Macrophage 12/15 lipoxygenase expression increases plasma and hepatic lipid levels and exacerbates atherosclerosis

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Abstract 12/15 lipoxygenase (12/15LO) oxidizes polyunsaturated fatty acids (PUFAs) to form bioactive lipid mediators. The role of 12/15LO in atherosclerosis development remains controversial. We evaluated atherosclerosis development and lipid metabolism in 12/15LO-LDL receptor (LDLr) double knockout (DK) vs. LDLr knockout (SK) mice fed a PUFA-enriched diet to enhance production of 12/15LO products. Compared with SK controls, DK mice fed a PUFA-enriched diet had decreased plasma and liver lipid levels, hepatic lipogenic gene expression, VLDL secretion, and aortic atherosclerosis and increased VLDL turnover. Bone marrow transplantation and Kupffer cell ablation studies suggested both circulating leukocytes and Kupffer cells contributed to the lipid phenotype in 12/15LO-deficient mice. Conditioned medium from in vitro incubation of DK vs. SK macrophages reduced triglyceride secretion in McArdle 7777 hepatoma cells. Our results suggest that, in the context of dietary PUFA enrichment, macrophage 12/15LO expression adversely affects plasma and hepatic lipid metabolism, resulting in exacerbated atherosclerosis.—Rong, S., Q. Cao, M. Liu, J. Seo, L. Jia, E. Boudyguina, A. K. Gebre, P. L. Colvin, T. L. Smith, R. C. Murphy, N. Mishra, and J. S. Parks. Macrophage 12/15 lipoxygenase expression increases plasma and hepatic lipid levels and exacerbates atherosclerosis. J. Lipid Res. 2012. 53: 686–695.

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12/15 lipoxygenase (12/15LO) belongs to a lipoxygenase family of enzymes that catalyze the stereospecific addition of molecular oxygen across double bonds in polyunsaturated fatty acids (PUFAs) to form a series of biologically active lipid mediators (1). Based on their phylogenetic classification, murine leukocyte-type 12LO and human reticuloocyte type 15LO (h15LO1) share 73% sequence similarity and belong to the same 12/15LO subfamily, but tissue distribution of 12/15LO is different among species (1). In mice, the expression of 12/15LO is limited to a few cell types, such as macrophages, endothelial cells, and adipocytes (2–4). Several studies reported that 12/15LO was highly expressed in macrophages (3–5). When bone marrow (BM) in apoE knockout (KO) mice was replaced with 12/15LO-apoE double KO BM, there was only residual 12/15LO expression in most of the organs compared with apoE KO recipient mice receiving BM from apoE KO mice (3), demonstrating that whole body 12/15LO expression originated primarily from BM-derived cells, presumably macrophages.

The role of 12/15LO in atherosclerosis development has been a focus of investigation since enzyme activity was first detected in human atherosclerotic lesions (6–8). Since 1996, atherosclerosis studies on 12/15LO gene-modified animals have been performed by several groups. However, the outcomes of these studies remain controversial; most studies have suggested that 12/15LO expression...
is atherogenic (3, 5, 9–15), but other studies, using similar experimental approaches, have concluded that 12/15LO expression is atheroprotective (16, 17). One possible explanation for the inconsistent results is that diets used in these studies varied in fatty acid composition, resulting in formation of different oxidative lipid mediators by macrophages in vivo, which had different biological effects on inflammation, cytokine production, and endothelial activation (18–20).

To date, no atherosclerosis study has addressed the role of 12/15LO expression in an experimental setting in which dietary substrate fatty acids are enriched to drive production of 12/15LO-catalyzed lipid mediators. To address this gap in knowledge, we compared atherosclerosis development in 12/15LO KO mice in the LDLrKO background [double knockout (DK)] to LDLrKO (SK) mice using an atherogenic diet enriched in PUFAs. For comparison, we also fed a control diet enriched in saturated and monounsaturated fatty acids (Sat/mono), similar to that used in previously published studies (11). Our results suggest a dietary fatty acid-gene interaction for 12/15LO that affects plasma and hepatic lipid metabolism as well as atherosclerosis.

**MATERIALS AND METHODS**

**Mice and diets**

LDLrKO and 12/15LOKO mice in the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were crossbred to generate heterozygotes at both targeted alleles, and heterozygous matings were used to generate 12/15LO-LDLrDK mice. Genotypes were ascertained by PCR analysis using tail DNA as described previously (21). For LDLr12/15LO-LDLrDK mice. Genotypes were ascertained by PCR geted alleles, and heterozygous matings were used to generate the mice were crossbred to generate heterozygotes at both targeted alleles.

