Cholesteryl Ester Synthesis in Normal and Atherosclerotic Aortas of Rabbits and Rhesus Monkeys

By Peter I. Brecher and Aram V. Chobanian

**ABSTRACT**

The formation of cholesteryl ester in aortic tissue was studied using subcellular fractions from normal and atherosclerotic rabbit and rhesus monkey aortas. The properties of two enzyme systems capable of esterifying 1-14C-oleic acid into cholesteryl ester in vitro were investigated, and increased activity was demonstrated for both systems as a result of cholesterol feeding. Microsomal preparations were used to study the ATP, CoA-dependent esterification which involves two enzymes, fatty acyl CoA synthetase and fatty acyl CoA:cholesterol acyltransferase. The properties of both enzymes were investigated, and an increase of about fourfold in activity of the acyltransferase was demonstrated in aortic microsomes as a result of cholesterol feeding for 3-6 months. Esterification of oleic acid into cholesteryl ester by aortic high-speed supernatant fractions at an acidic pH was also observed; the enzyme system involved did not require cofactors, and its activity greatly increased as a result of cholesterol feeding. Similar increases in the activity of both esterifying enzyme systems were found when normal and atherosclerotic rhesus monkey aortic fractions were compared. p-Chlorophenoxyisobutyrate (CPIB) and 2-methyl-2-[p-(1, 2, 3, 4-tetrahydro-1-naphthyl)-phenoxy]propionic acid (TPIA) produced inhibition of both cholesterol-esterifying enzyme systems. TPIA was a more effective inhibitor than CPIB on both enzyme systems by at least an order of magnitude. These studies suggest that increased intracellular synthesis of cholesteryl ester by aortic tissue may contribute to its accumulation in atherosclerosis.

**KEY WORDS** fatty acyl CoA:cholesterol acyltransferase cholesterol inhibition of cholesterol esterification fatty acid microsomal preparation oleic acid fatty acyl CoA synthetase high-speed supernatant fraction

Although the accumulation of arterial cholesteryl esters that occurs during the induction of atherosclerosis is a well-described process, the biochemical mechanisms responsible for this accumulation are not completely understood. Two major processes—infiltration of plasma cholesteryl esters and local synthesis of cholesteryl esters—are thought to be involved. In vivo studies in cholesterol-fed rabbits have suggested that plasma is the predominant source of both free and esterified aortic cholesterol (1, 2). Studies in rabbits fed labeled oleic acid and unlabeled cholesterol have suggested that a measurable fraction of atheroma cholesteryl oleate is derived by local synthesis (3). Several in vitro studies on intact arterial segments have shown that atherosclerotic tissue esterifies fatty acids more readily than do nonatherosclerotic arteries in pigeon (4), rabbit (5), monkey (6), and man (7). St. Clair et al. (8) have demonstrated an increase in the activity of long-chain fatty acyl CoA:cholesterol acyltransferase in the 1,000-g supernatant fraction from atherosclerotic aortas of White Carneau pigeons (8); this activity decreases somewhat during lesion regression (9). Homogenates from atherosclerotic rabbit aortas have also been shown to have an increased activity of this enzyme (10). Two different cholesterol-esterifying enzyme systems have been found in whole and fractionated homogenates of atherosclerotic rabbit aortic tissue; one has a pH optimum of about 7.5 and resembles fatty acyl CoA:cholesterol acyltransferase and the other has a pH optimum of about 5.0 (11). These studies have recently been extended to homogenates of isolated foam cells from atherosclerotic rabbit tissue; both types of activity were again found (12).

