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Inhibition of cardiac phosphatidylethanolamine *N*-methylation by oxygen free radicals

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This study was undertaken to examine the effects of oxygen free radicals on phosphatidylethanolamine (PE) *N*-methylation in rat heart sarcolemmal (SL) and sarcoplasmic reticular (SR) membranes. Three catalytic sites involved in the sequential methyl transfer reaction were studied by assaying the incorporation of radiolabeled methyl groups from *S*-adenosyl-L-methionine (0.055, 10, and 150 μM) into SL or SR PE molecules under optimal conditions. In the presence of xanthine + xanthine oxidase (superoxide anion radicals generating system), PE *N*-methylation was inhibited at site I and III in the heavy SL fraction isolated by the hypotonic shock-LiBr treatment method. In the light SL fraction isolated by sucrose-density gradient, a significant inhibition of PE *N*-methylation was seen at all three sites. These inhibitory effects of xanthine + xanthine oxidase on PE *N*-methylation were prevented by the addition of superoxide dismutase. Hydrogen peroxide showed a significant inhibition of PE *N*-methylation at site I in the heavy SL fraction, and at site I and II in the light SL fraction. Catalase blocked the inhibitory effects of hydrogen peroxide. The effects of both xanthine + xanthine oxidase and hydrogen peroxide on the SR membranes were similar to those seen for the heavy SL fraction. These results suggest that, in addition to lipid peroxidation, the oxygen free radicals may affect the function of cardiac membranes by decreasing the phospholipid *N*-methylation activity.

Introduction

It is now well known that phospholipids play an important role in maintaining the structure and function of cell membranes. The intramembranal rearrangement of the two major membrane phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), can be regulated by phospholipid *N*-methylation [1]. Conversion of PE to PC is catalyzed by the methyltransferase system, where phosphatidyl-*N*-monomethylethanolamine (PMME) and phosphatidyl-*N*,*N*-dimethylethanolamine (PDME) are formed as intermediates, and *S*-adenosyl-L-methionine (AdoMet) serves as a physiological donor for methyl groups [1]. We have previously reported the characteristics of three methyltransferase catalytic sites (I, II and III) for PE *N*-methylation in cardiac sarcolemma (SL) and sarcoplasmic reticulum (SR), which can be readily identified at 0.055, 10 and 150 μM concentrations of AdoMet [2–4]. Phospholipid *N*-methylation has been considered to have important implications for cell membrane properties such as membrane fluidity [5,6], membrane-bound enzyme activities [7–10] and β-adrenergic receptors [11]. Recently, oxygen free radicals have been suggested to be involved in myocardial damage in some pathophysiological conditions [12,13]. Cellular and subcellular membrane-bound enzyme proteins [15–17] as well as membrane phospholipids are targets of free radical damage [14]. Since membrane-bound *N*-methyltransferase catalyses the synthesis of phospholipid components of the membrane, it is possible that free radicals induced changes in membrane structure and function may be associated with alterations in the phospholipid *N*-methylation. Therefore, this study was undertaken to ex-

Abbreviations: [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; PE, phosphatidylethanolamine; PDME, phosphatidyl-*N*,*N*-dimethylethanolamine; PMME, phosphatidyl-*N*-monomethylethanolamine; SL, sarcolemma; SR, sarcoplasmic reticulum.

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amine the effects of oxygen free radicals on PE *N*-methylation in rat cardiac SL and SR membrane in vitro.

Methods and Materials

Isolation and characterization of cardiac SL and SR membranes

Male Sprague-Dawley rats weighing 250–300 g were used in this study. Purified sarcolemmal (SL) membranes were isolated from the fresh ventricular tissue by the hypotonic shock-LiBr treatment method [18]. This SL preparation containing plasma membrane with intact basement membrane has been shown to be of the cardiac cell membrane origin and devoid of endothelial cell membranes [18–21]. To exclude the possibility of contamination by subcellular organelles in the sarcolemmal preparation, different marker enzyme activities were measured according to the procedures used earlier [22]. Ouabain-sensitive Na^+/K^+-ATPase activity, a well recognized marker of the plasma membrane, exhibited about 8-fold increase in purity over the homogenate values. Cytochrome-*c* oxidase (a mitochondrial marker) and rotenone-insensitive NADPH–cytochrome-*c* reductase (a sarcoplasmic reticular marker) activities in this SL fraction showed 0.2–0.4-fold purification. These observations indicate that mitochondrial and sarcoplasmic reticular (SR) contaminations were minimal. K^+-EDTA-stimulated ATPase activity (a myofibrillar marker) was not detectable in the SL fraction. In some experiments, SL membranes isolated by the sucrose-density gradient method [23] were used for the purpose of comparison. Another membrane fraction enriched with SR fragments (microsomes) was obtained according to the method of Harigaya and Schwartz [24] with some modification as indicated elsewhere [25]. These preparations were used immediately after isolation. Protein concentration was estimated by the method of Lowry et al. [26].

