Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 710-716

INCREASED PHOSPHOLIPID METHYLATION IN THE MYOCARDIUM OF ALCOHOLIC RATS

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Received January 28, 1983

SUMMARY: Phospholipid methyltransferase activity is unevenly distributed throughout the contractile and the specialized regions of rat heart. Chronic alcohol treatment stimulated the incorporation of [^{3}H-methyl]-methionine but not [^{3}H-methyl]-choline into phosphatidylcholine by left ventricular slices. The increase in the incorporation of [^{3}H-methyl]-group from [^{3}H-methyl]-methionine into phosphatidylcholine was due to an increase in phospholipid methyltransferase activity.

Human as well as animal studies have shown that acute single-dose ingestion of alcohol produces deleterious yet reversible effects on cardiac performance (1,2); however, morphologic, biochemical, and physiologic studies conducted on hearts from animals consuming alcohol for prolonged periods suggest a number of irreversible changes in cardiac tissue structure and function (3,4).

The biochemical basis for these structural and functional abnormalities is not clear but probably involves changes in cell membranes concerned with the regulation of calcium fluxes in the myocardium as suggested by a marked depression of Ca^{2+} uptake by mitochondria and sarcoplasmic reticulum as well as Ca^{2+}-dependent myofibrillar ATPase in cardiac muscle from animals chronically ingesting alcohol (5-7). Phospholipids are closely linked with these changes because they are not only essential for the restructuring of membrane during alcohol-induced cardiomegaly but also for the optimal activity of membrane ATPase (8,9). Despite their importance, relatively little is known about cardiac phospholipid synthesis during chronic alcohol consumption.

Phosphotidylcholine (PC)[1] is the major phospholipid of the heart in all species examined (10). The bulk of myocardial PC is synthesized via the cytidinediphosphocholine

Abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidyl-N-monomethylethanolamine; PMME, phosphatidyl-N, N-dimethylethanolamine; AdoMet, S-adenosyl-L-methionine and TKM buffer, 40 mM tris (hydroxymethyl) aminomethane (pH 7.4) + 7.5 mM KCl + 2 mM $MgCl_2$.

0006-291X/83 $1.50

pathway (11). However, a small fraction is synthesized via sequential transmethylation of PE using AdoMet as the methyl donor (12-14). Such methylation reactions have been implicated as mediators of several membrane-associated functions (15), including regulation of Ca^{2+}-uptake and Ca^{2+}-ATPase (16). In this communication we examine the two pathways of PC synthesis in hearts from control and alcoholic rats and report an increase in phospholipid methylation in the myocardium of alcoholic rats.

MATERIALS AND METHODS

Materials:

S-Adenosyl-L-[methyl-^{3}H] methionine (14 Ci/mmol), [^{3}H-methyl]-L-methionine (5 Ci/mmol) and [^{3}H-methyl] choline were purchased from New England Nuclear (Boston, MA). PE, PME, PMME, and PC were obtained from GIBCO (Grand Island, NY), whereas AdoMet was a product of Sigma Chemical Co. (St. Louis, MO). All other reagents were from Aldrich Chemical Company.

Methods:

Animals: Male Sprague-Dawley rats (200-250 g) supplied by the Holtzman Company (Madison, WI) were maintained on a 12-h light-dark cycle at an ambient temperature of 23°C. Rats were given chronic treatment with alcohol by administering 7.5% ethanol (vol/vol) in the drinking water for one week, followed by 10% ethanol (vol/vol) for eleven successive weeks. Control animals received tap water ad libitum.

Regional dissection of heart: Rats were killed by decapitation, and the hearts were excised and dissected into seven regions (sinoatrial node, right atrium, left atrium, right venticle, arterio-ventricular node, left ventricle, and interventricular septum) as described by Schmid et al (17).

