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Phospholipid Synthesis in the Human Arterial Intima *

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The accumulation of lipids within the arterial intima represents an important feature in the development of the atherosclerotic lesion. Although much interest has been centered on the relationship between blood lipid levels and atherosclerosis, limited information is available in man concerning the intimal metabolism of lipids and its possible role in lipid accumulation within the vessel wall. Previous studies indicate, however, that the human intima is in fact a dynamic tissue involved in a number of metabolic processes (2), and preliminary observations suggest that the human intima can synthesize lipids, proteins, and lipoproteins (3, 4).

Interest in the arterial metabolism of phospholipids is derived in part from the apparent biological role of phospholipids in the transport of lipids (5) and from the observations that the total phospholipid content of the arterial wall increases with the severity of atherosclerosis (6, 7). All phospholipid groups appear to share in this increase, although the major rise of phospholipid in the atherosclerotic lesion occurs in the sphingomyelin fraction (7, 8). Studies in rabbits by McCandless and Zilversmit have demonstrated intimal phospholipid synthesis and have suggested that this local synthesis accounts for the major source of arterial phospholipid (9). Subsequent indirect studies in man have likewise suggested the presence of local intimal synthesis on the basis of higher phospholipid specific activities in the intima as compared with the plasma after infusions of inorganic ^{32}P (10). To approach the problem more directly, the present investigation has been undertaken to study the synthesis of phospholipids in isolated segments of human arterial intima and to characterize and compare the individual phospholipids formed *in situ*.

Methods

Human arterial tissue specimens were obtained either at postmortem examination within 4 hours of death or at the time of surgery for vascular disease or limb amputations. Segments of the descending portion of the thoracic aorta were used in all of the studies involving postmortem material (Patients 1, 2, 3, 8, and 10). The surgical specimens included aortic segments from areas surrounding atherosclerotic aneurysms of the thoracic and abdominal aorta and segments of femoral artery obtained from amputated limbs. The degree of atherosclerosis was graded by visual inspection as previously described (11). The adventitia and most of the media were carefully stripped away, and the remaining tissue, which was found by microscopic examination to be comprised of the entire intima and a small portion of adherent media, was divided into approximately 1- × 1-cm sections. The blood vessel segments thus prepared were suspended in 10 ml of incubation solution [1] containing inorganic ^{32}P (5.0 μc per ml, carrier free) and were incubated in an atmosphere of 95% oxygen and 5% carbon dioxide for 4 to 24 hours at 37° C in a Dubnoff shaker. In Patients 1, 2, 5, and 6, tissue aliquots were also incubated in solution containing acetate-1-^{14}C [2] (1.0 μc per ml, SA 53.0 mc per mmole). In Subjects 1, 2, 7, and 10, intimal segments were incubated in a medium containing albumin (50 mg per ml) and palmitate-^{14}C (0.5 μc per ml, SA 3 mc per mmole).[2, 3] All incubations were begun be-

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[1] Composition: Na 130 mEq per L, K 4.0 mEq per L, Ca 3.0 mEq per L, Cl 109 mEq per L, and lactate 28 mEq per L.

[2] Obtained from the New England Nuclear Corp., Boston, Mass.

[3] The radiopurity of the palmitate was determined by gas liquid chromatography after methylation with boron trifluoride. The analyses were carried out on a diethylene glycol succinate polyester column (20% wt/wt) us-

tween 3 and 6 hours after a patient's death or within 1 hour of surgical removal of tissue. Arterial tissue that could not be incubated within 6 hours of death was not utilized in these studies. Previous studies had demonstrated that when longer intervals (14 to 25 hours) were present between death and the beginning of incubation, the total phospholipid synthesis was negligible (less than 10% of the lowest value reported here). Cultures taken of selected samples of the incubation fluid at the end of incubation revealed no growth of bacteriological organisms.