In the present investigation we studied the properties of these two enzyme systems in arterial tissue from both the rabbit and the rhesus monkey. We examined the effect of cholesterol feeding on the cholesterol-esterifying activity of these enzyme systems, and we investigated the in vitro effects of certain hypocholesterolemic agents.
Methods

1-\(^{14}\)C-Oleic acid (specific activity 53.5 mc/mmole), 1-\(^{13}\)C-palmitic acid (specific activity 55.0 mc/mmole), and 1-\(^{13}\)C-palmitoyl CoA (specific activity 54.0 mc/mmole) (New England Nuclear Corp.) were routinely repurified prior to use by thin-layer chromatography on silica gel G with a petroleum ether-diethyl ether-glacial acetic acid (95:5:1) developing solvent system. Stock solutions of labeled and unlabeled (Sigma or Applied Sciences Laboratories, Woburn, Mass.) fatty acids were prepared as the ammonium salts in a 1% solution of Triton WR-1339 (Ruger Chemical Co.) as described by Suzue and Marcel (13). The sodium salt of p-chlorophenoxyisobutyrate (Clofibrate) was supplied by Ayerst Laboratories, and 2-methyl-[\(p\)-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionic acid (SU-13,437) (TPIA) was a gift from Ciba-Geigy.

Female New Zealand albino rabbits weighing approximately 3 kg were fed normal rabbit chow or chow containing 1% cholesterol for 1-6 months. Rhesus monkeys (36-45 months of age) were fed a diet providing 25% of the total calories as protein, 57% as carbohydrate, and 18% as fat. The atherogenic diet was supplemented by the addition of 1.0% cholesterol and 0.5% cholic acid. The monkeys were maintained on these diets for 2 years as part of a larger study on bile acid metabolism. For our study, 4 control and 3 cholesterol-fed monkeys were used. Plasma cholesterol levels for the control and the cholesterol-fed group ranged between 96 and 130 mg/dl and 420 and 530 mg/dl, respectively. Gross atherosclerotic lesions covering approximately 20-40% of the intimal surface were present in the hypercholesterolemic animals but not in any of the controls.

After the animals had been killed, both rabbit and monkey aortas were removed from the heart to the iliac bifurcation and transferred to cold saline. Subsequent steps were performed at 0-4°C. The adventitia and the outer portion of the media were dissected away, and the intima-media segments were minced and homogenized in a glass-glass apparatus containing ten volumes of 0.25M sucrose in 0.02M Tris buffer (pH 7.4). Samples of the homogenate were removed for protein and cholesterol determinations. The homogenates were then centrifuged at 9,000 g for 45 minutes to obtain the microsomal pellet and the high-speed supernatant fraction. Care was taken to separate this supernatant fraction. Care was taken to separate this supernatant fraction. The microsomal fractions were then centrifuged at 161,000 g for 45 minutes to obtain the microsomal pellet and the high-speed supernatant fraction. Care was taken to separate this supernatant fraction. The microsomal pellet was washed with buffer, and the microsomes were resuspended in 0.25M sucrose in 0.02M Tris buffer (pH 7.4) containing dithiothreitol (1 mg/ml). Protein concentrations in the subcellular fractions were determined using the method of Lowry et al. (14). The microsomal fractions were adjusted to a protein concentration of 330 \(\mu\)g/ml before the enzyme assays were carried out.

ASSAY FOR MICROSMAL CHOLESTERYL ESTER SYNTHETASE

The system used to determine fatty acyl CoA synthetase activity was also used to assay microsomal cholesteryl ester synthetase, although 50 \(\mu\)g of microsomal protein was routinely added. Following an incubation for 90 minutes at 37°C, the reaction was terminated by adding 5.0 ml of a chloroform-methanol (2:1) solution containing unlabeled oleic acid and cholesteryl oleate as carriers. The lipids were extracted and washed by the method of Folch et al. (15). The lipid extract was evaporated under a stream of nitrogen, redissolved in a small volume of chloroform, and spotted on Eastman chromatograms for thin-layer chromatography with a petroleum ether-diethyl ether-glacial acetic acid (95:5:1) developing solvent system (17). The lipids were visualized with iodine vapors, and the areas corresponding to different lipid classes were isolated and placed in scintillation vials for counting.