Incorporation of [^{3}H-methyl]-methionine and [^{3}H-methyl]-choline into phosphatidyl-choline by left ventricles: Left ventricular tissues from control and alcoholic rat hearts were dissected, rinsed with cold TKM buffer, and then sliced (average thickness 0.5 mm) using Stadie-Riggs Tissue Slicer (Arthur H. Thomas). Tissue slices (approx. 50 mg) were incubated for 30 min in 1.0 ml KRB buffer (18), pH 7.4 containing 5 µCi (62.5 pmol) of [^{3}H-methyl]-methionine or [^{3}H-methyl]-choline at 37°C in an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (120 vpm). At the end of the incubation period tissues were removed, rinsed with cold TKM buffer and frozen over dry ice. Frozen tissues were transferred to thick-walled glass tubes and then homogenized in 1.0 ml cold distilled water using a small glass homogenizer. A 10 µl aliquot of the homogenate was withdrawn for protein determination (19) and to the remainder, 1.0 ml of 20% (w/v) trichloroacetic acid was added. After 5 minutes, tubes were centrifuged and pellets collected. To extract phospholipids from the pellet, 3.0 ml of chloroform/methanol (3:1) containing 50 µg/ml of butylated hydroxytoluene, and 2 ml of 0.1 M KCl in 50% methanol were sequentially added. The mixture was vigorously vortexed twice and then centrifuged at 200 xg for 10 min. The aqueous phase was discarded; the chloroform phase was rewashed with 2 ml of 0.1 M KCl in 50% methanol. The chloroform phase was evaporated to dryness under nitrogen gas at 23°C and then stored overnight at -70°C. The residue was dissolved in 0.1 ml of chloroform, and then 80 µl of it was placed on a silica gel G plate and the chromatogram was developed in solvent 32 (chloroform/propionic acid/n-propyl alcohol/water, 3/2/6/1) at 23°C in ascending mode. The phospholipid standards were chromatographed simultaneously and their positions were visualized by spraying a saturated solution of iodine in chloroform. The area corresponding to PC (Rf=0.12) was scraped and transferred to a miniscintillation vial and the radioactivity was determined after adding 4 ml of scintillation fluid (Formula-9963, NEN). The values given are the mean ±SEM, and the differences between the treatment groups were determined by paired t-test (20).

Preparation of crude extract and assay of phospholipid methyltransferase activity: Tissues were homogenized in cold TKM-buffer (100 mg tissue/ml) using polytron (setting 4, 30 sec). The homogenate was filtered through double cheesecloth to remove any debris. The methylation of phospholipids was measured by incorporation of [^{3}H] methyl group from S-adenosyl-L-[methyl-^{3}H]-methionine into phospholipids. The reaction mixture (50 µl) contained: Tris-HCl buffer (5.0 µmol, pH 9.0), $MgCl_2$ (0.5 µmol), sodium EDTA (5 nmol), S-adenosyl-L-[methyl-^{3}H]-methionine (2 µCi, 10 nmol) and tissue extract (50-200 µg protein). The reaction was initiated by the addition of tissue extract and run in a 12-ml stoppered glass tube at 37°C for 5 min. To stop the reaction, 3 ml of chloroform/methanol/hydrochloric acid (2/1/0.02, v/v/v) containing BHT (50 µg/ml) followed by 2 ml of 0.1 M KCl in 50% methanol was added. The mixture was vigorously vortexed twice and then centrifuged at 200 x g for 10 min. The aqueous phase was aspirated, the chloroform phase was rewashed with 2 ml of 0.1 M KCl in 50% methanol, and 1 ml of the chloroform phase was transferred to a miniscintillation vial. After the solvent was evaporated to dryness at 80-85°C, 4 ml of scintillation fluid (Formula-963, New England Nuclear) was added and the radioactivity was measured in a Packard Liquid Scintillation Counter.

RESULTS

Differential Modulation of Two Pathways of Phosphotidylcholine Synthesis by Chronic Alcohol Consumption: To determine the effect of chronic alcohol ingestion on PC synthesis by CDP-choline and PE transmethylation pathways, left ventricular tissues from rat myocardium were incubated with [^{3}H-methyl]-methionine or [^{3}H-methyl]-choline as described in "Materials and Methods" and then the amount of radioactivity incorporated into PC was determined. The data presented in Table I show that chronic alcohol consumption led to an increase in PC synthesis by transmethylation (control=44.4±3.7, alcoholic=69.6±9.7 CPM/30 min/mg protein, $p<0.05$) but not CDP-choline pathway (control=2728.5±215.3, alcoholic=3104.6±382.6, $p>0.1$). This apparent increase in the incorporation [^{3}H-methyl] group from [^{3}H-methyl]-methionine into PC may be due to either an increase in the synthesis of methyl-donor [^{3}H-methyl]-AdoMet or an increase in

Table 1

Incorporation of [^{3}H-methyl]-methionine and [^{3}H-methyl]-choline into phosphatidylcholine by left ventricles of control and alcoholic rat hearts.

