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Dietary oxidized linoleic acid enhances liver cholesterol biosynthesis and secretion in rats

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Based on studies showing that excretion of cholesterol is elevated in rats fed oxidized linoleic acid, we hypothesized that cholesterol metabolism is enhanced under such oxidative stress. Liver cholesterol biosynthesis and secretion and fecal cholesterol excretion were studied in rats fed for 4 weeks diets containing 10% oxidized linoleic acid. Incubation of liver slices with 1-^{14}C acetate and intraperitoneal injection of 5-^{3}H-mevalonate showed the occurrence of enhanced hepatic cholesterol biosynthesis and elevated liver cholesterol secretion in animals subjected to oxidative stress. In addition, impaired liver cholesterol uptake was suggested. Higher levels of excreted cholesterol observed in the experimental animals were accompanied by augmented levels of liver phospholipids, primarily phosphatidylcholine, which most likely increased to enable the excessive cholesterol excretion. This study thus demonstrates that ingestion of oxidized lipids causes profound alterations in cholesterol metabolism. (J. Nutr. Biochem. 11: 176–180, 2000)

Keywords: dietary oxidized linoleic acid; rat liver microsomes; cholesterol biosynthesis; cholesterol secretion; excretion

Introduction

The phospholipid bilayer of membranes, which is composed largely of polyunsaturated fatty acids, is highly susceptible to peroxidation processes.[1] It is now well accepted that various membrane functions such as the activity of bound enzymes, the accessibility of hormone receptors, and the efficiency of transport systems are controlled by membrane fluidity, which is determined by membrane lipid composition and organization.[2] The major determinants that affect membrane fluidity are the levels of cholesterol and phospholipids and their molar ratio, as well as the degree of unsaturation of the phospholipid fatty acyl chains. The relative abundance of the phospholipid subclasses in the membrane is also of importance.[2]

In a recent study,[3] we demonstrated that feeding oxidized linoleic acid to rats resulted in profound differences in lipid composition of the hepatic microsomal membrane, namely, a low cholesterol to phospholipid molar ratio and a high arachidonic acid content. These changes, which were accompanied by in vivo peroxidation processes, were characterized by elevation of the microsomal membrane fluidity. In addition, increased cholesterol excretion suggested the occurrence of an enhanced cholesterol turnover in the rats fed oxidized linoleic acid compared with those fed the untreated preparation.[3]

The liver is the major site of cholesterol, bile acids and phospholipid synthesis and metabolism.[4] Most of the steps involved in these processes are catalyzed by membrane associated microsomal enzymes.[4] The function of these membrane bound enzymes most likely depends on the lipid composition and dynamics of the membrane.[5] Furthermore, it has been reported that the fatty acid composition of hepatic microsomal lipids is influenced by the dietary fat.[6]

The purpose of this investigation was to study the effects of consumption of oxidized linoleic acid by rats on some aspects of cholesterol and phospholipid metabolism. He-

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patic cholesterol biosynthesis as expressed by in vitro incorporation of 1-^{14}C acetate and following 5-^{3}H-mevalonate injection, and liver cholesterol uptake and secretion, as well as its fecal excretion in these animals, were evaluated.

Methods and materials

Preparation of oxidized linoleic acid

A preparation containing 60% linoleic acid (Sigma Chemical Co., St. Louis, MO USA) was oxidized by aeration at 37°C as previously described.[3] Following 7 days of oxidation, a peroxide value[7] of approximately 1,300 mEq oxygen (O_2)/kg oil and a content of the thiobarbituric acid reactive substances (TBARS)[8] of 2.4 mmol malondialdehyde/kg oil were achieved. A change in the fatty acid profile of the oxidized linoleic acid rich preparation, namely, a reduction in the linoleic and linolenic acid content and an elevation in the oleic acid level, was observed.[3]

