Enhanced solubilization and intestinal absorption of cholesterol by oxidized linoleic acid

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Abstract Solubilization of cholesterol in the intestinal lumen by bile acids and the subsequent formation of mixed micelles is an important step in the absorption of cholesterol. We propose that oxidized fatty acids (ox-FA) may mimic bile acids and form mixed micelles with cholesterol much more efficiently, as compared with unoxidized fatty acids, thereby increasing there absorption. In an in vitro assay at concentrations of 1, 5, and 10 mM, oxidized linoleic acid (ox-18:2) increased the solubilization of cholesterol (3.06, 8.16, and 15.46 nmol/ml) in a dose dependent manner compared with a 10 mM unoxidized linoleic acid (unox-18:2 at 0.97 nmol/ml). The uptake of cholesterol solubilized in the presence of ox-18:2 by Caco-2 cells and everted rat intestinal sacs was greater (1.78 and 1.95 nmol/ml respectively) as compared with the cholesterol solubilized in the presence of unox-18:2 (0.29 and 0.61 nmol/ml; $P = 0.05$). In addition, when LDL receptor deficient mice were fed a high fat diet along with ox-18:2 their plasma cholesterol levels were greater than animals fed the high fat diet alone (1290 mg/dl vs. 1549 mg/dl, $P = 0.013$). From these results, we suggest that ox-FA, by enhancing the solubilization of luminal cholesterol, increases the uptake of cholesterol that might lead to hypercholesterolemia and atherosclerosis.—Penumetcha, M., N. Khan-Merchant, and S. Parthasarathy. **Enhanced solubilization and intestinal absorption of cholesterol by oxidized linoleic acid. *J. Lipid Res.* 2002.** 43: **895–903.**

Supplementary key words bile acids • atherosclerosis • lipid peroxides • oxidized fatty acids

A plethora of studies have suggested that oxidized lipids may have atherogenic properties (1–5). Until recently, most of the attention has been primarily focused on oxidized free fatty acids derived endogenously from cellular sources. In the past decade, studies by Staprans et al. (6, 7) suggest that dietary peroxidized lipids can be secreted into chylomicrons and that these chylomicrons are more susceptible to in vitro oxidation. In addition, feeding oxidized oil accelerated the formation of fatty streak lesions in rabbits (8). In a human study, feeding oxidized fatty acids resulted in a decrease of plasma paraoxonase activity (9). Decreased activity of this enzyme is associated with increased risk of atherosclerosis (10, 11). All these studies centered on the premise that oxidized lipids might contribute to enhanced atherosclerosis by influencing the oxidative pathway.

Cells are constantly exposed to ox-FFA. The intestinal enterocytes are exposed to dietary ox-FFA during absorption. Studies in rodents by Kanazawa and others have shown that when animals are fed peroxidized fatty acids a certain percentage is found in the intestinal lumen and is incorporated into the liver and other organs (12–14). We recently reported the extent of absorption and subsequent metabolism of oxidized linoleic acid (ox-18:2) and unoxidized linoleic acid (unox-18:2) in Caco-2 cells and rat everted intestinal sacs (15). During the course of these experiments, we noted a similarity between ox-FFA and bile acids. Both have similar physio-chemical properties, such as a hydrophobic domain, carboxylic acid, and hydroxy functions. Bile salts are micelle forming agents because of their amphipathic properties.

Cholesterol is poorly soluble in aqueous solutions. Therefore, endogenous and dietary cholesterol has to undergo the obligatory step of micellar solubilization before being absorbed by the intestinal enterocyte. This is accomplished in the intestinal lumen by the formation of mixed micelles from bile and products of lipid digestion (16–18). Of the many components of bile, bile acids and phospholipids contribute to the formation of mixed micelles. During lipid digestion, pancreatic lipase and phospholipase A2 hydrolyze triglycerides to 2-mono-glyceride and fatty acids, and phospholipids to lysophospholipids and fatty acids. These products of lipid digestion along with bile acids form mixed micelles, which render the insoluble cholesterol soluble so that it can be presented to the enterocyte for absorption.

We propose that ox-18:2 is structurally similar to the bile acid lithocholic acid (**Fig. 1**), and its presence in the

Abbreviations: 13-HODE, 13-hydroxylinoleic acid; ox-FA, oxidized fatty acids; Lyso PtdCho, lyso phosphatidylcholine; ox-18:2, oxidized linoleic acid; unox-18:2, unoxidized linoleic acid; TC, taurocholate; 2-MOG, 2-mono-oleylglycerol.

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intestinal lumen might promote increased solubilization of cholesterol and thus its absorption. We realize that in vivo, lithocholic acid may not form micelles efficiently (19). Our intent is to show that, like lithocholic acid, ox-18:2 has one hydroxy group.

In this study, we tested the hypothesis that ox-18:2 solubilizes cholesterol better than unox-18:2 and this would lead to increased uptake of cholesterol by the intestine. Using an in vitro assay, we looked at the solubilization of cholesterol with ox- or unox-18:2 in the presence of other luminal components such as lysophosphatidylcholine (Lyso PtdCho) and 2-mono-oleoylglycerol (2-MOG). In addition, we also measured the uptake of cholesterol solubilized in the presence of ox-18:2 or unox-18:2 by Caco-2 cells and rat everted intestinal sacs. Finally, we fed LDL receptor deficient ($LDLr^{-/-}$) mice a high fat diet with or without ox-18:2 for 10 weeks and measured the plasma cholesterol levels.

MATERIALS AND METHODS

Fatty acids, soybean lipoxidase, taurocholate, Lyso PtdCho, and 2-MOG were purchased from Sigma (St. Louis, MO). [1-^{14}C]linoleic acid (1.9 GBq/mmol) and [4-^{14}C]cholesterol (1.8 GBq/mmol) were obtained from New England Nuclear (Boston, MA). Authentic hydroperoxyoctadecadienoic acid [13(s)-HPODE] was purchased from Cayman Chemicals (Ann Arbor, MI).

Caco-2 cells were cultured in 100-mm stock plates and in six-well plates for experiments in Eagle's minimum essential medium (EMEM) with nonessential amino acids (NEAA) and supplemented with FBS (10%) and 2 mM glutamine and 5% penicillin-streptomycin. Cells were maintained at a pH of 7.4 at 37°C with 95% air flow and 5% CO_2.

Four to six week old homozygous $LDLr^{-/-}$ mice (C57BL/6J-LDLr $^{tm1\ Her}$) (n = 22) were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed in 12 h light and dark cycles. Animals were treated in compliance with Emory University Animal Committee regulations.

