Oxidized Fatty Acids Promote Atherosclerosis Only in the Presence of Dietary Cholesterol in Low-Density Lipoprotein Receptor Knockout Mice\textsuperscript{1,2}

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ABSTRACT Studies suggest that heated oils contribute to the presence of oxidized components in the circulating lipoproteins and to the development of atherosclerosis in animals. We evaluated the effects of 11–13 wk of consumption of a well defined dietary oxidized fatty acid, 13-hydroxylinoleic acid (13-HODE) (8 mg), on athero-sclerotic lesion development and plasma cholesterol concentrations in mice fed diets varying in fat and cholesterol contents. LDL receptor knockout mice were used in two feeding studies. In study 1, oxidized fatty acid consumption in association with a high fat diet increased aortic lesion areas by \(>100\%\) (\(P < 0.05\)). Surprisingly, oxidized fatty acid intake also tended to increase plasma total cholesterol (\(P = 0.12\)) and LDL cholesterol (\(P < 0.05\)) as well as oxidative stress as measured by higher levels of autoantibodies to oxidatively modified proteins (\(P = 0.008\)). However, in mice fed a nonpurified diet, oxidized fatty acids were not atherogenic and may even have been beneficial, as indicated by a lower plasma triglyceride (TG) concentration (\(P < 0.05\)). In study 2, mice were fed either a high fat, medium fat or low fat diet to evaluate whether the increase in aortic lesions due to oxidized fatty acid consumption in study 1 was a result of the associated higher plasma total and LDL cholesterol concentrations. In study 2, 13-HODE-treated mice in the medium and low fat diet groups but not those fed the high fat diet had larger atherosclerotic lesions (\(P < 0.05\)). Additionally, plasma total and LDL cholesterol as well as TG were not affected by HODE treatment. However, the total cholesterol:HDL cholesterol ratio was higher in treated mice (\(P < 0.05\)) and HDL cholesterol was lower in HODE-treated mice that were fed the low fat diet (\(P < 0.05\)). Our results suggest that, in mice fed cholesterol, oxidized fatty acids may be atherogenic, both in terms of increased oxidative stress (as seen in study 1) and by increasing the atherogenicity of the plasma cholesterol profile. J. Nutr. 132: 3256–3262, 2002.

KEY WORDS: \lipid hydroxide \attherosclerotic lesions \plasma cholesterol \oxidized lipoproteins

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The typical Western diet contains large quantities of PUFA that are heated or processed to varying degrees (1). This is most apparent in fast food restaurants where oil is heated in commercial fryers for up to 18 h daily, at temperatures close to 180°C. Additionally, for cost-effectiveness, this oil is reused for up to a week before it is discarded and replaced with fresh oil. Frankel et al. (2) examined the frying oils used in a number of fast food restaurants and found that the majority of the oil samples, as well as foods fried in the oil, contained oxidized lipids. Yagi et al. (3) surveyed the peroxide content of 30 types of foods and found the peroxide content to be as high as 600 nmol per gram of food.

Staprans and colleagues (4–6) suggest that oxidized lipids from dietary heated oils are secreted into the VLDL + LDL and chylomicron fractions of plasma and they display increased susceptibility to CuSO\textsubscript{4}-induced in vitro oxidation. We have previously shown through cell culture and animal studies that the intestine efficiently absorbs oxidized linoleic acid, which can subsequently be esterified by enterocytes (7).

Oxidation has been implicated in the promotion of atherosclerosis. According to the oxidation hypothesis of atherosclerosis (8), oxidized LDL play a role in the initiation of the atherosclerotic lesion, and oxidized LDL appear to affect almost every step of the atherogenic process (reviewed in Ref. 9). This suggests that dietary oxidized lipids, if incorporated into LDL, could be proatherogenic.

