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Translational Studies of Lipoprotein-Associated Phospholipase A_2 in Inflammation and Atherosclerosis

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Objectives This study sought to examine the role of lipoprotein-associated phospholipase A_2 (Lp-PLA_2/*PLA2G7*) in human inflammation and coronary atherosclerosis.

Background Lp-PLA_2 has emerged as a potential therapeutic target in coronary heart disease. Data supporting Lp-PLA_2 are indirect and confounded by species differences; whether Lp-PLA_2 is causal in coronary heart disease remains in question.

Methods We examined inflammatory regulation of Lp-PLA_2 during experimental endotoxemia in humans, probed the source of Lp-PLA_2 in human leukocytes under inflammatory conditions, and assessed the relationship of variation in *PLA2G7*, the gene encoding Lp-PLA_2, with coronary artery calcification.

Results In contrast to circulating tumor necrosis factor-alpha and C-reactive protein, blood and monocyte Lp-PLA_2 messenger ribonucleic acid decreased transiently, and plasma Lp-PLA_2 mass declined modestly during endotoxemia. In vitro, Lp-PLA_2 expression increased dramatically during human monocyte to macrophage differentiation and further in inflammatory macrophages and foamlike cells. Despite only a marginal association of single nucleotide polymorphisms in *PLA2G7* with Lp-PLA_2 activity or mass, numerous *PLA2G7* single nucleotide polymorphisms were associated with coronary artery calcification. In contrast, several single nucleotide polymorphisms in *CRP* were significantly associated with plasma C-reactive protein levels but had no relation with coronary artery calcification.

Conclusions Circulating Lp-PLA_2 did not increase during acute phase response in humans, whereas inflammatory macrophages and foam cells, but not circulating monocytes, are major leukocyte sources of Lp-PLA_2. Common genetic variation in *PLA2G7* is associated with subclinical coronary atherosclerosis. These data link Lp-PLA_2 to atherosclerosis in humans while highlighting the challenge in using circulating Lp-PLA_2 as a biomarker of Lp-PLA_2 actions in the vasculature. (J Am Coll Cardiol 2012;59:764–72) © 2012 by the American College of Cardiology Foundation

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) has emerged as a potential therapeutic target in coronary heart disease (CHD) and phase III clinical trials are underway. Supporting evidence includes apparent atherogenic biochemical properties; Lp-PLA_2 cleaves oxidized phosphatidylcholine on modified low-density lipoprotein (LDL), producing inflammatory lysophosphatidylcholine and oxidized nonesterified fatty acids (1,2). In addition, enzymatic expression of Lp-PLA_2 is up-regulated in human atherosclerosis (3), whereas circulating levels are associated with incident CHD (4). Promising proof-of-principle preclinical and clinical trials have been carried out (5–7).

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However, whether Lp-PLA$_2$ is causal and whether its inhibition will prevent CHD events remain undetermined.

Data for Lp-PLA$_2$ in human atherosclerosis remain indirect and confounded by species differences in physiology and actions. Lp-PLA$_2$ circulates in blood bound to lipoproteins that modulate its actions. In rodents, Lp-PLA$_2$ is carried mostly on high-density lipoprotein particles, whereas in humans, the enzyme is bound to LDL particles. Thus, confounding may be particularly marked for plasma Lp-PLA$_2$ relative to other inflammatory markers, as regulation of atherogenic lipoproteins is a major influence on circulating Lp-PLA$_2$ levels and activity (8). Indeed, whether circulating Lp-PLA$_2$ is associated with CHD beyond a complete assessment of atherogenic lipoproteins remains uncertain (9).