	Incorporation of isotopes in PC CPM/30 min/mg protein	
Treatment	[^{3}H]-methionine	[^{3}H]-choline
Control (N=8)	44.4 ± 3.7	2728.5 ± 215.3
Alcoholic (N=8)	69.6 ± 9.7	3104.6 ± 382.6
P-value	<0.05	>0.1

Table 2

Regional distribution of phospholipid methyltransferase activity in control and alcoholic rat hearts.*

Region		pmols [^{3}H]-methyl incorporated into phospholipids/min/mg protein		
		Control	Alcoholic	p-value
1.	Sinoatrial node	9.6 ± 0.8	40.5 ± 6.1	<0.01
2.	Right atrium	7.1 ± 0.2	20.1 ± 2.0	<0.01
3.	Left atrium	6.5 ± 0.2	19.0 ± 4.9	<0.05
4.	Right ventricle	5.0 ± 0.2	8.2 ± 1.6	>0.05
5.	Arterioventricular node	5.9 ± 0.5	13.4 ± 3.0	<0.05
6.	Left ventricle	4.4 ± 0.4	9.1 ± 1.2	<0.01
7.	Interventricular septum	5.1 ± 0.3	8.6 ± 2.1	>0.1

* The data are presented as mean ±SEM (n=5).

phospholipid methyltransferase activity. Therefore, next we determined the characteristics of myocardial phospholipid methyltransferase and the possible changes in its activity after chronic alcohol consumption.

Characteristics of Myocardial Phospholipid Methyltransferase Activity: The rate of transfer of [^{3}H-methyl] group from [^{3}H-methyl]-AdoMet to phospholipids was linear with time up to 30 min at pH 6.0 and 8.0 and up to 15 min at pH 9.0 but remained constant with longer incubations. The nature of the products of the transmethylation reaction was studied by thin-layer chromatography as described in "Materials and Methods." A stepwise synthesis of PME, PMME, and PC was demonstrated. The sequence of methylated product formation was similar in control and alcoholic rat hearts; all steps were enhanced in the latter group. The pH optimum was found to be between 9.0 and 9.5. The activity at pH 8.5 and pH 10.0 was 73% and 30% of the maximal activity, respectively. The average initial rate of the reaction for whole heart extracts at pH 9.0 was 6.1±0.8 pmols/min/mg protein (n=8). This amounted to a transfer of about 0.07% of [^{3}H-methyl]-AdoMet to phospholipids during a standard 5-min incubation.

Uneven Distribution of Phospholipid Methyltransferase Activity in Rat Heart and Its Increase After Prolonged Alcohol Consumption: Phospholipid methyltransferase activity was ubiquitously but unevenly distributed throughout the specialized and the contractile regions of rat myocardium (Table II, left column). The rank order of tissues with decreasing enzyme activity is the sinoatrial node > right atrium > left atrium > AV node > interventricular septum > right ventricle > left ventricle. The enzymatic activity was significantly increased ($p<0.05$) in all regions of alcoholic heart except right ventricle and interventricular septum (Table II, right column).

DISCUSSION

The data presented here show for the first time an ubiquitous but uneven distribution of phospholipid methyltransferase activity in both the contractile and the specialized regions of rat heart. Chronic alcohol consumption leads to an increase in PC synthesis by transmethylation but not CDP-choline pathway. This is in contrast to the inhibitory effects of chronic alcohol consumption on myocardial Ca^{2+}-ATPase and citric acid cycle enzymes (7,21). The adverse effects of prolonged alcohol intake is not limited to myocardial tissues. It has been shown to alter structure and functions of tissues and organ systems such as endocrine system (22), muscle (23), pancreas (24), liver (25,26), gastrointestinal tract (27), and brain (28). Some of the effects of alcohol on synaptosomal membranes include increased membrane fluidity (29) and cholesterol content (30), attenuation of membrane fluidizing effects of ethanol (31), decreased muscarinic receptor numbers (32) and an increase in the density of α-adrenergic receptors (33).

Phospholipid methylation has been suggested to participate in a number of membrane-associated physiological processes including the regulation of membrane fluidity (34), hormone receptor number (35,36), and hormone receptor function (37-39). Although the possible physiological role of the stimulation of myocardial phospholipid methylation by chronic alcohol consumption remains to be established, a similarity between the effects of alcohol and increased phospholipid methylation on membrane fluidity and the membrane receptor numbers in neuronal systems must be pointed out. Since the stimulation of phospholipid methylation in cardiac membranes has been shown to increase the number of β-adrenergic receptors (12), it is possible that an increase in phospholipid

methylation after chronic alcohol consumption may affect β-adrenergic receptor-associated cardiac functions.

ACKNOWLEDGEMENTS

We thank Miss Virginia Howard for excellent editorial assistance. This research was supported by American Heart Association - Louisiana Affiliate.

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