Altered activities of anti-atherogenic enzymes LCAT, paraoxonase, and platelet-activating factor acetylhydrolase in atherosclerosis-susceptible mice

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Abstract **We examined whether the putative anti-atherogenic enzymes LCAT, paraoxonase (PON), and platelet-activating factor acetylhydrolase (PAF-AH) are impaired in 8 week old atherosclerosis susceptible apolipoprotein E (apoE)$^{-/-}$ and LDL receptor (LDLr)$^{-/-}$ mice and whether plasma concentrations of bioactive oxidized phospholipids accumulate in plasma. ApoE$^{-/-}$ mice had reduced (28%) LCAT activity and elevated lysophosphatidylcholine and bioactive oxidized phospholipids (1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine) compared with controls on the chow diet. Elevated oxidized phospholipids and reduced LCAT activity may, in part, contribute to spontaneous lesions in these mice on a chow diet. A Western diet decreased LCAT activity further (50% of controls) and PON activity was decreased 38%. The LDLr$^{-/-}$ mice showed normal LCAT activity on chow diet and little accumulation of oxidized phospholipids. On a Western diet, LDLr$^{-/-}$ mice had reduced LCAT activity (21%), but no change in PON activity. All genotypes had reduced PAF-AH activity on the Western diet. ApoE$^{-/-}$ and LDLr$^{-/-}$ mice, but not controls, had elevated plasma bioactive oxidized phospholipids on the Western diet. We conclude that impairment of LCAT activity and accumulation of oxidized phospholipids are part of an early atherogenic phenotype in these models.**—Forte, T. M., G. Subbanagounder, J. A. Berliner, P. J. Blanche, A. O. Clermont, Z. Jia, M. N. Oda, R. M. Krauss, and J. K. Bielicki. **Altered activities of anti-atherogenic enzymes LCAT, paraoxonase, and platelet-activating factor acetylhydrolase in atherosclerosis-susceptible mice.** ***J. Lipid Res.*** **2002.** 43: **477–485.**

Supplementary key words apoE deficient mice • LDL receptor deficient mice • high density lipoproteins • atherogenic diet • bioactive oxidized phospholipids

It is well known that there is an inverse relationship between HDL and risk for cardiovascular disease in humans. HDL transports at least three enzymes including LCAT, paraoxonase (PON), and platelet-activating factor acetylhydrolase (PAF-AH), which have putative protective roles in atherosclerosis.

LCAT, which is secreted by the liver, plays a critical role in reverse cholesterol transport, a process whereby excess cholesterol is removed from macrophage-foam cells and esterified on HDL and returned to the liver for catabolism. We have previously shown that LCAT is extremely sensitive to oxidation events in vitro and is rapidly inhibited by low levels of phospholipid hydroperoxides in plasma (1, 2). A deficiency of LCAT can predispose to atherosclerosis since it has been reported that two novel missense mutations in LCAT were associated with reduced LCAT activity and premature coronary artery disease (3). Protection against atherosclerosis by LCAT is supported by the observation that overexpression of human LCAT in the transgenic rabbit on an atherogenic diet reduced not only plasma cholesterol levels but also lesion formation (4). However, overexpression of LCAT in the transgenic mouse was found to be associated with the accumulation of dysfunctional HDL and increased diet induced atherosclerosis (5). By introducing the CETP gene into the LCAT transgenic mouse, pro-atherogenic effects of elevated LCAT were corrected (6). Taken together, these observations in humans and animal models suggest that LCAT has anti-atherogenic properties.

Paraoxonase (PON), which is also synthesized and secreted by the liver (7), is believed to inactivate phospholipid hydroperoxides formed during early events of lipoprotein oxidation (8–10). Low plasma PON activity has been demonstrated in C57BL/6 mice maintained on an atherogenic high fat, high cholesterol, cholic acid diet (11); moreover, PON deficient C57BL/6 mice have increased aortic lesions compared with wild-type (12). The deficiency in PON was also associated with an increase in

Abbreviations: DMPC, dimyristoylphosphatidylcholine; LysoPC, lysophosphatidylcholine; PON, paraoxonase; PAF-AH, platelet-activating factor acetylhydrolase; POVPC, 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine; PGPC, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine.

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HDL lipid hydroperoxides. Studies on PON-apolipoprotein E (apoE) double knockout mice suggested that PON deficiency promotes LDL oxidation and atherosclerosis; bioactive oxidized phospholipids were increased in the lipoproteins of these mice (13). These results, together with those from epidemiological studies in humans (14), suggested that there is an inverse relationship between PON activity and atherosclerosis.

The enzyme, PAF-AH, secreted by macrophages hydrolyzes platelet-activating-factor (PAF) and other PAF-like lipids that are potent mediators of inflammation (15). PAF-AH in humans is associated primarily with LDL (16), although a small fraction (15%) of the enzyme is found in the HDL density range; however, in mice this enzyme is transported almost exclusively on HDL (17, 18). Platelet activating factor and oxidatively fragmented phospholipids appear to be substrates for the enzyme (19). Watson et al. (10) have shown that this enzyme is able to hydrolyze oxidized phospholipids associated with minimally oxidized LDL, thus preventing monocyte binding to endothelial cells and production of monocyte chemotactic protein-1.