A number of animal studies have shown that feeding heated oils significantly increases atherosclerotic lesion development (10–13). In these studies, the source of the oxidized lipids was heated oil. Oil contains PUFA that are labile and can undergo peroxidative damage when subjected to high
temperatures in the presence of oxygen, resulting in the formation of lipid hydroperoxides. With further heating, the hydroperoxides decompose to form hydroxides and a number of decomposition products including aldehydes, ketones, hydrocarbons, acids and alcohols. Furthermore, the type of oil used, the length of time the oil is heated and the temperature to which the oil is heated are important factors that determine the composition of the products of oxidation. Therefore, the amounts and types of primary and secondary oxidation products can vary considerably, making it impossible to identify which components in the heated oils are responsible for the increased atherosclerotic development. Heated oils also contain trans fatty acids that also could have atherogenic effects. Our current feeding studies are unique in that heated oils are not used as the source of oxidized lipids. We enzymatically oxidized linoleic acid to form 13-hydroperoxylinoelic acid (13-HPODE), which is then converted into 13-hydroxylinoleic acid (13-HODE) (Fig. 1). The hydroxy form of linoleic acid, unlike the hydroperoxy form, is stable and does not decompose readily, especially when stored at low temperatures. Additionally, enzymatically oxidizing linoleic acid does not generate trans fatty acids. We can therefore control the amount and type of oxidized lipid that is fed. Linoleic acid was chosen for oxidation because it is the most common dietary PUFA. In two studies, we fed LDL r−/− mice 13-HODE along with diets containing different amounts of fat and cholesterol to examine the effects on aortic lesion development and the plasma cholesterol profile.

MATERIALS AND METHODS

Linoleic acid and soybean lipoxidase were purchased from Sigma-Aldrich (St. Louis, MO). LDL receptor knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The Emory Animal Care Committee approved all protocols and animals were treated in compliance with Emory University Animal Committee regulations. The high fat diet (TD88137), as well as the custom-made medium fat diet (TD01070) and low fat diet (TD01074), were purchased from Harland Teklad (Madison, WI). The nonpurified diet (LabDiet 5001) was obtained from Purina Mills (St. Louis, MO).

Linoleic acid oxidation formation of 13-HODE. Approximately 3.6 mmol/L of linoleic acid (3.6 mmol/L) were oxidized with 3 million units of soybean lipoxidase per gram of fatty acid (3 h at 37°C, pH 11). This resulted in the formation of oxidized linoleic acid (13-HPODE and 13-HODE), which was then extracted with ether and dried under liquid nitrogen (7,14). The resulting fatty acids were ~70% oxidized and in the form of 13-HODE, as monitored spectrophotometrically (model DB-3500; SLM-AMINCO, Urbana, IL) by scanning the absorption between 200 and 300 nm. Oxidized linoleic acid was stored in aliquots at ~80°C for daily feeding. At the time of feeding, the fatty acid was quickly thawed and fed through a 200-μL pipette tip to each mouse in the treatment group. The fatty acid was weighed daily to monitor the amount fed. Stored samples were analyzed by the leucemethethylene blue assay (15) and spectrophotometrically to confirm the presence of the HODE.

Collection of plasma, preparation of arterial samples and quantification of aortic lesion areas. Food-deprived mice were killed by CO₂ asphyxiation and blood was drawn into heparinized tubes from the inferior vena cava or by heart puncture. Red blood cells and plasma were immediately separated by centrifugation (2000 × g for 10 min at 4°C) and then frozen at −80°C. The aortic trunk was washed with cold PBS containing 10 mg/L aprotinin and 0.1 mmol/L phenylmethylsulfonyl fluoride through the left ventricle. The dissection of the aorta was performed under a stereomicroscope from the iliac bifurcation up to the heart, including the beginning of the carotid and subclavian arteries. The excess fat and connective tissue were carefully removed and the aorta was opened longitudinally and pinned up on black wax for en face observation (16). To use the aortas for future Western blot and enzyme analysis, no stains were used to visualize the lesions. After capturing different areas of the aorta, the lesions were circled on a printout under direct microscopic observation. Lesion areas were quantified using Adobe Photoshop (Adobe, San Jose, CA). Pixels were transformed to square millimeters by using a microscopic standard scale treated under the same condition as the aortas.

Cholesterol concentrations. Fast phase liquid chromatography was used to measure whole plasma total cholesterol (TC), LDL cholesterol, HDL cholesterol and triglyceride (TG) concentrations as described by Innis-Whitehouse et al. (17).