ASSAY FOR ACTIVITY AT pH 5.2

The high-speed supernatant fraction was diluted with a half volume of 0.05M sodium acetate buffer (pH 5.2). Then 0.2 ml of this diluted supernatant fraction was transferred to a 15-ml siliconized test tube, and 4 \(\mu\)l of an acetone solution of 1-\(^{14}\)C-oleic acid was added to achieve a final oleic acid concentration of 5.0 \(\mu\)M. Incubations were performed at 37°C for 60 minutes and terminated by adding 4 ml of a chloroform-methanol (2:1) solution. The lipids were extracted, and the major lipid groups were separated by thin-layer chromatography.

\(\mu\)oles of CoA, 1.25 \(\mu\)moles of 1-\(^{14}\)C-oleic acid (added as the ammonium salt in 10 \(\mu\)litters of a 1% aqueous Triton WR-1339 solution), and 20 \(\mu\)g of microsomal protein. The total incubation volume was 0.25 ml. Control incubations without ATP and CoA were routinely carried out.

The reaction was initiated by adding the microsomes to incubation tubes that had been preincubated for 1 minute at 37°C. Incubations were usually stopped after 2 minutes by adding 5.0 ml of a chloroform-methanol (2:1) solution. After 2 hours, 1.0 ml of 0.02% CaCl\(_2\) was added, and the tubes were shaken vigorously and allowed to stand overnight in the cold. Samples of the upper phase were removed and added to scintillation cocktail containing toluene (750 ml), Triton X-100 (250 ml), water (180 ml), PPO (50 g), and POPPOP (400 mg).

The following six criteria established that the radioactivity in the upper phase was fatty acyl CoA. (1) Labeled oleic acid standard was recovered almost exclusively in the lower phase (99% lower, 1% upper). (2) Labeled palmitoyl CoA standard was found primarily in the upper phase (98% upper, 2% lower). (3) Following a standard incubation with 1-\(^{14}\)C-oleic acid or 1-\(^{13}\)C-palmitic acid, the upper phase was removed, concentrated, and subjected to thin-layer chromatography, using a 1-butanol-acetic acid-water (5:2:3) solvent system; 95% of the counts originally present in the upper phase migrated with standard palmitoyl CoA. (4) Equivalent concentrations of 1-\(^{14}\)C-oleic acid or 9, 10-\(^{3}\)H-oleic acid produced identical results in the assay system. (5) Radioactivity in the upper phase was precipitated in 1.0% perchloric acid. (6) Incorporation of radioactivity into the upper phase required ATP, CoA, and an enzyme source; it was a temperature-dependent reaction.
**ANALYTICAL METHODS**

Thin-layer chromatography on glass plates coated with silica gel G (0.25 mm) was used for a more complete separation of various lipid classes. Lipid classes were separated using a hexane-diethyl ether-acetic acid (70:30:1) developing solvent system (17). Monoglycerides were separated from phospholipids by a two-step development system (18). Argentation thin-layer chromatography with 0.7% AgNO₃ (19) was used to characterize further the labeled cholesteryl ester formed in the reactions described in the preceding sections. More than 90% of the radioactivity located in the cholesteryl ester region of the routinely used chromatograms migrated with cholesteryl olate on the AgNO₃-coated plates.

Total and free cholesterol determinations were performed chemically (20) or by gas chromatography in a Packard 7400 series gas chromatograph using a 2-foot column of 3.8% SE-30 coated on Diataport S. Column temperature was 250°C.

**Results**

Long-chain fatty acyl CoA synthetase activity was present in rabbit aortic microsomes. This activity increased linearly with time through 5 minutes (Fig. 1A). No activity was seen in the absence of ATP and CoA. Most determinations were made at 2 minutes, and the radioactivity present in the upper phase, after subtracting the amount present in control tubes lacking ATP and CoA, was assumed to be fatty acyl CoA. Enzymatic activity was proportional to microsomal protein concentration (Fig. 1B) and followed normal Michaelis-Menten kinetics when l-14C-oleic acid was used as the substrate (Fig. 1C). Additional studies showed that the enzymatic activity was destroyed by heating at 70°C for 15 minutes, reduced by preincubation (in the absence of cofactors) at 37°C for 15 minutes, and unaffected by storage at -20°C for 2 weeks.