Two strains of mice have been extensively used to study atherosclerosis: the apoE deficient strain that is an inflammatory model demonstrating spontaneous atherosclerosis on a chow diet and exacerbated lesion formation on an atherogenic Western diet (20, 21), and the LDL receptor deficient model that exhibits severe atherosclerosis on the Western diet, but not on chow (22). In the present study we examined these two atherosclerosis-prone mouse strains to test the premise that short-term exposure of the mice to an atherogenic Western diet will alter LCAT, PAF-AH, and PON activities. Although there are reports that PON activity is reduced in these mice (11, 23, 24), there is little information on effects of pro-inflammatory conditions on LCAT and PAF-AH. The effect of pro-inflammatory/pro-atherogenic events in atherosclerosis-prone mouse models has not been previously examined in young (8 week old) mice after only a short exposure to an atherogenic diet. Elucidation of early changes in the activities of the putative anti-atherogenic enzymes could be helpful in understanding the atherosclerosis susceptibility in these strains. It is likely that decreases in the functionality of the anti-atherogenic enzymes will be associated with the accumulation of bioactive oxidized phospholipids in the plasma.

MATERIALS AND METHODS

Mice

C57BL/6 (controls), and apoE deficient (apoE$^{-/-}$) and LDL receptor deficient (LDLr$^{-/-}$) mice, the latter two strains in the C57BL/6 background, were obtained from Jackson Laboratory at 6 to 7 weeks of age. Only male animals were utilized. All mice were acclimated for 1 week before use.

Diet protocols

To test whether there were major differences in the response of LCAT, PON, and PAF-AH activities between different genetic strains of atherosclerosis-prone mice, animals were maintained on a chow diet and then switched to a high fat Western diet (Teklad, 42% fat, 0.15% cholesterol) for 2 weeks. The 2 week feeding period was chosen to be more representative of early events that would predispose to atherosclerosis; this diet is atherogenic for both apoE$^{-/-}$ and LDLr$^{-/-}$ mice but not for the C57BL/6 controls, since there is no cholate in the diet. Fasted blood samples were obtained before and 2 weeks after the onset of the diet and were used to determine plasma lipid concentrations and LCAT, PON, and PAF-AH activities. After the 2 week Western diet period, the mice were switched back to a chow diet for 2 weeks (assessment of plasma lipid concentrations verified that these had returned to pre-Western diet levels) and subsequently each group of 10 animals was separated into two groups of five; one group continued on the chow diet and the other was given the Western diet for 2 weeks. At the end of this period, blood was obtained for determining accumulation of bioactive oxidized phospholipids. The animals were sacrificed and their livers removed and snap frozen in liquid nitrogen.

Plasma lipids

Plasma lipid concentrations were determined enzymatically as previously described (17). Agarose gel electrophoresis, using Lipogels (Beckman) essentially as described by the manufacturer, was used to determine the distribution of α, β, and pre-β lipoproteins.

Enzyme activities

For PON activity, blood samples were drawn into heparinized tubes and activity was assayed with both phenylacetate (arylesterase activity) and paraoxon as substrate according to the procedure of Gan et al. (25). The data presented is based on arylesterase activity where one unit = 1 μmol phenylacetate hydrolyzed per min. For PAF-AH activity, blood samples were drawn into EDTA tubes and plasma was diluted 1:150 (v/v) before carrying out the assay. PAF-AH activity was determined by the release of [^{3}H]acetate from 2-acetyl-[^{3}H]PAF essentially as described by McCall et al. (16). Results are expressed as nmol acetate released per h per ml. LCAT activity was measured using the exogenous proteoliposome substrate containing [^{14}C]cholesterol as described by Chen and Albers (26). Samples were incubated for 0.5 h and cholesterol and cholesteryl esters extracted and separated on silica gel plates. Results are expressed as the amount of cholesterol converted to cholesteryl ester in 0.5 h.

Quantification of plasma bioactive oxidized phospholipids

Animals were fasted before blood was obtained. Mouse plasmas from wild-type (WT) control and LDLr$^{-/-}$ and apoE$^{-/-}$ mice on chow or Western diet were prepared by the immediate addition of butylated hydroxytoluene, EDTA, and 4(2-aminoethyl)benzenesulfonyl fluoride (0.3 mM, 100 mM, and 3 mM, respectively). Plasma samples were extracted with chloroform-methanol (2:1, v/v) and the chloroform layer collected.

Dimyristoylphosphatidylcholine (DMPC) was used as an internal standard. Extracted lipids were subjected to quantitative electrospray mass spectrometric analysis as described (27–29). Phospholipids were quantified based on their ion intensity relative to the internal DMPC standards; results are expressed as μg DMPC equivalents/100 μl plasma. The phospholipids quantified included: lysophosphatidylcholine (LysoPC), 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine (POVPC), and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC). A total of five animals per group were analyzed.

