Short-Term Feeding of Atherogenic Diet to Mice Results in Reduction of HDL and Paraoxonase That May Be Mediated by an Immune Mechanism

Catherine C. Hedrick, Kholood Hassan, Greg P. Hough, JiHyung Yoo, Soheil Simzar, Cindy R. Quinto, Sul-Min Kim, Alek Dooley, Sesilia Langi, Susan Y. Hama, Mohamad Navab, Joseph L. Witztum, Alan M. Fogelman

Abstract—Short-term feeding (up to 7 days) of an atherogenic diet to C57BL/6 low density lipoprotein receptor–deficient mice did not result in decreased hepatic paraoxonase (PON) mRNA but caused a dramatic decrease in plasma PON activity and mass. The decreased activity and mass were temporally related to an increase in plasma and high density lipoprotein (HDL) lipid hydroperoxides and to a decrease in HDL cholesterol and native apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II). After mice consumed an atherogenic diet for 1 or 3 days, switching the mice to a low fat chow diet for 3 days resulted in a return to baseline levels of lipid hydroperoxides but only a small return toward baseline for HDL cholesterol, with no significant increase in apoA-I mass or PON activity and mass. After mice consumed an atherogenic diet for 3 days, switching to the chow diet for 3 days did not significantly alter the high molecular weight forms of apoA-I or the signal generated by EO6. In marked contrast, after mice consumed an atherogenic diet for 7 days, switching to the chow diet for 3 days resulted in a dramatic increase in native apoA-I to baseline levels, with virtual disappearance of the high molecular weight forms of apoA-I, including the form recognized by EO6. After mice consumed an atherogenic diet for 7 days, switching to the chow diet for 3 days also resulted in significant increases in HDL cholesterol and PON activity and mass, although baseline levels were not reached. IgG and IgM antibodies were found to be associated with apoA-I in control animals, were minimally decreased after the 3-day atherogenic diet, were dramatically decreased after the 7-day atherogenic diet, and returned to near or above baseline levels after a return to the chow diet for 3 days. We conclude that the atherogenic diet rapidly induces lipid hydroperoxide formation and apoA-I oxidation with the formation of high molecular weight forms of apoA-I. Concomitant with these changes in apoA-I levels, HDL cholesterol and PON activity and mass declined without changes in mRNA levels for apoA-I or PON, suggesting increased clearance of these altered HDL particles. We further conclude that between the third and seventh day of the atherogenic diet, an as-yet-unidentified mechanism for clearing the high molecular weight forms of apoA-I is induced and that this mechanism may be related to the clearance of immune complexes. (Arterioscler Thromb Vasc Biol. 2000;20:1946-1952.)

Key Words: diet ■ paraoxonase ■ HDL ■ apolipoproteins

We previously reported that feeding an atherogenic diet to mice (C57BL/6J) that were genetically susceptible to atherosclerosis resulted in a decrease in hepatic paraoxonase (PON) mRNA levels after 5 weeks on the diet.1 Mice that were genetically resistant to atherosclerosis (C3H/HeJ) did not show a decrease in hepatic PON mRNA levels. Recombinant inbred strains of mice that were created by crossbreeding the C57BL/6J and C3H/HeJ parental strains for generations showed a heterogeneous response in hepatic PON mRNA levels on being fed the atherogenic diet.1 On analysis, there was a significant inverse correlation between the level of hepatic PON mRNA in these new recombinant inbred strains placed on an atherogenic diet and in the development of aortic atherosclerotic lesions.1 Thus, these data demonstrate a genetically determined inverse relationship between the dietary responsiveness of hepatic PON mRNA and atherosclerosis.

In another study,2 we demonstrated that the injection of mildly oxidized LDL or oxidized 1-α-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (Ox-PAPC) into the atherosclerosis-sensitive C57BL/6J mice significantly reduced PON activity within 24 hours. In contrast, injection
of mildly oxidized LDL or Ox-PAPC into the atherosclerosis-resistant C3H/HeJ mice did not cause a decrease in PON activity under these conditions.  