Arterial Lp-PLA$_2$ biosynthesis by macrophages and foam cells, rather than circulating levels or activity, may determine its atherogenicity (10). Lp-PLA$_2$ expression within the necrotic core and surrounding macrophages of vulnerable and ruptured plaques, compared with less-advanced lesions, is increased (11), suggesting a potential role in promoting plaque instability. The extent to which human Lp-PLA$_2$ is regulated in circulation by systemic inflammation, however, versus locally controlled in arterial macrophage-foam cells is uncertain. Further, lesion biosynthesis is difficult to measure in humans, limiting our ability to monitor Lp-PLA$_2$ activity in disease-relevant tissue and to assess vascular efficacy of pharmacological inhibition.

In this report, we examined inflammatory regulation of circulating Lp-PLA$_2$ during experimental endotoxemia in humans, probed the source of Lp-PLA$_2$ in human leukocytes under inflammatory conditions, and determined the relationship of genetic variation in phospholipase A$_2$, group VII (*PLA2G7*), the gene encoding Lp-PLA$_2$, to coronary artery calcification (CAC) as well as plasma levels of Lp-PLA$_2$ mass and activity. We found that, unlike blood tumor necrosis factor (TNF)-alpha and C-reactive protein (CRP), circulating Lp-PLA$_2$ did not increase during the acute phase response in humans; that inflammatory macrophages and foam cells, but not circulating or ex vivo monocytes, are primary leukocyte sources of Lp-PLA$_2$; and that common genetic variation in *PLA2G7* is associated with subclinical coronary atherosclerosis. These data link Lp-PLA$_2$ to atherosclerosis in humans while providing a human physiological context for the difficulty in using circulating Lp-PLA$_2$ as a biomarker of disease or pharmacological efficacy in atherosclerosis.

Methods

Clinical studies. HUMAN ENDOTOXEMIA. Healthy volunteers on no medications and no significant medical history (n = 32, 50% women; mean age 25.7 ± 3.90 years) were studied as described previously (12,13) and in the Online Appendix. Serial blood samples were collected before and after intravenous bolus infusion of 3 ng/kg U.S. standard reference endotoxin and were prepared for plasma, whole-blood ribonucleic acid (RNA) and monocyte RNA (12).

Abbreviations and Acronyms

CAC = coronary artery calcification

CHD = coronary heart disease

CRP = C-reactive protein

CT = cycle threshold

eQTL = expression quantitative trait locus

LDL = low-density lipoprotein

Lp-PLA$_2$ = lipoprotein-associated phospholipase A$_2$

LPS = lipopolysaccharide

mRNA = messenger ribonucleic acid

PCR = polymerase chain reaction

RNA = ribonucleic acid

SNP = single nucleotide polymorphism

TNF = tumor necrosis factor

GENETIC ASSOCIATION STUDIES. The PennCAC (Penn Coronary Artery Calcification) resource included European-ancestry subjects recruited to 3 separate studies at the University of Pennsylvania: the SIRCA (Study of Inherited Risk of Coronary Atherosclerosis) (n = 799), the PDHS (Penn Diabetes Heart Study) (n = 782), and the PAMSyN (Philadelphia Area Metabolic Syndrome Network) (n = 480). These studies are described in detail previously (14,15) and in the Online Appendix. In each study, subjects with clinical atherosclerotic cardiovascular disease were excluded. *PLA2G7* single nucleotide polymorphisms (SNPs) were genotyped in all 3 studies. Plasma Lp-PLA$_2$ mass and activity data were available in SIRCA and PDHS. Global CAC scores were determined by electron beam tomography (Imatron, San Francisco, California) according to the method of Agatston et al. (16). For all human studies described, the University of Pennsylvania Institutional Review Board approved each study, and written informed consent was provided by all participants.

Laboratory methods. HUMAN MONOCYTE, MACROPHAGE, AND FOAM CELL STUDIES. Human monocyte isolation, macrophage (M1 and M2 phenotype) differentiation (17), and "foam cell" preparation were performed as described elsewhere (12) and in the Online Appendix. Experiments were performed in batches using freshly isolated monocytes, macrophages, and foam cells derived from the same human volunteer.