Real time quantitative mRNA for PON, apoA-I, and LCAT

Total RNA was determined for three of each WT, LDLr$^{-/-}$, and apoE$^{-/-}$ mice on chow and Western diets. The total RNA fraction was extracted from frozen, pulverized tissue using RNeasy

Miniprep Kits (Qiagen, CA). The concentration of RNA was obtained from A_{260} measurements and stored at −70°C. The primers were designed for products 300 bp in length; LCAT: forward 5′-tatgtgatggggctgcctg-3′, reverse 5′-gctgtggttgtagacaatcctg-3′; apoA-I: forward 5′-ttggatatctcgcactttagc-3′, reverse 5′-agggaa gagaacagtgggaat-3′; PON: forward 5′-tcagccactagtcctgtctcag-3′, reverse 5′-tgcctgcagctggcttgtcacag-3′. The β-actin gene, forward 5′-gtccacacccgcc accaattcgccatg-3′, reverse 5′-ggtgtaaaacggatcccag taacagtccg-3′) was quantified and used as an internal standard .

Real time RT-PCR was performed in triplicate on total RNA samples or mRNA standards in 96-well optical plates on the iCycler Thermal Cycler (Bio-Rad, CA); data was obtained using the iCycler iQ Real-Time Detection System Software (version 2.1). Real-time one-step RT-PCR kits were purchased from Qiagen and used as directed. Each 50 μl reaction contained 25 μl 2× QuantiTect SYBR Green RT-PCR Master Mix, 5 μl forward primer (0.5 μM), 5 μl reverse primer (0.5 μM), 0.5 μl QuantiTect RT Mix, 1 μl Uracil-N-glycosylase (heat-labile), 0.5–2.5 μl Template RNA (200 ng), and 10–12 μl RNase-free water. RT-PCR parameters were as follows: reverse transcription at 50°C for 30 min, PCR initial activation at 95°C for 15 min, 45 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Standard curves, in triplicate, were generated using serial dilutions ($10–10^6$) of known quantities of mouse liver mRNA (Clontech, CA). The number of standard RNA molecules was calculated essentially as described by Medhurst et al. (30).

Statistical analysis

Statistical analyses were performed using ANOVA with Scheffe's method for multiple comparisons between groups. All significance levels are two-sided. The paired *t*-test was used for within group comparisons. Results are expressed as mean ± SD. Statistical significance is defined as $P \leqslant 0.05$.

RESULTS

Plasma lipoprotein distribution and lipid concentrations in control, apoE$^{-/-}$, and LDLr$^{-/-}$ mice

The electrophoretic mobility of lipoproteins from mice maintained on the chow diet is shown in **Fig. 1**. Characteristically, apoE$^{-/-}$ plasma shows a substantial increment in the pre-β (VLDL-IDL) region and a decrement in α (HDL) migrating particles compared with control C57BL/6 mice. LDLr$^{-/-}$ mice, on the other hand, show a pronounced increase in β (LDL) particles and an increase in HDL. A similar pattern persists after the animals are placed on a Western diet, except that the lipid stain is more intense (data not shown). Plasma lipid concentrations are shown in **Table 1**. On a chow diet, triglyceride concentrations are significantly increased in both apoE$^{-/-}$ and LDLr$^{-/-}$ mice compared with controls; however, the greatest differences are in total cholesterol, where there is a 4.4-fold and a 2.6-fold increase in apoE$^{-/-}$ and LDLr$^{-/-}$, respectively, compared with controls. These increases are consistent with those previously reported (20–22). There is a small (15%) but not significant decrease in HDL cholesterol (HDL-C) in apoE$^{-/-}$ mice on the chow diet compared with controls; on the other hand, HDL-C concentrations are significantly increased (67%, $P < 0.0001$) in LDLr$^{-/-}$ mice. The Western diet increased total cholesterol (75%) and HDL-C (53%) in the control mice compared with the chow diet. Two weeks maintenance on a Western diet, however, had a profound effect on total cholesterol in apoE$^{-/-}$ and LDLr$^{-/-}$ mice where total cholesterol doubled in the former and increased 3.6-fold in the latter over chow values. HDL-C in apoE$^{-/-}$ mice shows a 50% decrease after 2 weeks on the Western diet while HDL-C concentrations in LDLr$^{-/-}$ mice were not affected. It is also apparent in Table 1 that triglyceride increases almost 6-fold in LDLr$^{-/-}$ mice. The data indicate that short term feeding of the Western diet is associated with substantial changes in plasma lipids in the atherosclerosis-prone mouse models and in modest changes in the control mice.

TABLE 1. Plasma lipid concentrations (mg/ml) in WT, apoE$^{-/-}$, and LDLr$^{-/-}$ mice on chow and Western diet

Genotype	Diet	TG	TC	HDL-C
WT	Chow	0.29 ± 0.06	1.13 ± 0.07	1.08 ± 0.07
apoE$^{-/-}$	Chow	0.80 ± 0.31[b]	5.01 ± 1.01[c]	0.92 ± 0.16
LDLr$^{-/-}$	Chow	0.74 ± 0.28[b]	2.92 ± 0.47[c]	1.80 ± 0.23[c]
WT	Western	0.26 ± 0.08	1.98 ± 0.30	1.66 ± 0.25
apoE$^{-/-}$	Western	0.49 ± 0.25	11.46 ± 2.56[c]	0.47 ± 0.24[a]
LDLr$^{-/-}$	Western	4.22 ± 2.42[c]	10.50 ± 3.78[c]	1.97 ± 0.40

Data represent the mean ± SD of 10 mice per group. Compared to WT, ANOVA:
[a] $P = 0.003$.
[b] $P < 0.001$.
[c] $P < 0.0001$.