**Plasma and liver lipid analysis**

Plasma cholesterol and triglyceride (TG) concentrations were measured using enzymatic assays with commercially available reagents (cholesterol: Wako; TG: Roche). Plasma lipoproteins were separated by size using fast protein liquid chromatography, and cholesterol distribution among lipoprotein factions was determined by enzymatic cholesterol assays. After 16 weeks of atherogenic diet consumption, mice were euthanized, and liver was harvested for measurement of lipid content as previously described (23). Liver samples were also fixed in 10% formalin and embedded in paraffin for hematoxylin and eosin staining.

**Quantification of aortic lesion area and lipid content**

Whole aorta (from sinotubular junction to iliac bifurcate) was fixed in 10% formalin, and the adventitia was cleaned. Aortas were opened along the longitudinal axis, pinned onto black silicon elastomer, and photographed with a Sony DXC-S500 digital camera. The digital images were used to quantify the percentage of total aortic surface covered with lesion using Image Pro 6.2 software. After surface lesion quantification, aortas were transferred to a glass tube containing 10 μg of 5a-cholesterol as internal standard for gas-liquid chromatographic analysis of cholesterol content as previously described (24).

**Real-time PCR and Western blotting**

Real-time PCR and Western blot analysis were performed as described previously (25). Primer sequences used for real-time PCR were summarized in Supplementary Table IV.

**In vivo quantification of TG and apoB secretion rate**

Detergent block to inhibit TG lipolysis in vivo was used to quantify the rate of TG and apoB secretion into plasma of SK and DK mice after 12 weeks of PUFA diet consumption (25, 26). Another detergent block study was performed with BM transplanted mice after 15 weeks of PUFA diet consumption.

**VLDL turnover study**

After feeding mice the PUFA diet for 16 weeks, plasma was collected from SK and DK mice after a 4 h fast. VLDL was separated from plasma by ultracentrifugation (d = 1.006, 100,000 rpm for 4 h with a Beckman Coulter TLA100.2 rotor). VLDL chemical composition was determined using enzymatic assays, and particle size was quantified with a Zetasizer Nano S dynamic light scatter instrument (Malvern Instruments Ltd., Worcestershire, UK). VLDL from SK and DK mice were radioiodinated with 125I and 131I, respectively, as described (27). Ninety-eight percent of the radiolabel was trichloroacetic acid precipitable (i.e., protein bound), and 80% was associated with apoB, as determined by isopropanol extraction (28). VLDL tracers (125I and 131I; 350,000 cpm each) were mixed, diluted to a volume of 200 μl with saline, and injected into PUFA-diet fed SK- and DK-recipie mice (fasted 4 h) through a jugular vein catheter (29). Plasma samples were taken periodically after injection (5 and 30 min and 1, 2, 3, 5, 8, and 24 h), and 125I and 131I radioactivity in isopropanol-extracted plasma was quantified with a Perkin Elmer automatic γ counter. The fractional catabolic rate (FCR) of VLDL in plasma was calculated using SAAM II software (SAAM Institute, Seattle, WA) as described previously (21).

**BM transplantation**

BM was harvested from cleaned femurs and tibias of female SK and DK mice and resuspended in RPMI 1640 medium for injection. SK and DK male recipient mice were fasted overnight and received a lethal dose of radiation (900 rads) 4 h before BM injection. Mice were anesthetized with isoflurane, and 5 × 10⁶ BM cells were injected into the retro-orbital venous plexus. Three days before and 2 weeks after the BM transplantation, recipient mice received autoclaved acidified (pH 2.7) water supplemented with 100 mg/l neomycin and 10 mg/l polymyxin B sulfate. After
that, mice were given acidified water until the end of the experiment. The replacement of hematopoietic cells by donor BM was evaluated by quantifying the remaining relative copy number of male DNA in hematopoietic genomic DNA 4 weeks after BM transplantation. Briefly, genomic DNA was extracted from whole blood with a Wizard Genomic DNA Purification kit (Promega). The y-chromosome associated sex-determining region Y gene (Sry) was amplified by PCR to a linear amplification phase with the following conditions: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 61°C for 1 min, and 72°C for 1 min. The amplified 380 bp PCR product was separated on 0.8% agarose gels and visualized with ethidium bromide. Acyl-CoA:cholesterol acyltransferase 2 (ACAT2) was amplified by PCR as an internal control. A series of male and female genomic DNA mixtures (100, 50, 25, and 0% male DNA) were made and used to construct a standard curve of male:female DNA ratio vs. Sry:ACAT2 ratio, determined by quantification of density of PCR bands. Male genomic DNA in the whole blood samples of transplanted mice was estimated using the standard curve.