After incubation, the tissues were washed five times, blotted, minced, and then homogenized in chloroform-methanol 2:1 (vol/vol). Aliquots of the minced tissue were dried to a constant weight, and all results were expressed in terms of the dry tissue weight. The lipids were extracted from the homogenate by the method of Folch, Lees, and Sloane Stanley (12) and were separated by silicic acid chromatography into fractions containing neutral lipids and individual phospholipid groups as described by Newman, Liu, and Zilversmit (13). Serial elutions were carried out using 50 ml chloroform (vol/vol), 50 ml 20% methanol in chloroform, 150 ml 40% methanol in chloroform, 50 ml 60% methanol in chloroform, and 50 ml methanol to yield, respectively, neutral lipids, noncholine-containing phospholipids, lecithins, sphingomyelins, and lysolecithins. The eluates from the column were collected in five major fractions according to the different solvents used. Duplicate aliquots of each fraction were analyzed for lipid phosphorus content by the method of Bartlett (14) and for lipid-^{32}P or ^{14}C radioactivity in a liquid scintillation spectrometer after addition of 18 ml scintillation solution (11). Because analyses of the column eluates revealed incomplete separation of the noncholine-containing phospholipids from lecithins and of lecithins from sphingomyelins, further separation of the phospholipids was carried out by thin layer chromatography. The latter technique could not be used alone, since the large amounts of lipid in the tissue extracts overloaded the capacity of the thin layer system. Duplicate aliquots of the column extracts were reduced to a smaller volume, transferred to pointed tubes, and then taken to dryness at room temperature under a stream of nitrogen. The residue was redissolved in 0.2 ml of chloroform-methanol 2:1 (vol/vol), and the phospholipids in each duplicate fraction were separated by two dimensional thin layer chromatography according to the method of Skidmore and Entenman (15). The plates were exposed to iodine vapors, and the individual phospholipid spots were circled and identified by comparison with known phospholipid standards.[4] Excellent separation of varying mixtures of standards of phosphatidyl serine, phosphatidyl ethanolamine, lecithin, sphingomyelin, and phosphatidyl inositol was achieved. The iodine was allowed to evaporate, and the phospholipid spots and surrounding 0.5 cm of silica gel were scraped individually from the plate to weighing papers. When a given phospholipid group was present on more than one plate as usually occurred with the lecithins and sphingomyelins, the comparable spots were combined. The silica gel fractions containing phosphatidyl serine and phosphatidyl ethanolamine were combined and analyzed together for total radioactivity and lipid phosphorus content. The silica gel samples from one of the duplicate sets were suspended in toluene scintillation solution containing 4 g per 100 ml of thixotropic gel[5] and were counted directly in the liquid scintillation spectrometer. The silica gel fractions from the remaining set were transferred to conical tubes, and the phospholipids were extracted by the method of Abramson and Blecher (16). Lipid phosphorus content and radioactivity of the extracted material were measured as previously noted. The lipid phosphorus values were multiplied by 25 to convert to phospholipid. To detect possible contamination of the sphingomyelin by other phosphatides, we treated aliquots of the extract from the sphingomyelin-containing silica gel with KOH and separated the fractions according to the method of Schmidt, Benotti, Hershman, and Thannhauser (17). The total phosphorus content and radioactivity of the extracts were confined to the alkali-resistant sphingomyelin-containing fraction. Analyses of the silica gel in the areas intervening between the individual phospholipid spots revealed only trace amounts of phospholipid content or radioactivity. No significant radioactivity was present in the areas of the chromatogram where phosphatidyl inositol would have been expected to migrate. Small amounts of phospholipid ranging between 0.5 and 3.5% of the total applied to a plate remained at the origin. The recovery of phospholipid from the silicic acid column averaged 98% (range 94 to 101%). The recovery from the silica gel of lecithin averaged 86% (range 80 to 89%), of sphingomyelin 84% (range 80 to 86%), and of phosphatidyl serine and phosphatidyl ethanolamine 69% (range 64 to 71%). The results were corrected to allow for the decreased recovery of the phospholipids from the silica gel. All samples were counted to a statistical accuracy of 4% (18). The silica gel and thixotropic gel mixtures did not produce significant quenching of either lipid-^{14}C or ^{32}P radioactivity.

In Patients 1 and 10, grossly normal portions of aorta were dissected away from atherosclerotic sites, and the tissues thus divided were analyzed separately.

In Patient 1, the cephalin- and lecithin-containing fractions of tissue incubated with acetate-^{14}C were saponified according to the method of Albrink (19), and the fatty acid- and glycerol-containing moieties were counted separately for ^{14}C radioactivity.

Control samples of intima, heat killed by immersion in boiling water for 2 minutes, were also incubated with the radioactive phospholipid precursors and analyzed for phospholipid radioactivity. None of the heat-killed specimens contained significant lipid-^{32}P or ^{14}C radioactivity.