Oxidation of linoleic acid

The linoleic acid (3 mM) solution was oxidized with soybean lipoxidase (60 U/100 nmol, 2 h at 37°C) to produce ox-18:2 [hydroperoxy octadecadienoic acid (HPODE) and hydroxy octadecadienoic acid (HODE)] (15, 20). The formation of HPODE was determined by Leucomethylene blue (LMB) (21). Briefly, 40 μl of the ox-18:2 was added to 100 μl of LMB reagent in a micro titer plate and incubated at room temperature for 10 min. Then the sample was read in an Anthos htll microtiter reader. The amount of peroxide generated was quantitated against a standard curve generated by authentic 13-HPODE. The oxidized fatty acid was extracted with ether, dried under nitrogen, and stored at −20°C. The conversion of HPODE to HODE after extraction by ether was confirmed by LMB and a reading at 234 nm by a UVIKON (Research Instruments International, San Diego, CA) spectrophotometer.

Treatment of Caco-2 cells

Caco-2 cells were seeded at an initial density of 2×10^4. Experiments were carried out on days 18–21 for Caco-2 cells. On the day of the experiment, the medium was removed and the cells were washed thrice with serum free medium. Labeled cholesterol containing serum free medium (250–10,000 DPM/nmol) was applied to cells in triplicate and incubated for the indicated time at 37°C. An aliquot of the labeled cholesterol was added to EcoLume to determine radioactivity. After incubation, the medium was collected and the cells were washed twice with PBS. An aliquot of the medium and the PBS used for washing were each added to EcoLume to count the label remaining in the medium and the wash respectively. The cells were then solubilized in 2 ml of deoxycholic acid solution (0.5 mg/ml) and the radioactivity was determined in 100 μl of the cell lysate. After acidification, by adding 20 μl of 6N HCl, 4 ml of chloroform-methanol (1:1, v/v) was added to the cell lysate to extract cellular lipids by the method of Bligh and Dyer (22). After centrifugation (5 min, 2,000 rpm), 500 μl of the upper phase was gently dried under nitrogen and the radioactivity was determined. The lower chloroform phase was collected and 100 μl was gently dried under nitrogen and the cell-associated radioactivity was determined. The remaining chloroform phase was gently dried under nitrogen and was stored at −80°C for further analyses.

In vitro cholesterol solubilization

[4-^{14}C]cholesterol (specific activity 250–10,000 DPM/nmol) was placed (5 nmol) at the bottom of a glass test tube. Ox-18:2 or unox-18:2 (1,000 nmol) in ether was added followed by 2-MOG (150–300 nmol), after which the test tubes were dried under a gentle stream of nitrogen. Ether was added to the test tubes and the tubes were rolled gently to allow all the ingredients to mix well. Next, 1 ml of serum free EMEM was added and the ether was dried. This was followed by the addition of Lyso PtdCho (50–200 nmol), and the test tubes were vortexed vigorously for 2 min. Following this, the test tubes were incubated in a 37°C water bath for 30 min. After incubation the EMEM was filtered through a Millipore filtering apparatus (Millipore Corporation, Bedford, MA) equipped with a 0.22 μM filter. The radioactivity in the filter and the filtrate was determined by scintillation counting.

Cryo-high resolution-scanning electron microscopy

Cryo-high resolution-scanning electron microscopy (cryo-HR-SEM) was performed to characterize the particles in the filtrates obtained after solubilizing cholesterol with ox-18:2 or unox-18:2. The general method for solubilizing cholesterol is as mentioned above. The final concentrations of the various components in the incubation mixture were 5 μM cholesterol, 1 mM of ox- or unox-18:2, 0.2 mM Lyso PtdCho, and 0.3 mM 2-MOG. After incubation the mixture was filtered through a 0.22 μM filter and the filtrates were maintained at 37°C before they were cryo frozen for electron microscopy. Cryo-HR-SEM was performed by the method of Apkarian et al. (23). The frozen specimen was cracked, coated with chromium, and observed under the electron microscope while the samples were maintained at −115°C.

Preparation of rat everted intestinal sacs and incubation

Male Sprague-Dawley rats (250–300 gm) were obtained from Charles River Laboratories and were allowed to acclimatize for a week. They were fed normal rat chow (Harlan Teklad, Madison, Wisconsin). Rat everted sacs were made according to the method of Wilson and Weisman (24). Briefly, after an overnight fast rats were euthanized by CO_2 asphyxiation and 10 cm segments of the intestine were harvested and sacs were prepared. The sacs were incubated with solubilized cholesterol in 3.5 ml vol of serum-free EMEM. After incubation, the serosal contents were emptied and the radioactivity remaining in the medium was determined. The sac was washed with 4 ml of PBS for 1 min. The mucosa was gently scraped into a test tube and the volume was adjusted to 2 ml by adding 0.25 M sucrose at pH of 7.4. Tissue lipids were extracted by the method of Bligh and Dyer as mentioned above. In

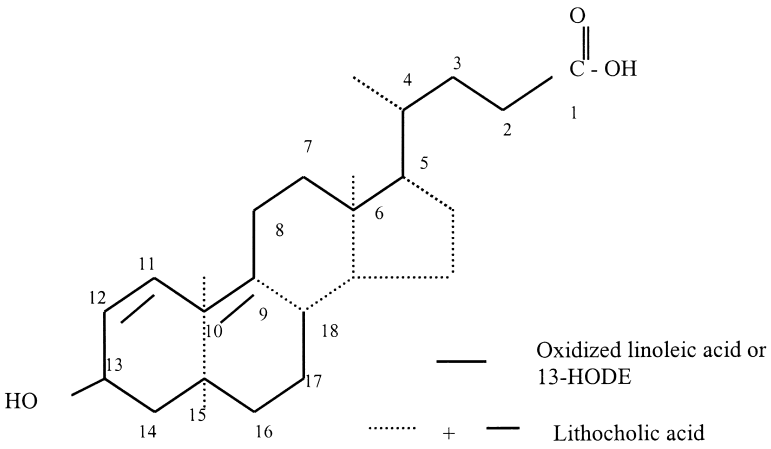

Fig. 1. Structural similarity of lithocholic acid and 13-hydroxyoctadecadienoic acid (13-HODE). The dotted lines together with the solid lines represent the structure of lithocholic acid while the solid lines alone represent 13-HODE.

all experiments, two adjacent sacs from the same rat were used to test for the uptake of cholesterol in the presence of ox- or unox-18:2.

In vivo study with LDLr$^{-/-}$

Four to six week old male, LDLr$^{-/-}$ mice were fed an atherogenic high fat diet (n = 12) containing 21.2% fat and 0.15% cholesterol but no cholic acid (TD 88137; Harlan Teklad). Another group (n = 10) was fed the atherogenic diet along with ox-18:2 (8.0 mg/day/mouse). The mice were fed ox-18:2 five times a week for a total of 10 weeks. Both groups stayed on the high fat diet for the entire study period of 10 weeks. At the end of 10 weeks the mice were euthanized and the tissues harvested.

Mice were sacrificed by CO_2 asphyxia, and then the blood was drawn in heparinized tube from the inferior venacava. Red blood cells and plasma were separated by centrifugation (3,000 rpm, 10 min at 4°C) and then frozen.

