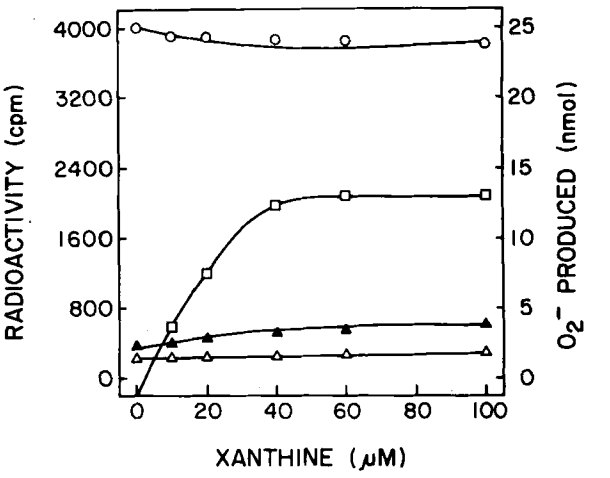

FIGURE 2 Effect of varying concentrations of xanthine on the generation of $O_2^{\cdot-}$ and degradation of membrane PC. The reaction mixture contained 10 mM tris (pH 7.4), 150 μg [1-^{14}C]arachidonate-labeled membrane, 0–100 μM xanthine, and 6 mU XO in a total volume of 0.8 ml. After incubating 30 min at 37°C, the lipids were extracted and subjected to t.l.c. as described under Methods. The radioactivities in LPC (△—△), FFA (▲—▲), and PC (○—○) were determined. The nmol of $O_2^{\cdot-}$ (□—□) produced by an identical reaction mixture was measured spectrophotometrically by following cytochrome C reduction.

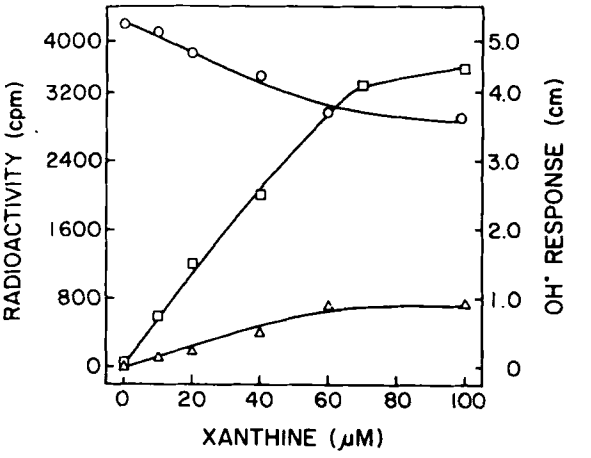

FIGURE 3 Effect of varying concentrations of xanthine on the generation of HO· and degradation of membrane PC. The reaction mixture contained 10 mM tris (pH 7.4), 150 μg [1-^{14}C]arachidonate-labeled membrane, 0–100 μM xanthine, 6 mU XO, and 100 μM each of $FeCl_3$ and EDTA in a total volume of 0.8 ml. The rest of the procedure is as described under Figure 2. The radioactivities of LPC (△—△) and PC (○—○) were determined. The reaction mixture for the measurement of HO· (□—□) by HPLC is similar to that described above, except for the omission of the labeled membrane.

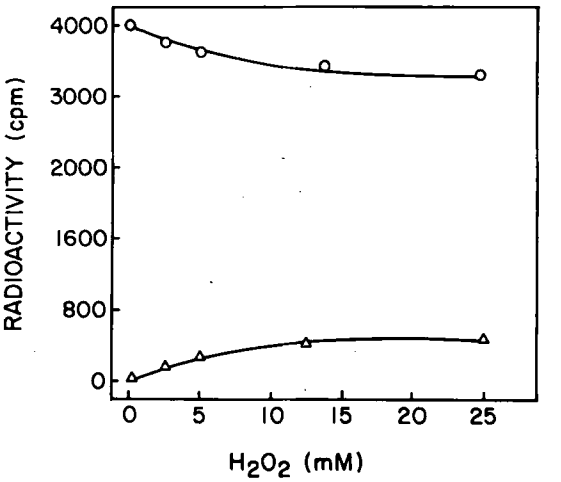

FIGURE 4 Effect of varying concentrations of H_2O_2 on the degradation of membrane PC. The reaction mixture and procedure are the same as described under Figure 3, except for the replacement of xanthine and XO by)–25 uM H_2O_2. The radioactivities in PC (○—○) and LPC (△—△) were determined.

When the degradations of membrane PC were compared by exposing them to $O_2^{\cdot -}$, hydroxyl radicals induced most of the acculation of radiolabel in LPC (Figure 5). H_2O_2 also induced a significant amount of accumulation in LPC, whereas $O_2^{\cdot -}$ was least effective in degrading the membrane PC.