Assay for phospholipid N-methyltransferase

Phosphatidylethanolamine *N*-methyltransferase activity was assayed by measuring the incorporation of ^{3}H-labeled methyl groups into membrane phospholipids in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) as described earlier [4]. Unless otherwise indicated, incubation of the membrane preparations was carried out in the presence of 1 mM $MgCl_2$, 0.055 μM [^{3}H]AdoMet (65.7 Ci/mmol) at pH 8.0 (50 mM Tris-hydroxymethyl)aminomethane-glycylglycine buffer) for the catalytic site I. The incubation was performed without $MgCl_2$ at 10 μM [^{3}H]AdoMet (200 μCi/μmol), pH 7.0 (50 mM imidazole buffer) for the catalytic site II and at 150 μM [^{3}H]AdoMet (200 μCi/μmol), pH 10.0 (50 mM sodium hydroxide-glycine buffer) for the catalytic site III. These incubation conditions have been found to be optimal for three catalytic sites in the methylation reaction as well as typical for the synthesis of PMME, PDME, and PC as major methylated lipid products at sites I, II and II, respectively [2–4]. After a 10 min incubation period at 37 °C, the reaction was initiated by adding [^{3}H]AdoMet as a methyl donor and terminated 30 min later with the addition of 3 ml of chloroform/methanol/2 M HCl (6 : 3 : 1, by vol.). The detailed procedure for the measurement of total and intermediate methylated phospholipids has been reported earlier [4].

Free radical generating system

Superoxide anion radicals ($O_2^{\cdot-}$) were generated by the xanthine oxidase reaction by using the xanthine as a substrate [15,27]. The concentrations of xanthine and xanthine oxidase in the incubation medium were 2 mM and 0.03 U/ml, respectively. Since it has been reported that trypsin inhibited methyltransferase activity [28,29], xanthine oxidase was pretreated with 0.4 mM phenylmethylsulforyl fluoride (PMSF) in order to inhibit a trypsin-like activity present in the commercial product as a contaminant. PSMF had no effect by itself on the methyltransferase activity under the experimental conditions of this study. Hydrogen peroxide (1 mM) (an activated oxygen, not free radical per se) was also used. Superoxide dismutase (SOD), and catalase were used as scavengers at a concentration of 80 and 10 μg/ml, respectively [14]. The concentrations of the above chemicals used in this study were based on previous experiments [17,30].

Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine (spec. act., 65.7 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL (U.S.A.). Phosphatidylcholine, xanthine, SOD, and catalase were purchased from Sigma Chemical, St. Louis, MO (U.S.A.). *S*-Adenosyl-L-methionine was from RBI, Natick, MA (U.S.A.). Phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine, and xanthine oxidase were obtained from Calbiochem-Behring, San Diego, CA (U.S.A.). Silica gel 60 F-254 thin-layer chromatography plates were obtained from E. Merck, Darmstadt, F.R.G.). Chromatographic analysis indicated that the radiochemical purity of *S*-adenosyl-L-[*methyl*-^{3}H]methionine was 99.9% and that *S*-adenosyl-L-methionine purity was > 98%. All other reagents were of analytical grade.

Statistical analysis

Results are presented as means ± S.E. For statistical evaluation, multiple analysis of variance was carried out, and Duncan's multiple range test was used to determine differences between the means within the population. P level < 0.05 was taken to reflect a significant difference.

Results

To study the effects of $O_2^{\cdot-}$ or H_2O_2 on methyltransferase activities, sarcolemmal membranes were preincubated without (control) or with 2 mM xanthine + 0.03 U/ml xanthine oxidase or 1 mM H_2O_2 for 10 min at 37 °C in a medium containing 5 mM Tris-HCl (pH 7.4). Table I shows the effects of $O_2^{\cdot-}$ or H_2O_2 on the catalytic activities at three sites involved in PE *N*-methylation in the heavy sarcolemma isolated by LiBr-treatment method. In the presence of xanthine + xanthine oxidase, catalytic activities at site I and III were inhibited by 27% and 28% of control values, respectively; these inhibitory effects of xanthine + xanthine oxidase were blocked by the addition of 80 μg/ml SOD. Catalytic activity at site II was not affected by xanthine + xanthine oxidase system. Hydrogen peroxide showed a significant inhibition of catalytic activity at site I (67% of control values), whereas site II and III remained unaffected. Catalase (10 μg/ml) prevented the reduction of catalytic activity reduced by H_2O_2. It should be noted that the control values of total *N*-methylated phospholipids at catalytic site I, II, and III were in agreement with those reported earlier [2–4]. Furthermore, xanthine, xanthine oxidase, SOD or catalase alone did not show any significant changes in catalytic activities at any of the three sites.

