Coronary Artery Disease–Associated LIPA Coding Variant rs1051338 Reduces Lysosomal Acid Lipase Levels and Activity in Lysosomes

Gavin E. Morris, Peter S. Braund, Jasbir S. Moore, Nilesh J. Samani, Veryan Codd, Tom R. Webb

Objective—Genome-wide association studies have linked variants at chromosome 10q23 with increased coronary artery disease risk. The disease-associated variants fall in LIPA, which encodes lysosomal acid lipase (LAL), the enzyme responsible for lysosomal cholesteryl ester hydrolysis. Loss-of-function mutations in LIPA result in accelerated atherosclerosis. Surprisingly, the coronary artery disease variants are associated with increased LIPA expression in some cell types. In this study, we address this apparent contradiction.

Approach and Results—We investigated a coding variant rs1051338, which is in high linkage disequilibrium ($r^2=0.89$) with the genome-wide association study lead–associated variant rs2246833 and causes a nonsynonymous threonine to proline change within the signal peptide of LAL. Transfection of allele-specific expression constructs showed that the risk allele results in reduced lysosomal LAL protein ($P=0.004$) and activity ($P=0.005$). Investigation of LAL localization and turnover showed the risk LAL protein is degraded more quickly. This mechanism was confirmed in disease-relevant macrophages from individuals homozygous for either the nonrisk or risk allele. There was no difference in LAL protein or activity in whole macrophage extracts; however, we found reduced LAL protein ($P=0.02$) and activity ($P=0.026$) with the risk genotype in lysosomal extracts, suggesting that the risk genotype affects lysosomal LAL activity. Inhibition of the proteasome resulted in equal amounts of lysosomal LAL protein in risk and nonrisk macrophages.

Conclusions—Our findings show that the coronary artery disease–associated coding variant rs1051338 causes reduced lysosomal LAL protein and activity because of increased LAL degradation, providing a plausible causal mechanism of increased coronary artery disease risk.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1050-1057. DOI: 10.1161/ATVBAHA.116.308734.)

Key Words: coronary artery disease | genome-wide association study | lipase | macrophage

Coronary artery disease (CAD), the most common cause of death worldwide, is caused by the build-up of atherosclerotic plaques within the artery wall. Plaques develop from endothelial damage, with subsequent inflammatory cell infiltration from circulating blood and migration/proliferation of surrounding structural cells, including medial vascular smooth muscle cells into the vessel’s intima. Initial extracellular lipid deposition occurs in the deep intima prior to macrophage recruitment. Subendothelial modified lipids are ingested predominantly by macrophages and vascular smooth muscle cells in an unregulated process, leading to excessive cholesteryl ester (CE) and triglyceride accumulation. Excess lipid accumulation in macrophages in the form of lipid droplets leads to differentiation into foam cells, which are abundant within atherosclerotic plaques, although new evidence suggests that a significant number of foam cells may be smooth muscle derived. Foam cells secrete proinflammatory cytokines and growth factors causing additional macrophage recruitment and matrix deposition, contributing to atherosclerotic lesion development.

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Genome-wide association studies have identified variants at chromosome 10q23, which associate with CAD. The lead variant, rs2246833, is associated with an $≈9\%$ increased risk per copy of the CAD-associated allele. This variant and those in high linkage disequilibrium (LD) with it lie within or in close proximity to LIPA, which encodes lysosomal acid lipase (LAL). LAL is the key lysosomal enzyme for hydrolyzing CEs and triglycerides into free cholesterol (FC), glycerol, and free fatty acids and prevents lipid accumulation in various tissues and cell types, including macrophages.

Loss-of-function mutations in LIPA cause either the rare autosomal recessive LAL deficiencies Wolman disease, where
LAL signal peptide processing. SignalP 4.1 predicted that how the threonine to proline amino acid change might affect cleavage site. We used in silico analyses tools to investigate the cytosol to the endoplasmic reticulum, and a C-terminal hydrophilic N-terminus, a central \( \alpha \)-helical hydrophobic core, which is required for cotranslational translocation from the cytosol to the endoplasmic reticulum, and a C-terminal cleavage site. We used silico analyses tools to investigate how the threonine to proline amino acid change might affect LAL signal peptide processing. SignalP 4.1 predicted that the signal peptide would be reduced by 2 amino acids in LAL\textsuperscript{Pro} compared with that in LAL\textsuperscript{Thr} (Figure 1A through 1C). PSIPRED \( 3.3,12,13 \) predicted the LAL\textsuperscript{Thr} residue to contribute to an \( \alpha \)-helix of length 13 residues, whereas the LAL\textsuperscript{Pro} residue was predicted to truncate the \( \alpha \)-helix to 11 residues and introduce a 2 residue \( \beta \)-strand conformation at the carboxyl end of the \( \alpha \)-helix, disrupting the hydrophobic core (Figure 1D).