PLASMA LP-PLA$_2$, INFLAMMATORY, AND METABOLIC MARKERS. Plasma and cell-media levels of Lp-PLA$_2$ mass and activity, TNF-alpha, and CRP, as well as lipid and biochemical markers were measured as described elsewhere (13,14,18) and in the Online Appendix.

REAL-TIME QUANTITATIVE PCR AND EXPRESSION QUANTITATIVE TRAIT LOCUS ANALYSIS. Whole-blood, isolated circulating monocyte, and human cultured monocyte, macrophage, and foam cell messenger ribonucleic acid (mRNA) were subjected to quantitative polymerase chain reaction (PCR) using primers and probes (Applied Biosystems 7300 Real-Time PCR System, Foster City, California) as previously described (12) for measurement of Lp-PLA$_2$, TNF-alpha, and beta-actin mRNA (Online Appendix). The relative quantitation $2^{-(\Delta\Delta Ct)}$ method was used to determine fold-change from baseline (19). Explor-

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atory expression quantitative trait locus (eQTL) analysis is described in the Online Appendix.

GENOTYPING. As described previously (20) and in the Online Appendix, PennCAC participants were genotyped using the ITMAT Broad Care CARDIOVASCULAR DISEASE candidate gene array, which surveys ~50,000 SNPs in ~2,000 candidate genes (21). SNP data for *PLA2G7* (n = 19) and *CRP* (n = 16) were selected for the present analysis.

Statistical analysis. The effect of endotoxemia on plasma Lp-PLA$_2$ mass and activity, TNF-alpha, and CRP, as well as whole blood and monocyte mRNA was tested by repeated-measures analysis of variance. Analysis of variance was applied also to in vitro cell data. Post hoc *t* tests were used to compare specific time points and treatments. We observed heterogeneity of variance in several variables following lipopolysaccharide (LPS) challenge, which was to be expected given the known variation in responses to endotoxin. We tested for homogeneity of variance using Levene test, and in cases where the assumption of homogeneity of variance was violated, we confirmed whether the group differences were significant using Tamhane post-hoc test.

In PennCAC, CAC scores were transformed by the natural log after adding 1, [Ln(CAC + 1)], to correct for skewed distribution. This variable was used as the outcome in a linear regression model, with *PLA2G7* and *CRP* SNPs, adjusting for age, sex, and age–sex interaction. For linear regression analysis of SNP associations with plasma proteins, Lp-PLA$_2$ mass and activity were normally distributed and, therefore, used as outcomes, whereas CRP was log-transformed. The linear regression model included adjustments for age, sex, and smoking. Analysis used PLINK (version 1.06, Shaun Purcell, Boston, Massachusetts). Analyses of CAC and plasma proteins were performed separately in each sample and then subjected to meta-analysis. Meta-analysis applied a weighted *Z*-score method using METAL (Center for Statistical Genetics, University of Michigan, Ann Arbor, Michigan) (22,23) as we applied it in (24) as described in the Online Appendix. In analysis of SNP data, we corrected for the number of independent tests within each gene (10 tests for 19 *PLA2G7* SNPs, unadjusted p value threshold of 0.005, and 15 tests for 15 *CRP* SNPs, unadjusted p value threshold of 0.0033) using the method of Nyholt (25).

Results

Lp-PLA$_2$ is not induced in a human model of acute phase response. As we described previously (13,26), endotoxemia produced an acute, febrile illness associated with a marked, transient induction of plasma TNF-alpha ($p < 0.001$), followed by a delayed ~100-fold induction of plasma CRP at 24 h ($p < 0.001$) (Fig. 1A). In contrast, plasma Lp-PLA$_2$ mass and activity did not increase following LPS (Fig. 1B). Indeed, levels of Lp-PLA$_2$ mass tended to decline (by 18% at 6 h, $p < 0.01$). The mRNA response to LPS in whole blood for TNF-alpha (Fig. 1C) and Lp-PLA$_2$

