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Fatty Acid Incorporation into Cholesteryl Ester and Phospholipid by Arterial Subcellular Fractions of Swine (39733)

B. H. SIMON CHO AND F. A. KUMMEROW[1]

The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801, and The Harlan E. Moore Heart Research Foundation, Champaign, Illinois 61820

Since the accumulation of cholesteryl ester in the arterial wall is a characteristic feature of atherosclerosis, the origin and the mechanism of formation of this lipid has received a great deal of attention (1–5). The cholesteryl ester has been reported to originate from both filtration and deposition of serum cholesteryl ester (2, 3) and by local synthesis within the arterial wall (1, 4, 5). Several investigators have demonstrated that there is an enhanced cholesterol esterifying activity in the atherosclerotic intima compared to normal intima (1, 6, 7). An accumulation of cholesteryl ester may also result from a decrease in the rate of cholesteryl ester hydrolysis. Cholesteryl esterase activity has been reported to decrease in experimental lesions when compared to esterase activity in normal vessels (8).

An increased rate of fatty acid synthesis and incorporation of these synthesized fatty acids into various lipid classes has been demonstrated in the atheromatous aortas of pigeons and rabbits (9, 10). In the normal artery, newly synthesized and added exogeneous fatty acids have been shown to be incorporated actively into phospholipids (1, 11, 12). The rate of fatty acid esterification to cholesterol is low in the normal artery but, in the artery with atherosclerotic lesions, incorporation into cholesteryl ester is much greater and is probably due to the enhanced esterification induced by free cholesterol accumulation.

The predominant cholesterol esterifying activity in the aortas of rabbits and pigeons was attributed to acyl-CoA cholesterol acyltransferase (6, 13). Abdulla *et al.* (14) have presented data suggesting the presence of lecithin–cholesterol acyltransferase activity in atheromatous lesions from human and rabbit aortas; however, other investigators have not confirmed the presence of this enzyme in arteries (6, 15).

One of the aims of the present study was to determine the cholesterol esterifying activities between the arteries which reveal differences in susceptibility to atherosclerosis. Since the extent of cholesterol esterification has been affected by the isomers and homologs of the commonly found oleic and linoleic acid (16), several dietary sources of fatty acids were tested *in vitro* for incorporation into cholesteryl esters in the arterial subcellular fractions of swine fed a regular grain diet. In standardized incubation conditions for studying cholesterol esterifying activity, the utilization of these fatty acids for phospholipid synthesis was also determined.

Materials and methods. Crossbred (New Hampshire × Duroc) 6-month-old swine weighing 100–110 kg were used. The swine were fed *ad libitum* a grain diet which consisted of 87.25% ground yellow corn, 10% solvent-extracted defatted soybean meal, and 2.75% premixed multiple vitamins and minerals (17). Within 30 min of slaughter, the whole lengths of the aortas and the right and left coronary arteries (CA) were removed and kept in ice-cold physiological saline solution.

The intima and innermedia portions of aortic tissues were peeled off from the thoracic (TA) and abdominal aorta (AA), weighed quickly, and transferred into ice-cold homogenization medium (pH 7.4) containing 0.25 M sucrose, 0.001 M EDTA, and 0.001 M dithiothreitol. In the case of coronary arteries, any fatty material on the surface of the adventitia was thoroughly cleaned off and the coronary artery was used without separation of the intima and media. A fine mince was made with a scissors and homogenized in a 5 ml/g of tissue

[1] Send reprint requests to Dr. F. A. Kummerow, The Burnsides Research Laboratory, University of Illinois, Urbana, Ill. 61801.

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homogenization medium in an ice-cooled Kontes glass homogenizer adapted to a hand drill and driven with varying speeds until no large particles remained.

The crude tissue homogenate was centrifuged to sediment cellular debris at 1500 *g* for 20 min. The resulting supernatant was centrifuged at 10,000*g* for 30 min and the 10,000*g* pellet was washed by resuspension in an equal volume of 0.25 *M* sucrose and recentrifuged. The 10,000*g* supernatant was centrifuged at 105,000*g* for 60 min and the resulting 105,000*g* pellet was resuspended in the same homogenization medium, recentrifuged, and the supernatant was discarded. The washed 10,000 and 105,000*g* pellets, referred to as mitochondrial and microsomal fractions, respectively, were then resuspended in the original volume of homogenization medium by gentle hand homogenization. The protein content of both subcellular fractions were determined by the method of Lowry *et al.* (18).