**Effect of rs1051338 on LAL Localization**

To investigate the functional effects of, rs1051338 we generated allele-specific C-terminal FLAG-tagged LAL expression constructs. COS7 cells were transfected with either FLAG-LAL\textsuperscript{Thr} or FLAG-LAL\textsuperscript{Thr} 48 hours prior to sample collection and analysis. Because LAL is only functional in lysosomes, we enriched the cellular lysosomal fraction by differential centrifugation (Figure I in the online-only Data Supplement) and measured LAL protein expression and activity in whole cell, lysosomal, and conditioned media fractions from the same cell preparations. LAL protein levels were assessed using an anti-FLAG antibody and found to be borderline reduced within LAL\textsuperscript{Thr} whole cell extracts when compared with LAL\textsuperscript{Thr} (\( P=0.066; \) Figure 2A and 2D) and significantly reduced within the lysosomal fraction of LAL\textsuperscript{Thr} compared with LAL\textsuperscript{Thr} (\( P=0.004; \) Figure 2B and 2E), resulting in significantly reduced LAL activity within the whole cell (\( P=0.027; \) Figure 2G) and lysosomal fraction (\( P=0.005; \) Figure 2H). LAL\textsuperscript{Pro} protein levels (\( P=0.184; \) Figure 2C and 2F) and activity (\( P=0.069; \) Figure 2I) in the conditioned media of transfected cells also showed trends toward lower levels compared with LAL\textsuperscript{Thr}, mirroring the trend seen intracellularly and suggesting a global reduction in LAL protein levels in LAL\textsuperscript{Pro} transfected cells. Of note, the difference in LAL protein levels was not caused by construct-specific transfection efficiency; there were equivalent levels of cotransfected eGFP protein expression in both conditions after 48 hours (\( P=0.858; \) Figure IA and IB in the online-only Data Supplement), and relative mRNA expression of each construct was equivalent after 24 hours (\( P=0.519; \) Figure IIC in the online-only Data Supplement).

**Materials and Methods**

Materials and Methods are provided in the online-only Data Supplement.

**Results**

**Coding Variant rs1051338 Is Predicted to Alter Processing of LAL Signal Peptide**

Examination of rs2246833, the CAD-associated \( \text{LIPA} \) variant from genome-wide association studies, indicated a total of 13 proxy single nucleotide polymorphisms in high LD (\( r^2>0.8; \) Table I in the online-only Data Supplement). One variant, rs1051338 minor allele frequency of 34%), causes a nonsynonymous threonine (LAL\textsuperscript{Thr} nonrisk) to proline (LAL\textsuperscript{Pro} risk) change at amino acid position 16 in the signal peptide of LAL. Signal peptides are found in the N-terminus of certain newly synthesized proteins and are responsible for targeting proteins for secretion or to specific organelles. Signal peptides comprise a hydrophilic N-terminus, a central \( \alpha \)-helical hydrophobic core, which is required for cotranslational translocation from the cytosol to the endoplasmic reticulum, and a C-terminal cleavage site. We used silico analyses tools to investigate how the threonine to proline amino acid change might affect LAL signal peptide processing. SignalP 4.1\textsuperscript{11} predicted that