Previously, we also demonstrated that in rabbits and humans PON activity changed dramatically over the course of a few days in response to the induction of an acute phase reaction. Because studies in mice and humans have suggested that PON may be important in determining susceptibility to atherosclerosis, we performed studies to determine whether short-term dietary interventions could alter PON activity. To accentuate the effects of the diet and to determine the mechanisms involved, we chose to feed an atherogenic diet to LDL receptor–deficient mice with an atherosclerosis-susceptible C57BL/6J genetic background. As reported in the present study, periods of feeding the atherogenic diet for as little as 3 days resulted in profound changes in plasma lipid hydroperoxides, HDL cholesterol, apoA-I, apoA-II, and PON levels.

Methods

Materials

The atherogenic diet (TD 90221) and the rodent chow containing 0.5% sodium cholate (TD 99057) were purchased from Teklad, and the rodent chow diet (Purina Rodent Chow) was from Ralston-Purina Co. Trizol reagent was from BRL Life Technologies, Inc. Taqman reverse transcription (RT)–polymerase chain reaction (PCR) kits and rodent GAPDH primers and probe were from Perkin-Elmer, Inc. Other Taqman primers and probes were from IDT Technologies. NuPage polyacrylamide gels were purchased from Novex, Inc. Cholesterol-20 and a cholesterol calibrator were from Sigma Chemical Co. Enhanced chemiluminescence reagents were from Amer sham Pharmacia, Inc. Monospecific polyclonal antibodies to mouse apoA-I, mouse apoA-II, and mouse PON were a kind gift from Dr Aldons J. Lusis (University of California, Los Angeles).

Mice

LDL receptor knockout mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Me). All animals were female and aged 4 to 6 months at the time of the experiments. The atherogenic diet contained 15.75% fat, 1.25% cholesterol, and 0.5% sodium cholate. The rodent chow diets contained 4% fat in the absence or presence of 0.5% sodium cholate. The protocol used to test the diets entailed feeding the mice the atherogenic diet for 1, 3, or 7 days, followed by the chow diet for 3 days. Mice fed the atherogenic diet for 1, 3, or 7 days were identified as groups 1.0, 3.0, and 7.0, respectively. Mice fed the atherogenic diet for 1, 3, or 7 days, followed in each case by 3 days on the rodent chow diet, were identified as groups 1.3, 3.3, and 7.3, respectively. Groups of mice were also maintained for 7 days on either the rodent chow diet or the rodent chow diet containing 0.5% sodium cholate to serve as controls.

Lipoproteins

With the mice under mild isoflurane anesthesia, blood was obtained from the retro-orbital sinus, with heparin used as an anticoagulant. Plasma lipoproteins were separated by fast performance liquid chromatography (FPLC) with the use of methods previously described. HDL cholesterol levels in the plasma of mice were determined after heparin and manganese chloride precipitation of VLDL and LDL. The concentration of lipoproteins reported in the present study is based on cholesterol content, unless otherwise specified.