For the assay of cholesterol esterifying activity, [1-^{14}C]palmitic acid (sp act, 58 Ci/mole), [1-^{14}C]oleic acid (sp act, 59 Ci/mole), [1-^{14}C]elaidic acid (sp act, 52 Ci/mole), and [1-^{14}C]linoleic acid (sp act, 52.5 Ci/mole) (Amersham/Searle, Arlington Heights, Ill.) were used as radioactive substrates. ^{14}C-labeled fatty acids were developed on glass fiber paper impregnated with silica gel ITLC SG (Gelman Instrument Company, Ann Arbor, Mich.) in a solvent system consisting of petroleum ether:benzene:acetic acid (95:3:0.4, v/v/v). Radioactive lipids were found to be 99% radiochemically pure. An aliquot of benzene solution containing from 0.37 to 0.41 μCi of ^{14}C-labeled fatty acid (6.9–7.1 nmole) was transferred into incubation tubes with a disposable micropipet. Solvents were evaporated with a stream of N_2 and 50 μl of acetone was added to each tube to dissolve the radioactive material. Incubation conditions were similar to those described for arterial and liver microsomal fractions (16, 19). The incubation medium was freshly prepared to contain in micromoles: potassium phosphate, 250; coenzyme A (Nutritional Biochemicals Co., Cleveland, Ohio), 0.3; ATP (NBC), 6.0; $MgCl_2$, 6.0; fatty-acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 0.3 mg in each 2.0 ml of buffer solution (pH 7.4). To each tube, 2.0 ml of incubation medium in phosphate buffer (pH 7.4) was added and preincubated for 1 hr at room temperature to equilibrate the fatty acid and bovine serum albumin.

After preincubation, 0.5 ml of subcellular preparation (0.3–0.5 mg of protein) was added to each tube and incubated for 1 hr at 37° in a metabolic shaker. For each experiment, blank tubes, without tissue homogenate, were included and examined at both zero-time and after 1 hr of incubation. The reaction was stopped by adding 10 ml of chloroform–methanol (2:1, v/v). After mixing and sometimes standing overnight at 5°, the lipids were extracted three times with 10 ml of chloroform–methanol. Solvents were evaporated under a stream of N_2 and the residue was redissolved in a small amount of chloroform. A lipid standard mixture (ca. 50 μg) containing free cholesterol, cholesteryl oleate, mixed triglycerides, and oleic acid was added to each sample as carrier for thin-layer chromatography. An aliquot of lipid extracts containing radioactive compounds and standard lipid carriers was applied with a disposable pipet to an ITLC-type SG sheet. The glass fiber paper impregnated with silica gel (10 × 20 cm) was then developed in a solvent system consisting of petroleum ether (Skelly B):benzene:acetic acid (95:3:0.4, v/v/v). After development, the chromatogram was dried briefly in the oven (110°) and lipid spots were visualized with iodine vapor. After visualization of lipid spots, the individual spot corresponding to the known standard was circled with a pencil, cut with a scissors, and placed in a scintillation vial.

Toluene scintillation solution (0.5% PPO and 0.03% dimethyl POPOP) was added to vials containing neutral lipid spots. For phospholipid spots, a cocktail of 5 g of PPO, 0.3 g of POPOP, 130 ml of methanol, and 100 ml of Bio-Solv Solubilizer (Beckman Instruments, Inc., Palo Alto, Calif.) in 1 liter of toluene was used (20). Each vial containing 10 ml of scintillation cocktail was shaken occasionally during 24–48 hr of the extraction period, and the ^{14}C radioactivity was counted in a Tri-Carb liquid scintillation

spectrophotometer. Quench corrections were made by the external standardization method.

Results. Esterification of ^{14}C-labeled fatty acids into cholesteryl ester of arterial microsomal and mitochondrial fractions is shown in Table I. Cholesterol esterifying activity occurred predominantly in the microsomal fraction. A considerable amount of activity was also present in the mitochondrial fraction. However, no significant differences in overall cholesteryl ester synthesizing activities were observed in either subcellular fractions between thoracic, abdominal, and coronary arteries. In the microsomal fraction, oleic, palmitic, and linoleic acid were readily utilized for cholesteryl ester synthesis. Elaidic acid was utilized to a lesser extent. With the mitochondrial fraction, the levels of incorporation of different fatty acids into cholesteryl ester were relatively similar, although palmitic and oleic acids esterified cholesterol slightly more than elaidic or linoleic acids.

