A sensitive method for the quantitation of lysophosphatidylcholine in canine heart

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Abstract We have developed a procedure for the determination of small amounts of lysophosphatidylcholine in cardiac tissue. Lysophosphatidylcholine from canine heart was separated from the major phospholipids by column chromatography, and then acetylated with labeled acetic anhydride. The acetylated lysophosphatidylcholine was isolated by thin-layer chromatography and the lysophosphatidylcholine content was calculated from the radioactivity associated with the acetylated product. Although the sensitivity of the assay depends on the specific radioactivity of the acetic anhydride used, as low as 0.5 nmol of lysophospholipid in tissue samples can be readily quantitated. The results obtained from the control and ischemic canine cardiac tissues by this assay compares favorably with those obtained by lipid-phosphorus assay. The sensitivity and specificity of the present procedure allows us and other investigators to assay for lysophosphatidylcholine content in very small (10 mg wet weight) tissue samples. — **Wientzek, M., G. Arthur, R. Y. K. Man, and P. C. Choy.** A sensitive method for the quantitation of lysophosphatidylcholine in canine heart. *J. Lipid Res.* 1985. **26:** 1166–1169.

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Lysophospholipids are ubiquitously distributed in all mammalian tissues (1). The majority of these lipids are formed from the hydrolytic action of intracellular phospholipase A on the parent phospholipids. Lysophosphatidylcholine (LPC) is the major lysophospholipid in mammalian tissues. It is regarded as an important intermediate in the catabolism of phosphatidylcholine (PC) (2) and in the resynthesis of PC in most tissues by the deacylation–reacylation cycle (3). Due to its cytolytic property, the level of LPC in all tissues is under rigid control (4).

Recently, LPC has been implicated as a major biochemical factor in the production of cardiac arrhythmias subsequent to the development of cardiac ischemia (5–7). Addition of exogenous LPC has been shown to cause depression of transmembrane potential in cardiac fibers (8) and cardiac arrhythmias in the isolated perfused heart (9, 10), but the physiological importance of this lysolipid in the ischemic tissue remains undefined. In order to further elucidate the role of LPC in the genesis of cardiac arrhythmias, the tissue level of LPC at different stages of cardiac ischemia must be accurately assessed. In this communication, we report a new procedure for the determination of LPC in the cardiac tissue that is highly sensitive and specific.

METHODS AND MATERIALS

Materials

L-α-Lysophosphatidylcholine (egg yolk) and phospholipase C (*Clostridium welchii*) were purchased from Sigma Chemical Co. Phosphatidylcholine (pig liver) and sphingomyelin (beef brain) were obtained from Serdary Labs. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[methyl-^{14}C]choline was obtained from New England Nuclear. 1-[1-^{14}C]palmitoyl,2-lyso-*sn*-glycero-3-phosphocholine, [1-^{14}C]acetic anhydride, and [^{3}H]acetic anhydride were all purchased from Amersham. Acetic anhydride of analytical grade was obtained from BDH Chemicals and kept desiccated over sodium sulfate at 4°C. Pyridine was obtained from Fisher Scientific Company and kept desiccated over barium oxide at 4°C. Anhydrous ether, 69–72% perchloric acid, chloroform, methanol, and all other chemicals and solvents used were of certified A.C.S. grade from Fisher Scientific Company. Aqueous counting scintillant was purchased from Amersham and thin-layer chromatographic plates (SIL-G25) were from Brinkman. Silicic acid (Bio-Sil A) 100–200 mesh was obtained from Bio-Rad Laboratories. All glassware was treated with dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane, BDH Chemicals) before use.

Methods

Mongrel dogs of either sex, weighing 8–15 kg, were

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; TLC, thin-layer chromatography.

used throughout this study. Ischemia in the heart was produced by surgical occlusion of the left anterior descending coronary artery by the Harris two-stage technique (11). The heart was excised 24 hr after surgery and ischemic areas of tissue from the left ventricle were removed and placed on ice. Control cardiac tissue was obtained from the nonischemic areas of the left ventricle of the same heart as described previously (12). All tissue samples were homogenized immediately in chloroform–methanol 1:1 (v/v), with a Polytron homogenizer (Brinkman PT10/35). Lipids were extracted from the homogenate as previously reported (13) and the lipid extracts were redissolved in a volume (ml) of chloroform–methanol 2:1 (v/v) equivalent to the gram wet weight of the tissue in the homogenate. Total lipid phosphorus was determined by the method of Bartlett (14), using perchloric acid digestion as described by King (15).

LPC was isolated from the total lipid extract on silicic acid columns according to the protocol of Sheltawy and Dawson (16). Silicic acid (0.5 g) was suspended in chloroform and packed into a 10 × 0.5 cm column. Lipid extract (20–100 μl) was applied to the column. The lipids were eluted from the column by the sequential application of 5 ml of chloroform, 5 ml of chloroform–methanol 4:1 (v/v), 25 ml of chloroform–methanol 1:1 (v/v), and 40 ml of chloroform–methanol 1:9 (v/v). LPC was eluted from the column with the last eluant and the volume of the LPC-containing fraction was reduced by evaporation in vacuo. The content was transferred to a test tube and the solvent was totally removed by evaporation under nitrogen.