Statistical analyses

Differences between the in vitro solubilization of cholesterol by ox-18:2 or unox-18:2, the uptake of solubilized cholesterol by CaCo-2 cells, and the plasma cholesterol levels of mice fed high fat diet versus high fat diet and ox-18:2 were analyzed by the student's *t*-test. Differences in the uptake of solubilized cholesterol by rat everted intestinal sacs was assessed by using the Proc Mixed model statistical software. Statistical significance was set at $P < 0.05$. Values are expressed as mean and SE.

RESULTS

We determined the solubilization of [4-^{14}C]cholesterol (in vitro) by ox-18:2 and unox-18:2 as shown in **Fig. 2**. Unox-18:2 at a 10 mM concentration incorporated cholesterol into micelles poorly. On the other hand, increasing amounts of ox-18:2 (1–10 mM) solubilized cholesterol in a dose dependent manner.

In the subsequent experiments we added Lyso PtdCho and 2-MOG along with ox-18:2 or unox-18:2 to the solubilization experiments because they are important components of mixed micelles formed in the intestinal lumen. Micelles composed of bile salts alone solubilize cholesterol poorly. However, their ability to solubilize cholesterol increases in the presence of luminal contents such as Lyso PtdCho and 2-mono-glyceride. Taurocholate was used to demonstrate the ability of bile acids to solubilize cholesterol, as it is representative of a typical human bile acid (25). As shown in **Fig. 3**, taurocholate alone solubilizes only about 7% of the added cholesterol, while in the presence of Lyso PtdCho and 2-MOG, it solubilizes 10 times more cholesterol. In a similar way, ox-18:2 also solubilizes seven times more cholesterol in the presence of Lyso PtdCho and 2-MOG, while solubilization by unox-18:2 is poor. Further more, **Fig. 4** shows that ox-18:2 solu-

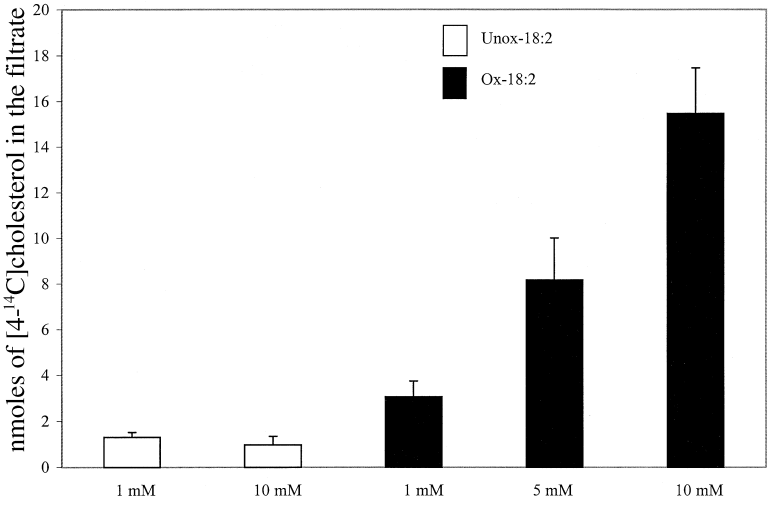

Fig. 2. In vitro solubilization of [4-^{14}C]cholesterol by unoxidized linoleic acid (unox-18:2) (white bar) or oxidized linoleic acid (ox-18:2) (black bar). One hundred nanomoles of [4-^{14}C]cholesterol were dried in a test tube to which ox-18:2 (1 mM, 5 mM, or 10 mM) or unox-18:2 (1 mM or 5 mM; in ether) were added and dried. Then 1 ml of PBS (pH 7.4) was added and the contents were vortexed vigorously for 2 min. Then the test tubes were incubated for 30 min at 37°C. After incubation, the contents of the test tube were filtered through a 0.22 μM filter and the radioactivity in the filtrate and the filter was determined. Each experiment was repeated three times in triplicate each time. Data represent the mean amount of cholesterol in the filtrate (solubilized) ± SE, $P < 0.001$.

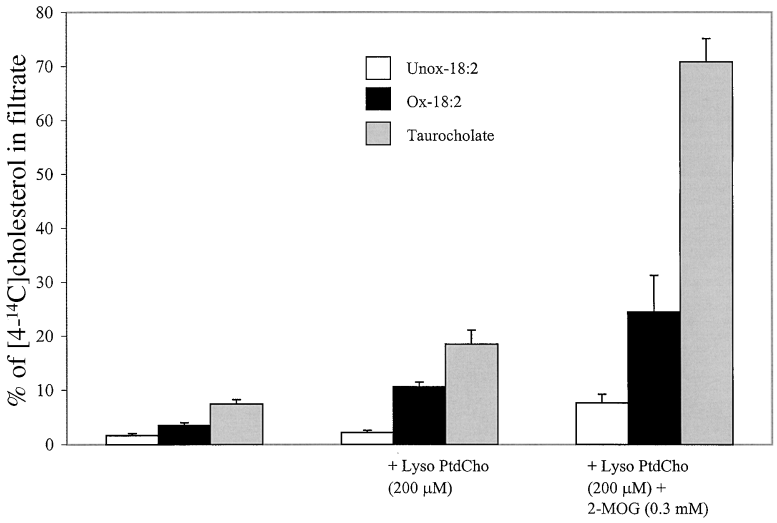

Fig. 3. In vitro solubilization of [4-^{14}C]cholesterol with unox-18:2 (white bar), or ox-18:2 (black bar), or taurocholate (gray bar) (5 mM) in the presence of lyso phosphatidylcholine (Lyso PtdCho) (0.2 mM) or Lyso PtdCho (0.2mM) and 2-mono-oleylglycerol (2-MOG) (0.15 mM). The solubilization was performed as mentioned in Materials and Methods. Briefly, 5 nmol of [4-^{14}C]cholesterol was dried in a test tube to which either ox-18:2, unox-18:2, or taurocholate (ratio of cholesterol-fatty acid/taurocholate was 1:200) together with 2-MOG (in chloroform) were added and dried. This was followed by Lyso PtdCho and 500 μl of serum free Eagle's minimum essential medium (EMEM). The contents of the test tube were vortexed vigorously for 2 min. After the addition of 500 μl of serum free EMEM, the test tubes were incubated for 1 h at 37°C. After incubation, the contents of the test tube were filtered through a 0.22 μM filter and the radioactivity in the filtrate and the filter was determined. Each experiment was repeated three times, in triplicate each time. Data represent the mean amount of cholesterol in the filtrate (solubilized) ± SE, $P < 0.01$.

bilizes cholesterol better than unox-18:2 both at low and high concentrations of Lyso PtdCho and that the amount of cholesterol solubilized by ox-18:2 increases as 2-MOG is added to the system (0.55 nmol with Lyso PtdCho vs. 1.22 nmol with Lyso PtdCho and 2-MOG). Similarly, in vitro studies by Simmonds et al. have also demonstrated that the solubilization of cholesterol increases proportionally to the amount of fatty acid or 1-mono-glyceride or both added, and that the solubility effects of 1-mono-glyceride and fatty acid are additive (26). Finally, we also demonstrate (**Fig. 5**) that at a pH of 6.5, 6.7, or 7.0 the ability of ox-18:2 to solubilize cholesterol is greater (1.37, 2.58, and 3.66 nmol, respectively) as compared with the amount solubilized by unox-18:2 (0.16, 0.55, and 1.31 nmol) at the same levels of pH.