Determination of mRNA Levels

Total cellular RNA was isolated from tissue with the use of Trizol reagent according to the manufacturer’s protocol. Levels of mRNA abundance were determined by Taqman real-time RT-PCR as described previously. The RT-PCR reaction was as follows: 50°C for 2 minutes and 60°C for 30 minutes, followed by 40 cycles of 95°C for 5 minutes, 94°C for 20 seconds, and 62°C for 1 minute. The mRNAs of interest were normalized to GAPDH mRNA with the use of rodent GAPDH Taqman primers and probe (Perkin Elmer, Inc). The sequences used for detection of murine apoA-I mRNA were as follows: sense 5'-tccaggggcttctcag-3', antisense 5'-ggcagatgattatccc-3', and the Taqman hydrolysis probe 5'-6FAM-ccctcgttgaattgatttcgca-TAMRA-3'. The sequences used for detection of murine PON mRNA were as follows: sense 5'-tcgctgggatgcc-3', antisense 5'-ctcgccggctgactt-3', and the Taqman hydrolysis probe 5'-6FAM-accttcctgaccaaaagttc-TAMRA-3'. The sequences used for detection of murine SR-B1 mRNA were as follows: sense 5'-gggctctcgggagatg-3', antisense 5'-cctgctgggatgcc-3', and the Taqman hydrolysis probe 5'-6FAM-accttcctgaccaaaagttc-TAMRA-3'. The sequences used for detection of murine cubilin mRNA were as follows: sense 5'-tggtctgctaatggagg-3', antisense 5'-gtcggcaggctgtagta-3', and the Taqman hydrolysis probe 5'-6FAM-cacgagggctctgcatc-TAMRA-3'.

SDS-PAGE and Western Blotting

Polyacrylamide gel electrophoresis in SDS was performed as previously described. FPLC-isolated HDLs (25 μg of total cholesterol) from each group of mice were run on 2% to 16% NuPage gels (NoveX, Inc) with the use of MOPS buffer at 125 V for 45 minutes according to the manufacturer’s protocol. The proteins were then transferred to nitrocellulose at 200 V for 2 hours with the use of a Novex transfer apparatus. Protein bands were visualized by a chemiluminescence procedure and quantified by densitometry (ZeroScan, Stratagene). The resulting signals were shown to be in the linear portion of the response curve with the use of apolipoprotein standards run in parallel at various dilutions.

Antibodies

Monospecific polyclonal antisera to mouse apoA-I, mouse apoA-II, and mouse PON were used. Quantitative estimates of the levels of the apolipoproteins were performed as previously described by using apolipoproteins isolated from mouse lipoproteins.

Immunoassays

Autoantibody titers against malondialdehyde-modified LDL, an important epitope of oxidized LDL, were determined by ELISA as previously described, except that a highly sensitive chemiluminescent detection system was used. In brief, malondialdehyde-modified LDL in 50 mmol/L Tris-buffered saline (TBS) at 5 μg/mL was plated in flat-bottomed high-binding Microfluor microtiter plates (Dynex Technologies, Inc) and incubated overnight at 4°C. The plates were then washed with PBS containing 0.27 mmol/L EDTA, 0.02% NaN3, 0.05% Tween 20, and 0.001% aprotinin, with use of a microtiter plate washer. Fifty microliters of a 1:400 dilution of plasma in TBS/5% BSA was incubated with each well for 1 hour at room temperature. After 3 additional washes, the amount of bound immunoglobulin was detected by the addition of alkaline phosphatase–linked secondary antibodies against murine IgG or IgM, which were then detected by chemiluminescence techniques. To measure the amount of plasma immunoglobulin specifically associated with HDL (ie, apoA-I), 5 μg/mL of an affinity-purified goat anti-mouse IgG (or anti-mouse IgM) was used to coat the bottom of microtiter wells. Varying dilutions of murine plasma in 1% BSA/TBS were then incubated in the wells for 90 minutes at room temperature. After 3 independent wash steps with an automated plate washer, a 1:10 000 dilution of a rabbit anti-mouse A-I antibody was added for a 1-hour incubation at room temperature. After 4 additional washes with TBS, the amount of rabbit IgG (or IgM) bound was detected by use of the alkaline phosphatase–linked secondary antibody as described above. In similar assays, we sought to detect the amount of apoA-I bound on particles containing EO6 epitopes. Microtiter wells were coated with 5 μg/mL of purified EO6 and incubated with varying dilutions of plasma, and then the amount of apoA-I bound was detected by use of the rabbit anti-mouse A-I antibody as described above. For each of these assays, the
Results

Plasma Lipid Hydroperoxide Levels in Mice on the Atherogenic Diet

As shown in Figure 1, plasma lipid hydroperoxide levels increased after 1 day on the atherogenic diet and continued to increase to almost 10-fold above baseline by 7 days on the diet. In each instance (ie, after 1, 3, or 7 days), the atherogenic diet to a chow diet for 3 days resulted in a return to or below baseline in plasma lipid hydroperoxide levels. Adding 0.5% cholate to the rodent chow diet for 7 days did not significantly change plasma lipid hydroperoxide levels.