PON, PAF-AH, and LCAT activities in control, apoE$^{-/-}$, and LDLr$^{-/-}$ mice

Figure 2 reveals that in chow fed mice, there is no difference in PON activity between controls and apoE$^{-/-}$ mice; but LDLr$^{-/-}$ mice have significantly increased

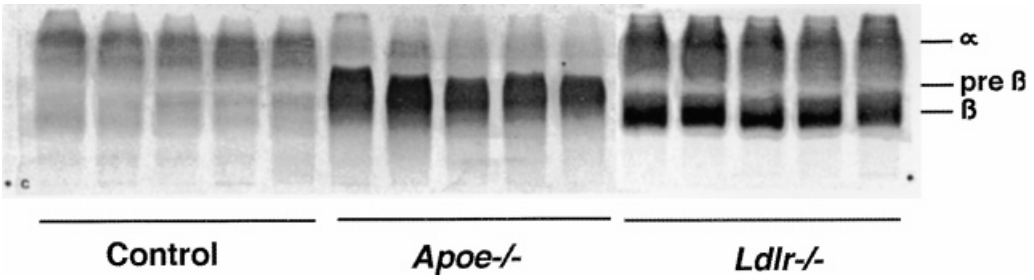

Fig. 1. Agarose gel electrophoresis profiles of plasma from control, apolipoprotein E (apoE)$^{-/-}$ and LDL receptor (LDLr)$^{-/-}$ mice. The figure shows the lipid profile from five mice of each genotype on the chow diet.

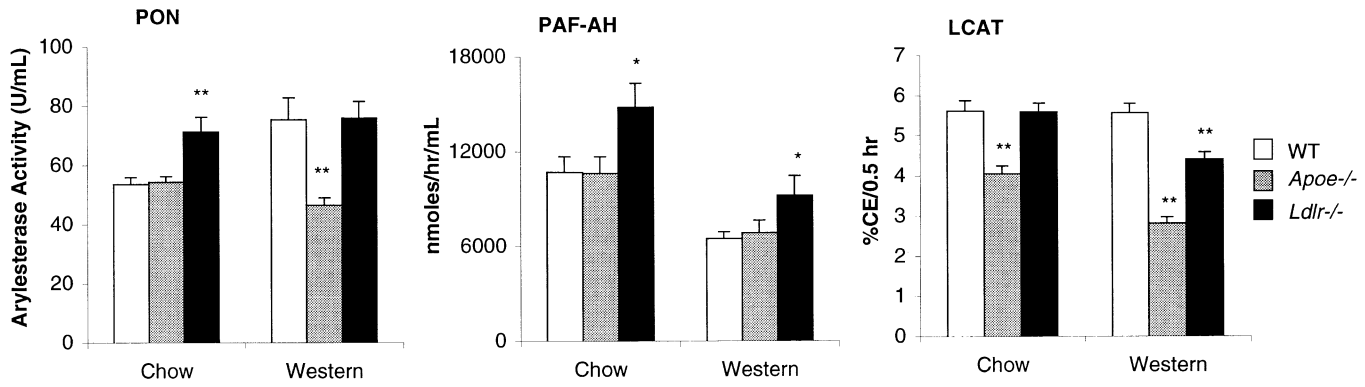

Fig. 2. Changes in paraoxonase (PON), platelet-activating factor acetylhydrolase (PAF-AH), and LCAT activities as a function of genotype and diet. Data represent the mean ± SD of 10 animals. ANOVA: * $P < 0.01$ and ** $P < 0.0001$ compared with wild type (WT) controls within each diet group.

(33%) activity compared with controls concomitant with elevated HDL-C (Table 1). On the Western diet, PON activity increases 40% in control animals and appears to parallel the increase seen in HDL-C (Table 1). PON activity in LDLr$^{-/-}$ mice on the Western diet did not increase in activity mirroring the lack of change in HDL-C. There was a modest (17%) but significant ($P < 0.05$) decline in PON activity in apoE$^{-/-}$ mice after 2 weeks on the Western diet. However, compared with controls on the Western diet, apoE$^{-/-}$ mice exhibit a substantial decrease (38%) in PON activity on the atherogenic diet.

PAF-AH activity in apoE$^{-/-}$ mice on either chow or Western diet was no different from controls; however, on each diet, LDLr$^{-/-}$ mice had activity levels significantly exceeding that of controls (Fig. 2). It is important to note, however, that PAF-AH activity was significantly lower (35–40%, $P < 0.01$) on the Western diet compared with chow for all genotypes.

Unlike the other two anti-atherogenic enzymes, LCAT is significantly lower (28%, $P < 0.001$) in chow fed apoE$^{-/-}$ mice than in controls; on a Western diet there is a 50% loss of activity (Fig. 2). In contrast, the LDLr$^{-/-}$ mice on a chow diet show no change in LCAT activity compared with controls but have a significant decrease (21%, $P < 0.0001$) in LCAT activity on the Western diet. Taken together, the data suggests that LCAT activity may be more sensitive to potential oxidative stress than either PON or PAF-AH.