Microsomal preparations from aortic tissue produced labeled cholesteryl ester when 1-14C-oleic acid was used as the substrate. Figure 2 illustrates the effects of incubation time and protein concentration on the rate of incorporation of labeled oleic acid into cholesteryl ester for both control and atherosclerotic preparations. Incorporation into cholesteryl ester proceeded linearly throughout the 180-minute incubation period. A greater rate of incorporation was observed with atherosclerotic microsomes. Incorporation was proportional to protein concentration in the ranges used for these studies, and atherosclerotic microsomes contained more activity per milligram of protein than did microsomes from control tissue. When high-speed supernatant fractions were assayed in this system, some activity was also observed, although it corresponded to less than 10% of the activity observed in the microsomal fraction.

Figure 3 illustrates the relationship between substrate concentration and cholesteryl ester incorporation when labeled palmitic and oleic acid were used. Both fatty acids were incorporated into cholesteryl ester. However, cholesteryl olate was formed at a more rapid rate than was the palmitate ester at all concentrations tested. Inhibition was observed at fatty acid concentrations greater than 40 μM. Similar results were obtained with control and atherosclerotic preparations.

Figure 4 illustrates the rate of incorporation of oleic acid into oleyl CoA, polar lipids, and cholesteryl ester under the standardized incubation conditions used to determine microsomal cholesteryl...
ester activity. Fatty acyl CoA was formed rapidly and did not appear to be rate limiting with respect to cholesteryl ester formation. Incorporation into the phospholipid-containing fraction was always observed. Incorporation into cholesteryl esters continued linearly with time, whereas the net incorporation into the phospholipid moiety and into oleyl CoA leveled off or decreased at the later time periods. Omission of ATP and CoA resulted in negligible incorporation into all lipid fractions. Similarly, omission of microsomes from the incubation mixture or addition of microsomes preheated at 70°C for 15 minutes resulted in no incorporation of radioactivity into the fatty acid esters.

Incorporation of labeled fatty acid into diglycerides and triglycerides was negligible. Although monoglycerides migrated with the phospholipids in

**Figure 2**
Effect of time and protein concentration on \(^1\)\(^4\)C-oleate incorporation into cholesteryl ester in microsomal fractions of normal and atherosclerotic rabbit aortas.

**Figure 3**
Effect of substrate concentration on incorporation of \(^1\)\(^4\)C-oleate and \(^1\)\(^4\)C-palmitate into cholesteryl ester in a microsomal fraction from a control rabbit aorta.

**Figure 4**
Incorporation of \(^1\)\(^4\)C-oleate into oleyl CoA, phospholipid, and cholesteryl ester by a microsomal fraction from an atherosclerotic rabbit aorta.
the thin-layer chromatograms routinely used, less than 10% of the total radioactivity in the polar lipid region was present in the monoglyceride fraction when a chromatographic system that separates monoglycerides from phospholipids was used.

Under standardized incubation conditions, we compared the cholesteryl ester synthetase activities of normal and atherosclerotic rabbit aortas. Table 1 summarizes data from 24 rabbits with various degrees of atherosclerosis. Total cholesterol content of the aorta was used as an indicator of the degree of atherosclerosis. The free cholesterol content of all microsomal preparations was measured, since it presumably serves as the cholesterol source for subsequent esterification. A significant increase ($P < 0.001$) in cholesteryl-esterifying activity was measured in the severely atherosclerotic rabbit aortas. However, rabbits on the high-cholesterol diet for 4-10 weeks exhibited no significant increase in enzymatic activity over that in the control rabbits. The correlation between total aortic cholesterol content and enzymatic activity was highly significant ($r = 0.87$), whereas a similar comparison between microsomal free cholesterol content and enzymatic activity was less significant ($r = 0.66$).