**GdCl₃-induced ablation of Kupffer cells**

GdCl₃·6H₂O (Sigma Aldrich) powder was reconstituted to 6 mg/ml with saline and sterilized by passing through a 0.22 µm filter. After 16 weeks of PUFa diet consumption, mice were anesthetized with isoflurane, and the GdCl₃ solution (25 µg/g body weight) was injected into mice through the retro-orbital venous plexus. Injections were repeated every 4 days for a total of three injections.

**Hepatocyte and Kupffer cell isolation**

Hepatocytes and Kupffer cells were harvested from collagenase-perfused livers (30). Nonparenchymal cells were separated from hepatocytes by spinning at 50 × g for 5 min and loaded onto the top of a Percoll gradient. Kupffer cells were harvested at a density of 1.037–1.066 g/ml. Cells were immediately treated with Trizol to isolate RNA. Gene expression was quantified by real-time quantitative PCR.

**Macrophage-conditioned medium preparation and McArdle RH7777 TG secretion studies**

Resident peritoneal macrophages were collected from mice fed the PUFa diet for 12–16 weeks by flushing the peritoneal cavity with PBS. The macrophages were pelleted by low-speed centrifugation; resuspended in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% of FBS; and seeded onto cell culture plates. After a 2 h incubation, floating cells were removed by washing with PBS and adherent macrophages were stimulated with ±2 µM Ca²⁺ ionophore A23187 for 20 min. Then, cells were changed to fresh medium (RPMI 1640, DMEM equal volume) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% Nutridoma-SP (Roche Applied Science) and cultured overnight. Macrophage-conditioned medium was transferred to McArdle RH7777 cells along with 5 µCi ³H-oleate, 0.8 mM oleate, and 5% BSA and incubated for 4 h. After incubation, media lipids were extracted (31), TG was separated by TLC, and radiolabel in TG was quantified by liquid scintillation counting and expressed as dpm/mg cell protein.

**Macrophage lipid mediator analysis**

Macrophages were stimulated with Ca²⁺ ionophore (A23187) for 20 min. Then, cells and medium were collected together to separate and quantify lipid mediator concentration by LC/MS/MS as previously described (32).

**Statistical analyses**

Data were presented as mean ± SEM and were analyzed for statistical significance using a two-tailed unpaired Student’s t-test (i.e., SK vs. DK comparisons). PCR values for different radiiodinated VLDL tracers in the same recipient mice were compared using a two-tailed paired t-test. All the analyses were performed with GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA).

**RESULTS**

**12/15LO expression is pro-atherogenic in SK mice**

To determine whether 12/15 LO expression is pro-atherogenic, we fed DK and SK mice atherogenic diets that were enriched in Sat/mono (i.e., palm oil) or PUFa (i.e., safflower oil) for 16 weeks (Supplementary Table 1). Atherosclerosis was evaluated by two measurements: the percentage of aortic surface covered with lesion and aortic cholesterol content. DK mice fed either diet developed less atherosclerotic surface lesion area compared with SK mice (Fig. 1A, B). The attenuation of atherosclerotic surface lesion area in DK vs. SK mice was more robust in the PUFa diet group compared with the Sat/mono diet group (68.9% vs. 35.7%). Aortic cholesterol content was lower in PUFa-fed DK vs. SK mice but was similar for Sat/mono-fed mice (Fig. 1C). To determine the extent to which aortic surface lesion area and cholesterol content were associated,

**Fig. 1.** 12/15LO-deficient mice developed less atherosclerosis. A: Representative images of aortic surface lesions after mice were fed atherogenic diets enriched with Sat/mono or PUFa for 16 weeks. Aortas were fixed in 10% formalin and opened along the longitudinal axis and pinned for images. B: Aortic surface lesion area was normalized to percentage of total aortic surface area. C: Aortic total cholesterol content per mg protein. In B and C, mean ± SEM; n = 10–13. * P < 0.05 for SK vs. DK. D: Regression analysis of aortic lesion area vs. aortic cholesterol content. Each point represents an individual animal of the indicated genotype and diet group. Regression coefficient is shown for entire data set. SK = LDLr KO; DK = 12/15LO/LDLr double KO.
we performed linear regression analysis and found a highly significant association between the two measurements of atherosclerosis (Fig. 1D).