In order to determine the identity of methylated phospholipids that resulted from the above mentioned changes in sarcolemmal PE *N*-methylation, radiolabeled phospholipids of the PE methyltransferase pathway were examined under optimal conditions for site I (Table II) and site III (Table III). It can be seen that PMME level was significantly decreased at site I in both xanthine + xanthine oxidase and H_2O_2-treated groups (Table II). Although site I assay conditions are not selective for a specific analysis of PDME and PC, a reduced formation of PDME and PC was observed under these conditions

TABLE I

Effects of xanthine + xanthine oxidase and H_2O_2 on PE N-methylation in heavy sarcolemma isolated from the rat heart by the hypotonic shock-LiBr treatment method

Values are means ± S.E. of 5 or 6 different membrane preparations and are expressed as pmol total [^{3}H]methyl groups incorporated/mg per 30 min. Purified heavy sarcolemmal membranes were isolated according to the method described by Dhalla et al. [18]. Assays at catalytic sites I, II, and III were performed in the presence of 0.055, 10, and 150 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine, respectively. X, xanthine; XO, xanthine oxidase; SOD, superoxide dismutase; CAT, catalase. * Significantly different from control values ($P < 0.05$).

Treatment	Catalytic site		
	I	II	III
Control	0.51 ± 0.04	7.61 ± 0.52	129 ± 9
X	0.57 ± 0.03	8.02 ± 0.44	138 ± 9
XO	0.50 ± 0.04	7.35 ± 0.48	127 ± 10
X + XO	0.37 ± 0.02 *	7.47 ± 0.50	93 ± 8 *
X + XO + SOD	0.47 ± 0.03	7.60 ± 0.77	124 ± 11
SOD	0.50 ± 0.05	7.49 ± 0.95	123 ± 12
H_2O_2	0.35 ± 0.03 *	7.98 ± 0.63	136 ± 14
H_2O_2 + CAT	0.49 ± 0.04	7.74 ± 0.85	124 ± 9
CAT	0.49 ± 0.05	7.36 ± 0.54	123 ± 12

TABLE II

Catalytic site I associated incorporation of [^{3}H]methyl groups into individual N-methylated phospholipids of the heavy sarcolemmal fractions obtained from rat heart

Values are means ± S.E. of 5 or 6 different membrane preparations. Assays were performed in the presence of 0.055 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine. PMME, phosphatidyl-*N*-monomethylethanolamine; PDME, phosphatidyl-*N*,*N*-dimethylethanolamine; PC, phosphatidylcholine. Other abbreviations are same as described in the legend for Table I. * Significantly different from control values ($P < 0.05$).

Treatment	pmol [^{3}H]methyl groups incorporated/mg per 30 min		
	PMME	PDME	PC
Control	0.216 ± 0.015	0.124 ± 0.011	0.103 ± 0.007
X	0.242 ± 0.017	0.143 ± 0.009	0.114 ± 0.004
XO	0.214 ± 0.022	0.126 ± 0.007	0.098 ± 0.005
X + XO	0.155 ± 0.013 *	0.084 ± 0.008 *	0.076 ± 0.004 *
X + XO + SOD	0.199 ± 0.011	0.114 ± 0.012	0.092 ± 0.005
SOD	0.203 ± 0.019	0.120 ± 0.015	0.105 ± 0.008
H_2O_2	0.149 ± 0.015 *	0.081 ± 0.010 *	0.078 ± 0.003 *
H_2O_2 + CAT	0.205 ± 0.019	0.118 ± 0.009	0.101 ± 0.007
CAT	0.211 ± 0.017	0.115 ± 0.008	0.100 ± 0.009

TABLE III

Catalytic site III associated incorporation of [^{3}H]methyl groups into individual N-methylated phospholipids of the heavy sarcolemmal fraction obtained from the rat heart

Values are mean ± S.E. of 5 or 6 different membrane preparations. Assays were performed in the presence of 150 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine. All abbreviations are same as described in legends for Tables I and II. * Significantly different from control values ($P < 0.05$).