In order to further characterize the physical dimen-

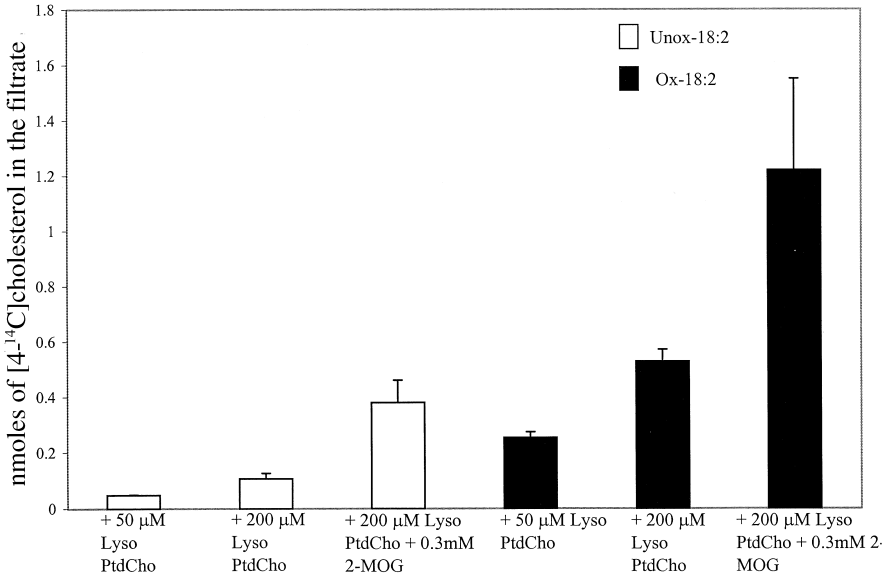

Fig. 4. In vitro solubilization of [4-^{14}C]cholesterol with unox-18:2 (white bar) or ox-18:2 (black bar) (1 mM of fatty acid) in the presence of Lyso PtdCho (0.05–0.2 mM) or Lyso PtdCho (0.2 mM) and 2-MOG (0.15 mM). The solubilization was performed as mentioned in Materials and Methods. Briefly, 5 nmol of [4-^{14}C]cholesterol was dried in a test tube to which either ox-18:2 or unox-18:2 together with 2-MOG (in chloroform) was added and dried. This was followed by Lyso PtdCho and 500 μl of serum free EMEM. The contents of the test tube were vortexed vigorously for 2 min. After the addition of 500 μl of serum free EMEM, the test tubes were incubated for 1 h at 37°C. After incubation, the contents of the test tube were filtered through a 0.22 μM filter and the radioactivity in the filtrate and the filter was determined. Each experiment was repeated three times in triplicate each time. Data represent the mean amount of cholesterol in the filtrate (solubilized) ± SE, $P < 0.01$.

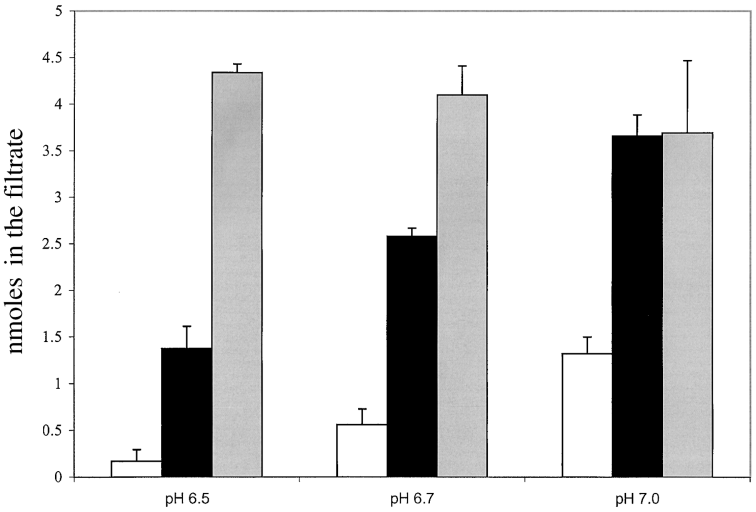

Fig. 5. In vitro solubilization of [4-^{14}C]cholesterol with unox-18:2 (white bar), or ox-18:2 (black bar) (1 mM fatty acid), or taurocholate (gray bar) [5 mM; ratio of cholesterol-fatty acid/taurocholate (TC) = 1:200] in the presence of Lyso PtdCho (0.2 mM) and 2-MOG (0.3 mM). The solubilization was performed as mentioned in Materials and Methods. Briefly, 5 nmol of [4-^{14}C]cholesterol was dried in a test tube to which either ox-18:2, unox-18:2, or taurocholate, together with 2-MOG (in chloroform) were added and dried. This was followed by the addition of Lyso PtdCho and 500 μl of PBS at a pH of 6.5, 6.7, or 7.0. The contents of the test tube were vortexed vigorously for 2 min. After the addition of 500 μl of PBS, the test tubes were incubated for 1 h at 37°C. After the incubation, the contents of the test tube were filtered through a 0.22 μM filter and the radioactivity was determined. Data represent the mean amount of cholesterol in the filterate ± SE. The experiments were repeated in triplicate two times.