**PUFA-fed DK mice have reduced plasma lipid concentrations**

Previous studies in different mouse genetic backgrounds (i.e., apoE-KO and LDLrKO) have not observed an effect of 12/15LO expression on plasma lipid concentrations (9, 11). However, none of those studies used an atherogenic diet enriched in PUFA or substrate fatty acids for 12/15LO enzymatic activity. To determine whether the additional reduction in aortic surface lesion area observed for PUFA-fed vs. Sat/mono-fed DK mice (Fig. 1B) was due to a reduction in plasma lipids, we measured total plasma cholesterol (TPC) and TG concentrations and lipoprotein cholesterol distribution. Unexpectedly, DK mice fed the PUFA diet had significantly lower TPC and TG concentrations compared with SK mice (Fig. 2A–D). However, Sat/mono-fed SK and DK mice had similar TPC values, in agreement with previously published studies (9,11).

Given the critical role of liver in lipoprotein and lipid metabolism and the dramatic reduction in plasma VLDL and LDL cholesterol in PUFA-fed DK mice, we examined hepatic lipid content of the mice. Liver weight was similar among the four groups (data not shown). Liver cholesteryl ester (CE) content was significantly lower in PUFA-fed DK compared with SK mice (Fig. 3B). Interestingly, liver TG content was 50% to 60% lower in DK vs. SK mice in both diet groups (Fig. 3D), suggesting a major effect of 12/15LO expression on liver TG storage. Hepatic free cholesterol and phospholipid were similar among the groups (Fig. 3A, C). Finally, hematoxylin and eosin staining showed fewer and smaller neutral lipid droplets in livers of DK mice in both diet groups compared with SK mice (Fig. 3E), indicating improved hepatic steatosis in the absence of 12/15LO expression.

**Liver lipogenic gene expression is reduced in 12/15LO-deficient mice**

Because PUFA diet feeding resulted in reduced plasma (Fig. 2) and liver (Fig. 3) cholesterol in DK mice, whereas the Sat/mono diet did not, the remainder of the studies was focused on PUFA-fed DK and SK mice. To investigate the underlying mechanisms contributing to the protective role of 12/15LO deficiency on lipid metabolism and atherosclerosis, expression of key hepatic lipid regulatory genes was analyzed by real-time PCR after the mice were fed the PUFA diet for 16 weeks. Hepatic expression of fatty acid and TG synthesis-related genes, such as sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC-1), and stearoyl-CoA desaturase-1, were decreased in DK mice (Fig. 4A). mRNA level of cholesterol synthetic regulatory genes, such as SREBP-2 and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthetase and reductase, also trended lower in DK mice. Acyl-CoA oxidase 1 and carnitine palmitoyltransferase 1, two genes involved in regulation of fatty acid β-oxidation, had similar expression in both genotypes of mice. Consistent with the decreased hepatic mRNA expression of lipogenic genes, FAS and stearoyl-CoA desaturase-1 protein expression was 56% and 33% lower in DK compared with SK mice (Fig. 4B, C). Expression of proteins involved in removal of cholesterol from the liver, such as ATP-binding cassette transporter A1, liver X receptor...
after mice were fed the PUFA diet for 16 weeks. Plasma VLDL from both genotypes of mice were similar in size (49 nm diameter), but VLDL from DK mice were relatively enriched in TG (45% vs. 36% of total mass) and poor in CE (23% vs. 33%) compared with those isolated from SK mice (Supplementary Table II). Because the core lipid compositions were different, VLDLs from SK and DK mice were used as tracers for the turnover studies. VLDLs from DK and SK mice were radiolabeled with $^{131}$I and $^{125}$I, respectively, mixed together, and injected into PUFA-fed recipient mice intravenously. $^{131}$I-VLDL tracer from DK mice was cleared significantly faster ($P < 0.05$) from plasma in both genotypes of recipient mice compared with $^{125}$I-VLDL tracer from SK mice (Supplementary Fig. I; FCR, 2.0 ± 0.3 vs. 1.0 ± 0.2 pools/day in SK recipients and 2.81 ± 0.45 vs. 1.85 ± 0.18 pools/day in DK recipients, n = 5). There was also a trend toward faster plasma clearance of both VLDL tracers in DK recipient mice that reached statistical significance for the $^{125}$I-VLDL tracer from SK mice. These data support the conclusion that 12/15LO deficiency in PUFA-fed mice results in hypercatabolism of plasma VLDL particles, which partially explains the reduced plasma apoB lipoprotein concentrations in DK mice.