Attempts were made to estimate the pool size of endogenous cholesterol or fatty acid participating in the formation of cholesteryl ester. No significant change was detected in the free or total cholesterol content of the microsomes after incubation, suggesting that the microsomal pool of fatty acid available for esterification into cholesteryl ester may be negligible. If only the added labeled oleic acid was incorporated into cholesteryl ester, the amount would be too small to detect by the gas chromatographic technique utilized. It is possible that exchange mechanisms could play a role in this process.

To further study the possibility of exchange mechanisms in the system, we performed experiments on the effects of exogenously added cholesterol or cholesteryl ester on the incorporation of oleic acid into cholesteryl ester. Addition of free cholesterol to the incubation system, introduced in a small volume of acetone, was without effect when 0.5-300 µg was added. Similarly, cholesteryl ester added in amounts up to 30 µg was also without effect on oleic acid incorporation. Addition of 4-14C-cholesterol, in the presence or the absence of unlabeled oleic acid or oleyl CoA, resulted in no incorporation of radioactivity into cholesteryl ester.

**ACTIVITY AT pH 5.2**

Incorporation of labeled oleic acid into the cholesteryl ester fraction was observed using aortic high-speed supernatant fractions adjusted to pH 5.2. This activity increased linearly as a function of both time and protein concentration. ATP, CoA, Mg$^{2+}$, and dithiothreitol were not necessary in this system and were omitted from the incubations. Preincubation of the supernatant fraction at 80°C for 15 minutes eliminated subsequent activity; however, preincubation at 37°C for 60 minutes had no effect. Triton WR-1339 at a final concentration of 0.01% reduced the reaction rate. Therefore, labeled oleate was added as an aceton solution rather than as the ammonium salt dissolved in Triton WR-1339; the latter procedure was used for the acyltransferase system. Microsomal fractions contained less than 10% of the activity found in high-speed supernatant fractions when they were assayed in this system.

Table 2 summarizes the data obtained using control and cholesterol-fed rabbits (12-20 weeks). Increased incorporation into cholesteryl ester was seen in the cholesterol-fed group. However, the interpretation of these results was complicated by the fact that larger free cholesterol contents were also observed in these fractions. Furthermore, precipitation of protein occurred during the incubation period, producing a heterogeneous system.

Further studies were performed in which the aortic high-speed supernatant fraction was adjusted to pH 5.2 and incubated in the absence of oleic acid at 37°C for 60 minutes. The solution was

<table>
<thead>
<tr>
<th>Duration of cholesterol feeding (weeks)</th>
<th>N</th>
<th>Total aortic cholesterol content (mg/100 mg wet wt)</th>
<th>Microsomal free cholesterol content (µg/mg protein)</th>
<th>Enzymatic activity (dpm/90 min 50 µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11</td>
<td>0.07 ± 0.02</td>
<td>0.031 ± 0.014</td>
<td>1750 ± 370</td>
</tr>
<tr>
<td>4-10</td>
<td>7</td>
<td>0.40 ± 0.14</td>
<td>0.077 ± 0.013</td>
<td>1816 ± 402</td>
</tr>
<tr>
<td>12-24</td>
<td>6</td>
<td>2.30 ± 0.47</td>
<td>0.162 ± 0.048</td>
<td>7900 ± 554</td>
</tr>
</tbody>
</table>

All data are means ± SE; N refers to the number of rabbits tested.
TABLE 2
Effect of Cholesterol Feeding on 1-14C-Oleate Incorporation by High-Speed Supernatant Fractions (pH 5.2) of Rabbit Aortas

<table>
<thead>
<tr>
<th>Duration of cholesterol feeding (weeks)</th>
<th>Total aortic cholesterol content (mg/100 mg wet wt)</th>
<th>Supernatant free cholesterol content (µg/mg protein)</th>
<th>Enzymatic activity (dpm/hour mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>0.07 ± 0.01</td>
<td>11.0 ± 4.1</td>
</tr>
<tr>
<td>12-20</td>
<td>7</td>
<td>1.14 ± 0.18</td>
<td>106.2 ± 16.9</td>
</tr>
</tbody>
</table>