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ing a modular chromatograph with an ionization detector. The column effluents were collected at the gas liquid chromatography outflow portal under liquid nitrogen and analyzed for ^{14}C radioactivity. The radiopurity as determined by this method varied between 97 and 99%.

[4] Obtained from Sigma Chemical Co., St. Louis, Mo.

[5] Cabot Corp., Boston, Mass.

TABLE I

Incorporation of inorganic ^{32}P into intimal phospholipids

Patient	Age and sex	Tissue	Degree athero-sclerosis (0–4+)	Phospholipid content				^{32}P incorporation into phospholipid			
				Total	Ceph-alin	Leci-thin	Sphingo-myelin	Total	Ceph-alin	Leci-thin	Sphingo-myelin
				mg/g dry wt				mμc/g/hour			
1	72 M	Thoracic aorta	0–1+	9.0	1.5	3.8	3.3	10.8	3.9	6.9	0
2	3 M	Thoracic aorta	0	11.0	2.5	4.3	2.5	49.2	24.1	25.1	0
3	39 M	Thoracic aorta	0–1+	23.0	4.0	11.3	5.3	18.4	8.5	9.9	0
4	72 M	Abdominal aneurysm	4+	32.3	2.3	8.8	17.2	86.1	23.6	57.6	4.90
5	70 F	Thoracic aneurysm	4+	34.3	9.8	11.7	12.0	14.5	4.7	9.3	0.46
6	48 F	Femoral artery	3–4+	51.0	7.3	14.5	24.5	5.8	2.0	3.7	0.10
7	66 F	Femoral artery	4+	33.0	6.0	10.5	13.0	59.8	6.7	52.7	0.38
8	68 F	Thoracic aorta	4+	50.3	7.5	18.0	19.5	14.4	11.5	2.8	0.08
9	62 M	Femoral artery	4+	112.8	22.5	38.8	43.8	189.6	25.3	162.0	2.30
10	57 M	Thoracic aorta	3–4+	45.8	5.0	11.1	27.0	10.2	2.8	7.1	0.25

Results

Table I summarizes the results of the incubation studies when inorganic ^{32}P was used as the labeled phospholipid precursor.

Intimal phospholipid content. The phospholipid content of the intima averaged 40.3 mg per g intima (range from 9.0 to 112.8). All atherosclerotic segments contained greater amounts of phospholipid (mean 51.4, SE ± 10.9 mg per g) than the normal tissue (mean 14.3, SE ± 5.4 mg per g). Lecithin was the major phospholipid of normal intima, averaging 43% of total intimal phospholipids as compared with respective values of 27% and 19% for the sphingomyelins and cephalins. In atherosclerotic tissue, sphingomyelin was the predominant phospholipid fraction, representing 45% of total phospholipid, whereas the lecithins comprised 31% and the cephalins 16% of the total. Eighty-four to 98% of the total phospholipids present were in the lecithin, cephalin, and sphingomyelin fractions. No attempt was made to quantitate the other intimal phospholipids, since none of these showed significant lipid-^{32}P radioactivity after incubation.

Incorporation of ^{32}P. The rate of incorporation of inorganic ^{32}P into phospholipids averaged 26.1 mμc per g per hour (SE ± 11.7) in normal intima (Patients 1 to 3) and 54.3 mμc per g per hour (SE ± 25.3) in the atherosclerotic segments (Patients 4 to 10). Two to four time points were used to calculate the incorporation rates. The major incorporation of ^{32}P into phospholipids was in the lecithins and cephalins. The lecithin fraction contained the greatest ^{32}P radioactivity when

TABLE II

Incorporation of inorganic ^{32}P into adjacent normal and atherosclerotic areas of thoracic aorta

Patient	Degree athero-sclerosis (0–4+)	Phospholipid content				^{32}P incorporation into phospholipid			
		Total	Cephalin	Lecithin	Sphingo-myelin	Total	Cephalin	Leci-thin	Sphingo-myelin
		mg/g dry wt				mμc/g/hour			
1	0–1+	9.0	1.5	3.8	3.3	10.8	3.9	6.9	0
	4+	30.3	2.8	9.3	14.5	13.1	3.0	9.6	0.51
10	0–1+	17.5	2.4	6.9	6.4	11.0	3.2	7.8	0
	3–4+	45.8	5.0	11.1	27.0	10.2	2.8	7.1	0.25