\[ P \text{ values: } 0.027, 0.005, 0.004, 0.184, 0.069, 0.858, 0.519, 0.547 \]
carrying different genotypes of **LIPA**, circulating monocytes were isolated from healthy adult individuals homozygous for either the risk (n=4) or nonrisk alleles (n=4) of rs1051338 and differentiated into macrophages. **LIPA** expression was measured using quantitative real-time polymerase chain reaction in the isolated monocytes and the derived macrophages. Monocytes with the risk genotype showed a nonsignificant trend toward higher LAL mRNA level compared with non-risk monocytes (P=0.097; Figure 4A). However, there was no difference in **LIPA** mRNA levels in macrophages with the contrasting **LIPA** genotypes (P=0.925; Figure 4B). Consistent with this, there was no genotype-related difference in LAL protein levels in whole macrophages (P=0.667; Figure 4C and 4E); however, there was significantly reduced lysosomal LAL protein in risk compared with nonrisk macrophages (P=0.02; Figure 4D and 4F). LAL activity was not significantly different between risk and nonrisk genotype in whole cells (P=0.164; Figure 4G), but in the lysosomal fraction, LAL activity was significantly reduced in the risk genotype compared with that in nonrisk genotype (P=0.026; Figure 4H). The rate of LAL degradation on cycloheximide treatment was significantly greater in macrophages with the risk genotype compared with those with nonrisk genotype (P=0.004; Figure 4I through 4K), while no difference in the levels of LAL protein were identified after inhibition of protein degradation (P=0.299; Figure 4I through 4L). To examine whether the reduced lysosomal expression and activity of LAL in macrophages from those carrying the risk allele had functional downstream consequences, macrophages from risk and nonrisk genotypes were incubated with the modified lipoprotein acetylated LDL.
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and subsequent cholesterol efflux to apolipoprotein A1 measured. Macrophages from subjects carrying the risk genotype showed a ≈25% reduced efflux capacity compared with those with the nonrisk genotype, although this difference was not significant (P=0.187; Figure 4M).

**Discussion**

One of the major challenges in understanding the disease-associated loci found through genome-wide association studies is the identification of the causal variant and how it alters the function of the affected gene. In the majority of loci, the disease-associated variants fall in noncoding regions and affect gene expression rather than altering the coding sequence of a gene; however, the 10q23 CAD risk locus is more complex because it is both associated with increased expression of LIPA in some cells and tissues and contains a variant that changes the amino acid sequence of the encoded LAL protein. In this study, we investigated the effect of the CAD-associated coding variant, rs1051338, and found that the risk allele caused an increased degradation of LAL, resulting in reduced lysosomal levels of LAL protein and reduced activity. These results are consistent with the early onset atherosclerosis seen with loss of function LIPA mutations in CE storage disease15–21 and with data from mouse models.22–26

The coding variant causes a threonine to proline change within the signal peptide of LAL. Signal peptides are necessary for the correct and efficient targeting of certain proteins to specific organelles, and the CAD-associated variant is in the hydrophobic core of the signal peptide, a region essential for cotranslational translocation into the endoplasmic reticulum by the signal recognition particle.34 The α-helical structure and hydrophobicity of the hydrophobic core are known to increase the efficiency of protein secretion.35,36 Prolines are known α-helix breakers,37,38 and our in silico analysis predicted that

![Figure 2](image-url)
the risk allele of rs1051338 disrupts the α-helix, reducing the hydrophobic core, and changes the predicted signal peptide cleavage site. Although these in silico findings indicate a clear alteration in the signal peptide, such findings should be confirmed using structural biology approaches. In addition, several studies have reported that mutations within signal peptide hydrophobic core regions impair translocation and result in protein degradation.39–42 Overexpression of plasmids containing either the risk or nonrisk allele of rs1051338 confirmed that LALPro had significantly reduced lysosomal protein levels and activity. We also found a trend toward reduced LAL protein levels and activity in both whole cell and extracellular fractions, suggesting that the single nucleotide polymorphism does not result in mistrafficking of LAL. Instead, by treating cells with the protein synthesis inhibitor cycloheximide or the proteasome inhibitor bortezomib, we were able to show that LALPro is directed toward the proteasome. Analyses of primary macrophages homozygous for either the risk or nonrisk genotype supported the results of the overexpression system.