All data are means ± SE; N refers to the number of rabbits tested.

centrifuged at 10,000 g for 20 minutes, the supernatant fraction was removed, and the precipitate was resuspended in 0.17 M sucrose in 0.017 M acetate buffer (pH 5.2). Labeled oleic acid was added to both the supernatant fraction and the resuspended precipitate; the fractions were then assayed for cholesterol-esterifying activity. Enzymatic activity per milligram of protein was comparable in both fractions.

Addition of unlabeled cholesteryl ester or free cholesterol had no effect on the enzyme system, and labeled palmitoyl CoA was not incorporated into cholesteryl ester under the assay conditions used.

**ENZYMATIC ACTIVITY IN RHESUS MONKEY AORTAS**

Enzymatic activity in both the microsomal (pH 7.4) and the high-speed supernatant (pH 5.2) fractions was measured in the aortas of rhesus monkeys. The data obtained are summarized in Figure 5. Significantly increased activity for both enzyme systems was observed in atherosclerotic tissue preparations, consistent with the findings in the rabbit. However, the minor differences in incubation procedure and the small number of monkeys available make quantitative comparisons with the rabbit impractical.

**EFFECTS OF CPIB AND TPIA**

**Microsomal System.—** Addition of either CPIB (Na salt) or TPIA reduced both the rate and the extent of oleic acid incorporation into cholesteryl ester in a concentration-dependent manner. TPIA was the more potent inhibitor; it reduced incorporation 50% at 0.5 mM, whereas 5.0 mM CPIB was required to achieve a similar level of inhibition (Fig. 6). Although some variability in the absolute amounts of the drugs required for 50% inhibition was observed using different animal preparations, TPIA was invariably the more effective inhibitor.

**pH 5.2 System.—** Addition of CPIB or TPIA to aortic high-speed supernatant fractions in acetate buffer also produced an inhibition of fatty acid incorporation into cholesteryl ester (Fig. 7). TPIA was more effective in reducing the rate of incorporation, although CPIB did reduce esterification slightly.

**Discussion**

The objective of this study was to characterize two separate enzyme systems in subcellular fractions of aortic tissue capable of intracellular cholesteryl ester formation. Both the microsomal cholesteryl ester synthetase and the soluble enzyme active at acid pH exhibited greater activity in atherosclerotic subcellular fractions than they did in nonatherosclerotic control tissues. The study also demonstrated for the first time that the activity of these enzymes could be reduced by the addition, in vitro, of CPIB or TPIA, substances which have been utilized clinically for their hypcholesterolemic effect.

Our assay system for measuring fatty acyl CoA: cholesterol acyltransferase activity included labeled oleic acid as the substrate; therefore, the formation of oleyl CoA was a necessary prerequisite.

**FIGURE 5**

Incorporation of 1-14C-oleic acid into cholesteryl ester in subcellular fractions from normal and atherosclerotic rhesus monkey aortas. C = control, and A = atherosclerotic. Results are expressed as the percent of added substrate incorporated into cholesteryl ester in a 2-hour incubation period. Substrate concentration varied from 3 to 5 µM in different experiments.
for cholesteryl ester incorporation. The microsomal enzyme responsible for fatty acyl CoA formation, long-chain fatty acyl CoA synthetase, has been described in several tissues (13, 21, 22), and its properties have recently been described in liver (13, 15), using an assay system that eliminates the high concentrations of fatty acid needed in previously described methods. With this method, the concentrations of both fatty acids and fatty acyl CoA can be maintained below their critical micellar concentrations. Using a slight modification of this technique, we showed that both control and atherosclerotic aortic tissues have sufficient enzymatic activity to ensure that the formation of oleyl CoA proceeds readily and at a more rapid rate than the subsequent esterification to cholesteryl esters.

