Oxidative Stress Is Markedly Elevated in Lecithin:Cholesterol Acyltransferase-deficient Mice and Is Paradoxically Reversed in the Apolipoprotein E Knockout Background in Association with a Reduction in Atherosclerosis*

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Complete lecithin:cholesterol acyltransferase (LCAT) deficiency is a rare cause of severe hypoalphalipoproteinemia, but the affected subjects are surprisingly not particularly prone to premature coronary heart disease. We studied oxidative stress in lcat−/− mice and their cross-bred with apolipoprotein-E knockout mice (apoE−/−x lcat−/−) by measuring vascular ring superoxide production and plasma phospholipid (PL)-bound F2-isoprostane levels and their relationship with aortic atherosclerosis. Compared with wild type control (lcat+/+), lcat−/− and lcat+/− mice showed a 4.9 (\(p = 0.003\)) and a 2.1-fold (\(p = 0.04\)) increase in plasma PL-F2-isoprostane levels, respectively. There was also a 3.6- (\(p < 0.0001\)) and 2.9-fold (\(p = 0.003\)) increase in the area under the curve for the aortic ring superoxide excursion by lucigenin-derived chemiluminescence. A comparison of apoE−/−x lcat+/+ mice with wild type control mice showed a more modest 2.1- (\(p = 0.04\)) and 2.2-fold (\(p < 0.00001\)) increase in these respective markers. Surprisingly, the apoE−/−x lcat−/− mice showed a paradoxical normalization in both oxidation markers. Furthermore, by fast protein liquid chromatography separation, we observed an associated retention and redistribution of serum paraoxonase activities to the non-high density lipoprotein fractions in both the apoE−/−x lcat−/− and apoE−/−x lcat+/+ mice. Aortic atherosclerotic lesions in male apoE−/−x lcat−/− and apoE−/−x lcat+/+ mice were reduced by 52 (\(p = 0.02\)) and 24% (\(p = 0.46\)), respectively. Our data suggest that LCAT-deficient mice are associated with an increased oxidative stress that is paradoxically reversed in a hyperlipidemic background, possibly due to the redistribution of paraoxonase. This modulation of oxidative stress may in part contribute to the reduced atherosclerosis seen in the apoE−/−x lcat−/− mice.

Lecithin:cholesterol acyltransferase (LCAT)1 plays a central role in the reverse cholesterol transport process by mediating the esterification of tissue-derived free cholesterol (FC) and is responsible for the majority of esterified cholesterol (CE) in the circulation (1). Subjects with LCAT deficiency as a result of mutations of the LCAT gene invariably develop severe HDL deficiency, but surprisingly, these subjects do not seem to be particularly prone to premature coronary heart disease (2). The role of LCAT in atherosclerosis remains controversial.

Several lines of experimental evidence suggest that HDL may partially confer its anti-atherogenic action as an antioxidant through activities of its associated enzyme, paraoxonase (PON1) (3). Recent studies on PON1 ko mice (4, 5) suggest that PON1 plays a major role as antioxidant in the prevention of atherosclerosis. The PON1 ko mice were found to develop accelerated atherosclerosis with and without the apoE deficiency background. We reported recently (6) that LCAT-deficient mice have significantly lower levels of plasma PON1 arylesterase activities. A number of in vitro studies have also suggested that the LCAT enzyme itself may have intrinsic anti-oxidant properties (7, 8). It is therefore of considerable interest to understand better the in vivo effect of LCAT deficiency on oxidative stress and atherosclerosis in this mouse model.

Vascular oxidant stress due to superoxide anion (O2−) and other reactive oxygen species has been implicated in the development of atherosclerosis (9, 10), possibly through being a major source of free radicals in the oxidative modification of low density lipoproteins (LDL) in the arterial wall (11). Excessive production of vascular O2− also attenuates the bioavailability of endothelial derived nitric oxide (NO), which may contribute to endothelial dysfunction and atherosclerosis (12). Increasing evidence, both in vivo and in vitro, suggests that NAD(P)H oxidase is a major contributor to the generation of O2− anions in the vascular wall (9, 13). In addition to oxidized lipids, the NAD(P)H oxidase activity has also been shown to be modulated by a number of vasoactive peptides, cytokines, and growth factors (9).

F2-isoprostanes are chemically stable prostaglandin isomers

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‡ The abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; apoE, apolipoprotein E; ko, knockout; LDL-CL-AUC, lucigenin-derived chemiluminescence-area under the curve; PL-F2-isop, phospholipid (glycerol-phosphocholine)-bound F2-isoprostane; PON, paraoxonase; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; FC, free cholesterol; CE, cholesterol ester; FPLC, fast protein liquid chromatography; ANOVA, analysis of variance; PAF-AH, platelet-activating factor acetylhydrolase; VLDL, very low density lipoprotein; LpX, lipoprotein X.