The maximum levels of lipid hydroperoxides in LDL differed in that they were seen after day 3 of the atherogenic diet and actually declined by day 7 (data not shown). The lipid hydroperoxide levels in HDL paralleled the data in Figure 1 for plasma, with dramatic elevations in lipid hydroperoxide levels in HDL after the 7-day atherogenic diet (data not shown). The majority of plasma lipid hydroperoxides were associated with HDL in all instances (data not shown).

PON Activity in Mice on the Atherogenic Diet

PON activity in serum declined slightly \((P<0.01)\) when 0.5% cholate was added to the rodent chow diet for 7 days (Figure 2). However, there was a progressive and dramatic decline in PON activity after days 1, 3, and 7 of the atherogenic diet. At 7 days, PON activity was only \(\approx 25\%\) of control values (Figure 2). Changing the atherogenic diet to chow for 3 days did not significantly increase HDL cholesterol levels if the atherogenic diet had been fed for 1 day. However, after the mice were fed the atherogenic diet for 3 or 7 days and then switched to the chow diet for 3 days, there was a significant increase in HDL cholesterol levels, although the levels of HDL cholesterol remained substantially below the levels on the chow or chow plus cholate diets (Figure 3).

FPLC profiles of lipoprotein cholesterol confirmed the data in Figure 3 and further demonstrated the increase in VLDL.
A dramatic decrease in native apoA-I in HDL and a dramatic
increase in high molecular weight proteins in HDL that were
recognized by a mouse apoA-I antibody in a Western blot.
Changing to a chow diet for 3 days after the 3-day atherogenic
diet did not result in a significant change in these high
molecular weight proteins in HDL that were recognized by an
antibody to apoA-I (Figure 5). Despite the decrease in these
high molecular weight proteins in HDL after the 7-day atherogenic
diet, there was no return of native apoA-I (Figure 5).

Oxidized Phospholipid Epitopes Appear in HDL
After 3-Day Atherogenic Diet
To look for the presence of oxidized phospholipids in HDL,
we used the monoclonal antibody E06. This antibody has
been well characterized and recognizes oxidized phospholipids
that have been shown to play a role in atherosclerosis.15,16,24
The E06 antibody recognized an epitope in HDL that mildly increased after the 3-day atherogenic diet (data
not shown). Interestingly, after switching to the chow diet for 3
days after the 3-day atherogenic diet, the signal for this
epitope showed little change (data not shown). After the
7-day atherogenic diet, the signal for the E06 epitope in HDL
was maximal (data not shown). On changing to the chow diet
for 3 days after the 7-day atherogenic diet, the signal for the
E06 epitope dramatically declined (data not shown).

Search for Mechanisms for Clearance of HDL
Because scavenger receptor B1 (SR-B1) has been implicated
in the removal of cholesteryl esters from HDL,25–27 we
determined hepatic SR-B1 protein levels. After the 7-day
atherogenic diet with or without a further 3 days of chow
diet, there was no significant change in SR-B1 protein levels (data
not shown).

Recent publications28,29 have reported that disruption of the
gene for the ATP-binding cassette transporter-1 (abc-1)
reproduced the manifestations of Tangier disease. Alterations
of this gene in patients with Tangier disease were also seen. It has been concluded that abc-1 is critical for the export of cholesterol from cells to nascent HDL and that its absence results in low levels of HDL cholesterol. The low levels of HDL cholesterol are presumed to occur because of decreased clearance of the nascent HDL compared with mature HDL. A chow diet supplemented with 0.5% cholate caused a small but significant increase in hepatic mRNA levels for abc-1 (data not shown). This increase was comparable to the small increase seen after a 1-day atherogenic diet (data not shown). The 3- and 7-day atherogenic diets progressively increased hepatic mRNA levels for abc-1, and changing to the chow diet for 3 days after the 3- or 7-day atherogenic diet resulted in a significant decline in abc-1 hepatic mRNA levels but not to baseline levels (data not shown).