Under the standardized incubation conditions used to determine the cholesterol esterifying activity, incorporation of fatty acid substrate into phospholipid also took place. The incorporation of labeled fatty acid into di- and triglycerides was negligible. Variations in the phospholipid snythesis with different fatty acids by microsomal and mitochondrial fractions from arterial tissues are given in Table II. Phospholipid synthesis was very active in both arterial subcellular fractions, and the bulk of fatty acid substrates preferentially incorporated into phospholipid instead of cholesteryl ester. Synthesis of phospholipid was most active in the microsomal fraction, and considerable synthesizing activity was also found in the mitochondrial fraction.

Among the fatty acids tested, linoleic acid was the most preferential substrate for phos-

TABLE I. ESTERIFICATION OF FATTY ACIDS INTO CHOLESTERYL ESTER BY ARTERIAL SUBCELLULAR FRACTIONS OF SWINE.[a]

Substrate	Palmitic acid	Oleic acid	Elaidic acid	Linoleic acid
Microsomal fraction[b]				
Thoracic artery	37.5 ± 1.8[c]	41.4 ± 1.3	25.8 ± 2.4	35.1 ± 2.0
Abdominal artery	38.5 ± 2.0	43.8 ± 1.2	25.3 ± 0.6	32.6 ± 2.4
Coronary artery	38.0 ± 2.0	40.2 ± 2.6	29.5 ± 1.1	30.8 ± 2.3
Mitochondrial fraction				
Thoracic artery	25.6 ± 1.6	26.5 ± 1.2	20.4 ± 1.4	20.3 ± 1.8
Abdominal artery	27.1 ± 1.1	26.5 ± 1.6	23.8 ± 1.4	23.7 ± 1.6
Coronary artery	28.9 ± 2.4	29.6 ± 1.1	25.3 ± 2.7	24.2 ± 0.8

[a] Incubation medium consisted of 0.1 *M* phosphate buffer (pH 7.4) containing 0.3 μmole of CoA, 6 μmole of ATP, 6 μmole of $MgCl_2$, 0.3 mg of fatty-acid-free bovine serum albumin, ^{14}C-labeled fatty acid (0.37–0.41 μCi; 6.9–7.1 nmole), and 0.3–0.5 mg of protein. Reaction mixture was shaken for 1 hr at 37° under air.

[b] Microsomal fraction, 105,000*g* pellet; mitochondrial fraction, 10,000*g* pellet.

[c] Picomoles of fatty acid esterified per milligram of protein per hour; mean ± SEM of duplicate experiments from five animals, calculated assuming no equilibrium with endogenous substrates.

TABLE II. INCORPORATION OF FATTY ACIDS INTO PHOSPHOLIPID BY ARTERIAL SUBCELLULAR FRACTIONS OF SWINE.[a]

Substrate	Palmitic acid	Oleic acid	Elaidic acid	Linoleic acid
Microsomal fraction[b]				
Thoracic artery	239.8 ± 17.8[c]	118.9 ± 13.2	123.7 ± 4.6	535.9 ± 18.7
Abdominal artery	257.2 ± 15.9	99.1 ± 2.6	101.7 ± 8.5	501.9 ± 17.6
Coronary artery	240.1 ± 17.2	129.4 ± 7.8	120.7 ± 7.9	548.3 ± 19.3
Mitochondrial fraction				
Thoracic artery	159.6 ± 5.0	51.9 ± 1.7	46.3 ± 2.4	240.8 ± 13.7
Abdominal artery	141.9 ± 6.1	45.8 ± 2.8	70.9 ± 3.9	224.6 ± 18.6
Coronary artery	134.0 ± 6.6	67.4 ± 4.9	87.5 ± 4.1	255.2 ± 10.5

[a] Incubation conditions were the same as described in Table I.

[b] Subcellular fractions were the same as described in Table I.

[c] Picomoles of fatty acid incorporated per milligram of protein per hour; mean ± SEM of duplicate experiments from five animals.

pholipid synthesis in both microsomal and mitochondrial fractions. A considerable amount of palmitic acid was utilized for phospholipid synthesis, but incorporation of oleic and elaidic acids into phospholipid was several times less than that of linoleic acid. Generally, phospholipid synthesizing activities between both subcellular fractions were similar in the thoracic, abdominal, and coronary arteries.