Plasma oxidation with CuSO₄ lag time. Plasma was diluted 1/150 in PBS to which 50 μL of 1 mmol/L CuSO₄ was added. Oxidation was monitored as the increased absorbance at 234 nm for 10 h at room temperature (SLM-AMINCO spectrophotometer, DB-3500) (18). PBS was used as a blank. Lag time for oxidation was calculated as the intercept of the slopes of the lag phase and the propagation phase.

Plasma apolipoprotein A1 (ApoA1). An ApoA1 kit (Sigma-Aldrich) was used to immunoturbidimetrically measure plasma ApoA1.

Preparation of lipid peroxide (LOOH)-modified rabbit serum albumin (RSA). Linoleic acid was converted to 13-HPODE by treatment with soybean lipoxidase and was immediately reacted with immunoglobulin G-free RSA and incubated at 37°C for 2 d (19).

Detection of plasma autoantibodies against LOOH-modified proteins (LOOH-RSA). Ninety-six-well ELISA plates were coated overnight at 37°C with a solution of LOOH-RSA (5 μg/well). Every step of the following method was separated by six washes with 1× PBS using a plate washer. Plates were blocked for 2 h with 30 g/L bovine serum albumin in PBS and incubated with 100 μL of 1/50 diluted plasma (1 h at 37°C). The wells were then incubated for 1 h at 37°C with 100 μL of anti-mouse immunoglobulin G-conjugated alkaline phosphatase (1/10,000 dilution), followed by the substrate (p-nitrophenyl phosphate) for 30 min. Plates were read at 405 nm and repeated measurements were made after 1 h. Results are expressed as optical density (OD) equivalents of p-nitrophenol formed.

Study 1. LDL receptor knockout male mice (n = 37; The Jackson Laboratory) between 4 and 6 wk of age were randomly divided into four groups. Blood for baseline data were collected by retro-orbital bleeding after overnight food deprivation. Two groups were fed a high fat diet (TD88137; 210 g/kg fat and 1.5g/kg cholesterol), and two were fed the nonpurified diet (LabDiet 5001; 45 g/kg fat, 0.002 g/kg cholesterol) (Table 1). Within each diet group, mice in one subgroup were individually fed 8 mg of 13-HODE 5 d/wk, for an average duration of 11 wk (range, 10.5–11.5 wk because mice were fed until the day of the aortic dissection, and it took 7 d to perform the procedure on all of the mice). The amount of 13-HODE that was fed is equivalent to 0.26 g/100 g diet and 1.3 g/100 g total fat consumption. Weekly food intakes and body weights were measured. The diets

**FIGURE 1** Chemical structures of linoleic acid (18:2), 13-hydroperoxylinoelic acid (13-HPODE) and 13-hydroxylinoleic acid (13-HODE).
were stored in tightly sealed containers away from light at 4°C and were replenished in the cages on a regular basis to prevent oxidation of the fatty acids in the diets.

Study 2. LDL receptor knockout male mice (n = 60) from 4 to 6 wk of age were randomly divided into six groups. Blood for baseline procedures were collected after overnight food deprivation by retro-orbital bleeding. Two groups composed each of three diets: high fat diet (TD88137; 210 g/kg fat and 1.5 g/kg cholesterol), medium fat diet (TD01073; 140 g/kg fat and 0.98 g/kg cholesterol) and low fat diet (TD01074; 70 g/kg fat and 0.51 g/kg cholesterol) (Table 1). The medium and low fat diets were custom-made by modifying the high fat diet. These diets varied in their fat and cholesterol contents by substituting corn oil and sucrose for milk fat. Within each diet group, mice in one subgroup were individually fed 8 mg of 13-HODE 5 d/wk for 13 wk (range, 12.5–13.5 wk for the reason given in study 1). Precautions were taken to prevent oxidation of the fatty acids in the diet as mentioned in study 1.

Statistical analysis. Data are expressed as sample mean ± SD. Outliers were defined as data points greater than ± 2 SD from the mean. Data were analyzed as 2 × 2 (study 1) and 3 × 2 (study 2) factorial designs using Statistical Analysis System. ANOVA and least-square means were calculated using the general linear model procedure. Differences with P < 0.05 were accepted as significant. When significant effects were found, post hoc comparisons of means was done using the Tukey test.