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that result from non-enzymatic, free radical-mediated oxidative modification of arachidonic acid. These compounds can be found in tissues and many other body fluids including plasma and urine. These analyses have been shown to be excellent in vivo markers of oxidative stress both in human and in animal models (14).

In this study, by using the LCAT ko mouse model, we tested the hypothesis that LCAT deficiency is associated with increased oxidative stress by measuring plasma levels of glycerophosphocholine-bound F2-isoprostanes (PL-F2-isoP) and vascular O2 oxidative stress by measuring plasma levels of glycerophosphocholine-bound F2-isoprostanes (PL-F2-isoP) and vascular O2

**EXPERIMENTAL PROCEDURES**

**Materials**—Lucigenin, NADH, CuSO4, and butylated hydroxytoluene were purchased from Sigma, and coelenterazine was purchased from Molecular Probes (Cedarlane Lab, Ontario, Canada). Free cholesterol, phospholipid, and cholesterol kits were from Wako (Neuss, Germany).

**Animals**—Lcat−/− mice were created in Dr. Rubin’s laboratory as reported previously (6, 15). ApoE−/−/xcat−/− mice were generated by first cross-breeding apoE−/− mice in C57BL/6 background (The Jackson Laboratory) with lcat−/− mice to yield apoE−/−/xcat−/−. Brother-sister matings of the F1 double heterozygous siblings were carried out. ApoE−/−/xcat−/+ were selected by PCR screening for subsequent se-rial breeding for 5 generations. All studies were carried out with the mice being fed a chow diet, and litters were used as controls. All mouse protocols were approved by the Animal Care Committee at St. Michael’s Hospital, Toronto, Canada.

**Plasma Lipid Analyses**—Plasma lipid analyses were performed on mice 7–10 weeks of age. Plasma was obtained as described previously (6). The 1.019–1.063 g/ml ("LDL") fraction was obtained through ultra-centrifugation (16). Fast protein liquid chromatography with on-line electrospray/mass spectrometry as described previously (20).

**Lipid Analyses**—Lipoprotein analyses of the LCAT-deficient mice in this report were carried out as described previously (20).

**Aortic Atherosclerosis Measurement**—Measurements of aortic atherosclerotic lesions in mice was carried out by the en face method (22). Male mice in apoE ko background fed a chow diet were sacrificed at 8–9 months of age. Aortae were dissected out intact from the aortic root to the femoral bifurcation, fixed overnight with formalin, followed by staining with Oil Red-O. Digital images were obtained with a Nikon Coolpix 880 camera (Nikon, Ontario, Canada). The Scion imaging soft-ware (Scion Inc.) was used to compute the lesion area, and all results were means of triplicate measurements. The severity is expressed as percent surface occupied by Oil Red-O-stained lesions.

**RESULTS**

**Production of the lcat−/−/xapoE−/− Double Knockout Mice**—ApoE−/−/xcat−/+ parents were used to generate off-springs with genotypes apoE−/−/xcat−/+ and apoE−/−/xcat−/−. The double ko mice grew normally with no apparent physical defects.

**Lipid Analyses**—Lipoprotein analyses of the LCAT-deficient mice are summarized in Table I. The lipoprotein profile of the lcat−/+ and lcat−/− mice agreed with those reported previously (15). In the apoE−/− background, LCAT deficiency preserved the severely elevated plasma cholesterol. We also observed an LCAT gene dose-dependent decrease in the FC/CE ratio with and without the apoE−/− background. Lipoprotein analyses on the FPLC fractions showed a preservation of the accumulation of "VLDL" fractions in the apoE−/−/xcat−/+ and apoE−/−/xcat−/− mice in comparison with the apoE−/−/xcat−/+ control mice (Fig. 1). HDL-C was also severely re-duced in apoE−/−/xcat−/− mouse plasma, but unlike the lcat−/+ mice, its level was also markedly decreased in the apoE−/−/xcat−/− mice (Table I and Fig. 1). On the other hand, the IDL/IDL shoulder (fraction 8) showed a 23% reduction in the apoE−/−/xcat−/+ mice compared with the apoE−/−/xcat−/+ and apoE−/−/xcat−/+ littermates (Fig. 1), consistent with the significant up-regulation of the LDL receptor found in LCAT ko mice (23).