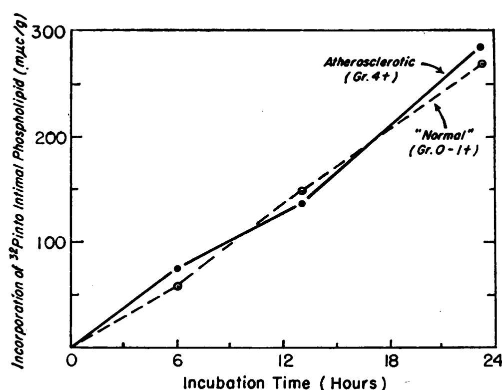

FIG. 1. INCORPORATION OF INORGANIC ^{32}P INTO INTIMAL PHOSPHOLIPIDS IN NORMAL AND ATHEROSCLEROTIC AORTIC INTIMA.

compared according to tissue weight in nine of ten tissue studies. The phospholipid-^{32}P specific activity was highest in the cephalin fraction in all normal tissues and in three of seven atherosclerotic segments. Significant incorporation of ^{32}P into sphingomyelin was evident in only atherosclerotic intima. In these atherosclerotic segments, the labeled sphingomyelin represented 0.5 to 5.7% of the total lipid-^{32}P radioactivity, and the sphingomyelin specific activity was 1.4 to 10.4% of that of the total phospholipids in the intima.

In Patients 1 and 10, in whom adjacent normal and atherosclerotic tissues were available for analysis (Table II), the total phospholipid radioactivity in the relatively normal areas (10.8 and 11.0 mμc per g per hour) was similar to that observed in the severely atherosclerotic segments (13.1 and 10.2 mμc per g per hour). These results in Patient 1 are illustrated in Figure 1. The incorporation of ^{32}P into sphingomyelin was measurable in the atherosclerotic segments but not in the adjacent relatively normal intima.

Incorporation of acetate-^{14}C. Table III summarizes the results when acetate-^{14}C was used as the phospholipid precursor. The rates of incorporation of acetate into phospholipid ranged from 1.88 to 5.02 mμc ^{14}C per g per hour. The major phospholipid-^{14}C radioactivity was associated with the cephalin and lecithin fractions. A small amount of labeled sphingomyelin (0.95% of the total phospholipid radioactivity) was present in one of the two normal vessels. In the two atherosclerotic tissues, sphingomyelin radioactivity represented 4.5 and 8.4% of the total phospholipid radioactivity. In Patient 1, after alkaline hydrolysis of the intimal phospholipid isolated by column chromatography, 76% of the ^{14}C radioactivity was present in the fatty acid and the remainder in the glycerol portion of the phospholipid molecule.

Incorporation of palmitate-^{14}C. The results of the incubation studies with palmitate-^{14}C are summarized in Table IV. Significant incorporation of labeled palmitate into intimal phospholipids was observed in all tissues studied. The major incorporation of palmitate into phospholipid was present in the cephalins and lecithins with the former accounting for the major ^{14}C radioactivity in three of four instances. No significant sphingomyelin synthesis was demonstrable in either of the normal specimens. In the atherosclerotic intima, 2.9 and 8.4% of the total palmitate incorporated into phospholipid was present in the sphingomyelin fraction.

TABLE III

Incorporation of acetate-^{14}C into intimal phospholipids

Patient	Tissue	Degree atherosclerosis (0–4+)	Acetate-^{14}C incorporation into phospholipid			
			Total	Cephalin	Lecithin	Sphingomyelin
			mμc ^{14}C/g/hour			
1	Thoracic aorta	0	2.11	1.48	0.61	0.02
2	Thoracic aorta	0	1.88	1.27	0.61	0
5	Thoracic aneurysm	4+	5.02	1.40	3.20	0.42
6	Femoral artery	3–4+	2.68	0.97	1.59	0.12

TABLE IV

Incorporation of palmitate-^{14}C into intimal phospholipids

Patient	Tissue	Degree athero-sclerosis (0–4+)	Palmitate-^{14}C incorporation into phospholipid			
			Total	Cephalin	Lecithin	Sphingo-myelin
			mμc ^{14}C/g/hour			
1	Thoracic aorta	0–1+	32.51	24.50	8.01	0
2	Thoracic aorta	0	2.81	1.10	1.71	0
7	Femoral artery	4+	1.71	1.46	0.20	0.05
10	Thoracic aorta	4+	1.19	0.84	0.25	0.10