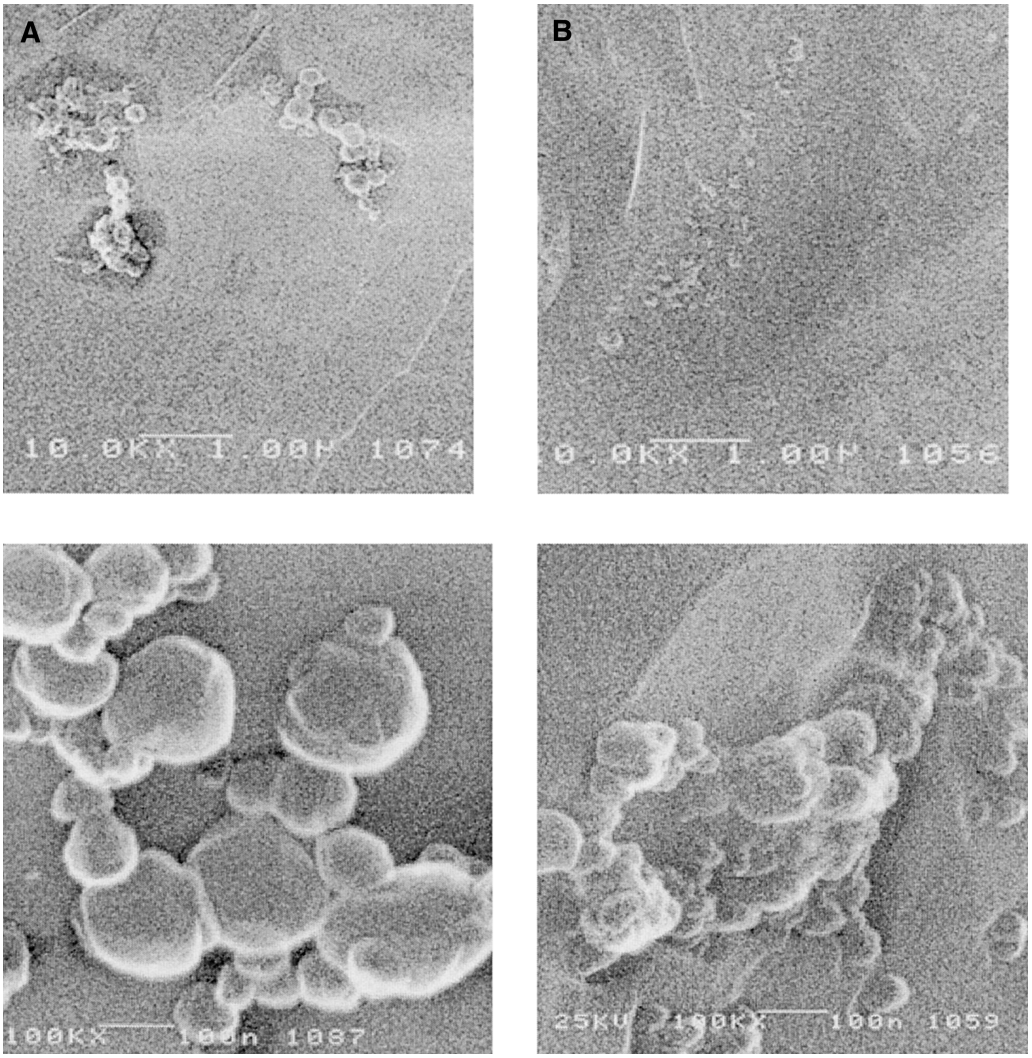

Fig. 6. Micrographs of filtrates obtained by solubilizing cholesterol (5 nmol) with unox-18:2 (A, top and bottom) or ox-18:2 (B, top and bottom) (1 mM) along with Lyso PtdCho (0.2 mM) and 2-MOG (0.3 mM). After obtaining the filtrates they were maintained at 37°C until they were cryo frozen and coated with chromium. Micrographs were then taken with an electron microscope according to the protocol mentioned in Materials and Methods. Micrographs in the top represent a magnification of 10 KX while those at the bottom represent a magnification of 100 KX.

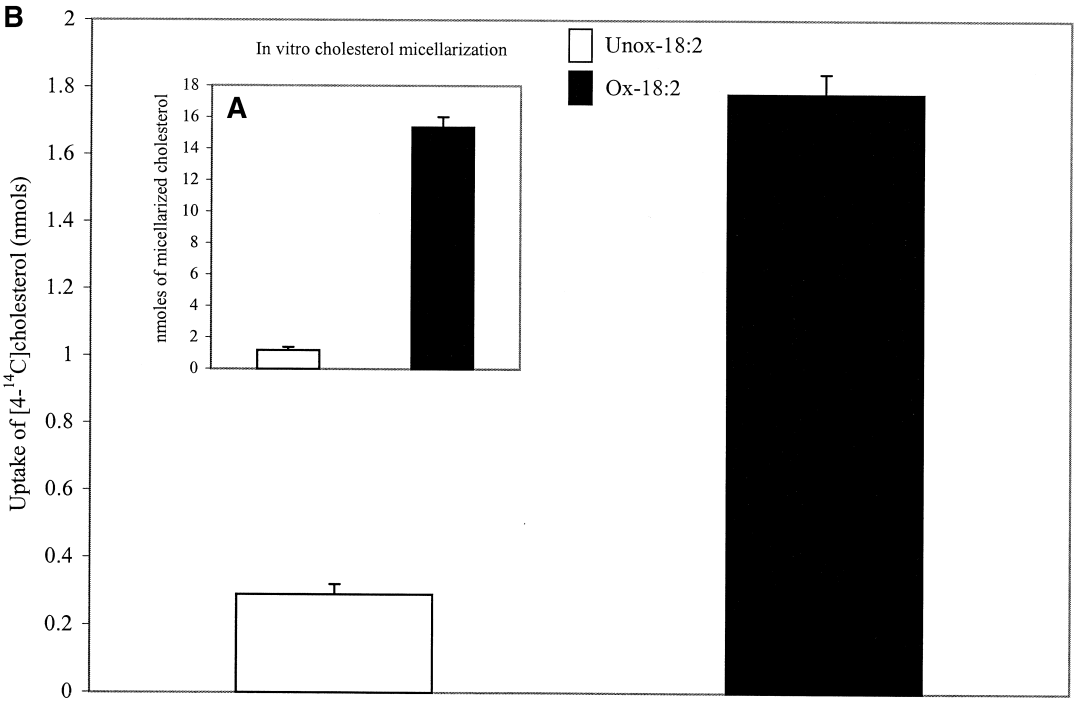

Fig. 7. The uptake of [4-^{14}C]cholesterol solubilized by Lyso PtdCho, 2-MOG, and unox-18:2 (white bar) or ox-18:2 (black bar) in Caco-2 cells. This experiment had two steps: the in vitro solubilization (A) of 25 nmol of [4-^{14}C]cholesterol with Lyso PtdCho (0.16 mM) and 2-MOG (0.25 mM) and ox-18:2 or unox-18:2 (1 mM) followed by the incubation of Caco-2 cells (B) with the solubilized cholesterol in serum free medium for 1 h at 37°C. After incubation, medium was removed and the cells were washed with PBS and then solubilized in 2 ml of deoxycholicacid (0.5 mg/ml). The cellular lipids were then extracted as mentioned in Materials and Methods and the radioactivity was determined. Each experiment was repeated thrice, in triplicate each time. Data represent the mean amount (nmol) of cholesterol ± SE, $P < 0.001$ in the cells.

sions of filtrates, we obtained electron micrographs of filtrates produced by the solubilization of cholesterol in the presence of ox-18:2 or unox-18:2 along with Lyso PtdCho and 2-MOG. As shown in **Fig. 6A** and **B**, at two different magnifications the spherical particles created in the presence of ox-18:2 (Fig. 6B, top and bottom) have a diameter that is between 25–75 nm range (250–750 Å) while the particles in the filtrates from unox-18:2 (Fig. 6A top and bottom) had a diameter of up to 150 nm (1,500 Å) suggesting that the physical dimensions of particles created by ox-18:2 are smaller than those created by unox-18:2.