**PON1 Aryl esterase Activities**—The total plasma PON1 aryl esterase activities in different genotype groups are shown in Fig. 2A. A 46.5% reduction in PON1 activity was noted in the lcat−/+ mice as compared with those of the lcat−/− mice, in agreement with our previous observation (8). Its reduction in the lcat−/+ mice was not significant, suggestive of an autosomal recessive pattern in relation to the lcat mutant allele, which mirrors that of the plasma HDL-C levels. On the contrary, despite significantly lower HDL-C levels in both apoE−/− xcat−/+ and apoE−/−/xcat−/− mice, their total plasma PON1 activities were not significantly different from the apoE−/−.
was determined using LDCL on all six genotype groups (same mice as those in Fig. 1): lcat+/+ (n = 3), lcat+/− (n = 3), and lcat−/− (n = 3); apoE−/− xcat+/+ (n = 6), apoE−/− xcat+/− (n = 4), and apoE−/− xcat−/− (n = 5). Inset, enlargement of the IDL/LDL and HDL fractions.

Fig. 2. a, total plasma paraoxonase (PON1) arylesterase activities in six different genotype groups (same mice as those in Fig. 1): lcat+/+, lcat+/−, lcat−/−, apoE−/− xcat+/+, apoE−/− xcat+/−, and apoE−/− xcat−/−. b, FPLC profile of PON1 arylesterase activities from pooled plasma. Animals used are identical to those in Fig. 1.

xcat+/+ control. Analysis of the PON1 activities in FPLC fractions showed that nearly half of the total plasma PON1 activity eluted in the non-HDL fractions of the apoE−/− xcat−/− mice. A lesser extent of redistribution was also observed in the plasma of apoE−/− xcat+/− mice (Fig. 2B).

Plasma PL-F2-isoprostane (F2-isoP) Levels—Because nearly 90% of the plasma isoprostanes exist in esterified form bound to phospholipids (14), the plasma level of F2-isoprostanes was determined by measuring the content of PL-F2-isop in all six genotypic groups (Fig. 3). Compared with the wild type mice, plasma from lcat−/− mice showed a 4.9-fold increase (p = 0.003) in plasma PL-F2-isop. The lcat+/− mice showed intermediate levels but were not statistically different from those of the lcat−/− mice. In contrast, apoE−/− xcat+/+ mice showed a 2.1-fold increase (p = 0.04) in plasma PL-F2-isop as compared with the wild type mice, in excellent agreement with the 2-fold increase in the plasma level of iPF2α-VI as reported previously (24). Surprisingly, the apoE−/− xcat−/− mouse plasma PL-F2-isop level was normalized. It is also of interest to note that the PL-F2-isop level of apoE−/− xcat+/− mice was comparable with that of the apoE−/− xcat−/− mice, suggestive of an autosomal dominant effect.

CuSO4-induced Oxidizability of LDL—We observed a comparable lag time between the LDL fraction from apoE−/− xcat+/+ and that of a pooled normal human LDL. However, apoE−/− xcat−/− LDL showed a prolongation of the lag time by 60.0 min. The rate of rise of the absorbance and the level of inflection point were similar between the two mouse strains.

Aortic Ring Superoxide Production Using LDCL—Vascular ring O2− production was determined using LDCL on all six genotypic groups of mice (Fig. 4). Compared with lcat+/+ mice, the AUC for lcat−/− mice was found to be increased 3.6-fold (p = 0.00006) and that for lcat+/− mice 2.9-fold (p = 0.003). Likewise, we also observed a 2.2-fold increase (p = 0.00001) in LDL-AUC of the apoE−/− xcat+/+ mice when compared with the lcat+/+ mice. Again, the LDL-AUCs for the apoE−/− xcat−/− mice were paradoxically normalized (1.26-fold change from the lcat+/+ mice, p = 0.1), which paralleled the normalization of the plasma PL-F2-isop in the same strain. The LDL-AUC for apoE−/− xcat+/− mice was found to be subnormal (0.6-fold, p = 0.01). This also correlates well with the normal PL-F2-isoprostane level observed in this same genotypic group as stated above.

The validity of using lucigenin at high concentrations raised concern because of the potential for superoxide recycling, and this potential artifact is absent when using coelenterazine as a marker (25). All but one genotype group of mice were evaluated using coelenterazine as a superoxide sensor with n ≥ 3 in each group. We observed a near-unity correlation (r² = 0.9265, p = 0.00006) between the LDCL-AUC and coelenterazine-derived chemiluminescence-AUC, thus validating the use of lucigenin.