Discussion

These studies suggest that the human arterial intima has the capacity to synthesize phospholipid. Phospholipid formation did not seem limited to any single portion of the phospholipid molecule. Synthesis of the glycerol and fatty acid moieties was suggested by the incorporation studies with acetate, formation of the phosphoric acid complex was indicated by the utilization of inorganic ^{32}P, and incorporation of long chain fatty acid was apparent from the observations with labeled palmitate. The major phospholipids that appeared to be formed in both normal and atherosclerotic intima were the lecithins and cephalins. Most atherosclerotic intima incubated with either ^{32}P, acetate, or palmitate synthesized more lecithins than cephalins. The results were more variable in normal intima, where cephalin synthesis predominated with acetate as a precursor, but no definite pattern was present when either ^{32}P or palmitate was used. Because of the limited number of patients studied, it is unclear whether the qualitative differences in phospholipid production observed with the different isotopic precursors in the normal and atherosclerotic tissues are indicative of actual differences in the rates of turnover of various portions of the phospholipid molecule.

The apparent rate of phospholipid synthesis in atherosclerotic tissue, when related to tissue weight, was similar to or more rapid than that observed in normal intima (depending upon whether the comparisons were made between adjacent normal and atherosclerotic areas of the same vessel or between different vessels). Since total intimal weight increases with atherosclerosis, the results suggest that total intimal phospholipid synthesis is greater in atherosclerotic than in normal tissue. The present findings are in agreement with those previously reported in rabbits by McCandless and Zilversmit (9).

Sphingomyelin synthesis appeared to be greater in atherosclerotic than in normal intima. All atherosclerotic tissue showed some incorporation of labeled precursor into sphingomyelin, whereas none of the normal tissues incubated with either ^{32}P or palmitate and only one incubated with acetate contained measurable quantities of labeled sphingomyelin after incubations lasting as long as 24 hours. A similar increased capacity for sphingomyelin synthesis in atherosclerotic tissue has previously been demonstrated in rabbits (9). The importance of these findings in relationship to the accumulation of sphingomyelin in atherosclerotic tissue is unclear. Indirect evidence does suggest that the plaque sphingomyelin may originate at least in part from local synthesis. Although a minor phospholipid constituent of the plasma, representing from 10 to 25% of the total plasma phospholipid (20, 21), sphingomyelin is the major phospholipid of the atherosclerotic lesion (7, 8) and accumulates predominantly at the base of the atherosclerotic plaque rather than the superficial areas of intima in closer proximity to the circulating blood (22). However, even in atherosclerotic lesions, the apparent rate of sphingomyelin formation was relatively slow compared to the rate of total phospholipid synthesis, suggesting that other factors might also contribute to intimal sphingomyelin accumulation. Relatively low synthesis rates for sphingomyelin have also been de-

scribed in other human tissues by Crocker and Mays (23).

The effects of sphingomyelin accumulation within the intima are unclear. Published information is not as yet available concerning the tendency to develop atherosclerosis in patients with Niemann-Pick disease who have sphingomyelin accumulation within the body tissues. The physicochemical properties of sphingomyelin may differ somewhat from other phospholipids (24), but the biological implications of these differences are unknown.

These experiments have been concerned only with samples of arterial intima that were either severely atherosclerotic or relatively normal by gross appearance. Large amounts of tissue were needed for the analyses, and sufficient quantities of tissue from areas with intermediate degrees of atherosclerosis were not available for comparison. The present results, therefore, do not provide information concerning the possible sequence of changes in phospholipid synthesis that might occur in the development of the atherosclerotic lesion.

Summary

Phospholipid synthesis in the arterial wall has been studied in isolated human blood vessels that have been incubated with inorganic ^{32}P, acetate-^{14}C, and palmitate-^{14}C. The results indicate that intimal phospholipid synthesis does occur and that the major phospholipids synthesized are the lecithins and cephalins. Sphingomyelin synthesis appears increased in atherosclerotic as compared with normal intima and may contribute to the observed accumulation of sphingomyelin in atherosclerotic plaques.

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