α, and scavenger receptor B1, was similar between the two genotypes (Fig. 4B, C).

VLDL secretion is reduced in PUFA-fed DK mice

Decreased liver lipid synthetic gene and protein expression in PUFA-fed DK mice suggested that lower plasma lipid levels might result from decreased hepatic lipid synthesis and secretion. To investigate this possibility, we measured the VLDL secretion rate in vivo after intravenous injection of detergent to inhibit TG lipolysis in mice fed the PUFA diet for 12 weeks. TG secretion rates were measured as an increase in plasma TG concentration (Fig. 5A, C) or as $^3$H-oleate incorporation into plasma TG (Fig. 5B and D); both measures of TG production were significantly reduced in DK mice. There was a significant reduction in apoB 48 secretion rate (Fig. 5G) and a trend toward decreased apoB100 secretion rate (Fig. 5F) in PUFA-fed DK vs. SK mice.

VLDL clearance is increased in PUFA-fed 12/15LO-deficient mice

To evaluate plasma VLDL clearance rate as a possible mechanism for reduced plasma VLDL and LDL concentrations in DK mice, VLDL turnover studies were performed after mice were fed the PUFA diet for 16 weeks. Plasma VLDL from both genotypes of mice were similar in size (~49 nm diameter), but VLDL from DK mice were relatively enriched in TG (45% vs. 36% of total mass) and poor in CE (23% vs. 33%) compared with those isolated from SK mice (Supplementary Table II). Because the core lipid compositions were different, VLDLs from SK and DK mice were used as tracers for the turnover studies. VLDLs from DK and SK mice were radiolabeled with $^{131}$I and $^{125}$I, respectively, mixed together, and injected into PUFA-fed recipient mice intravenously. $^{131}$I-VLDL tracer from DK mice was cleared significantly faster ($P < 0.05$) from plasma in both genotypes of recipient mice compared with $^{125}$I-VLDL tracer from SK mice (Supplementary Fig. I; FCR, 2.0 ± 0.3 vs. 1.0 ± 0.2 pools/day in SK recipients and 2.81 ± 0.45 vs. 1.85 ± 0.18 pools/day in DK recipients, n = 5). There was also a trend toward faster plasma clearance of both VLDL tracers in DK recipient mice that reached statistical significance for the $^{125}$I-VLDL tracer from SK mice. These data support the conclusion that 12/15LO deficiency in PUFA-fed mice results in hypercatabolism of plasma VLDL particles, which partially explains the reduced plasma apoB lipoprotein concentrations in DK mice.
BM transplanted into DK recipients (DK→DK) resulted in significantly reduced TPC and TG concentrations compared with SK recipients transplanted with SK BM (SK→SK) (Fig. 6A–D), similar to results observed for non-transplanted SK and DK mice (Fig. 2A–D). However, when SK or DK mice received BM from the opposite genotype (i.e., SK→DK or DK→SK), plasma lipid levels were intermediate between those of SK→DK and DK→DK recipients. This was also the case when we evaluated liver TG secretion using the detergent block method (Fig. 6E–F). These data suggested that transplanted BM cells could only partially explain the hepatic and plasma lipid lowering observed in PUFA-fed DK mice.

Atherosclerosis, measured as a percentage of aortic surface lesion area, was reversed by transplantation of DK BM

Transplantation of DK mouse BM into SK mice reversed atherosclerosis but only partially restored plasma lipid phenotype

Our data indicated that the lower TPC and TG in PUFA-fed DK mice was the result of altered hepatic lipid metabolism. However, 12/15LO is mainly expressed in BM-derived cells, such as macrophages (3, 4). We also could find no detectable 12/15LO mRNA expression in isolated hepatocytes from SK mice, but we found abundant expression in Kupffer cells of SK vs. DK mice (Supplementary Fig. II). We hypothesized that macrophages were affecting hepatic lipid metabolism, presumably by secreting a 12/15LO product that directly or indirectly affected hepatic lipid metabolism. To investigate this possibility, we transplanted BM from SK and DK donor mice into lethally irradiated SK and DK recipient mice. More than 98% of the hematopoietic cells in the circulation were replaced by the donor BM 4 weeks after transplantation (Supplementary Fig. III). Eight weeks after transplantation, mice were fed the PUFA diet for 16 weeks to induce atherosclerosis. DK