RESULTS

Study 1. Mice in the high fat diet group weighed 32.45 ± 3.2 g and those in the nonpurified diet group weighed 22.04 ± 1.6 g (P < 0.001) at the end of the study. Consumption of 13-HODE did not influence body weights. Daily food intake was not affected by diet, but in the high fat diet groups untreated mice consumed more food than those receiving 13-HODE (2.81 ± 0.17 vs. 2.38 ± 0.12 g/d; P = 0.01). In those fed the nonpurified diet, intake was lower in the controls than in the 13-HODE-treated mice (2.74 ± 0.11 vs. 2.95 ± 0.11 g/d; P = 0.04). Oxidized fatty acid feeding was not accompanied by any obvious physical distress.

Control mice that were fed the high fat diet had higher plasma TC levels than those fed the nonpurified diet (P < 0.001) (see Table 2). Consumption of 13-HODE by the high fat diet group tended to increase plasma TC levels (P = 0.12) and did increase LDL cholesterol (P < 0.05) and the TC:LDL cholesterol ratio (P < 0.05). In the nonpurified diet-fed mice, oxidized fatty acid consumption decreased plasma TG concentration (P < 0.05) but did not affect total, HDL or LDL cholesterol concentrations.

13-HODE treatment of mice fed the high fat diet increased aortic lesion area by 100% (Fig. 2, upper panel). The nonpurified diet-fed, untreated mice had no aortic lesions and 13-HODE also did not cause lesion formation (Fig. 2, lower panel).

Mice fed the high fat diet had higher titers of plasma autoantibodies to oxidatively modified proteins than the mice that were fed the nonpurified diet (0.88 ± 0.21 vs. 0.61 ± 0.24 OD; P < 0.05). In mice that were fed 13-HODE with the high fat diet, autoantibody levels were greater than in the untreated mice (1.19 ± 0.26 vs. 0.88 ± 0.21 OD; P = 0.008). Autoantibody levels did not differ between the treated and untreated mice fed the nonpurified diet.

Plasma ApoA1 levels were elevated in the high fat diet-fed groups compared with the nonpurified diet groups (1.31 ± 0.11 vs. 0.96 ± 0.04 mmol/L; P < 0.001). Administration of 13-HODE did not affect ApoA1 levels.

Study 2. Two mice in the high fat diet treatment group died during retro-orbital blood collection. Weight gain was greater (P < 0.01) in the high fat diet group than in the medium and low fat diet groups (34.3 ± 3.6, 27.9 ± 1.74 and 26.0 ± 2.6 g, respectively). Oxidized fatty acid consumption reduced body weight at the end of the study in the high (34.3 ± 3.6 vs. 30.6 ± 2.5 g; P = 0.02) and low fat diet groups (26.0 ± 0.17 vs. 22.38 ± 0.12 g; P = 0.04).
Lesion areas are mean ± SD (n = 7–10). Means without a common letter differ (P < 0.05).

**TABLE 2**

Plasma lipids in LDL r^{-/-} mice fed a high fat or nonpurified diet, with or without 13-HODE treatment (study 1)^1

<table>
<thead>
<tr>
<th>Diet</th>
<th>13-HODE</th>
<th>TC (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>TC:HDL-C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>High fat</td>
<td>-</td>
<td>11.97 ± 2.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.65 ± 2.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.86 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.32 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.15 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14.20 ± 2.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.90 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.90 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.01 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.89 ± 0.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonpurified</td>
<td>-</td>
<td>2.99 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.64 ± 0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.02 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.02 ± 0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.93 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.90 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.76 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.05 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.55 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**P-Value**^2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13-HODE</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diet × 13-HODE</td>
<td>NS</td>
</tr>
</tbody>
</table>

1 Values are means ± so (n = 7–10). Means in a column without a common letter differ (P < 0.05).
2 P-Values from two-way ANOVA. NS, P = 0.05.

± 2.6 vs. 23.5 ± 2.1 g; P = 0.03) but not in the medium fat group. Daily food intake was less (P < 0.05) in the medium fat diet-fed group (2.22 ± 0.45 g/d) than in the high fat (2.51 ± 0.45 g/d) and low fat (2.40 ± 0.42 g/d) diet groups. There were no differences in food intake between the controls and 13-HODEx-treated mice within each diet group.