The labeled fatty acyl CoA formed by the microsomal preparation was incorporated into several lipid classes. The present study focused on its incorporation into cholesteryl ester, but we also examined the incorporation into phospholipid, which is considerably greater than that into other lipid classes. The phospholipid incorporation observed could be due to the combined effects of acyl CoA:phospholipid acyltransferases and phospholipase activity. The properties of such enzymes have recently been reviewed (23). Phospholipase activity, which has been demonstrated in aortic microsomes (24), could form lysophosphatides from membrane-associated phospholipids, and the resulting lysophosphatides could serve as acceptors for labeled oleyl CoA. We did not observe any significant increase in oleic acid incorporation into phospholipids induced by microsomes from atherosclerotic tissue. Since our system did not include several substances required for de novo phospholipid synthesis, the data on polar lipid incorporation may not accurately reflect changes in phospholipid synthesis occurring in vivo. St. Clair et al. (8), using a 10,000-g supernatant fraction from pigeon aortic homogenates, have observed a relatively small increase in fatty acid incorporation into lecithin in atherosclerotic fractions, although they have reported a much greater percent increase for incorporation into the cholesteryl ester fraction.

The incorporation of labeled oleate into cholesteryl ester by the microsomal fraction was completely dependent on the presence of ATP and CoA, suggesting that the long-chain fatty acyl CoA: cholesterol acyltransferase was the enzyme catalyzing this reaction. This enzyme has been well characterized in liver (25) and adrenal glands (26) and is believed to be responsible for the intracellular formation of cholesteryl ester by these tissues. Recent reports have shown that this enzyme is present in aortic tissue from pigeons (8) and rabbits (10, 11); moreover, its activity increases as a result of cholesterol feeding.

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We carefully studied the properties of this enzymatic activity in microsomes from normal and atherosclerotic tissue with respect to reaction rate, substrate and enzyme concentration, and the effect of various inhibitors. The increased activity we observed as a result of atherosclerosis is in agreement with the findings of other workers (8, 10, 27). Added exogenous cholesterol did not increase oleic acid esterification and did not become esterified to endogenous fatty acids. In contrast, other workers have shown that preparations from liver and adrenal glands esterify both exogenous fatty acid and cholesterol (25, 26). The reason for the difference between these latter tissue preparations and those from the aorta is not clear.

Another possible explanation for the increased cholesterol esterification in atherosclerotic tissue would be that oleic acid binds to atherosclerotic microsomes more readily than it binds to control microsomes. This hypothesis is unlikely, since both normal and atherosclerotic tissue form oleyl CoA readily through the action of fatty acyl CoA synthetase. However, it is conceivable that the oleyl CoA formed could be hydrolyzed or bound differently by the aortic preparations, resulting in greater accessibility of the fatty acyl CoA to the atherosclerotic microsomal enzymes.

Our results suggest that the reason for the increased cholesterol-esterifying activity is the presence of more enzyme in the atherosclerotic preparations. Parker et al. (28) have observed a proliferation of endoplasmic reticulum in aortic smooth muscle cells as a result of cholesterol feeding. Portman and Alexander (29) have reported that the microsomal fraction from nonatherosclerotic aortic tissue largely contains plasma membrane fragments, which proliferate as a result of aging or hyperlipemia. If the fatty acyl CoA:cholesterol acyltransferase is localized in endoplasmic reticulum, one might expect more activity from atherosclerotic tissues than from normal aorta.

Control and atherosclerotic preparations clearly possess the enzyme systems necessary for making cholesteryl esters intracellularly. Considering the ATP, CoA-dependent system, the capacity of microsomal fractions from nonatherosclerotic rabbits to esterify oleic acid with cholesterol would be about 100–150 nmol/g wet weight aorta day⁻¹, whereas fractions from atherosclerotic animals would have severalfold greater activity. The difficulty in interpreting such calculations is compounded by the facts that activity is found in other subcellular fractions, the calculations assume a steady supply of available free fatty acid and cholesterol, cholesterol ester hydrolysis is not considered, and the potential inhibiting or enhancing effects of vigorous homogenization of the intact tissue are ignored.