Acetylation of LPC was conducted in the following manner. The reaction mixture contained 24 μl of pyridine, 36 μl of [^{3}H]acetic anhydride (500–2000 dpm/nmol) and 0.6 μl of perchloric acid. These were added sequentially to each sample tube containing dry lipid sample. The tubes were sealed with Teflon-coated stoppers and the contents were mixed vigorously. Tubes were then incubated at 70°C for 30 min, mixed for 10 sec, and reincubated for another 30 min. After incubation, the solvents in the reaction mixture were evaporated with nitrogen. The residue was dissolved in 1 ml of chloroform followed by 1.25 ml of methanol–water 2:3 (v/v) to cause phase separation. The upper phase was removed, and the lower phase was reextracted with 1.25 ml of methanol–water 2:3 (v/v). The solvents in the lower phase were evaporated to dryness with nitrogen, and the residue in each tube, including reagent blanks, was redissolved in 25 μl of chloroform–methanol 2:1 (v/v). The acetylated LPC was isolated by TLC with a solvent system containing chloroform–methanol–water–acetic acid 70:30:4:2 (v/v) and was visualized by exposure of the TLC plate to iodine vapor. In some experiments, 10 nmol of non-labeled 1-acyl, 2-acetyl-*sn*-glycerophosphocholine was used as a carrier during TLC separation. The amount of radioactivity in the acetylated LPC fraction was determined by scintillation counting. Radioactivity was calculated from cpm by the Channel Ratio Calibration method. The theoretical yield on the acetylation of LPC was calculated from one-half the specific radioactivity of [^{3}H]acetic anhydride used in the assay. Student's *t* test was used for statistical analysis and significance was defined as $P < 0.05$.

Hydrolysis of the acetylated LPC by phospholipase C (*C. welchii*) was conducted as previously described (13). In order to detect any radioactivity associated with the long chain acyl groups, both the acyl and acetyl groups of the acetylated LPC (acetylated by [^{3}H]acetic anhydride) were converted into methyl esters (17). The labeled methyl acetate formed from the reaction was removed from the long chain methyl esters by evaporation at 50°C under a stream of nitrogen. The acyl composition of LPC was determined by the methyl esters of the fatty acyl groups with gas–liquid chromatography.

RESULTS

In a preliminary study, 10 nmol of LPC was acetylated with [^{3}H]acetic anhydride (2,000 dpm/nmol) and the product formed was isolated by TLC. The acetylated LPC migrated as a single band which was found to correspond to the standard 1-acyl, 2-acetyl-*sn*-glycerophosphocholine (R_f = 0.11). The site of acetylation of LPC was investigated by treatment of the acetylated LPC with phospholipase C (13). The reaction was terminated and the radioactivity in the aqueous and organic phases was analyzed. No radioactivity was detected in the aqueous phase which indicates that the phosphocholine moiety in LPC was not acetylated. Over 98% of the original radioactivity was found in the organic phase. Analysis of the organic phase by TLC (13) revealed that all the radioactivity in this fraction migrated close to the solvent front, which indicates that the acetylated LPC was completely hydrolyzed by phospholipase C and the lipid moiety of LPC after hydrolysis was acetylated. In order to demonstrate that the acyl groups were not acetylated, the acyl groups of LPC before and after acetylation were methylated and analyzed by gas–liquid chromatography. Comparison of the methyl esters showed identical acyl contents before and after acetylation, and no significant radioactivity was detected in the long chain methyl esters. From the results obtained, it is clear that the acyl group of the LPC was not acetylated, and there was no significant ^{3}H exchanged onto the acyl group.

Acetylation of LPC was shown to be linear from 0.5–100 nmol of LPC (**Fig. 1**) and the yield was greater than 90% of the theoretical yield calculated from the specific radioactivity of the [^{3}H]acetic anhydride. Identical results were obtained with [1-^{14}C]acetic anhydride. Both pyridine and perchloric acid were required for the reaction and the ratio of acetic anhydride–pyridine–perchloric acid was

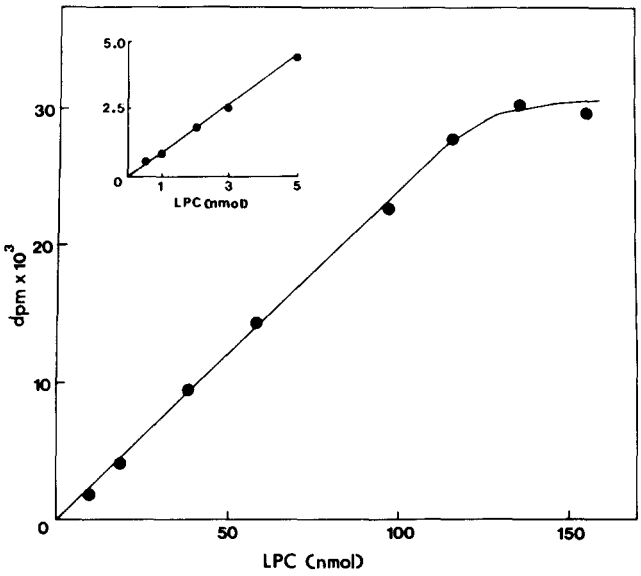

Fig. 1. Quantitation of lysophosphatidylcholine by acetylation. The formation of 1-acyl, 2-[^{3}H]acetyl-*sn*-glycerophosphocholine (acetylated LPC) with 0.5–160 nmol of LPC was determined as described in Methods. Specific radioactivity of [^{3}H]acetic anhydride was 500 dpm/nmol in each assay except in the inset where 2,000 dpm/nmol was used. Each point represents the mean of triplicate determinations.