Animals and diets

Male rats of the Charles River CD strain (animal colony, Department of Food Engineering and Biotechnology, Technion, Haifa, Israel) weighing 93 ± 7 g were fed for 4 weeks AIN[9] diets containing 10 g/100 g fresh (LA) or air oxidized (LAOx) linoleic acid rich preparation as previously described.[3] The facilities met the requirements of the Institutional Animal Care and Use Committee. The animals were divided into two groups of 20 rats each and individually housed in stainless steel wire cages and maintained in a temperature controlled room (23°C) operated with a light-dark cycle of 12 hours. Food was supplied at 6:00 PM and deprived at 6:00 AM with free access to water. The fatty acid free diets were stored at 4°C. A mixture of each of the fatty acid preparations and an appropriate amount of starch stored at −18°C was added to the designated diet daily and provided to the animals, discarding the residue of the previous ration. These steps were undertaken to insure minimal additional oxidation of dietary components. After 1 week of acclimation to the diet, the light-dark cycle was inverted and the room was lighted from 6:00 PM to 6:00 AM, and darkened from 6:00 AM to 6:00 PM. Food was supplied for additional 3 weeks during the dark period.

At the end of the feeding period, food was withheld for 14 hours, and the rats were taken for the cholesterol metabolism studies.

In vivo study

The animals were injected intraperitoneally with 5-^{3}H mevalonic acid lactone (RS-[5-^{3}H], Du Pont, Wilmington, DE USA), 370 KBq (in 1 mL saline) per 100 g body weight. At time intervals of 0.75, 1.5, 3, 7, and 24 hours, four rats from each group were sacrificed by carbon dioxide (CO_2) asphyxiation, and portal vein blood was collected in tubes containing ethylendiamine-tetraacetic acid (EDTA). The rats sacrificed 24 hours after the injection of the radioactive mevalonic acid also served for feces collection for the determination of cholesterol excretion. Livers were removed after perfusion with saline and most of the organ was kept frozen at −70°C for analysis of cholesterol and phospholipid classes.

In vitro study

A small part of the liver from eight animals of each group was immersed in ice-cold saline, and slices of 0.4 to 0.6 mm were cut with a Stadie-Rigges hand microtome (No 7120-A, Arthur H. Thomas Co. Philadelphia, PA USA). Samples of 250 to 300 mg of the liver slices from each animal, in duplicates, were suspended in 3 mL of ice-cold Krebs Ringer bicarbonate buffer (pH 7.4) in 20 mL scintillation vials containing 18.5 KBq of 1-^{14}C sodium acetate (Sigma Chemical Co.). The vials, kept in an ice bath, were flushed for 30 minutes with a mixture of CO_2:O_2 (95:5). At the end of the treatment, the vials were stoppered and incubated for 3 hours in a 37°C water bath with mild shaking. Enzymatic activity was terminated by the addition of a 15 mL solution of chloroform: methanol (2:1).

Lipid composition and radioactivity analyses

For the determination of the specific activity of hepatic cholesterol, liver samples were homogenized and extracted with chloroform: methanol (2:1) according to Folch et al.[10] The lipid extract was subjected to thin layer chromatography (TLC) separation on silica gel, using a solvent mixture of hexane:diethyl ether:acetic acid (80:20:1.5), and the spots corresponding to free and esterified cholesterol were scraped and assayed by the method of Kates[11] and by scintillation counting for the determination of the incorporation of 1-^{14}C acetate and 5-^{3}H-mevalonate into cholesterol in the in vitro and in vivo studies, respectively. Serum cholesterol was assayed enzymatically using Sigma kit No. 352 (Sigma Chemical Co.), and the radioactivity of cholesterol following extraction according to Bligh and Dyer[12] was determined. Feces were extracted with petroleum ether:diethyl ether (1:1; v/v) for 5 hours using a Soxhlet apparatus. The lipid extract was used for assaying fecal cholesterol radioactivity. For the determination of the various phospholipid classes, the liver lipid extract was subjected to two consecutive TLC separations[13] and the spots presenting the main phospholipid classes were scraped and assayed according to Rouser et al.[14]

Statistical methods

Data were analyzed statistically by the Student's *t*-test. Differences between groups were considered significant at a *P*-value of less than 0.05.