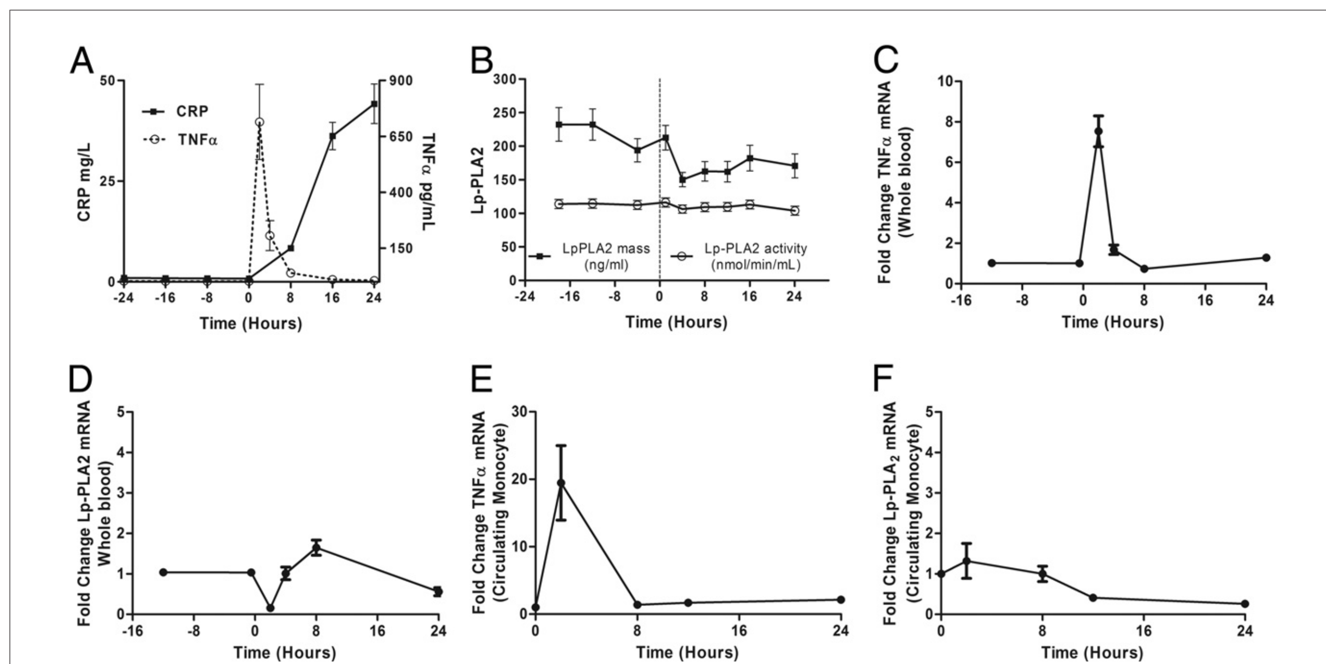

Figure 1 Human Endotoxemia Does Not Induce Circulating Lp-PLA$_2$ Protein or Leukocyte Lp-PLA$_2$ mRNA In Vivo

Endotoxemia (3 ng/kg lipopolysaccharide [LPS] intravenously) markedly increased plasma levels of **(A)** tumor necrosis factor (TNF)-alpha and C-reactive protein (CRP) ($p < 0.001$) but not **(B)** lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) mass or activity, which declined transiently ($p < 0.01$). Following LPS, whole blood **(C)** TNF-alpha messenger ribonucleic acid (mRNA) was markedly induced but **(D)** Lp-PLA$_2$ mRNA is not. Similarly, LPS increased circulating monocyte mRNA levels of **(E)** TNF-alpha but not of **(F)** Lp-PLA$_2$.

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(Fig. 1D), as well as in circulating monocytes for TNF-alpha (Fig. 1E) and Lp-PLA$_2$ (Fig. 1F), was similar to that of plasma proteins. The mRNA levels of Lp-PLA$_2$ in circulating monocytes were low but detectable (baseline cycle threshold [CTs] ~30, varying from CTs of 28 to 32 post-LPS).