optimized at 6:4:0.1 (v/v). The optimum reaction volume for 20 nmol of LPC was 60 μl and further increases in volume did not improve the yield. The reaction was essentially completed when incubated at 70°C for 60 min, but the yield was dramatically reduced when the reaction was incubated at 37°C, regardless of the incubation period.

Since acetic anhydride has the ability to acetylate other lysophospholipids and lipids containing a hydroxyl group (18), the LPC fraction must be isolated from the total tissue lipid extract prior to the acetylation reaction. Isolation by silicic acid column chromatography was rapid and provided good yield. When 1-[1-^{14}C]palmitoyl glycerophosphocholine was added to the total lipid extract prior to column chromatography, 92% of the radioactivity was recovered in the LPC fraction. Analysis of this fraction by TLC showed that it contained only lysophosphatidylcholine, sphingomyelin, and phosphatidylcholine. Although sphingomyelin was also acetylated during the reaction, its acetylated product migrated differently (R_f = 0.19) and was readily separated from the acetylated product of LPC by TLC. No radioactivity was detected in phosphatidylcholine after the reaction, which indicates that it was not acetylated and there was no isotope exchanged onto the acyl chains.

LPC content in the control and ischemic canine heart tissue was determined by the acetylation method. The amount of radioactivity associated with the acetylated LPC was used to determine the LPC content from a standard curve. The data obtained are shown in **Table 1.** In a control experiment, an internal standard of 10 nmol of unlabeled LPC was added to each sample prior to the acetylation. With the aid of this internal standard, the yield of the acetylation reaction was determined to be better than 90%. The results obtained from the acetylation reaction were compared with those obtained by determination of lipid-P content (Table 1). A 2.5-fold increase in LPC content in the ischemic cardiac tissue was observed by both determinations.

DISCUSSION

The involvement of LPC in several molecular events during arrhythmia after myocardial ischemia (5–10, 12) has put a focus on the need to accurately and specifically quantitate this lysolipid in order to examine its physiological role in the genesis of cardiac arrhythmia. In the last several years, the concentration of LPC in cardiac tissues has been subject to much debate (19). The discrepancies were probably caused by *1*) mode of extraction and *2*) mode of isolation and quantitation after isolation. It has been generally agreed that extraction of tissue LPC in an acidic medium will cause hydrolysis of the parent phospholipids, which will artificially cause the generation of LPC during extraction (19, 20). Hence, LPC was extracted in this study with a neutral solvent, which has been shown to be a preferred mode of extraction (20). Although the method of isolation by TLC and the quantitation of the LPC fraction by lipid-P content have been used in all previous studies, there are a number of difficulties with this mode of determination. The quantitation of LPC by lipid-P content is subject to interference from other phosphate-containing substances and a substantial amount of tissue (200 mg wet weight) is required for each determination because of the sensitivity of the lipid-P assay (20–50 nmol) (20). With the present method, as little as 10 mg of cardiac tissue (0.5–1.0 nmol of LPC) can be accurately determined and the sensitivity of the assay can be further enhanced by increasing the specific radioactivity of the labeled acetic anhydride. The specificity of

TABLE 1. Lysophosphatidylcholine (LPC) content in control and ischemic canine heart

Mode of LPC Determination	LPC Content (nmol/g wet weight) Control (C) Tissue	Ischemic (I) Tissue	Ratio (I/C)
Acetylation	92 ± 4 (15)	229 ± 26 (9)	2.5
Lipid-P	103 ± 7 (30)	255 ± 25 (6)	2.5

Ischemic tissue from canine heart was obtained by a Harris two-stage occlusion of the left anterior coronary artery (11, 12). The levels of LPC in both control and ischemic tissues were assayed by the acetylation procedure as described in Methods and the lipid-P determination. The results are expressed as mean ± standard error of the mean (number of determinations).

the assay alleviates extensive purification of LPC, thus allowing the rapid isolation of the lipid by column chromatography.

The tissue LPC content obtained by lipid-P determinations is slightly but consistently higher than that obtained by the radioactive labeling method. Although the difference is not statistically significant in all cases, the higher mean values obtained by lipid-P analysis are probably caused by small amounts of other phosphate-containing compounds that may co-chromatograph with LPC. The specificity of the present assay eliminates this possibility and allows an accurate assessment of the changes in LPC levels during cardiac ischemia and other cardiac disorders. The sensitivity of the assay also provides the ability to determine the lysolipid content in small biopsy samples and in small volumes of ischemic blood.

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