Plasma total and LDL cholesterol were not affected by dietary fat level or HODE treatment (Table 3). The plasma TG concentration was higher in the mice fed the high fat diet than in mice fed the other two levels of fat but was not affected by HODE. HDL cholesterol was lower in HODE-treated mice fed the low fat diet than in all other groups, and the TC:HDL cholesterol ratio was greater because of HODE treatment.

Untreated mice fed the low fat diet had lower atherosclerotic lesion areas than the high fat diet and medium fat diet group controls (Fig. 3). Oxidized fatty acid consumption by the high fat diet-fed mice did not affect lesion area. However, in the medium fat and the low fat diet groups, 13-HODE consumption increased lesion area (P < 0.05).

Within the high fat diet-fed groups, untreated mice tended to have longer plasma oxidation lag times than the 13-HODE-fed mice (333.7 ± 98.4 vs. 224.7 ± 135.9 min; P = 0.12), suggesting increased plasma and lipoprotein oxidizability with oxidized fatty acid feeding. However, there was no effect on plasma oxidizability with 13-HODE consumption in the medium fat and low fat diet groups.

There were no differences among groups in plasma autoantibodies to oxidatively modified proteins and plasma ApoA1 concentrations.

**DISCUSSION**

Study 1 suggests that oxidized fatty acids are atherogenic when consumed with a diet that contains high amounts of fat and cholesterol. This was shown by the increased aortic lesion development that accompanied a more atherogenic cholesterol profile. However, in mice fed the nonpurified diet that is extremely low in fat and lacking cholesterol, oxidized fatty acids did not increase lesion development and plasma cholesterol levels, suggesting that dietary cholesterol must be present for 13-HODE to have proatherogenic effects. Additionally, oxidized fatty acid intake in the nonpurified diet-fed mice lowered plasma TG levels, which may be beneficial in reducing the risk of diabetes and heart disease. Lowering of TG levels has also been shown in previous studies with heated oil feeding (20,21). The mechanism by which oxidized fatty acids may reduce plasma TG levels is still unclear, and we speculate...
that this may result from changes in endogenous cholesterol metabolism and VLDL secretion. Additionally, studies have suggested that moderate oxidative stress from LOOH can also be beneficial by inducing cellular antioxidant responses such as catalase (22), heme oxygenase (23), nitric oxide synthase (24), manganese superoxide dismutase (25) and glutathione (26).

We speculate that the proatherogenic effects of oxidized fatty acids in the high fat diet group may arise by two mechanisms. First, dietary oxidized fatty acids may directly or indirectly increase oxidative stress and the presence of oxidized LDL and other lipoproteins in the plasma and arterial walls, thereby initiating fatty streak formation. It has been previously shown that there is a 10-fold increase in the uptake of chylomicrons by macrophages after consumption of heated oil (27). Also, free radical scavenging activity is depleted with ingestion of oxidized lipids, as seen by lower serum and tissue α-tocopherol levels (28). This increased oxidative stress is supported by the raised titer of autoantibodies to oxidatively modified proteins in the mice that consumed oxidized fatty acids with the high fat diet in study 1.

The second possible mechanism for the action of oxidized fatty acids is based on the observation that 13-HODE shares structural similarities with the monohydroxy bile acid, lithocholic acid. Bile acids are essential for the absorption of cholesterol in the intestinal lumen. Our in vitro data (29) suggest that dietary oxidized fatty acids may act as bile salt enhancers and thereby increase the solubilization and absorption of dietary cholesterol, leading to higher plasma cholesterol levels. The increased plasma cholesterol levels may be a mechanism by which oxidized fatty acids are atherogenic, because hypercholesterolemia has been shown to be a risk factor for atherosclerosis (30–32). This hypothesis is supported by our observation in study 1 that, in the absence of dietary cholesterol (nonpurified diet group), oxidized fatty acids did not induce atherogenic changes such as increased oxidative stress and plasma cholesterol levels. Additionally, the oxidized fatty acid-fed mice in the high fat diet group in study 1 had plasma LDL cholesterol levels and TC:HDLC cholesterol ratios that were higher than those in the untreated mice.