Results and discussion

As reported in our previous study,[3] which involved feeding oxidized linoleic acid to rats, the animals subjected to the oxidative stress exhibited growth retardation. The decrease in growth was accompanied by significant liver hypertrophy, as indicated by the ratio of liver to body weight of 6.07 ± 0.32 and 4.89 ± 0.13 observed for the experimental and control groups, respectively. A ratio of 4.75 ± 0.30, which was obtained for a third pair-fed group, indicated that the hypertrophy was associated with oxidative stress rather than with a low food consumption and reduced weight gain, as was observed for the experimental rats. In our previous study,[3] the occurrence of marked changes in liver microsomal lipid composition led us to hypothesize that the observed low cholesterol levels in these membranes may originate from alterations in one or more cholesterol metabolic processes: biosynthesis, liver uptake and secretion, and fecal excretion.

To gain better insight into the factors leading to the changes in membrane lipid composition and fluidity induced by the dietary LAOx, several aspects of cholesterol metabolism were studied. Low hepatic cholesterol levels are known to enhance the activity of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the rate-limiting step of cholesterol biosynthesis, due to the feedback mechanism characteristic to the regulation of

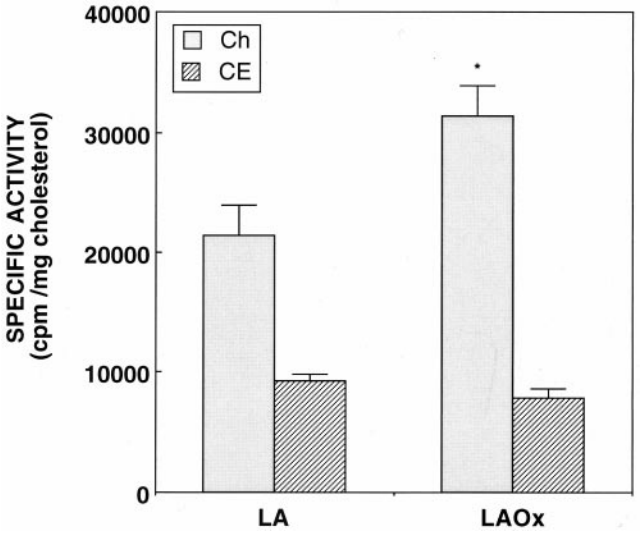

Figure 1 Cholesterol biosynthesis as expressed by 1-^{14}C acetate incorporation into liver slices (mean ± SEM, $n = 4$) derived from rats fed untreated (LA) and oxidized (LAOx) linoleic acid rich fraction. Ch, cholesterol ; CE, cholesterol ester. *Values are significantly different from those of the controls at $P < 0.05$.

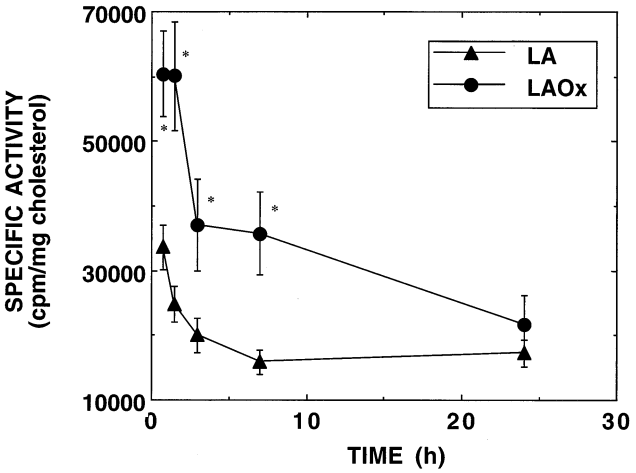

Figure 2 Specific activity of liver cholesterol of rats fed untreated (LA) and oxidized (LAOx) linoleic acid rich preparation following intraperitoneal injection of 5-^{3}H-mevalonate (mean ± SEM, $n = 4$). *Values are significantly different from those of the controls at $P < 0.05$.

cholesterol synthesis. Indeed, the inclusion of oxidized linoleic acid in the diet resulted in enhanced hepatic cholesterol biosynthesis, as demonstrated by both in vitro and in vivo studies. Thus, incorporation of 1-^{14}C acetate into cholesterol in liver slices indicated the occurrence of a significantly higher biosynthetic capacity in the livers of the experimental animals compared with those of the controls. This elevated synthesis was expressed by a 46% increase in the level of the labeled free cholesterol, whereas essentially no change was observed in the amount of the labeled esterified derivative (*Figure 1*).