Lp-PLA$_2$ expression is induced in inflammatory human macrophages and foam cells. Lp-PLA$_2$ mRNA levels were low (CTs ~30) in freshly isolated human monocytes but increased markedly (CTs ~20) following 6 days of differentiation to mature macrophages ($p < 0.0001$) (Fig. 2A) and increased modestly during further polarization to M1 ($p < 0.0001$) but not M2 macrophages (Fig. 2B). Lp-PLA$_2$ protein mass also was induced during differentiation to macrophages, with increases in both the cell-associated protein ($p < 0.0001$) and the secreted protein ($p = 0.0004$) (Fig. 2C). Following loading of human monocyte-derived macrophages with acetylated LDL-cholesterol for 48 h, cholesterol ester (128 vs. 0.6 μg cholesterol ester /mg protein) and total cholesterol (422 vs. 316 μg cholesterol/mg protein) were significantly higher in loaded versus unloaded cells, which is consistent with findings for in vivo foam cells (27). Lp-PLA$_2$ mRNA levels were significantly greater in foam cells than in mature macrophages ($p < 0.01$) (Fig. 3A). Similarly, cell-associated ($p = 0.05$) and secreted ($p = 0.008$) Lp-PLA$_2$ protein levels were higher in foam cells than in macrophages (Fig. 3B). There was no Lp-PLA$_2$ protein detectable in the media or acetylated LDL used to treat cells. Overall, these data are consistent with lack of in vivo increase in plasma or monocyte levels of Lp-PLA$_2$ during the acute phase and suggest that, in human atherosclerosis, Lp-PLA$_2$ may be generated by macrophages and foam cells rather than by circulating leukocytes.

Exploratory interrogation of *PLA2G7* SNP eQTLs for Lp-PLA$_2$ mRNA expression in publicly available data revealed nominal associations of several SNPs in the *PLA2G7* region with exon probe levels in peripheral blood mononuclear cells (best $p = 0.0059$, rs12181971) and brain (best $p = 0.008$, rs12195701) (28), as well as skin (best $p = 0.021$, rs16874962), fat (best $p = 0.019$, rs16874962), and lymphoblastoid cells (best $p = 0.037$, rs7745519) (MuTHER [Multiple Tissue Human Expression Resource] twin2 study (29)). These modest associations, however, were not significant after correction for multiple testing (see the Online Appendix). Macrophage and foam cell expression datasets were not available for testing a more atherosclerosis-relevant cell type.

Common polymorphisms in *PLA2G7* are associated with coronary calcification but only weakly with plasma Lp-PLA$_2$ mass or activity. Individually in SIRCA or PDHS samples, there were no significant associations between PLA2G7 SNPs and Lp-PLA$_2$ mass or activity. In the combined meta-analysis, only 1 SNP (rs1805017) had nominal association with Lp-PLA$_2$ mass ($p = 0.02$; $p = 0.2$ after Bonferroni correction) (Table 1A). As a positive control, we performed similar analysis of plasma CRP using common CRP SNPs. In contrast to findings for PLA2G7-Lp-PLA$_2$, there were significant associations between numerous CRP SNPs and plasma CRP in the SIRCA and PDHS samples and in the overall meta-analysis (Table 1B). Of 16 CRP SNPs, 9 had nominal ($p < 0.05$) associations with CRP levels and 8 of these SNPs had significant associations after Bonferroni correction.