Next, we determined the uptake of cholesterol by Caco-2 cells in the presence of Lyso PtdCho, 2-MOG, and ox-18:2 or unox-18:2. As shown in **Fig. 7A**, ox-18:2 solubilized about 15 nmol of cholesterol compared with 1.2 nmol by unox-18:2. When this solubilized cholesterol was added to Caco-2 cells, 1.6 nmol of cholesterol solubilized by ox-18:2 was associated with the cells compared with 0.29 nmol of cholesterol solubilized by unox-18:2 (Fig. 7B). This suggests that if more cholesterol is solubilized then more is likely to be taken up by the intestine. We also tested the above-mentioned hypothesis in the rat everted intestinal sac model. As shown in **Fig. 8B**, when rat everted intestinal sacs were incubated with cholesterol solubilized by ox-18:2, Lyso PtdCho, and 2-MOG the uptake of cholesterol was significantly greater in each of the segments tested compared with cholesterol solubilized by unox-18:2, Lyso PtdCho, and 2-MOG. Similar results in both models demonstrate that if oxidized fatty acids can solubilize cholesterol as bile acids do, then the uptake of cholesterol by the intestine is increased.

Finally, we evaluated whether oxidized fatty acid consumption would result in elevated plasma cholesterol levels, possibly as a result of increased dietary cholesterol absorption by the intestine. We fed 8 mg of ox-18:2 along with a high fat diet to LDLr$^{-/-}$ mice for 10 weeks. Plasma cholesterol levels of mice fed the high fat diet with or without ox-18:2 are shown in **Fig. 9**. The mice that were fed ox-18:2 along with the high fat diet had a mean cholesterol level that was significantly higher than the mice that were fed the high fat diet alone (1549 mg/dl vs. 1290mg/dl; $P = 0.013$). This shows that feeding ox-18:2 increased plasma cholesterol levels.

DISCUSSION

In this study, we demonstrate a cooperative association between ox-18:2 and micellar bile salts that results in a marked enhancement of cholesterol solubilization. Oxidized lipids in foods arise from food processing such as heating and frying (27). So far, most of the studies with di-

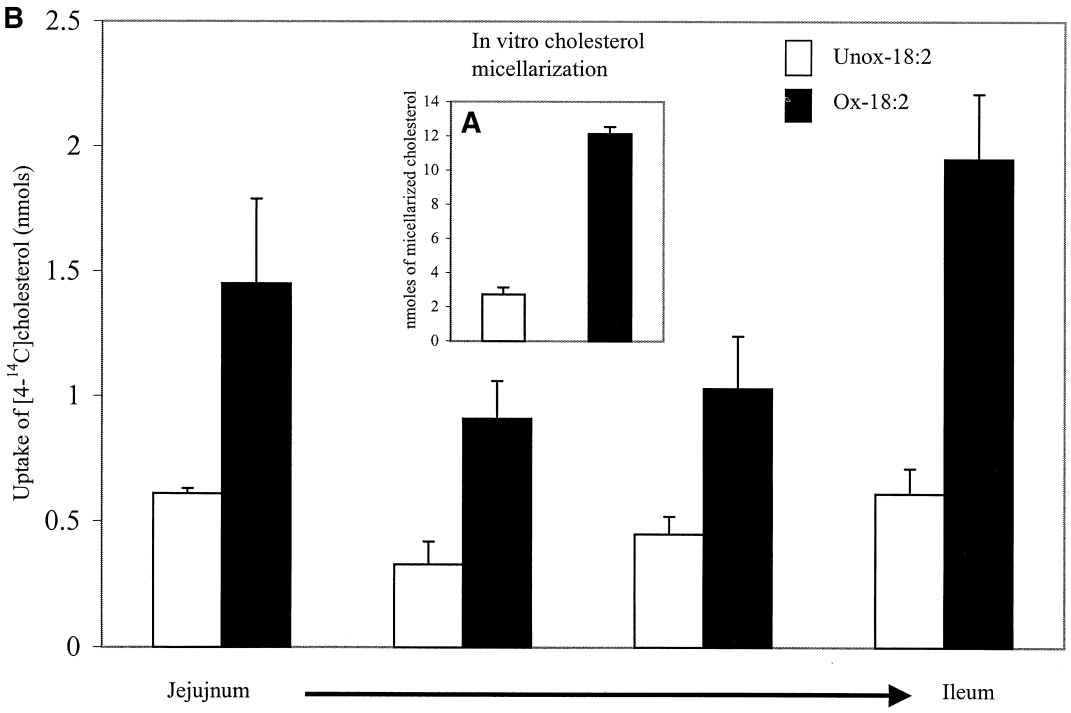

Fig. 8. The uptake of [4-^{14}C]cholesterol solubilized by Lyso PtdCho, 2-MOG, and unox-18:2 (white bar) or ox-18:2 (black bar) in rat everted intestinal sacs. This experiment had two steps: the in vitro solubilization (A) of 18 nmol of [4-^{14}C]cholesterol with Lyso PtdCho (0.16 mM) and 2-MOG (0.3 mM) and ox-18:2 or unox-18:2 (0.5 mM) followed by the incubation of rat everted sacs (B) with the solubilized cholesterol in 3.5 ml of serum free medium for 1 h at 37°C. After incubation, medium was removed and each sac was washed with 3 ml of PBS for 1 min. Then the mucosa from the sac was scraped, weighed, and placed in 2 ml of 0.25 M sucrose, pH 7.4. Then cellular lipids were extracted as mentioned in Materials and Methods and the radioactivity was determined. Each experiment was repeated four times. Data represent the calculated mean amount (as nmol) of cholesterol/1 gm of mucosa ± SE, $P < 0.05$.

etary oxidized fatty acids (hydroperoxides) were geared toward elucidating their effect on atherosclerosis. Most of these studies looked at the effect of dietary oxidized fatty acids on the oxidation potential of lipoproteins. In this study, we considered that the "atherogenicity" of oxidized fatty acids might go beyond their potential to induce oxidative stress. This is important to consider because the pH in the intestinal lumen converts the hydroperoxy fatty acids to hydroxy-fatty acids. These hydroxy-fatty acids are not oxidants themselves and therefore we speculated that they should have other pro-atherogenic effects.

Oxidation of linoleic acid results in the formation of both primary and secondary oxidation products. We decided to use the primary oxidation product of linoleic acid (13-hydroxyoctadecadienoic acid) because its structure is similar to that of a bile acid. More over, linoleic acid is the most abundant fatty acid in the Western diet. While the chemical comparison of lithocholic acid to ox-18:2 in Fig. 1 is simplistic, it must be kept in mind that the presence of the hydroxy group in the ox-18:2 could certainly effect the amphipathic nature of the molecule. Unox-18:2, like all FFAs, is an amphiphile and thus can form micelles. We suspect that the critical micellar concentration of ox-18:2 might be lower than the unox-18:2 and this might solubilize cholesterol more efficiently than unox-18:2. However using traditional methods we were unable to quantify the critical micellar concentration of ox-18:2 (data not shown). Using a higher pH range (around 8.5 to 9.0) might allow us to measure the critical micellar concentration.