Treatment	pmol [^{3}H]methyl groups incorporated/mg per 30 min		
	PMME	PDME	PC
Control	22.9 ± 1.6	27.7 ± 1.9	47.8 ± 3.2
X	24.7 ± 1.1	31.3 ± 1.9	47.5 ± 4.3
XO	20.2 ± 2.1	28.5 ± 2.2	50.2 ± 3.9
X + XO	16.3 ± 1.4 *	19.8 ± 1.7 *	32.8 ± 3.8 *
X + XO + SOD	20.8 ± 0.9	28.0 ± 1.8	46.4 ± 5.4
SOD	22.9 ± 2.3	26.3 ± 2.3	43.9 ± 4.9
H_2O_2	24.9 ± 2.5	27.4 ± 3.6	52.1 ± 3.7
H_2O_2 + CAT	20.6 ± 3.0	27.1 ± 2.5	46.4 ± 3.5
CAT	20.4 ± 1.6	26.3 ± 1.9	48.8 ± 4.1

TABLE IV

Effects of xanthine + xanthine oxidase and H_2O_2 on PE N-methylation in the light sarcolemmal fraction isolated by the sucrose-density gradient method

Values are means ± S.E. of five different membrane preparations and are expressed as pmol total [^{3}H]methyl groups incorporated/mg per 30 min. Sarcolemmal membranes were isolated according to the method described by Pitts [23]. Assays at catalytic sites I, II, and III were performed in the presence of 0.055, 10, and 150 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine, respectively. All abbreviations are same as described in the legend for Table I. * Significantly different from control values ($P < 0.05$).

Treatment	Catalytic site		
	I	II	III
Control	0.49 ± 0.03	5.21 ± 0.37	119 ± 8
X + XO	0.25 ± 0.04 *	3.07 ± 0.36 *	88 ± 9 *
X + XO + SOD	0.42 ± 0.05	4.22 ± 0.40	101 ± 7
H_2O_2	0.37 ± 0.03 *	3.59 ± 0.15 *	123 ± 14
H_2O_2 + CAT	0.50 ± 0.04	4.53 ± 0.26	115 ± 7

(Table II). This may reflect the decreased availability of methylated precursors to sites II and III due to the depression of site I which is the rate-limiting step for the methylation process [31]. Table III shows that [^{3}H]methyl incorporation into PC was significantly reduced by xanthine + xanthine oxidase; under this assay condition, low intramembranal levels of PMME and PDME reflected the inhibition of site I. The changes in *N*-methylated phospholipids due to xanthine + xanthine oxidase were prevented by the addition of SOD. H_2O_2 had no effect on the formation of PMME, PDME or PC under assay conditions for site III (Table III).

The possibility that the observed changes in PE *N*-methylation were influenced by the SL isolation procedure was examined by employing the light sarcolemmal membranes isolated by another method. The sucrose-density gradient method of Pitts [23] was used since it yields a sarcolemmal preparation which has been well characterized in terms of PE *N*-methyltransferase activities and is relatively free from contamination by other subcellular organelles [4]. In this membrane preparation the catalytic activities were reduced at all three sites by xanthine + xanthine oxidase whereas the activities at site I and II were depressed by H_2O_2, (Table IV). SOD and catalase showed a protective effect on methyltransferase activities due to xanthine + xanthine oxidase or H_2O_2, respectively.

Since PE *N*-methylation has been shown to be present in cardiac microsomes (sarcoplasmic reticulum fragments) [4], we examined the effects of xanthine + xanthine oxidase and H_2O_2 on the PE *N*-methylation of this subcellular fraction (Table V). The combination of xanthine + xanthine oxidase showed significant inhibition at site I and III, whereas H_2O_2 inhibited the activity only at site I. The inhibitory effect of xanthine + xanthine oxidase was prevented by SOD, whereas that of H_2O_2 was prevented by catalase. These results on total methyl group incorporation were confirmed by the study of radiolabeled intermediates of PE *N*-methylation pathway (data not shown).

TABLE V

Effects of xanthine + xanthine oxidase and H_2O_2 on PE N-methylation in the rat heart microsomal fraction containing fragments of the sarcoplasmic reticulum

Values are mean ± S.E. of six different membrane preparations and are expressed as pmol total [^{3}H]methyl groups incorporated/mg per 30 min. Microsomal membranes containing fragments of the sarcoplasmic reticulum were isolated according to the method described by Harigaya et al. [24]. Assays at catalytic site I, II, and III were performed in the presence of 0.055, 10, and 150 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine, respectively. All abbreviations are same as described in the legend for Table I. * Significantly different from control values ($P < 0.05$).