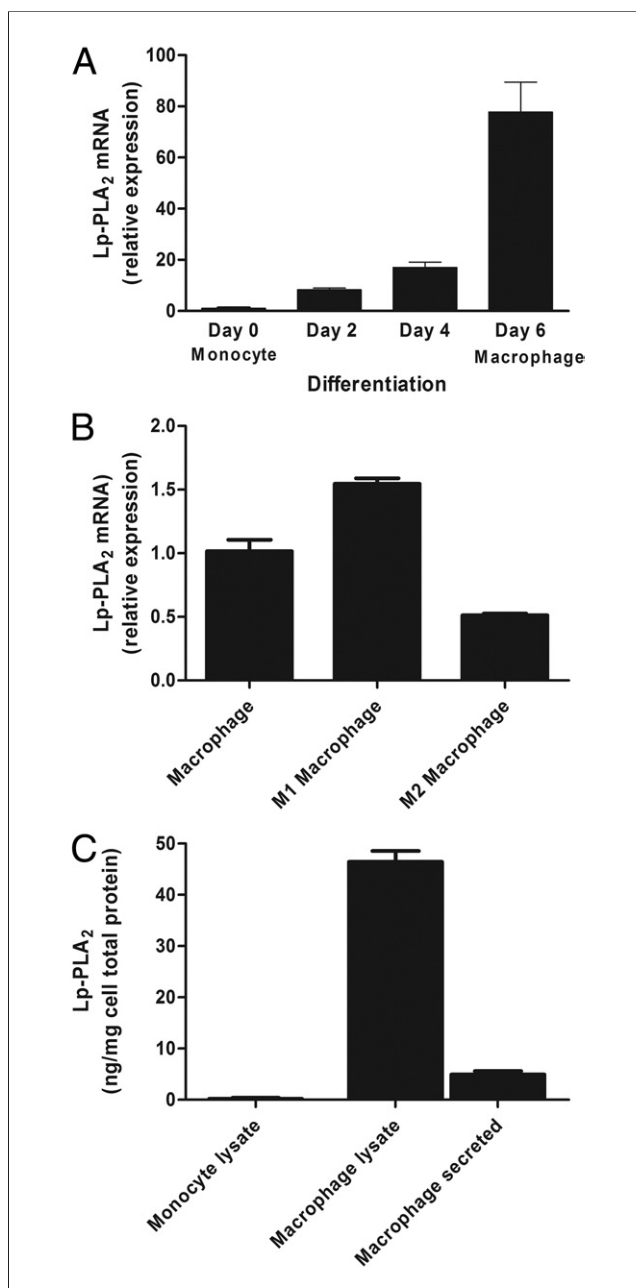

Figure 2 **Lp-PLA$_2$ mRNA and Protein Increase During Differentiation of Human Monocytes to Macrophages In Vitro**

Lp-PLA$_2$ mRNA levels increased markedly during differentiation from monocytes to mature macrophages ($p < 0.0001$) **(A)** and increased modestly during further polarization to M1 macrophages ($p < 0.001$) but fell during M2 polarization ($p < 0.001$) **(B).** Lp-PLA$_2$ protein mass also increased significantly during differentiation to macrophages, with increases in both the cell-associated protein ($p < 0.0001$) and the secreted protein ($p = 0.0004$) **(C).** *Note:* As monocytes were grown in suspension, protein levels were measured in monocyte cell lysates but could not be measured in media. (Analysis of variance and Bonferroni post hoc tests). Abbreviations as in Figure 1.

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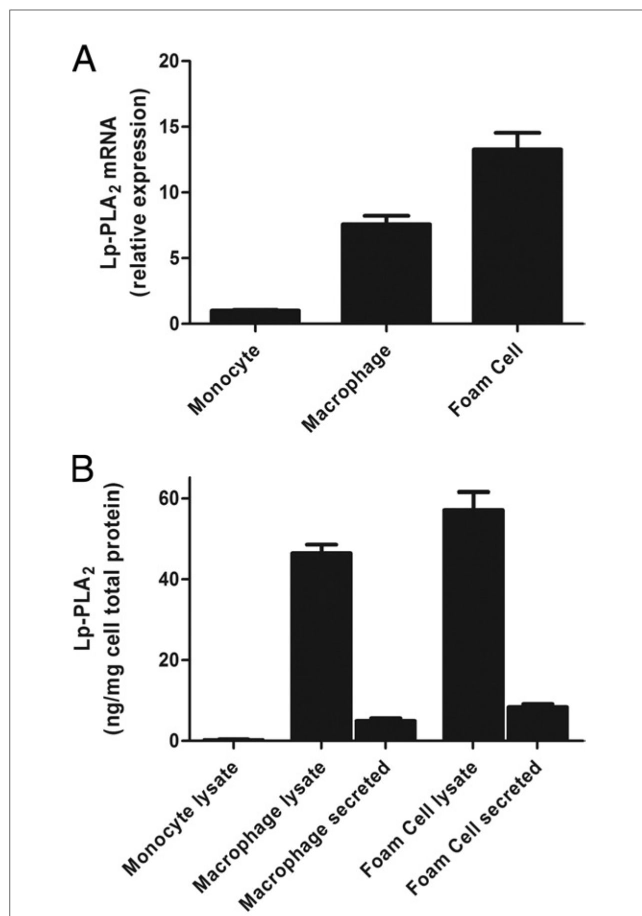