This study contrasts the ability of the oxidized form of linoleic acid to its unoxidized form in solubilizing cholesterol. To begin with, we show that ox-18:2 solubilizes cholesterol better than unox-18:2, both by itself and in the presence of potential luminal contents such as Lyso PtdCho and 2-MOG. Then we show that the cholesterol that is solubilized in the presence of ox-18:2 is absorbed better in Caco-2 cells and rat everted intestinal sacs. Finally, we show that feeding ox-18:2 to LDLr$^{-/-}$ mice increases their plasma cholesterol levels.

Under normal circumstances, cholesterol in the intestinal lumen forms mixed micelles with fatty acids, Lyso PtdCho, 2-mono-glyceride, bile acids, and other components of bile. Cholesterol is then absorbed by passive diffusion, although a receptor mediated uptake was suggested more recently (28). Based on studies by Mansbach (29), the concentration of Lyso PtdCho in human duodenal fluid in the micellar phase is 0.12–0.81 μmol/ml up to 4 h after a meal in humans. Based on regression lines, the researchers also calculated the stochiometry of 0.15 mol of Lyso PtdCho/mol of bile salt in mixed lipid micelles. We substituted ox-18:2 for

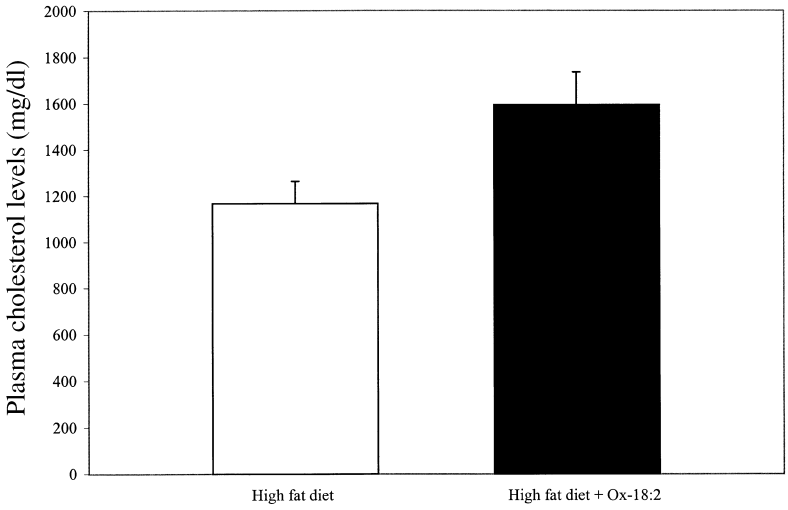

Fig. 9. Plasma cholesterol (mg/dl) levels in LDL receptor deficient mice that were fed a high fat diet (white bar) or a high fat diet + ox-18:2 (black bar). Mice in the treatment group were fed 8 mg/day of ox-18:2, 5 days a week for a period of 10 weeks. At the time of sacrifice, blood was drawn from the inferior vena cava and the plasma separated and frozen at –80°C. Cholesterol levels were assayed by using a cholesterol reagent kit from Sigma. Data represent the mean plasma cholesterol levels (mg/dl) ± SE, $P = 0.013$.

bile acids in our experiments and had 0.1–0.2 mol of Lyso PtdCho/mol of fatty acid. In general, we used Lyso PtdCho concentrations of 25–200 μM in all of our experiments. The concentration of mono-glyceride in Mansbach's paper was 0.31–0.93 mM 4 h postprandially. In our experiments, the concentration of 2-MOG was 0.15–0.3 mM.

Studies by Sallee and Mansbach have shown that the concentration of FFAs in mixed micelles is 0.01–8 mM (29, 30). There are only a few studies that have looked at the concentration of oxidized fatty acids in the intestinal lumen in humans (14). Kanazawa et al. showed that feeding 18 μmol of linoleic acid hydroperoxide to male Wistar rats resulted in the presence of 3 μmol of linoleic acid hydroxide (16% of dose) in the intestinal lumen. The amount of peroxidized lipids in one medium serving of french-fried potatoes is estimated to be about 8,000 μmol (8). Since hydrolysis of oxidized triacylglycerol is just as efficient as the unoxidized form (31, 32) it is possible that concentrations of these oxidized fatty acids is high enough to promote solubilization of luminal cholesterol. Under normal circumstances, when luminal cholesterol undergoes solubilization, it forms mixed micelles that have been known to have a radius of 25–40 Å. We used cryo-HR-SEM to ascertain the physical dimensions of these particles. Based on these pictures the size of the spherical particles is too big to be micelles. All that we can conclude is that the cholesterol that passes the 0.22 μM filter is increased by oxidized 18:2 (at pHs between 6.5–7.4) compared with similar systems with unoxidized 18:2.

We wanted to see if the presence of greater amount of solubilized cholesterol in the lumen/medium leads to an increased uptake of cholesterol. Studies by Simmonds et al. in humans suggest that the micellar solubilization of luminal cholesterol is the rate limiting step in the absorption of cholesterol. We studied the uptake of solubilized cholesterol in two models, Caco-2 cells and rat everted intestinal sacs. Adding cholesterol solubilized by ox-18:2 led to an increased uptake in both systems compared with the uptake of cholesterol solubilized by unox-18:2. This is not surprising, because the greater the amount of cholesterol in the micellar phase, the greater the uptake. This suggests that in the presence of ox-18:2, if more cholesterol gets solubilized then more of it gets absorbed.

Studies have shown that when there is an increase in dietary or luminal cholesterol, then the activity of ACAT in the enterocyte also increases (33–35) and results in the formation of cholesteryl esters (CE). These CE are then packaged into lipoproteins and secreted into the lymph. In a cell culture model, Field et al. showed that inhibition of intestinal ACAT results in a decreased secretion of lipoproteins (36), suggesting that an increased ACAT activity in the presence of luminal fat could enhance the secretion of lipoproteins that could ultimately lead to hypercholesterolemia. Feeding LDLr$^{-/-}$ mice with ox-18:2 raised plasma cholesterol levels. However, this increase in plasma cholesterol levels could have been due to factors other than increased luminal absorption of cholesterol. For example, oxidized fatty acids could increase the synthesis of cholesterol, or could decrease its clearance from plasma.

Taking into consideration the data from the in vitro solubilization and uptake studies, we cannot rule out the possibility that the hypercholesterolemia in mice fed ox-18:2 is due to increased absorption of cholesterol from the intestinal lumen. Increasing the solubilization of cholesterol and increasing its absorption could be one more mechanism by which oxidized fatty acids might promote atherosclerosis.

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REFERENCES

1. Esterbauer, H. 1993. Cytotoxicity and genotoxicity of lipid-oxidation products. *Am. J. Clin. Nutr.* **57:** 779S–785S; discussion 785S–786S.