The rapid initial accumulation of labeled cholesterol in the livers of the experimental animals 1 hour after injection of ^{3}H-mevalonate, which was 90% higher than that observed in the control animals, also supports a high capacity for cholesterol biosynthesis (*Figure 2*). However, the relatively steep decline of the labeled hepatic cholesterol in the experimental animals within 3 hours, which at 24 hours reached a level similar to that found in the control group, suggests that the enhanced biosynthesis was accompanied by elevated secretion of cholesterol from the liver. This secretion can be in the form of very low density lipoprotein (VLDL) cholesterol or of biliary cholesterol and bile acids.[15]

The high level of labeled cholesterol found in the plasma (*Figure 3*) of the experimental animals 45 to 90 minutes after the injection of ^{3}H-mevalonate suggests an elevated secretion of VLDL cholesterol. In the case of the experimental animals, the labeled cholesterol peaked 7 hours after 5-^{3}H-mevalonate injection, whereas for the control rats a respective peak was observed after 3 hours. Nonetheless, similar levels of total plasma labeled cholesterol were observed following 24 hours of ^{3}H-mevalonate administration (*Figure 3*). When examining the build up and the decline of plasma free and esterified labeled cholesterol in the experimental and control groups, a pattern similar to that observed for the total labeled cholesterol was found (*Figure 4*).

Elevated levels of plasma cholesterol in rats subjected to adriamycin induced oxidative stress were reported by Huertas et al.[16] These authors proposed that the observed high serum cholesterol levels resulted from enhanced hepatic cholesterol biosynthesis, accompanied by elevated cholesterol mobilization toward its plasma compartment, and by diminished hepatic membrane cholesterol levels. Although the oxidative stress induced in the present study was of a different nature from the one described by these authors,[16] the outcome with respect to cholesterol metabolism appears remarkably similar. This may suggest the existence of a common underlying mechanism for alterations in cholesterol metabolism when different types of oxidative stress are induced.

The late peaking and slow decline of the labeled plasma cholesterol levels substantiates the possible occurrence of

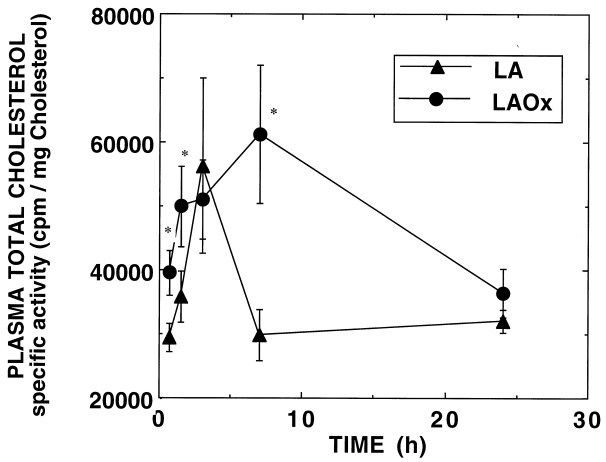

Figure 3 Specific activity of plasma total cholesterol of rats fed untreated (LA) and oxidized (LAOx) linoleic acid rich preparation (mean ± SEM, $n = 4$) following intraperitoneal injection of 5-^{3}H-mevalonate. *Values are significantly different from those of the controls at $P < 0.05$.