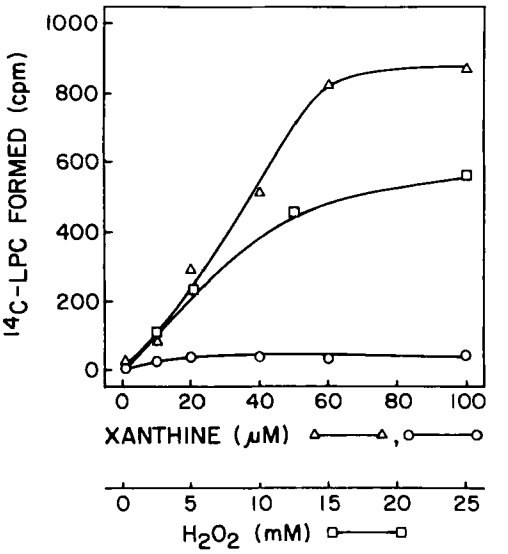

FIGURE 5 Comparison of formation of ^{14}C-LPC in the membranes exposed to $O_2^{\cdot -}$ (○—○), $HO^{\cdot}$ (△—△), and H_2O_2 (□—□). The assay mixtures and conditions are as described under Figures 2, 3, and 4.

Effects of Free Radical Scavengers on Free Radical Mediated Phospholipid Degradation

The effect of scavengers of $O_2^{\cdot -}$ (SOD), $HO^{\cdot}$ (DMTU) and H_2O_2 (catalase) on the free radical induced degradation of membrane [^{14}C]PC is shown in Table II. DMTU (25 μM) scavenged about 50% of the hydroxylated salicylate peak and about 40% of the formation of [^{14}C]LPC. However, the combination of SOD and catalase not only scavenged 100% of the $HO^{\cdot}$ formation, but also totally inhibited formation of [^{14}C]LPC. The above combination was also effective in inhibiting formation of [^{14}C]LPC by exposure to H_2O_2.

Among the O_2-derived free radials/oxidents tested here, $O_2^{\cdot -}$ caused no significant breakdown breakdown of any membrane phospholipid This observation is consistent with Starke and Farber,[16], who observed no cellular damage by $O_2^{\cdot -}$ in the presence of a ferric iron chelator. As shown below, iron is essential for the conversion of $O_2^{\cdot -}$ into $HO^{\cdot}$, which appears to be the most deleterious free radical that is produced biologically.

$$O_2^{\cdot} + Fe^{+++} \rightarrow O_2 + Fe^{++}$$

$$O_2^{\cdot} + O_2^{\cdot -} + 2H^{+} \rightarrow H_2O_2 + O_2$$

$$Fe^{++} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-}$$

Between $HO^{\cdot}$ and H_2O_2, $HO^{\cdot}$ caused a significantly higher degradation of membrane phospholipids. PC, PE, and PI (but not PS) wer susceptible to free radical attack. The formation of archidonate (or peroxidized product) containing [^{14}C]LPC from 2-[1-^{14}C]arachidonyl phosphatidylcholine suggests that l-oxygen ester of PC rather than 2-oxygen ester of PC ws hydrolyzed. This interpretation is based on the fact that arachidonate primarily occupies position 2 of cellular phospholipid.[17] This hydrolysis of 1-oxygen ester bond could be arising from the peroxidation of unsaturated fatty acid at position 2, which may render the 2-oxygen ester bond resistant to hydrolysis or may facilitate hydrolysis of 1-oxygen bond.

TABLE 2
Effect of Scavengers of $O_2^{\cdot -}$, $OH^{\cdot}$, and H_2O_2 on the Degradation of ^{14}C-Phosphatidylcholine

SYSTEM	^{14}C-LPC Formed[a] (%)	$HO^{\cdot}$ Peak (cm)
Hydroxyl Radical		
Control ($FeCl_3$ + EDTA)	1.45	0.00
$HO^{\cdot}$ generating system	11.45	2.5
$HO^{\cdot}$ + DMTU (25μM)	7.30	1.20
$HO^{\cdot}$ + SOD + catalase	1.50	0.10
H_2O_2		
Control	0.80	
H_2O_2(2 mM)	2.75	
H_2O_2	1.50	
H_2O_2 + SOD + catalase	1.05	

[a]Percent ^{14}C-LPC was calculated by dividing the radioactivity in LPC by the total radioactives present in LPC and PC. The values are the mean of 3 independent experiments. 100 units/ml each of SOD and catalase were used.

The breakdown of phospholipids catalyzed by H_2O_2 appears to be of lesser degree compared to $HO^{\cdot}$. Cellular H_2O_2 concentration is known to be about 10 nM, and the mitochondrial production may increase as much as 4–10 times under pathophysiological conditions.[18] cellular H_2O_2 may be increased to as high as 100 nM. However, mM amounts of H_2O_2 were required to bring about the degradation of membrane phospholipids, while $HO^{\cdot}$ produced from μM amounts of $O_2^{\cdot-}$ were enough to catalyze such a process. Therefore, between H_2O_2 and $HO^{\cdot}$, the latter may be the major deleterious O_2—derived free radical that can affect the membrane during ischemic-reperfusion of the hyocardium. This suggestion is also consistent with the observation of Starke and Farber,[16]where in the presence of deferoxamine, a ferric iron chelator (see equation 1), H_2O_2 failed to produce and cellular damage.

In summary, the present results suggest that during reperfusion of ischemic myocardium, $HO^{\cdot}$ can directly cause the degradation, H_2O_2 present in the cell may not be sufficient in concentration to degrade membrane phospholipids during ischemic-reperfusion of myocardium.

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