2. Parthasarathy, S., N. Santanam, S. Ramachandran, and O. Meilhac. 2000. Potential role of oxidized lipids and lipoproteins in antioxidant defense. *Free Radic. Res.* **33:** 197–215.
3. Khan, B. V., S. S. Parthasarathy, R. W. Alexander, and R. M. Medford. 1995. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J. Clin. Invest.* **95:** 1262–1270.
4. Thomas, C. E., R. L. Jackson, D. F. Ohlweiler, and G. Ku. 1994. Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. *J. Lipid Res.* **35:** 417–427.
5. Wen, Y., S. Scott, Y. Liu, N. Gonzales, and J. L. Nadler. 1997. Evidence that angiotensin II and lipoxygenase products activate c-Jun NH2-terminal kinase. *Circ. Res.* **81:** 651–655.
6. Staprans, I., X. M. Pan, M. Miller, and J. H. Rapp. 1993. Effect of dietary lipid peroxides on metabolism of serum chylomicrons in rats. *Am. J. Physiol.* **264:** G561–G568.
7. Staprans, I., J. H. Rapp, X. M. Pan, and K. R. Feingold. 1993. The effect of oxidized lipids in the diet on serum lipoprotein peroxides in control and diabetic rats. *J. Clin. Invest.* **92:** 638–643.
8. Staprans, I., J. H. Rapp, X. M. Pan, D. A. Hardman, and K. R. Feingold. 1996. Oxidized lipids in the diet accelerate the development of fatty streaks in cholesterol-fed rabbits. *Arterioscler. Thromb. Vasc. Biol.* **16:** 533–538.
9. Sutherland, W. H., R. J. Walker, S. A. de Jong, A. M. van Rij, V. Phillips, and H. L. Walker. 1999. Reduced postprandial serum paraoxonase activity after a meal rich in used cooking fat. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1340–1347.
10. Shih, D. M., Y. R. Xia, X. P. Wang, E. Miller, L. W. Castellani, G. Subbanagounder, H. Cheroutre, K. F. Faull, J. A. Berliner, J. L. Witztum, and A. J. Lusis. 2000. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J. Biol. Chem.* **275:** 17527–17535.
11. Mackness, B., M. I. Mackness, S. Arrol, W. Turkie, K. Julier, B. Abuasha, J. E. Miller, A. J. Boulton, and P. N. Durrington. 1998. Serum paraoxonase (PON1) 55 and 192 polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus. *Atherosclerosis.* **139:** 341–349.
12. Eder, K. 1999. The effects of a dietary oxidized oil on lipid metabolism in rats. *Lipids.* **34:** 717–725.
13. Kanazawa, K., E. Kanazawa, and M. Natake. 1985. Uptake of secondary autoxidation products of linoleic acid by the rat. *Lipids.* **20:** 412–419.
14. Kanazawa, K., and H. Ashida. 1998. Catabolic fate of dietary trilinoleoylglycerol hydroperoxides in rat gastrointestines. *Biochim. Biophys. Acta.* **1393:** 336–348.
15. Penumetcha, M., N. Khan, and S. Parthasarathy. 2000. Dietary oxidized fatty acids: an atherogenic risk? *J. Lipid Res.* **41:** 1473–1480.
16. Ganguly, J., P. V. Subbaiah, and S. Parthasarathy. 1972. Intestinal absorption of lipids. *Biochem. Soc. Symp.* **35:** 67–80.
17. Carey, M. C., D. M. Small, and C. M. Bliss. 1983. Lipid digestion and absorption. *Annu. Rev. Physiol.* **45:** 651–677.
18. Borgstrom, B. 1985. The micellar hypothesis of fat absorption: must it be revisited? *Scand. J. Gastroenterol.* **20:** 389–394.
19. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28:** 589–595.
20. Fruebis, J., S. Parthasarathy, and D. Steinberg. 1992. Evidence for a concerted reaction between lipid hydroperoxides and polypeptides. *Proc. Natl. Acad. Sci. USA.* **89:** 10588–10592.
21. Auerbach, B. J., J. S. Kiely, and J. A. Cornicelli. 1992. A spectrophotometric microtiter-based assay for the detection of hydroperoxy derivatives of linoleic acid. *Anal. Biochem.* **201:** 375–380.
22. Bligh, E. G., and W. J. Dyer. 1959. Rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917
23. Apkarian, R. P., K. L. Caran, and K. A. Robinson. 1999. Topographic imaging of chromium-coated frozen-hydrated cell and macromolecular complexes by in-lens field emission scanning electron microscopy. *Microsc. and Microanal.* **5:** 197–207.
24. Wilson, T. H., and G. Wiseman. 1954. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. *J. Physiol.* **123:** 116–125.
25. Hofmann, A. F. 1999. The continuing importance of bile acids in liver and intestinal disease. *Arch. Intern. Med.* **159:** 2647–2658.
26. Simmonds, W. J., A. F. Hofmann, and E. Theodor. 1967. Absorption of cholesterol from a micellar solution: intestinal perfusion studies in man. *J. Clin. Invest.* **46:** 874–890.
27. Kubow, S. 1992. Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radic. Biol. Med.* **12:** 63–81.
28. Hernandez, M., J. Montenegro, M. Steiner, D. Kim, C. Sparrow, P. A. Detmers, S. D. Wright, and Y. S. Chao. 2000. Intestinal absorption of cholesterol is mediated by a saturable, inhibitable transporter. *Biochim. Biophys. Acta.* **1486:** 232–242.
29. Mansbach, C.M. II, R. S. Cohen, and P. B. Leff. 1975. Isolation and properties of the mixed lipid micelles present in intestinal content during fat digestion in man. *J. Clin. Invest.* **56:** 781–791.
30. Sallee, V. L. 1974. Apparent monomer activity of saturated fatty acids im micellar bile salt solutions measured by a polyethylene partitioning system. *J. Lipid Res.* **15:** 56–64.
31. Miyashita, K., T. Takagi, and E. N. Frankel. 1990. Preferential hydrolysis of monohydroperoxides of linoleoyl and linolenoyl triacylglycerol by pancreatic lipase. *Biochim. Biophys. Acta.* **1045:** 233–238.
32. Henderson, R. J., I. C. Burkow, and R. M. Millar. 1993. Hydrolysis of fish oils containing polymers of triacylglycerols by pancreatic lipase in vitro. *Lipids.* **28:** 313–319.
33. Field, F. J., A. D. Cooper, and S. K. Erickson. 1982. Regulation of rabbit intestinal acyl coenzyme A-cholesterol acyltransferase in vivo and in vitro. *Gastroenterology.* **83:** 873–880.
34. Field, F. J., E. J. Albright, and S. N. Mathur. 1987. Effect of dietary n-3 fatty acids on HMG-CoA reductase and ACAT activities in liver and intestine of the rabbit. *J. Lipid Res.* **28:** 50–58.
35. Stange, E. F., K. E. Suckling, and J. M. Dietschy. 1983. Synthesis and coenzyme A-dependent esterification of cholesterol in rat intestinal epithelium. Differences in cellular localization and mechanisms of regulation. *J. Biol. Chem.* **258:** 12868–12875.
36. Kam, N. T., E. Albright, S. N. Mathur, and F. J. Field. 1989. Inhibition of acylcoenzyme A: cholesterol acyltransferase activity in CaCo-2 cells results in intracellular triglyceride accumulation. *J. Lipid Res.* **30:** 371–377.