Treatment	Catalytic site		
	I	II	III
Control	2.43 ± 0.16	4.76 ± 0.34	121 ± 8
X	2.53 ± 0.11	4.69 ± 0.16	114 ± 11
XO	2.30 ± 0.18	4.48 ± 0.46	117 ± 8
X + XO	1.39 ± 0.12 *	4.46 ± 0.42	88 ± 6 *
X + XO + SOD	2.02 ± 0.13	4.36 ± 0.27	107 ± 9
SOD	2.33 ± 0.23	4.39 ± 0.25	109 ± 7
H_2O_2	1.51 ± 0.17 *	4.51 ± 0.39	106 ± 8
H_2O_2 + CAT	2.53 ± 0.17	4.50 ± 0.21	124 ± 12
CAT	2.75 ± 0.21	4.34 ± 0.23	116 ± 10

Discussion

In the present study we observed that both xanthine + xanthine oxidase and H_2O_2 decreased the PE *N*-methylation process in cardiac subcellular fractions; these effects were due to the generation of oxygen free radicals because they were prevented by SOD and catalase, respectively. In both heavy sarcolemmal and sarcoplasmic reticular membranes, xanthine + xanthine oxidase showed a significant inhibition of the methyltransferase activity at sites I and III, whereas site II remained unaffected. Interestingly a similar type of alteration of the methyltransferase system was seen by Jaiswel et al. [29] who reported that the treatment of aortic microsomes with phospholipase C induced a partial decrease of PMME and PC (first and third methyl transfer reaction) without any effect on PDME (second methyl transfer reaction). It should be pointed out that H_2O_2 affected methyltransferase in both heavy sarcolemmal and sarcoplasmic reticular membranes at site I only. On the other hand, xanthine + xanthine oxidase was found to depress all three sites, whereas H_2O_2 depressed sites I and II of PE *N*-methylation process in the light sarcolemmal fraction obtained by the sucrose density gradient method. Although the exact reasons for

the higher sensitivity of site II in the light sarcolemmal membrane to the oxidative stress are not known, differences in the properties of these membrane preparations may account for such a diversity in their response. Particularly, varying amounts of antioxidants and scavengers for free radicals in different membrane preparations may be important in determining their response to oxygen free radical generating system. The precise molecular mechanisms by which oxygen free radicals inhibit the methyltransferase activities are still unclear; however, two possibilities can be considered. The first may be a direct modification of the sulfhydryl groups of the enzyme by oxygen free radicals [14,30]. The second possibility may be a peroxidation of membrane lipids which affect the membrane-bound enzyme activities by changing the lipid microenvironment of the protein [14,32]. The oxygen free radical generating systems employed in this study have been shown to promote lipid peroxidation [16]. Nonetheless, the results presented in this study have indicated that oxygen free radicals may also affect the membrane lipids by altering the PE *N*-methylation process.

The methylation of PE polar head group is considered to have important effects on the physicochemical properties of the cellular membranes [5–11]. Furthermore, Ca^{2+} transport systems of the cardiac cell have been shown to be influenced by PE *N*-methylation where both sarcolemmal and sarcoplasmic reticular Ca^{2+} pumps were stimulated [8,9]. Evidence has also been provided to show that an increase in the intracellular level of methyl donor, AdoMet, induces a powerful and long-lasting positive inotropic effect in which PE *N*-methylation of the sarcoplasmic reticulum plays an important role [33,34]. Since PE *N*-methylation activities have been reported to be decreased in different types of diseased hearts where oxygen free radicals are suspected to be involved in the pathogenesis of cardiac dysfunction [35–37], it is possible that the formation of oxygen free radicals may adversely affect the process of PE *N*-methylation. In this regard, it should be noted that oxygen free radicals have been shown to be involved in myocardial damage in different pathophysiological conditions [12,13,38]. In fact, oxygen free radicals have been reported to increase the membrane permeability and result in the loss of the membrane integrity [32]. Furthermore, membrane-bound enzyme activities such as sarcolemmal and sarcoplasmic reticular Ca^{2+} pump activities were depressed by oxygen free radicals [16,17]. Contractile activity of the perfused heart with oxygen radicals generating systems has also shown to be decreased [39]. These observations are consistent with results of a previous study in which an inverse relationship between activation of the superoxide anion radicals production and the extent of incorporation of methyl groups into cellular lipids in human monocytes has been shown [40].

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