We went on to test whether the changes in lysosomal LAL activity had a functional effect on cholesterol homeostasis. In atherosclerosis, modified LDLs are endocytosed in an unregulated process, leading to dramatic increases in intracellular FC concentrations. Excess FC is potentially cytotoxic to cells, so inherent mechanisms limit intracellular FC concentration build-up, including upregulation of cholesterol efflux43,44 and re-esterification of FC to form nontoxic, neutral pH, lipid droplets.45,46 Lipid droplet degradation was thought to be performed exclusively by cytoplasmic neutral CE hydrolases47; however, recent evidence indicates a significant role for lysosomal lipid autophagy in modified LDL metabolism in macrophages.48,49 A key role for LAL in this process was highlighted in cholesterol-loaded macrophages, when chemical inhibition of LAL reduced cholesterol efflux to apolipoprotein A1 by >50%.48,49 We, therefore, investigated whether rs1051338 genotype affects efflux to apolipoprotein A1 in acetylated LDL-loaded macrophages and found a reduction of ≈25% in efflux to apolipoprotein A1 (P=0.187) in macrophages isolated from individuals homozygous for the risk allele. Although not significant, the reduction is in the expected direction; however, interindividual variation means a much larger sample size is required to confirm the functional effects of rs1051338. In LAL deficiency (Wolman disease and CE storage disease), the reduced LAL activity can impair ATP-binding cassette transporter A1 expression in response to LDL loading, leading to reduced cholesterol efflux, an effect that is reversed on treatment with recombinant human LAL.50 Indeed, a Phase 3 trial of LAL replacement therapy has recently reported improvements in lipid profile in individuals with LAL deficiency.51

Our data suggest that the CAD-associated coding variant results in reduced LAL activity in the lysosomes of macrophages and, therefore, implies that raising LAL levels might be beneficial as a treatment for CAD. However, it is important to emphasize that the effects on LAL activity are complicated by the association between the CAD-risk genotype and LIPA expression, which have been identified by multiple studies.27–30 Ours is the first study to suggest that the CAD-risk genotype results in a reduction in LAL activity rather than an increase as indicated by gene expression analysis. At present, it is unclear if the association with LIPA expression is pertinent to CAD risk or how it relates to the functional effects of the coding variant.
The increased LIPA expression could be a bystander effect of a high LD variant that falls in a regulatory region or potentially a feedback mechanism caused by the reduced LAL activity induced by the coding variant. In our analysis, we observed a trend toward increased LIPA mRNA levels in primary monocytes homozygous for the risk allele but no difference between genotypes in the differentiated macrophages. These results reflect the effects seen in the Cardiogenics consortium’s gene expression analysis of monocytes and macrophages. In monocytes collected from control subjects, the risk genotype (rs2246833) is strongly associated with LIPA expression ($P=6.6\times10^{-64}$; $\beta=0.44$, n=395) but is attenuated in macrophages ($P=0.0001$; $\beta=0.06$, n=305). The difference in effect size between monocytes and macrophages suggests some cell type–specific regulation of LIPA expression, and further investigation of the CAD-associated single nucleotide polymorphisms at this locus is required to determine the relevance of LIPA expression and to confirm the effects of the coding variant.

Our findings provide a possible explanation for the association between chromosome 10q23 and an increased risk of CAD and is consistent with recent studies describing the role of LAL in the metabolism of modified LDL in macrophages. However, several limitations of our study and findings need to be highlighted. First, the majority of our findings are from experiments involving overexpression of LAL in COS7 cells and may not accurately reflect the action of the protein processing machinery or LAL activity of a disease-relevant cell type. Second, while our findings in primary macrophages are concordant with those in COS7 cells, the small sample size limited our analysis of the functional consequences of reduced lysosomal LAL activity, and our findings, therefore, require replication in larger cohorts to verify the effects of rs1051338 on lysosomal LAL expression, activity, and cholesterol homeostasis. Finally, we only investigated the coding variant rs1051338 and cannot exclude other high LD variants that affect LAL function and contribute to CAD risk. Indeed, the association between the CAD risk genotype and increased LIPA expression has not been studied, and to fully understand the mechanism underlying the disease association will require each of the variants at the locus to be examined under study to understand the mechanism underlying the disease association. Future work using genome editing could allow the generation of an allelic series comprising the coding variant and other high LD variants in induced pluripotent stem cells prior to differentiation into...
monocytes and macrophages. Such experiments would identify the relative contribution of each variant to LIPA expression and activity.

In conclusion, our findings suggest that the CAD-associated variant rs1051338, which causes a missense change in the signal peptide of LAL, disrupts the normal sorting and transport of LAL to the lysosome. LAL containing the risk allele is prone to cytosolic proteasomal degradation, reducing lysosomal LAL protein and activity. Understanding how this affects cholesterol metabolism may provide novel therapeutics for CAD through the modulation of macrophage cholesterol concentration.

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Disclosures

None.

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Highlights

• This study identifies a coding variant in LIPA as likely causal variant at the 10q23 coronary artery disease–associated locus.

• The variant increases lysosomal acid lipase protein degradation, causing reduced lysosomal lysosomal acid lipase activity in macrophages.