Fig. 5. Decreased VLDL secretion in PUFA-fed DK mice. Plasma TG (A) and 3H-TG (B) accumulation in plasma was measured after intravenous injection of detergent to inhibit lipolysis and 35S-Met/Cys and 3H-oleic acid to trace newly synthesized apolipoprotein and TG, respectively (mean ± SEM; n = 7). The line of best fit was determined by linear regression analysis. C, D: Plasma TG accumulation rate during detergent block of lipolysis was calculated for each mouse from the linear regression analysis of the time vs. TG concentration or 3H-TG plot (mean ± SEM; n = 7). E: Representative phosphorimager exposure of 35S-radiolabeled apoB at different time points after detergent injection. F, G: The density of each band in panel E was quantified, expressed as arbitrary units (AU), and plotted against time. ApoB secretion rate was calculated as described in panels A and B (mean ± SEM; n = 4). * P < 0.05. SK = LDLr KO; DK = 12/15LO/LDLr double KO.

Fig. 6. Transplantation of DK bone marrow does not recapitulate plasma lipid phenotype of PUFA-fed DK mice. Eight weeks after bone marrow transplantation, mice were started on the PUFA-enriched atherogenic diet for 16 weeks before termination for atherosclerosis evaluation. The first two letters denote donor genotype; the last two letters indicate recipient mouse genotype (i.e., SK→DK = SK donor BM into DK recipient). A, C: Blood samples were collected periodically during the atherosclerosis progression phase to measure TPC (A) and TG (C) concentrations (mean ± SEM; n = 7–8 mice). B, D:) Area under the curve for each animal was calculated from the TPC and TG data in panels of A and C, respectively. E: Plasma TG concentration at different time points after detergent injection to inhibit TG lipolysis (mean ± SEM; n = 7). The line of best fit, determined by linear regression analysis, is shown for each group of BM-transplanted mice. F: Each value represents the average TG secretion rate (mean ± SEM), calculated from the slope of linear regression plots of seven individual mice in each BM transplantation group. Values with different letters are significantly different (P < 0.05). SK = LDLr KO; DK = 12/15LO/LDLr double KO.
between Kupffer cell 12/15LO expression and lipid metabolism in vivo, a GdCl\textsubscript{3} solution was injected into 16-week PUFA-diet fed DK and SK mice three times at 4-day intervals to ablate hepatic Kupffer cells. Plasma cholesterol and TG concentrations were measured periodically for up to 32 days after the first GdCl\textsubscript{3} injection. Plasma cholesterol and TG levels in SK mice began to decrease 6 days after the first GdCl\textsubscript{3} injection (Fig. 8). The decrease in plasma lipid values continued for 12–14 days and then began to return to preinjection values, coincident with the reported time course of Kupffer cell repopulation by BM (34). Thirty-two days after the first injection, plasma lipid levels had recovered to preinjection values. Plasma lipid concentrations of DK mice did not change after GdCl\textsubscript{3} injection. These results support our hypothesis that Kupffer cells expressing 12/15LO regulate hepatic lipid metabolism, raising plasma cholesterol and TG concentrations, whereas ablation of Kupffer cells not expressing 12/15LO (i.e., DK) had no effect on plasma lipids.

Plasma lipid concentrations are decreased with ablation of Kupffer cells in SK mice

The BM transplantation experiment partially supported our hypothesis that 12/15LO expression in macrophages plays a role in regulation of liver lipid metabolism. However, resident macrophages, in general, and Kupffer cells, in particular, require a long time for complete replacement after BM transplantation (33). Incomplete replacement of Kupffer cells by donor BM in our study might explain why liver and plasma lipid levels were not completely reversed by BM replacement. GdCl\textsubscript{3} is a reagent that is specifically taken up by Kupffer cells and induces Kupffer cell apoptosis, resulting in temporary ablation of Kupffer cells (34). To further study the relationship between Kupffer cell 12/15LO expression and lipid metabolism in vivo, a GdCl\textsubscript{3} solution was injected into 16-week PUFA-diet fed DK and SK mice three times at 4-day intervals to ablate hepatic Kupffer cells. Plasma cholesterol and TG concentrations were measured periodically for up to 32 days after the first GdCl\textsubscript{3} injection. Plasma cholesterol and TG levels in SK mice began to decrease 6 days after the first GdCl\textsubscript{3} injection (Fig. 8). The decrease in plasma lipid values continued for 12–14 days and then began to return to preinjection values, coincident with the reported time course of Kupffer cell repopulation by BM (34). Thirty-two days after the first injection, plasma lipid levels had recovered to preinjection values. Plasma lipid concentrations of DK mice did not change after GdCl\textsubscript{3} injection. These results support our hypothesis that Kupffer cells expressing 12/15LO regulate hepatic lipid metabolism, raising plasma cholesterol and TG concentrations, whereas ablation of Kupffer cells not expressing 12/15LO (i.e., DK) had no effect on plasma lipids.