Cubilin, the intrinsic factor–vitamin B₁₂ receptor, is expressed in the terminal ileum of the intestine and in the kidney. Cubilin has been shown to be a high-affinity apoA-I and HDL receptor, which facilitates the endocytosis of HDL. We examined whether the expression of cubilin in the kidney was affected by the atherogenic diet. Expression of mRNA for cubilin in the kidney was not changed after the 7-day atherogenic diet (data not shown).

Autoantibodies to oxidized lipids have been found in a variety of species. Because dramatic changes in the levels of apoA-I and the apoA-I–associated enzyme PON were detected between days 3 and 7 of the atherogenic diet, we reasoned that an immune complex mechanism might account for some of the observed changes. First, we measured the titer of autoantibodies to a general epitope of oxidized LDL, malondialdehyde-modified LDL, in the plasma of control mice, mice fed the atherogenic diet for 3 or 7 days, and mice fed the atherogenic diet for 3 or 7 days and then switched to the chow diet for 3 days. The titers of the autoantibodies were similar in all groups (data not shown). Next, we determined the presence of specific apoA-I–IgG and apoA-I–IgM complexes at the various time points. As shown in Figure 6, these complexes were found in the control animals on a chow diet. After the 3-day atherogenic diet, there was a small increase in the level of these complexes, which returned to above baseline on switching to the chow diet for 3 days. After the 7-day atherogenic diet, there was a dramatic decrease in the level of these complexes, which returned toward baseline after switching to the chow diet for 3 days. Similar results were seen with apoA-I–EO6 complexes. Figure 6A shows the detection of apoA-I complexes with immunoglobulins and oxidized phospholipids (EO6). The amount of plasma IgG (A) or IgM (B) or EO6 epitopes (C) specifically associated with apoA-I was determined in LDL receptor–negative mice as described in Methods. RLU indicates relative light units; control, mice fed a chow diet for 7 days. Data are mean±SD of triplicate determinations. *Statistically significant differences by ANOVA compared with control (P<0.03).

Discussion

The data in the present study indicate that short-term feeding of an atherogenic diet to LDL-receptor–deficient mice with an atherosclerosis-susceptible genetic background (C57BL/6J) results in dramatic changes in lipid hydroperoxides, HDL cholesterol, apoA-I, apo-A-II, PON protein, and PON activity. Dueland et al have reported that cholate is required for the decline in HDL cholesterol levels that have been seen in susceptible mice fed the atherogenic diet used in the present study. The studies of Dueland et al, like those previously reported by this laboratory, were carried out for months. Our data demonstrate that the cholate component of the atherogenic diet had only minimal effects on HDL cholesterol levels in the short-term feeding studies that we performed and, thus, could not account for the dramatic changes in HDL in these studies.

These acute changes induced by the atherogenic diet include a virtual disappearance of HDL cholesterol (Figure 3), HDL apoA-I (Figure 4B), HDL apo-A-II (data not shown), HDL PON activity (Figure 2), and PON protein (Figure 4).
after the 7-day atherogenic diet. The return of these parameters after switching to a chow diet for 3 days was minimal after the 3-day atherogenic diet. In contrast, there was a much more robust return toward normal after changing to the chow diet for 3 days after the 7-day atherogenic diet.