Plasma accumulation of bioactive phospholipids

Because plasma lipid levels are markedly elevated in the atherosclerosis-susceptible mice on a Western diet, we asked the question whether elevation of lipids is associated with an accumulation of plasma bioactive oxidized phospholipids. **Figure 3** shows the concentrations of the bioactive oxidized phospholipids in mouse plasma from animals on a chow or Western diet. The data suggest that the apoE$^{-/-}$ mice have a particularly large burden of plasma oxidized phospholipids even on a chow diet because POVPC and PGPC are substantially elevated (288% and 80%, respectively) over controls. In contrast, LDLr$^{-/-}$ mice do not have elevated oxidized phospholipids on the chow diet. Both genotypes exhibit a 35% increase in plasma LysoPC concentrations compared with controls.

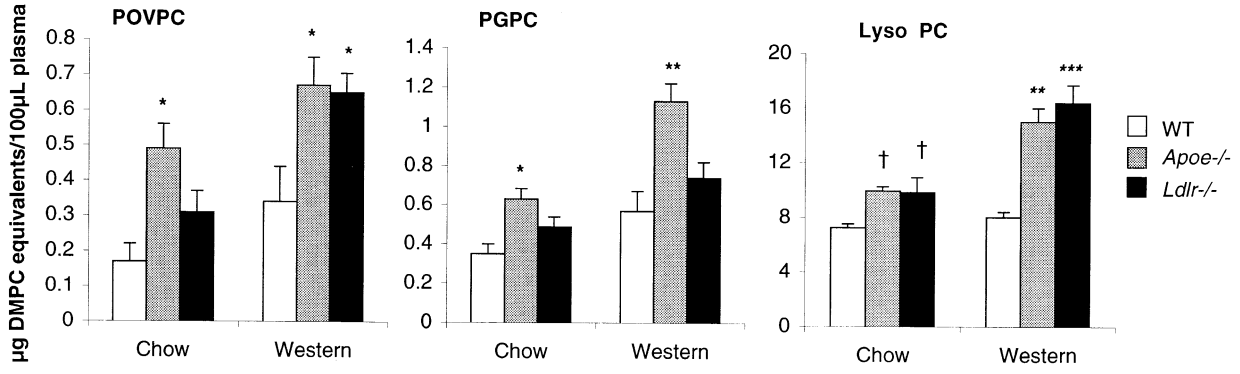

Fig. 3. Accumulation of bioactive oxidized phospholipids and lysolecithin in the plasma of control (WT), apoE$^{-/-}$, and LDLr$^{-/-}$ mice maintained on chow or Western diet. Values represent the mean ± SD of five animals. ANOVA: † $P < 0.05$, * $P \leq 0.01$, ** $P \leq 0.001$, and *** $P \leq 0.0001$ compared with WT within each diet group. The data also reveal that oxidized phospholipids are generally significantly higher on the Western diet than on chow where paired *t*-test *P* values are: controls; 0.05, <0.03, NS: apoE$^{-/-}$; 0.04, <0.0001, 0.0002: LDLr$^{-/-}$; 0.0003. <0.02, 0.0001 for POVPC, PGPC, and LysoPC, respectively.

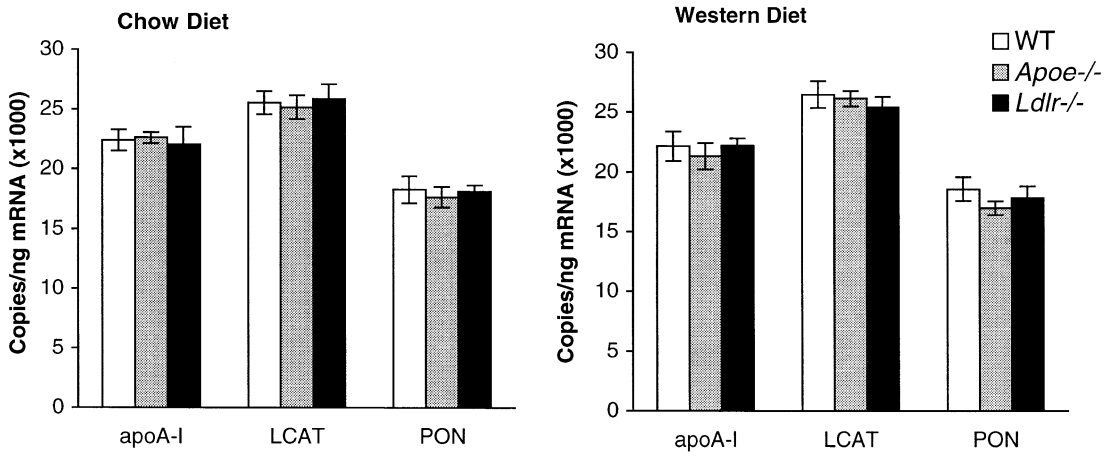

Fig. 4. ApoA-I, LCAT, and PON mRNA expression in mouse liver. The sensitivity of RT-PCR using different primer sets was established from the cycle threshold (Ct) values obtained using known quantities of mRNA. For the primers sets, a Ct of 22–25 was obtained with 22,000 copies. Copies/ng mRNA were calculated from standard curves constructed from serial dilutions of known quantities of mouse liver mRNA. Reverse transcription reactions from each RNA sample were carried out in triplicate. The values represent the mean ± SD from three mouse livers per group.