Profile of lipid mediators produced by activated macrophages

Because 12/15LO enzymatic activity generates bioactive lipid mediators, we profiled the lipid mediator content of macrophages (cells + conditioned media) 20 min after stimulation with the Ca\textsuperscript{2+} ionophore A23187. 12-HETE, the direct product of 12/15LO, was nearly absent in 12/15LO-deficient macrophages (Supplementary Table III). By comparison, nonenzymatic generation of 15-HETE was low and similar for both genotypes of macrophages. However, 5-HETE, the product of 5LO, was doubled in medium from 12/15LO-deficient macrophages compared with SK-conditioned medium, suggesting that PUFA substrate shunts to 5LO in the absence of 12/15LO. PGE\textsubscript{2} and...
PGF$_{2\alpha}$ levels were also doubled in 12/15LO-deficient macrophages, whereas the LTB$_{4}$ level was 50% lower. Thus, 12/15LO expression altered multiple bioactive lipid mediators generated from macrophages of mice fed the PUFA diet.

**Secretion products from DK macrophages attenuate TG secretion from McArdle cells**

Results from the BM transplantation and Kupffer cell ablation studies support our hypothesis that macrophage 12/15LO expression regulates hepatic lipid metabolism. We hypothesized that this likely resulted from a secretion product of macrophages that affected hepatic lipid synthesis/secretion. To test this concept, resident peritoneal macrophages were isolated from SK and DK mice fed the PUFA diet for 12 weeks. Conditioned medium was prepared by culturing the macrophages overnight after an acute (20 min) stimulation with Ca$^{2+}$ ionophore (A23187) to activate the macrophages. The macrophage conditioned medium was then added to McArdle cells, along with $^3$H-TG, to stimulate McArdle cell TG secretion (20 min) stimulation ± Ca$^{2+}$ ionophore (A23187) to activate the macrophages. The macrophage conditioned medium was then added to McArdle cells, along with $^3$H-oleate and 0.8 mM oleate to stimulate VLDL TG secretion, and $^3$H-TG in the medium was quantified after a 4 h incubation. Macrophage-conditioned medium from both genotypes stimulated McArdle cell TG secretion compared with unconditioned medium (Fig. 9). However, McArdle cells receiving conditioned medium from DK macrophages secreted less TG than those receiving conditioned medium from SK macrophages, suggesting that secretory products from SK macrophages stimulated McArdle cell TG secretion more than those of DK macrophages.

**DISCUSSION**

The role of 12/15LO expression in atherosclerosis development is controversial, with 12/15LO expression reported as pro- and antiatherogenic (3, 5, 9–17). However, no studies have been conducted in the context of 12/15LO substrate fatty acid enrichment. In this study, we demonstrate that DK mice fed a PUFA diet have reduced TPC and TG concentrations, hepatic cholesterol content, and atherosclerosis compared with SK mice with intact 12/15LO expression. The decrease of TPC in PUFA-fed DK mice that drives the reduction in atherosclerosis is primarily due to decreases in plasma VLDL and LDL cholesterol resulting from decreased hepatic lipogenic gene expression and VLDL TG secretion as well as increased VLDL turnover. BM transplantation and Kupffer cell ablation studies suggested that 12/15LO activity has different pathological outcomes depending on where it is expressed, with circulating leukocytes primarily affecting the atherosclerosis outcome, with minimal impact on plasma lipids, whereas Kupffer cells affected plasma lipid levels. Finally, macrophages from DK relative to SK mice fed the PUFA diet lack one or more components responsible for increased TG secretion in McArdle cells. These results suggest a dietary fatty acid-gene interaction for 12/15LO that affects plasma and hepatic lipid metabolism as well as atherosclerosis.