Using our experimental system, we observed no substantial increase in enzymatic activity during the early weeks of cholesterol feeding. In contrast, St. Clair et al. (8) have reported that cholesterol esterification by 900-g supernatant fractions from White Carneau pigeons increases tenfold after only 2 weeks of cholesterol feeding, a time when no visible lesions are present. A recent report by Day and Proudlock (30) demonstrated increased oleic acid incorporation into cholesteryl ester in intact arterial segments from rabbits fed a cholesterol diet for only 3 days. This increase was observed in the aortic arch but not in the thoracic or abdominal regions. Species differences between rabbit and pigeon, methodological differences, or different values for control tissues could explain the discrepancies in the rate of increase of cholesterol esterification by cholesterol feeding. Cholesterol-esterifying activity has recently been shown to differ in several species (31).

We are the first to report increased esterification of labeled oleic acid in acidified (pH 5.2) supernatant fractions from atherosclerotic aortic tissue. This activity has previously been reported to be present in atherosclerotic rabbit aortic tissue (11), although the experimental conditions used for measuring activity differed from the procedures described in this report. In confirmation of the findings of Proudlock and Day (11), the activity did not require ATP, CoA, or any other cofactors. Furthermore, following incubation, cholesteryl ester was the only lipid class found to contain radioactivity. The source of this activity within the smooth muscle cell is not clear; however, a reasonable site would be the lysosomes. Cholesteryl esterase activity has been reported in aortic lysosomes (32, 33), and this enzymatic activity may be reversible. The homogenization procedure used for our experiment may have disrupted aortic lysosomes, thereby causing the lysosomal enzymes to be present in the high-speed supernatant fraction. Utilizing cell suspension preparations from rabbit aortic tissues as an enzyme source, Peters et al. (33) have demonstrated increased cholesterol esterase activity in atherosclerotic lysosomes compared with controls. Furthermore, an enzyme from isolated rat liver lysosomes that catalyzes both the hydrolysis and the formation of cholesteryl ester has recently been described (34).

Exchange mechanisms could also explain this
increased activity in the high-speed supernatant fraction following cholesterol feeding. Increased amounts of both free and esterified cholesterol were present in atherosclerotic supernatant fractions; either of these factors could contribute to the increased activity. The difficulty in evaluating the meaning of this activity is further compounded by the fact that a precipitate forms when the 150,000-g supernatant fraction is adjusted to pH 5.2, thus forming a heterogeneous system. Studies from our laboratory (unpublished) indicate that the activity is distributed between the acidified (pH 5.2) supernatant fraction and the pellet. Thus, only a slight purification was achieved after separation by centrifugation. The inhibition of this activity by TPIA and to a lesser extent by CPIB is of interest, but further studies on a more purified fraction must be carried out before the inhibition can be evaluated and discussed.

The effects of CPIB and TPIA on microsomal activity and cholesterol esterification were of interest in view of the observed relationships between cholesterol ester metabolism and atherosclerosis and the fact that the mechanism of action of these hypolipidemic drugs is still not completely understood. These substances have been shown to inhibit several microsomal enzymes involved in lipid metabolism (35–38). In our studies, TPIA was more potent than CPIB in inhibiting the fatty acyl CoA:cholesterol acyltransferase. Other workers have observed that TPIA is a more effective inhibitor than CPIB of the rat liver acetyl CoA carbonylase (36) and of lipogenesis in rat mammary gland cell cultures (37). To gain further information on the interaction of these drugs with the fatty acyl CoA:cholesterol acyltransferase, kinetic analysis would be desirable. Since our system contains many enzymes which could act on the lipid metabolites involved, purification of the cholesterol-esterifying enzyme would be required to obtain meaningful kinetic parameters.

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References


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