As shown in Fig. 3, consumption of the Western diet is associated with significant increases in plasma bioactive oxidized phospholipids in all genotypes. ApoE$^{-/-}$ mice on the Western diet, however, have significantly higher concentrations of LysoPC, POVPC, and PGPC than controls on the Western diet, whereas LDLr$^{-/-}$ mice have greater quantities of LysoPC and POVPC than controls. The data demonstrate that the Western diet is associated with increased levels of plasma oxidized phospholipids, and that apoE$^{-/-}$ and LDLr$^{-/-}$ mice have especially elevated levels of these bioactive phospholipids.

mRNA levels of PON, apoA-I, and LCAT

LCAT is expressed primarily in the liver, which is also the major site of apoA-I and PON synthesis. Since apoE$^{-/-}$ mice have a significant loss of LCAT and PON activity, as well as low HDL-C concentrations on the Western diet, we examined, by real time quantitative PCR, whether mRNA levels for LCAT, PON, and apoA-I were altered in these mice compared with the other genotypes and whether diet had an impact on mRNA levels. The expression levels of apoA-I, LCAT, and PON in the liver of each genotype is shown as copies/ng total mRNA in **Fig. 4**. The data reveal that there are no differences in LCAT, PON, and apoA-I mRNA expression between the different genotypes, nor does the Western diet influence mRNA levels. The absence of any significant reduction in mRNA levels for LCAT and PON in apoE$^{-/-}$ mice suggests that post-transcriptional events are responsible for low enzyme activities.

Diet-related changes in the distribution of PAF-AH activity

Unlike humans where PAF-AH is associated primarily with LDL, PAF-AH in the mouse is associated almost exclusively with HDL particles. Earlier studies with LCAT deficient mice suggested to us that PAF-AH can associate with LDL-like particles under metabolic conditions where LDL is elevated (17). To test whether the change in lipoprotein distribution changes the distribution of PAF-AH activity, pooled plasma from control, apoE$^{-/-}$, and LDLr$^{-/-}$ mice on chow and Western diet were subjected to fast protein liquid chromatography and enzyme activity of the fractions determined. **Figure 5** summarizes the data and reveals that, on a Western diet, the LDLr$^{-/-}$ mice have a modest amount (16%) of PAF-AH activity associated with LDL (fractions 11–15), while only scant or no activity is in this fraction in apoE$^{-/-}$ and control mice, respectively. The cholesterol concentration in LDLr$^{-/-}$ mice increased 3.7-fold on the Western compared with chow diet, suggesting that large increases in LDL-C levels may coincide with the shift of PAF-AH into this density region.

DISCUSSION

The enzymes PON and PAF-AH are reputed to have antioxidative properties and, by virtue of this property, are thought to be athero-protective. PON has been shown to hydrolyze oxidized phospholipids that are pro-inflammatory agents (10) and to protect LDL from oxidation (8), thus preventing monocyte recruitment and cell adhesion (23). In human subjects, low PON activity has been associated with increased risk for coronary artery disease (31–34), whereas a deficiency of PON in C57BL/6 mice is coupled with increased lesion formation in mice maintained on an atherogenic diet (12). PAF-AH is also known to hydrolyze pro-inflammatory oxidized phospholipids (19, 35). Several recent studies have shown that PAF-AH is elevated in subjects who had coronary events, suggesting that PAF-AH is a marker for atherosclerosis and could potentially be a contributing factor to coronary artery disease (36, 37). However, it is unclear whether elevated PAF-AH activity is a contributing factor in atherosclerosis, because studies with Japanese subjects who are deficient in PAF-AH indicate that coronary artery disease (CAD) is increased in this population (38). A more direct approach with apoE$^{-/-}$ mice sub-