Figure 3 **Lp-PLA$_2$ mRNA and Protein Are Up-Regulated in Human Foamlike Cells In Vitro**

Lp-PLA$_2$ mRNA was significantly greater ($p < 0.01$) in foam cells than in mature macrophages **(A).** Similarly, cell-associated ($p = 0.05$) and secreted ($p = 0.008$) Lp-PLA$_2$ protein levels were higher in foam cells than in macrophages **(B).** (*Note:* As monocytes were grown in suspension, protein levels were measured in monocyte cell lysates but could not be measured in media. (Analysis of variance and Bonferroni post hoc tests). Abbreviations as in Figure 1.

Association of *PLA2G7* SNPs with CAC was assessed initially in SIRCA and PAMSyN together with follow-up in PDHS. Multiple *PLA2G7* SNPs had nominal associations with CAC in SIRCA and PAMSyN (11 with $p < 0.05$; lowest $p < 0.0001$ for rs1421378). Replication signals in PDHS were modest (strongest rs10948300, $p = 0.02$) likely due to limited power; however, in PDHS, 16 of 19 SNPs had effects in the same direction as in SIRCA or PAMSyN (chi squared: 8.9, $p = 0.003$). Meta-analysis of the combined sample found several SNP associations with CAC (rs9349373, $p = 0.002$; rs2216465, $p = 0.002$; rs12195701, $p = 0.004$) that were significant after Bonferroni correction (Table 2A). Including plasma Lp-PLA$_2$ mass or activity in the model did not attenuate the association between *PLA2G7* SNPs and CAC. These findings support recent associations of variation in *PLA2G7* with CHD (30–32).

As an expected negative control (14,33,34), we examined *CRP* variant associations with CAC in the same sample and found minimal signal, with 1 SNP having nominal association in SIRCA and PAMSyN (rs3093068, $p = 0.04$); however, there were no associations in PDHS nor in combined meta-analysis.

Discussion

We provide novel insight into the pathophysiology of Lp-PLA$_2$ in humans. First, we show that unlike TNF-alpha and CRP, circulating Lp-PLA$_2$ does not increase during experimental endotoxemia and, therefore, does not contribute to human acute phase response. Second, we found that inflammatory macrophages and foam cells, but not circulating monocytes or cultured primary monocytes, generate significant Lp-PLA$_2$. This is consistent with the concept that the majority of Lp-PLA$_2$ in atherosclerotic plaque is derived from local biosynthesis by inflammatory macrophage and foam cells rather than from circulating leukocytes. Third, we found that common variants in *PLA2G7* are associated with CAC but had limited relation to circulating Lp-PLA$_2$ mass or activity. This supports an atherogenic role for *PLA2G7*-Lp-PLA$_2$ in humans that may be independent of circulating Lp-PLA$_2$ mass or activity.