Discussion. Most of the cholesterol esterifying activity has been found in the microsomal and particulate fractions of normal and atherosclerotic aortas (6, 13, 21). Cholesterol esterification required ATP and CoA, when free fatty acid was provided as the acyl source, for the formation of the fatty acyl-CoA and its utilization in the acylation of cholesterol. This observation indicated that cholesterol esterification was probably accomplished by fatty acyl-CoA cholesterol acyltransferase by a mechanism similar to that described for liver and adrenal cortex (22, 23).

Cholesterol esterifying activity was influenced to some extent by the type of fatty acid substrate, as observed in the liver microsomes (16). Oleic acid (*cis*-9-octadecenoic acid) appeared to be more readily utilized for cholesteryl ester synthesis than elaidic acid (*trans*-9-octadecenoic acid). The noticeable difference in cholesterol esterification between *cis*- and *trans*-isomers of C18-monounsaturated fatty acid could be, in part, due to a reflection of the enzyme specificity toward the various fatty acid substrates. Differences between saturated and unsaturated fatty acids were less noticeable in cholesterol esterification than in phospholipid synthesis. Although the present data are insufficient to provide any functional specificity to cholesterol esterifying enzyme, it is quite possible that the enzyme responds to the structural features of the fatty acid substrate. Since free cholesterol acts as an acyl group receptor, its availability and the competitive nature of the receptors, i.e., lysoglycerophosphatides, will also affect the channeling of different fatty acids resulting in the accumulation of various cholesteryl esters in the aortic tissue. Different rates of hydrolysis of cholesteryl ester in the aortic tissue could also contribute to the difference in the accumulations of cholesteryl esters (24).

Under the standardized incubation conditions used to determine the cholesterol esterifying activity, the labeled fatty acyl-CoA formed by the subcellular preparations was incorporated into several lipid classes. However, other lipids formed were minor and the present study focused mainly on the fatty acid incorporation into two dominant lipids: cholesteryl ester and phospholipid. As demonstrated in the monkey and rat aortas (12) and in the rat liver (25), most of the enzyme activity responsible for phospholipid synthesis appeared to be associated with the microsomal fractions. A considerable phospholipid synthesizing activity was also found in the mitochondrial fractions and reports have appeared describing the biosynthesis of lecithin by the mitochondrial fractions from rat and monkey aortas (12) and mammalian liver (26).

The incorporation of fatty acids into phospholipid could involve the fatty acyl-CoA:lysophosphatide fatty acyltransferase mechanism (12). Phospholipase activity, which has been demonstrated in aortic homogenates (27), could form lysophosphatides from membrane-associated phospholipids and the resulting lysophosphatides could serve as acceptors for fatty acyl-CoA. The preferential incorporation of linoleic acid to oleic, palmitic, or elaidic acid into phospholipids indicates the competition of different fatty acids for some common site of the enzyme system. The level of endogenous lysophosphatides available may be an important determinant of the rate of fatty acid renewal in the phospholipid fraction (12).

A gradual increase in the total phospholipid content and changes in the composition of phospholipids with age and evolution of atherosclerotic lesions have been reported (27, 28). These changes could represent both deposition from plasma lipoproteins and local synthesis by the arterial wall. The present study indicated that phospholipid synthesis is very active in the normal aortic tissue. The incorporation of fatty acids into phospholipid was many times greater than that of fatty acids esterified to cholesterol. It would be of interest to identify the relative

role of local phospholipid synthesis in relationship to cholesterol metabolism in aortic tissue and its functional significance in the development of atherosclerosis.

Summary. Cholesterol esterifying and phospholipid synthesizing activities were measured with ^{14}C-labeled palmitic, oleic, elaidic, and linoleic acid by subcellular fractions from the thoracic, abdominal aorta, and the coronary arteries of swine. Cholesterol esterification was most active in the microsomal fraction and considerable esterification was also found in the mitochondrial fraction. Oleic, palmitic, and linoleic acid were readily utilized for cholesteryl ester synthesis and elaidic acid was least utilized. Incorporation of fatty acids into phospholipid was very active in the aortic tissue. Most of the phospholipid synthesizing activity was found in the microsomal fraction, although considerable activity was present in the mitochondrial fraction. Among the fatty acids tested, linoleic acid was the most preferential substrate for phospholipid synthesis and the relative order of incorporation into phospholipid was linoleate > oleate > elaidate. In subcellular fractions from grossly normal aortic tissue, the utilization of fatty acid for phospholipid synthesis was many times greater than that for esterification to cholesterol. However, in cholesterol esterifying and phospholipid synthesizing activities, no significant differences were found in subcellular fractions between the thoracic, abdominal, and coronary arteries.

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