After the 3-day chow diet that followed the 3-day atherogenic diet, there was virtually no decline in the high molecular weight proteins in HDL that were recognized by antibody to apoA-I (Figure 5). In contrast, lipid hydroperoxide levels, which progressively increased with the atherogenic diet from day 1 to day 7, returned to baseline levels after switching to the chow diet for 3 days at each time point studied (Figure 1). By day 7 of the atherogenic diet, there was a dramatic decrease in the high molecular weight proteins in HDL recognized by the antibody to apoA-I (Figure 5), and there was an increase in the EO6 signal (data not shown) with no return of native apoA-I (Figure 5). On changing to the chow diet for 3 days after the 7-day atherogenic diet, there was a dramatic decrease in the high molecular weight proteins in HDL recognized by the antibody to apoA-I (Figure 5) and in the signal for EO6 (data not shown) and a dramatic return of native apoA-I (Figure 5).

Despite the dramatic changes in apoA-I protein (Figures 4 and 5), PON activity (Figure 2), and protein (Figure 4), there was no change in hepatic mRNA for apoA-I or PON (data not shown).

These data suggest that between days 3 and 7 of the atherogenic diet, a mechanism was induced for clearing the abnormal high molecular weight proteins in HDL recognized by the antibody to apoA-I, including the proteins containing the oxidized phospholipid epitope recognized by the EO6 antibody. The abc-1 transporter has been demonstrated to be induced with cholesterol loading and reduced with cholesterol removal by apolipoproteins.30–32,37,38 Because hepatic mRNA levels for the abc-1 transporter were progressively induced from day 1 to 7 of the atherogenic diet and because these mRNA levels fell only modestly on switching to the chow diet, we suspect that the abc-1 protein does not account for the observed changes. We suspect that abc-1 mRNA levels were induced to compensate for the increased cellular cholesterol that must have resulted from the atherogenic diet.

The failure of SR-B1 protein levels to change suggests that this pathway is not the mechanism for the clearance of the abnormal HDL proteins. SR-B1 has been shown to remove abnormal HDL proteins. SR-B1 has been shown to remove abnormal HDL proteins. SR-B1 has been shown to remove abnormal HDL proteins. SR-B1 has been shown to remove abnormal HDL proteins.

These data suggest that in the LDL receptor–negative mice, there are always detectable levels of oxidized lipids in HDL and that these are associated with autoantibodies complexed to the modified HDL. In response to the atherogenic diet, there is an increase in oxidized lipids per HDL particle, which, in turn, may lead to an increased number of antibodies per particle. When some threshold is exceeded, this could lead to enhanced plasma clearance of the immune complexes. In turn, this could cause decreased absolute plasma levels of HDL cholesterol, apoA-I, apoA-II, and the associated oxidized lipids detected by EO6 as well as decreased levels of apoA-I–associated IgG, IgM, and EO6 epitopes (Figure 6). This scenario is very similar to that previously demonstrated by studying the turnover and tissue sites of degradation of nonenzymatically glyicated LDL in normal and immunized rabbits.39 In rabbits fed normal chow, the plasma clearance of glyicated LDL was retarded in proportion to the extent of lysine derivatization.39 In contrast, in animals immunized with the glyicated LDL, the clearance of the glyicated LDL was greatly accelerated.39 For example, when only 5% of the lysine residues were derivatized, glyicated LDL clearance was retarded by 20% when injected into a control nonimmunized rabbit, but when the same glylated LDL was injected into the immunized animals, there was an acceleration of 2- to 3-fold in its clearance.39 However, when 10% of the lysine residues were derivatized, the injected glycated LDL was cleared from the plasma of the immunized animals 50- to 100-fold faster than control.39 In the present study, switching back to the chow diet led to a rapid decrease in the content of lipid hydroperoxides per HDL particle and, presumably, a decrease in the number of antibodies bound per particle, with a net result that the rapid immune-mediated clearance was decreased with a consequent net increase in the apoA-I–IgG and apoA-I–IgM complexes found in the plasma (Figure 6). Further elucidation of the details of the mechanism of clearance of HDL and its associated proteins after a short-term atherogenic diet will likely yield new insights into lipoprotein metabolism.

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References


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