In this study, we show that 12/15LO expression is proatherogenic, in agreement with other studies (3, 5, 9–15). The increased atherosclerosis with 12/15LO expression in previous studies was not accompanied by a change in plasma cholesterol concentrations. We also observed a reduction in atherosclerosis in DK mice fed the Sat/mono diet that was independent of plasma cholesterol changes, in agreement with previous studies. A potential explanation for decreased atherosclerosis in the absence of plasma cholesterol changes is that 12/15LO expression primarily affects oxidation and inflammation in the artery wall. Cyrus et al. (12) reported that 12/15LO promoted atherosclerosis by increasing in vivo oxidative status. Additional support for this idea came from another study in which vitamin E, an antioxidant, attenuated atherosclerosis in SK mice to the same level as DK mice not treated with vitamin E (15). 12/15LO was also reported to increase endothelial expression of intercellular adhesion molecule 1, which in turn increased the recruitment of monocytes to atherosclerotic lesions, exacerbating the disease (2, 14).

However, Merched et al. (16) observed that 12/15LO expression systemically or in BM protected against atherosclerosis and proposed that the anti-inflammatory products of 12/15LO, such as lipoxins, resolvins, and protectins, protect mice from the development of atherosclerosis by dampening inflammation. Past studies have not used diets enriched in PUFA, which are the substrate fatty acids for 12/15LO generation of bioactive lipid mediators, which can be pro- or anti-inflammatory (18–20). In our study, dietary PUFA resulted in additional reduction of atherosclerosis in the absence of 12/15LO expression beyond that observed with a Sat/mono diet, suggesting a dietary fatty acid-gene interaction that resulted in this outcome. The additional reduction in atherosclerosis was directly attributable to a significant reduction in plasma VLDL and LDL cholesterol concentrations because there was a strong positive association between total plasma cholesterol and aortic surface lesion area (Fig. 2E). These results suggest another environmental variable, dietary fatty acid composition, to consider in interpreting the role of 12/15LO in
the pathogenesis of diseases such as atherosclerosis and obesity (35).

PUFA-fed DK mice had lower hepatic neutral lipid (TG and CE) content and hepatic lipogenic gene expression compared with their SK counterparts. A recent study of chow and high fat diet-fed 12/15LO knockout mice in the apoE knockout background also reported decreased hepatic Oil Red-O staining compared with apoE knockout controls and decreased expression of FAS but not SREBP-1c; however, no significant difference in total serum cholesterol or TG concentrations was observed (36). Our hepatic TG results are in agreement with this published study as our liver TG content was lower in DK vs. SK mice regardless of diet (Fig. 3), suggesting that dietary PUFA enrichment is not necessary for the reduced hepato steatosis in the absence of 12/15LO expression, whereas it is for decreased plasma cholesterol and TG concentrations.

The mechanisms by which 12/15LO expression affected plasma lipid levels in PUFA-fed mice in our study involved both production and catabolism. The reduced plasma lipid concentrations in PUFA-fed DK mice were due to reduced VLDL and LDL concentrations (Fig. 2F). Detergent block studies demonstrated reduced plasma accumulation of VLDL particles (i.e., apoB) and TG in PUFA-fed DK mice compared with SK mice (Fig. 5). Because the detergent block studies were performed in fasted mice, the reduced secretion was likely due to decreased hepatic, not intestinal, secretion. In addition, there was an increase in particle turnover for DK VLDL tracer in both genotypes of recipient mice, suggesting that the composition of the VLDL particle was affecting its clearance. The increased TG content in DK VLDL (Supplementary Table II) may have resulted in smaller VLDL remnants after intravascular TG lipolysis, with subsequent accelerated particle clearance from plasma.

Because the lipid phenotype of the PUFA-fed DK mice implicated decreased liver lipid production and 12/15LO is mainly expressed in macrophages, not hepatocytes (3) (Supplementary Fig. II), we hypothesized that macrophage 12/15LO expression was altering a secretory product(s) that affected hepatic lipid synthesis. Although transplantation of homologous BM (i.e., SK→SK or DK→DK) recapitulated the plasma phenotype of nontransplanted mice, heterologous BM transplantation (SK→DK and DK→SK) resulted in an intermediate phenotype (Fig. 6). Resident liver macrophages (i.e., Kupffer cells) are replaced slowly after BM transplantation (33), likely resulting in a chimeric (i.e., donor and recipient) Kupffer cell population after transplantation. Thus, failure to recapitulate the nontransplanted mouse lipid phenotype may be due to chimeric Kupffer cells exerting a paracrine effect on hepatic lipid synthesis. In support of this concept, ablation of Kupffer cells exerting a paracrine effect on hepatic lipid metabolism in a paracrine manner that may be related to inflammatory cytokines.

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