Gene-Gene Interactions on Oxidative Markers and PON1 Activity—Two-way ANOVA analyses on the six genotypic groups showed strong interaction between lcat and apoE alleles on LDL-AUC, F2-isop, and PON1 activities with their respective F values being 29.19 (p < 0.0001), 10.5 (p = 0.0003), and 22.73 (p < 0.0001). Correlation between LDL-AUC and PL-F2-isop showed an r² = 0.921 (p = 0.0024), but the r² for PON1 activity versus LDL-AUC and PON1 versus PL-F2-isop were 0.0582 and 0.1648, respectively, both being statistically insignificant.

Aortic Atherosclerosis Assay—Aortic lesion quantification using the en face method showed a 52% reduction (3.62 ± 0.96% versus 7.44 ± 3.35%; p = 0.02) in lesion area in the apoE−/− xcat−/− mice (n = 7) as compared with the apoE−/−
production. When bred into the apoE production in both apoE
reduction but was not statistically significant from either of the
abilities, although the latter two are affected in an autosomal
association with an inverse trend in both HDL-C and PON1 activ-
LDCL-AUC in the lcat mutant allele in an autosomal dominant fashion, in asso-
Fig. 5. En face aortic atherosclerosis quantitation in male
findings by Shih et al. (4, 5). The diverging effect of the lcat
mice is modulated by a significant reduction in serum PON3 levels and activities, contributing to the marked increase in oxidative stress seen in the lcat−/− mice. Platelet-activating factor acetylhydrolase (PAF-AH) is a 45-kDa protein secreted primarily by macrophages and circulates in both LDL and HDL in humans but exclusively in HDL in mice. This enzyme has the ability to hydrolyze not only PAF but also oxidized phospholipids (28).

Recent studies (29) demonstrated that overexpression of circulating PAF-AH in apoE knockout mice results in a reduction in oxidized β-VLDL and atherosclerosis. We reported previously (6) that PAF-AH activity was significantly reduced in the lcat−/− mice. Although the in vivo effect of PAF-AH deficiency in mice has not yet been reported, it is conceivable that a reduced plasma level of PAF-AH activity may contribute to the overall oxidative stress.

The lipid profiles of the apoE−/−xlcat+/+ and apoE−/−xlcat−/− mice were both characterized by a preservation of the severe hyperlipidemia of the apoE−/−xcat+/+ control. In the apoE ko background, the effect of lcat mutant allele on HDL-C, PON1 activities, and the oxidation markers are even more divergent. The lcat mutant gene dose leads to a lowering of HDL-C in an autosomal dominant fashion but has no effect on PON1 activity (Table I and Fig. 2). The surprising finding in the double ko mice is the paradoxical normalization of plasma PON3 levels and activities compared with its age- and sex-matched apoE−/−xcat−/− control. This is in agreement with that reported by Lambert et al. (23) despite a difference in genetic background. The apoE−/−xlcat+/+ (n = 8) showed a 24% reduction but was not statistically significant from either of the other two groups (Fig. 5).

**DISCUSSION**

In this paper, we report the observation of marked enhancement in oxidative stress in chow-fed lcat−/− mice using two independent measurements, namely plasma PL-F2-isoP level and aortic ring O$_2^-$ production. When bred into the apoE−/− mouse background, we observed a paradoxical normalization of the same oxidative markers. This paradoxical change was found to be associated with a retention and redistribution of PON1 arylesterase activities into the non-HDL fractions based on FPLC separation. Furthermore, the normalization of the oxidative markers in the apoE−/−xcat−/− mice was also found to be associated with a significant reduction in atherosclerotic lesions in the male apoE−/−xcat−/− mice as compared with its age- and sex-matched apoE−/−xcat−/+ control.

We present the first in vivo evidence showing a marked increase in plasma PL-F2-isoP level and aortic O$_2^-$ production (LDCL-AUC) in the lcat−/− mice. The increase is related to the lcat mutant allele in an autosomal dominant fashion, in association with an inverse trend in both HDL-C and PON1 activities, although the latter two are affected in an autosomal recessive fashion. Despite being severely hypolipidemic, the increases in both oxidation markers in the lcat−/− mice are substantially higher than those in the extremely hyperli-

![Image](https://via.placeholder.com/150)

**FIG. 4. Murine vascular ring superoxide production.** a, LDCL lcat+/+ (n = 8), lcat+/− (n = 4), and lcat−/− (n = 6); b, apoE−/−xlcat+/+ (n = 11), apoE−/−xlcat+/− (n = 8), and apoE−/−xlcat−/− (n = 7); c, bar graph of LDCL-AUC, comparing the same six genotypes.