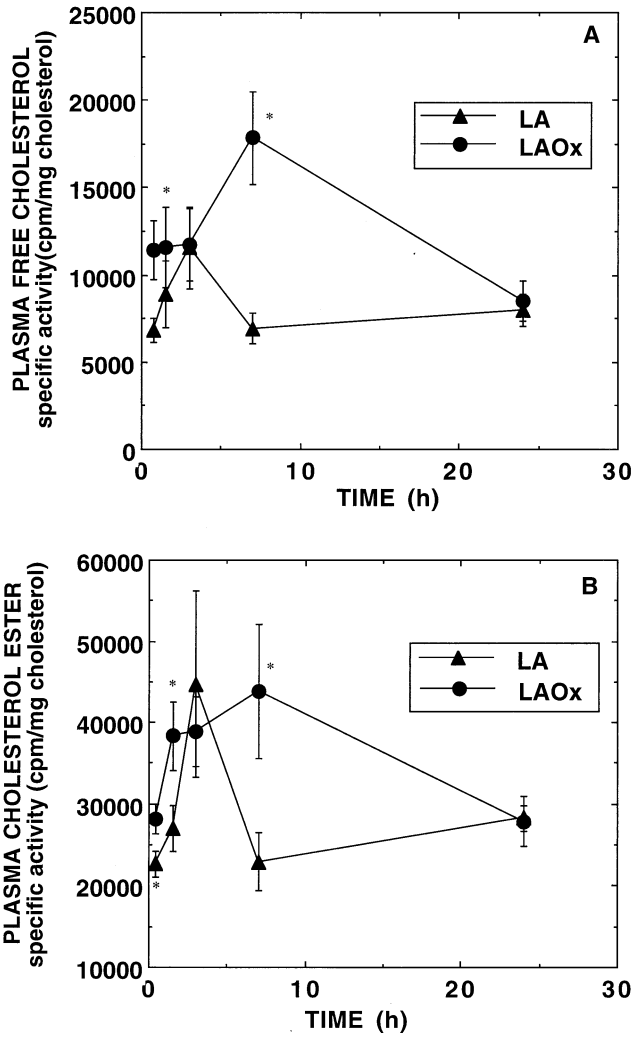

Figure 4 Specific activity of (A) plasma free and (B) esterified cholesterol of rats fed untreated (LA) and oxidized (LAOx) linoleic acid rich preparation (mean ± SEM, $n = 4$) following intraperitoneal injection of 5-^{3}H-mevalonate. *Values are significantly different from those of the controls at $P < 0.05$.

an impaired liver uptake of lipoprotein cholesterol in the rats subjected to oxidative stress. Indeed, in a study that involved oxidative stress caused by cigarette smoke, the binding affinity of the peroxidized low density lipoproteins (LDL) to the hepatic membrane receptors was found to be significantly lower than that of the control LDL.[17] In addition, a recent study[18] demonstrated that oxidative stress was associated with a low hepatic uptake of chylomicrons of rats fed highly oxidized corn oil.

Levels of labeled fecal cholesterol accumulated over a 24-hour period following 5-^{3}H-mevalonate injection were found to be significantly higher ($P < 0.05$) in the experimental animals than in those in the control group (233 ± 22 vs. 179 ± 7.8 cpm/g body weight, respectively). This is indicative of an enhanced biliary secretion of hepatic cholesterol and bile acids, in line with the increased amounts of fecal cholesterol previously observed for the LAOx fed rats.

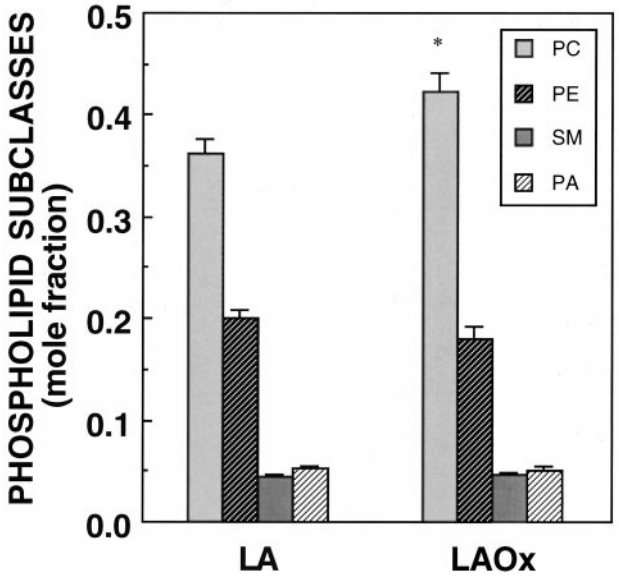

Figure 5 Liver composition of major phospholipid subclasses (mean ± SEM $n = 8$) of rats fed untreated (LA) and oxidized (LAOx) linoleic acid rich preparation. PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PA, phosphatidic acid. *Values are significantly different from those of the controls at $P < 0.05$.