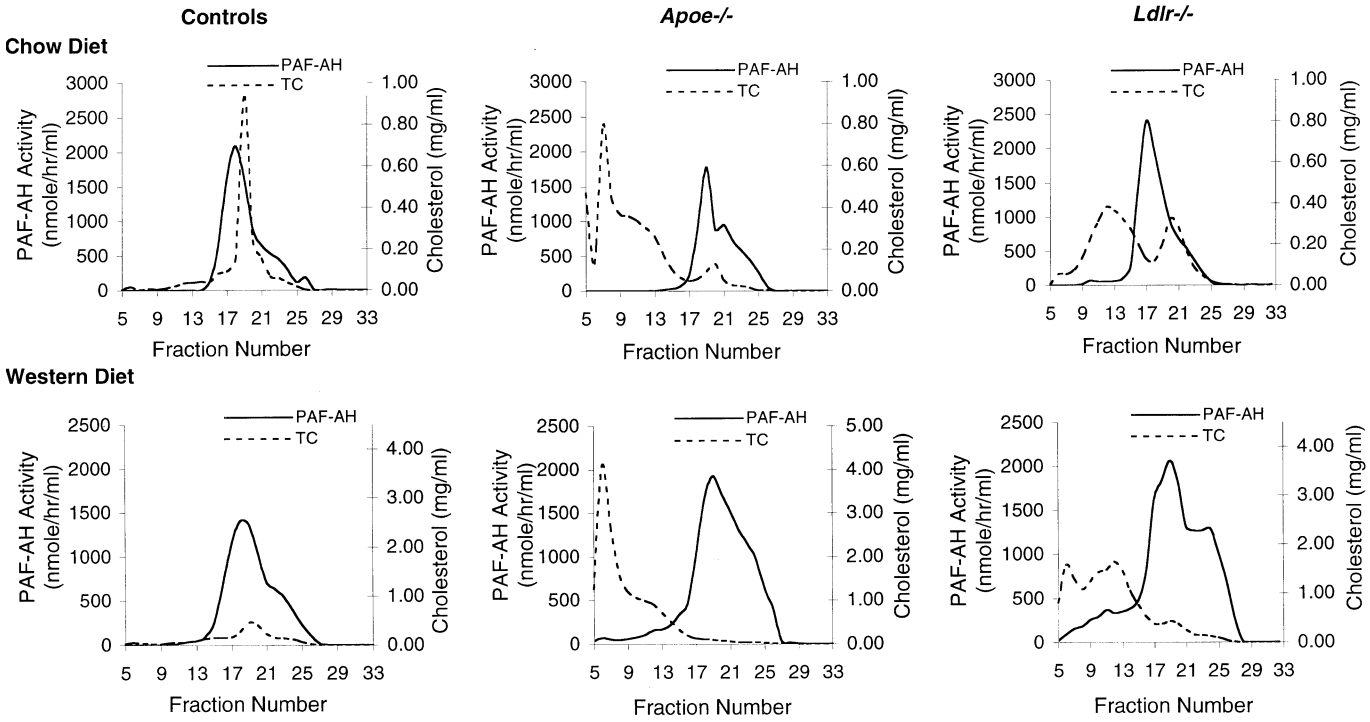

Fig. 5. Distribution PAF-AH activity and plasma cholesterol in control, apoE$^{-/-}$, and LDLr$^{-/-}$ mice on a chow or Western diet. Pooled plasmas (1.0 ml) were fractionated by FPLC; 0.5ml fractions were collected and assayed for cholesterol and PAF-AH activity. Comparison of the cholesterol and PAF-AH activity profiles indicate that most of the enzyme activity localizes to the HDL fraction except in the case of LDLr$^{-/-}$ mice on the Western diet. Calibration with isolated lipoproteins indicate that lipoproteins isolate as follows: VLDL, fr 5–6; IDL, fr 7–10; LDL, fr 11–15; HDL, fr 16–25. Note the change of scale for cholesterol concentrations in the Western diet profiles.

jected to adenovirus-mediated gene transfer of human PAF-AH indicated that over-expression of the enzyme lowered ICAM-1 and VCAM-1 expression and diminished βVLDL-induced macrophage adhesion, suggesting an atheroprotective role for PAF-AH in this model (39).

Although it has been suggested that LCAT, by virtue of its phospholipase activity, has anti-oxidative properties (40, 41), its primary function in athero-protection is thought to be through its participation in reverse cholesterol transport (42). In the present study we examined whether these three anti-atherogenic enzymes are impaired in atherosclerosis-susceptible mice placed on an atherogenic diet for a short duration, and whether changes in enzyme function are associated with an increase in plasma concentrations of pro-inflammatory bioactive oxidized phospholipids.

Our studies with apoE$^{-/-}$ mice reveal that even on a chow diet there is a significant elevation of LysoPC and the bioactive phospholipids POVPC and PGPC; such an accumulation may contribute to spontaneous lesion formation in these mice on a chow diet. Interestingly, LCAT activity was also significantly decreased; this loss of activity could, in part, be caused by the accumulation of oxidized lipids. We have previously shown that plasma LCAT activity in vitro is extremely sensitive to, and readily inhibited by, the accumulation of low levels of oxidation products (1, 2). Reduction in LCAT activity would be expected to exacerbate the atherosclerotic process as cholesterol efflux from macrophage-foam cells would likely be impaired.

The phospholipid composition of lipoproteins in the apoE$^{-/-}$ mouse is unusual because sphingomyelin is highly enriched in all lipoprotein fractions (43). It has also been reported that sphingomyelin is an inhibitor of LCAT activity (44); therefore, an alternative explanation for the loss of LCAT activity in apoE$^{-/-}$ mice on the chow diet is that some of this loss may be attributable to the increased sphingomyelin to phosphodidylcholine ratio. Although the major function of LCAT is the esterification of cholesterol, it is possible that the reduction of LCAT activity in these mice, as well as in LDLr$^{-/-}$ mice on the Western diet, may contribute to the accumulation of oxidized phospholipids since Goyal et al. (41) showed that LCAT has the capacity, albeit weak, to hydrolyze oxidized phospholipid in vitro.