**FIG. 5.** En face aortic atherosclerosis quantitation in male apoE−/−xlcat+/+ (n = 11), apoE−/−xlcat+/− (n = 8), and apoE−/−xlcat−/− (n = 7) mice sacrificed at 8–9 months.
The mechanism by which the non-HDL-associated PON1 may impart antioxidative action is unclear. The protective role of PON1 against Cu\(^{2+}\)-induced oxidation of LDL has been well established (26, 35). We observed a reaction of the 1.019–1.063 g/ml plasma fraction with the apoE–/– xcat–/– mice, we isolated LpX on the basis of an identification of an FC-, PL-rich, and CE-poor VLDL-like Superose peak in the 1.019–1.063 g/ml plasma fraction (33). In this plasma fraction, a small but definite quantity of PON1 activity co-eluted with this peak. However, inconsistent recovery of PON1 activity after ultracentrifugation precluded a more quantitative measurement. For the same reason, current data do not exclude association of PON1 with other non-HDL lipoprotein classes. Nonetheless, the redistribution of PON1 observed should be considered as one of the most biologically plausible explanations for the paradoxical normalization of oxidative stress in the double knockout mice. Similarly, redistribution of PON3 and/or PAF-AH in LCAT-deficient mice should also be considered. In the case of PON3, a redistribution of the protein to non-HDL particles has not been reported. Due to the lack of a specific assay for murine plasma PON3 (26), this possibility cannot be determined with certainty in the apoE–/– xcat–/– mice. In humans, it is the PAF-AH that is associated with circulating LDL that is biologically active. It has been shown that human PAF-AH associates with the C terminus portion of apoB, and the sequences mediating this binding are altered in murine PAF-AH, consistent with its absence from murine apoB lipoproteins (34). It is therefore unlikely that PAF-AH would associate with non-HDL in the apoE–/– xcat–/– mice.

The two oxidative markers, mean LDL-AUC and PL-F2-isop, are both inversely related to mean plasma PON1 activities among the six groups. However, based on the opposite effects of the two apoE alleles on the lac heterozygotes, the correlations between them are poor as expected. This is largely the result of the diverge influence of apoE genotype on the lac heterozygotes, as reflected by the strong gene–gene interactions based on the two-way ANOVA analyses. Exclusion of these two heterozygous groups would have unmasked a remarkably linear inverse relationship (r\(^2\) = 0.85 and p < 0.05) in both cases, underscoring the complexity of gene–gene interactions on oxidative stress in heterozygous LCAT-deficient mice. In addition, the PON1 in the HDL and non-HDL fractions are bound to very different lipoproteins, and their antioxidant activities may be differentially modulated, further contributing to the poor correlation.

Recent studies suggest that the NADPH oxidase system is a major barrier of arterial O\(_2^-\) (13), and oxidized LDL has been shown to be one of the potent stimulators of this enzyme (9). The strong correlation between PL-F2-isop and aortic O\(_2^-\) production in the LCAT-deficient mice therefore suggests a direct mechanistic link. It is conceivable that aortic vessel wall NADPH oxidase activity and circulating oxidized lipids may be fueling a self-perpetuating cycle, establishing a unique steady state for each genotype. The resistance of the apoE–/– xcat–/– mouse LDL to Cu\(^{2+}\)-induced oxidation may have contributed to the attenuation of the fueling of this cycle and the normalization of the oxidative markers in these mice.

Oxidative stress has been shown to be important in mouse models of atherosclerosis. In apoE ko mice, oral supplementation with vitamin E resulted in concomitant reductions in plasma, urinary, and arterial isoprostane levels in association with a significant reduction in aortic atherosclerosis, without altering the plasma cholesterol level (24). In the present report, our finding of a significant reduction in spontaneous aortic atherosclerosis in male apoE–/– xcat–/– mice, when compared with that of age- and gender-matched apoE–/– xcat+/+ mice, is in agreement with Lambert et al. (23) despite a difference in genetic backgrounds. Although we found a 23% reduction in plasma IDL and LDL levels in our apoE–/– xcat–/– mice, the extent is unlikely to account completely for the normalization of the aortic O\(_2^-\) production. Our data therefore suggest that the increased resistance of the LDL to oxidative modification in the apoE–/– xcat–/– mice is likely important for the observed reduction in atherosclerotic lesions, and this may in turn be a result of the retention and redistribution of PON1 in the hyperlipidemic LCAT-deficient mice. Furthermore, the non-linearity in the association between plasma PON1 activities and the oxidative stress markers in the heterozygous LCAT-deficient mice suggests that the role of the antioxidants, including PON1, in atherosclerosis is complex in LCAT deficiency.

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LCAT Deficiency, Oxidation, and Atherosclerosis

11719
LCAT Deficiency, Oxidation, and Atherosclerosis

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