We designed study 2 to test the cholesterol solubilization hypothesis by modifying dietary fat and cholesterol content. We thereby hoped to compare lesion development in mice that were fed oxidized fatty acids with untreated mice, all of whom had similar plasma cholesterol levels. We designed medium and low fat diets that were similar in energy but with two thirds and one third of the fat and cholesterol levels of the high fat diet. The low fat diet still contained higher amounts of fat and cholesterol than the nonpurified diet that was used in study 1 (Table 1).

The varying amounts of fat and cholesterol in the diets in study 2 did not affect plasma total or LDL cholesterol levels. This suggests that, for these LDL receptor knockout mice, dietary cholesterol, even in low amounts as seen in the low fat diet, increases plasma cholesterol levels compared with the nonpurified diet that contains no cholesterol.

Study 2 demonstrated that oxidized fatty acids can increase aortic lesion development independent of an increase in plasma cholesterol as seen in the mice that were fed the medium and low fat diets. In these two groups, oxidized fatty acid consumption increased atherosclerotic lesion development without changes in plasma cholesterol levels. However, study 2 did not replicate the 100% increase in lesion development in 13-HODE-fed versus untreated mice in the high fat diet group in study 1. This may have been due to

### Table 3

**Plasma lipid concentration in LDL r−/− mice fed a high fat diet, medium fat diet or low fat diet, with or without 13-HODE treatment (study 2)**

<table>
<thead>
<tr>
<th>Diet</th>
<th>13-HODE</th>
<th>TC</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>TG</th>
<th>TC: HDL-C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>High fat</td>
<td>−</td>
<td>12.31 ± 3.24</td>
<td>8.78 ± 2.43</td>
<td>3.05 ± 0.87a</td>
<td>2.40 ± 1.11a</td>
<td>4.10 ± 0.5b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16.44 ± 6.06</td>
<td>12.86 ± 5.44</td>
<td>3.25 ± 0.69a</td>
<td>2.05 ± 0.50a</td>
<td>5.10 ± 1.0a</td>
</tr>
<tr>
<td>Medium fat</td>
<td>−</td>
<td>13.40 ± 2.80</td>
<td>10.22 ± 2.40</td>
<td>2.86 ± 0.39a</td>
<td>1.60 ± 0.38b</td>
<td>4.64 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14.17 ± 3.57</td>
<td>10.94 ± 3.40</td>
<td>2.88 ± 0.38a</td>
<td>1.72 ± 0.74b</td>
<td>4.93 ± 1.1b</td>
</tr>
<tr>
<td>Low fat</td>
<td>−</td>
<td>11.61 ± 3.41</td>
<td>8.56 ± 2.83</td>
<td>2.72 ± 0.60a</td>
<td>1.64 ± 0.53b</td>
<td>4.24 ± 0.6b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>11.98 ± 5.97</td>
<td>9.24 ± 5.56</td>
<td>2.43 ± 0.54b</td>
<td>1.54 ± 0.63b</td>
<td>4.74 ± 1.7b</td>
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**P-Value**

<table>
<thead>
<tr>
<th>Diet</th>
<th>13-HODE</th>
<th>TC</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>TG</th>
<th>TC: HDL-C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.03</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Diet × 13-HODE</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1. Values are means ± SD (n = 8–10). Means in a column without a common letter differ (P < 0.05).
2. P-Values from two-way ANOVA. NS, P ≥ 0.05.

### Figure 3

Aortic lesion areas in LDL r−/− mice fed the high fat, medium fat and low fat diets with or without 13-hydroxylinoleic acid (13-HODE) for 13 wk (study 2). Lesion areas are represented as mean ± SD (n = 8–10). Means without a common letter differ (P < 0.05). Two-way ANOVA was performed: diet P < 0.0001; 13-HODE, P ≥ 0.05. Diet × 13-HODE P ≥ 0.05.
the difference in the study design, whereby the study duration was 11 wk in study 1 and 13 wk in study 2. It is possible that the extra 2 wk of consuming the high fat diet allowed the untreated mice to “catch up” to the 13-HODE-treated mice. Additionally, the treated mice may have reached a level in lesion development beyond which the rate of lesion development slows down. This is supported by the greater mean lesion size in study 2 in the high fat diet group (3.76 mm²) compared with the same group in study 1 (2.55 mm²).