On the Western diet, there were significant reductions in PON, PAF-AH, and LCAT activities in apoE$^{-/-}$ mice that were paralleled by large increases in the bioactive oxidized phospholipids. We examined mRNA levels for LCAT, apoA-I, and PON, all of which are synthesized by the liver, but found no change related to the atherogenic diet, suggesting changes in enzyme activity are due to post translational events. HDL-C concentration in these mice decreased 50% on the Western diet; as PON, PAF-AH, and LCAT enzymes are transported on HDL, this reduction could conceivably decrease enzyme activity. On the other hand, HDL was elevated in control mice on the Western diet, and concomitantly LCAT activity was not inhibited, suggesting that increased HDL levels may protect enzyme

function. It is interesting that Zhang et al. (20) reported a decrease in HDL in apoE$^{-/-}$ mice on an atherogenic diet but found that apoA-I concentrations were not affected, but rather that apoA-I distribution was altered. They found that a large proportion of apoA-I was associated with less dense lipoproteins rather than HDL. We too noted a similar phenomenon where, on a Western diet, apoA-I was associated with IDL/LDL and VLDL in apoE$^{-/-}$ mice (data not shown). It has been suggested that apoA-I may be required for optimal PON activity because this protein co-isolates with PON1 during purification of the enzyme (45). We have also demonstrated that the conformation of apoA-I appears to play a role in optimizing PON activity (46). Therefore, it is conceivable that an altered apoA-I conformation that can be expected by its transport on non-HDL particles may adversely affect PON activity in apoE$^{-/-}$ mice. Alternatively, PON activity may be reduced because the enzyme is impaired by the accumulation of oxidized phospholipids; indeed, inhibition of HDL-PON activity by copper oxidation was demonstrated in vitro by Aviram et al. (47). The redistribution of apoA-I can, as well, potentially contribute to the decreased LCAT activity, since apoA-I is a co-factor in activation of LCAT. Although speculative, it is possible that the concomitant decline in the activity of the anti-atherogenic enzymes, together with an increase in bioactive oxidized phospholipids, contributes to the pronounced lesions found in apoE$^{-/-}$ mice on an atherogenic diet. This speculation is supported by the observations of Watson et al. (27) that POVPC and PGPC are found in the atherosclerotic lesions of cholesterol fed rabbits and that both these bioactive oxidized phospholipids induce endothelial cells to bind monocytes in vitro.

Unlike apoE$^{-/-}$ mice that develop spontaneous lesions on a chow diet, the LDLr$^{-/-}$ mouse does not develop atherosclerosis under this condition. This may be explained, in part, by the observations that: *1*) the total plasma burden of bioactive oxidized lipids is less in the LDLr$^{-/-}$ genotype than in the apoE$^{-/-}$ genotype, and *2*) there is a significant increase in PON and PAF-AH activities compared with WT, which parallels the increase in HDL-C in the LDLr$^{-/-}$ mouse. Although speculative, the latter may be important in protecting the anti-atherogenic function of HDL such as LCAT activity that, unlike that of apoE$^{-/-}$ mice, is not reduced in chow fed LDLr$^{-/-}$ mice.

In the LDLr$^{-/-}$ mouse, consumption of the Western diet resulted in a markedly increased accumulation of POVPC compared with controls. Although oxidized phospholipids increased significantly, PON activity, unlike that seen in apoE$^{-/-}$ mice, was unaffected. In control mice on the Western diet there was actually an increase in PON activity; this increase mirrored an increase in HDL-C suggesting that increased HDL concentrations may preserve PON function.

LDLr$^{-/-}$ mice on the Western diet were unusual because PAF-AH activity was significantly elevated over controls even though both genotypes had similarly elevated HDL. Clearly, elevated HDL levels are not sufficient in themselves to maintain PAF-AH activity. It was previously demonstrated by Stafforini et al. (48) that, in humans, the lipoprotein environment in which PAF-AH exists influences its catalytic activity. They noted that, in humans, activity was most efficient on LDL particles. A possible explanation for the elevation of PAF-AH activity in LDLr$^{-/-}$ mice on the Western diet compared with that of controls is that redistribution of some of the enzyme's activity (16%) to LDL may be important in maintaining the stability and activity of the enzyme during fat feeding.

The reductions of PAF-AH and PON activities in apoE$^{-/-}$ and PAF-AH activity in LDLr$^{-/-}$ mice on the Western diet may, in part, be due to inactivation of these enzymes by the oxidized phospholipids in the plasma. This is supported by in vitro studies that have shown that oxygen free radicals generated by xanthine-xanthine oxidase (49) and copper-mediated oxidation of LDL (50) inhibit plasma PAF-AH, and that copper ion-induced oxidation products inhibit PON1 (47). In the present study, however, apoE$^{-/-}$ and LDLr$^{-/-}$ mice on the chow diet have a modest increase in bioactive oxidized phospholipids that is not associated with decreased PON1 and PAF-AH activity. It is likely that in the closed in vitro systems the response is more pronounced, and that in vivo the threshold for oxidative inhibition of the enzymes is increased.

The results from the two different atherosclerosis-susceptible mouse strains suggest that the response to an atherogenic diet is complex. However, it is likely that the total plasma burden of bioactive oxidized phospholipids is important in modulating the pro-inflammatory responses that predispose to atherosclerosis. Thus, it is not surprising that apoE$^{-/-}$ mice that have precocious atherosclerosis on the chow diet also have a greater burden of bioactive oxidized phospholipids than LDLr$^{-/-}$ mice that do not develop spontaneous atherosclerosis on chow. This study also suggests that the anti-atherogenic enzyme, LCAT, is more sensitive to plasma oxidative events than either PON or PAF-AH, because it was the only enzyme that was significantly decreased in both atherosclerosis-susceptible mouse models compared with controls. It is tempting to speculate that impairment of LCAT, and subsequently cholesterol efflux, by the accumulation of plasma bioactive oxidized phospholipids may be a key event in the development of atherosclerotic lesions in these models.

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