Enhanced secretion of cholesterol in the bile requires elevated levels of phosphatidylcholine in addition to bile acids for effective micellization of the excessive cholesterol.[15] Indeed, the high hepatic phospholipid levels previously observed in the experimental animals[3] are shown in the present study to be primarily due to increased phosphatidylcholine levels (*Figure 5*), whereas no significant differences in the other phospholipid subclasses were observed. This further supports the occurrence of a physiologic need to eliminate the surplus cholesterol synthesized in the livers of the animals subjected to oxidative stress. The elevated levels of excreted cholesterol might also suggest an impaired intestinal reabsorption of cholesterol and bile acids in these animals.

Hepatic cholesterol homeostasis is maintained by an equilibrium between the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase on the one hand, and that of acyl CoA:cholesterol acyl transferase (ACAT) activity on the other hand.[19,20] The enhanced biosynthesis of cholesterol in the experimental rats demonstrated by the 1-^{14}C acetate incorporation studies is indicative of elevated hepatic HMG-CoA reductase activity in the animals subjected to the oxidative stress. Furthermore, the apparent low liver uptake of LDL cholesterol in the LAOx rats may also contribute to the higher HMG-CoA reductase activity in these animals. This enzyme is an integral protein of the microsomal membrane, the activity of which is believed to be mediated by the fluidity of its microenvironment.[5] In that study it was suggested that one of the mechanisms by which cholesterol may modulate HMG-CoA reductase activity is by changing the fluidity of the membrane surrounding the enzyme. Indeed, our previous results, which showed that feeding oxidized linoleic acid was associated with low liver microsomal and cytosol cholesterol levels and elevated

microsomal membrane fluidity, coupled with the present observation that dietary LAOx apparently enhanced HMG-CoA reductase activity, further support this possible relationship between HMG-CoA reductase activity and membrane fluidity.

In addition, the observed elevated fecal radioactivity of cholesterol origin following ^{3}H-mevalonate injection in the LAOx fed rats indicates in these animals the occurrence of enhanced cholesterol 7α-hydroxylase activity, which is known to be coupled to that of HMG-CoA reductase.[21] A high activity of the 7-α-hydroxylase that results in enhanced hepatic bile acid production and secretion is needed to enable the elimination of the excessive cholesterol from the liver of the rats subjected to the oxidative stress.

As shown previously,[3] a high ratio of free to esterified cholesterol was observed in the livers of the LAOx fed rats. The high ratio may stem from two metabolic processes, namely, reduced hepatic ACAT activity and enhanced VLDL secretion. Reduced hepatic ACAT activity was shown to be associated with a low liver cholesterol influx[22] and enhanced biliary cholesterol secretion.[23] Both these processes appear to have taken place in the animals subjected to the oxidative stress, supporting the possible occurrence of a decreased ACAT activity in these animals. ACAT is a microsomal enzyme,[23] and as such its activity is likely to depend on the fluidity of its microenvironment.[2] It is therefore possible that the marked elevation of the microsomal membrane fluidity previously observed for the rats fed LAOx[3] might have resulted in reduced ACAT activity. In addition, the apparently enhanced secretion of VLDL cholesterol following ^{3}H-mevalonate administration (*Figure 3*) might have also contributed to the low level of hepatic esterified cholesterol.

In conclusion, the foregoing results suggest that under oxidative stress caused by peroxidized dietary lipids, the observed low hepatic and microsomal cholesterol levels are associated with elevated cholesterol biosynthesis and secretion and with impaired liver cholesterol uptake.

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