Some of the ingested dietary hydroperoxides are converted into hydroxides in the intestinal lumen (33,34). We administered the hydroxide form of linoleic acid to the mice. Hydroxides are not as capable of inducing oxidative damage as hydroperoxides, thereby suggesting that the effects are not a result of direct oxidation. Fatty acid hydroxides could undergo peroxisomal β-oxidation, resulting in the formation of hydrogen peroxide, which could promote oxidative stress within the cells. This suggests that dietary oxidized lipids may not themselves be present in lipoproteins. Rather, they may initiate secondary oxidation, which is then responsible for increased oxidative stress and the formation and presence of peroxides in lipoproteins, resulting in atherosclerosis. We measured the plasma peroxide concentrations of these mice to determine whether oxidized fatty acid consumption increases plasma peroxide levels. The plasma peroxide levels were extremely low and were not different in the untreated and 13-HODE-treated groups (data not shown).

The oxidized lipid used in this study was 13-HODE (S)-isomer. In contrast, heated oils that are exposed to air generate a mixture of oxidized lipids and their various isomers. Whether different isomers of the different types of oxidized lipids would have atherogenic effects cannot be answered unless they are all tested. However, at present there are no data to suggest that they would vary in their atherogenic properties.

Our method for oxidizing of linoleic acid yielded ∼70% oxidized fatty acids, resulting in 30% or 2.4 mg per mouse per day of the linoleic acid in the oxidized form. We speculate that the effects in our studies were a result of the oxidized fatty acids. It is highly unlikely that the unoxidized linoleic acid that accompanied the 13-HODE feeding could be responsible for the atherogenic effects in the treated mice, because this was a relatively small portion of the 7–15 mg of linoleic acid consumed daily by the untreated mice fed the various diets. Additionally, dietary linoleic acid has been suggested to be antiatherogenic because of its cholesterol-lowering properties (35).

Our study used a total of four diets, which differed not only in fat and cholesterol content but also in the vitamin and mineral contents. However, to determine the effects of 13-HODE, comparisons were made between treated and untreated mice within each dietary group, thus eliminating the need to equilibrate the diets. Additionally, in our study the mice were given oxidized fatty acids by gavage during the day, whereas the food was mostly consumed at night. We therefore speculate that the antioxidants in the diet did not affect the oxidized lipid treatments. Despite the high levels of antioxidants in the high fat, medium fat and low fat diets, oxidized fatty acid consumption with these diets resulted in proatherogenic changes such as higher cholesterol levels and/or increased aortic lesion development.

Thompson and Aust (36) showed that >15% of PUFA become oxidized in the process of preparing french-fried potatoes. Therefore, a medium serving of french fries, which contains 15 g of fat, could contain 2.3 g of oxidized fatty acids. We fed 8 mg of 13-HODE to the mice daily, which in humans is comparable with consumption of less than half of a medium serving of french fries daily. This level of oxidized fatty acid consumption is quite reasonable considering the Western diet that is commonly consumed.

The public health implications of this study, as well as studies that use heated oils, are quite strong. Fried foods are the predominant source of oxidized fatty acids, and at the present time there are no specific recommendations regarding the intake of oxidized fatty acids. Our study suggests that even small quantities of oxidized linoleic acid promotes an atherogenic lipoprotein profile and atherosclerosis in the presence of a cholesterol-rich diet. People with higher dietary fat and cholesterol intakes usually consume more fried foods. This combination of dietary cholesterol and oxidized fatty acids may be a dangerous one, leading to atherosclerosis. Additionally, fried foods contain trans fatty acids, which themselves pose atherogenic risks. Limiting the intake of oxidized PUFA, together with lower cholesterol intake, could be beneficial for those at high risk for cardiovascular disease. Additionally, increasing monounsaturated fatty acid intake may be recommended, because these fatty acids are not as likely to be oxidized.

**LITERATURE CITED**