Lp-PLA$_2$ does not contribute to human acute phase response. We demonstrate that Lp-PLA$_2$ is not an acute phase protein in humans. This is in contrast to rodent models in which LPS challenge was shown to induce a rapid increase in plasma and tissue levels of Lp-PLA$_2$ (35). This provides further evidence of fundamental differences between humans and rodents in the physiology and action of Lp-PLA$_2$ (36). Lack of induction in blood and circulating monocytes by endotoxemia in vivo also suggests limited, if any, role for circulating leukocyte production of Lp-PLA$_2$ in atherosclerosis. In contrast, marked in vitro up-regulation in macrophages and foam cells is consistent with a specific role for local vascular production of Lp-PLA$_2$ in atherosclerosis. Although it is possible that local macrophage Lp-PLA$_2$ production in plaque may contribute to a portion of circulating Lp-PLA$_2$, it is unlikely to render circulating levels useful as independent biomarkers of Lp-PLA$_2$ actions in atherosclerosis because published data show that circulating Lp-PLA$_2$ mass and activity do not correlate with plaque Lp-PLA$_2$ in patients undergoing elective carotid endarterectomy (37) and because there is substantial confounding of plasma Lp-PLA$_2$ by circulating lipoproteins regardless of tissue source. Overall, these data suggest that levels of Lp-PLA$_2$ mRNA and protein in blood may be poor surrogates of *PLA2G7* actions in arterial plaque.

Proinflammatory macrophages and foam cells, but not monocytes, generate significant Lp-PLA$_2$. We found that Lp-PLA$_2$ expression was markedly increased during the differentiation of monocytes to macrophages, and further induced in vitro in "foam cell–like macrophages. This is consistent with constitutive expression and activity in inflammatory macrophages (38) and foam cells in atherosclerosis. Indeed, Lp-PLA$_2$ expression is increased in athero-

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Table 1 Lack of Association Between SNPs in *PLA2G7* and Circulating Lp-PLA_2 Protein Mass or Activity But Significant Relation of Multiple SNPs in *CRP* With Circulating CRP Levels*

PLA2G7 SNP (Allele: Minor/Major)†	Type of SNP	MAF	Lp-PLA_2 Mass, Meta-Analysis (n = 1,723): Z-Score	Lp-PLA_2 Mass, Meta-Analysis (n = 1,723): p Value	Lp-PLA_2 Activity, Meta-Analysis (n = 1,683): Z-Score	Lp-PLA_2 Activity, Meta-Analysis (n = 1,683): p Value
rs1805017 (A/G)	Nonsynonymous coding	0.26	2.34	0.02	0.23	0.82
rs2216465 (C/G)	Intronic	0.34	1.54	0.12	0.66	0.51
rs1421378 (G/A)	Upstream	0.41	1.37	0.17	0.37	0.71
rs17288905 (G/A)	Intronic	0.08	1.74	0.08	0.79	0.43
rs10948300 (T/A)	Intronic	0.21	1.77	0.08	0.31	0.76
rs12195701 (A/G)	Intronic	0.21	1.46	0.14	0.77	0.44
rs1051931 (A/G)	Nonsynonymous coding	0.21	1.53	0.13	0.07	0.95
rs7756935 (C/A)	Intronic	0.21	1.53	0.13	0.07	0.95
rs12528807 (C/A)	Intronic	0.08	–0.36	0.72	0.44	0.66
rs1362931 (A/C)	Intronic	0.20	1.15	0.25	0.06	0.96
rs1421372 (A/G)	Intronic	0.20	1.15	0.25	0.06	0.96
rs3799861 (A/G)	Intronic	0.20	1.15	0.25	0.06	0.96
rs9472830 (A/G)	Intronic	0.20	1.07	0.29	0.08	0.94
rs16874962 (A/G)	Intronic	0.07	–1.12	0.26	–0.46	0.64
rs3799863 (A/T)	Intronic	0.05	–0.71	0.48	1.01	0.31
rs1421368 (G/A)	Intronic	0.10	0.42	0.68	0.02	0.99
rs9349373 (A/G)	Upstream	0.20	0.23	0.82	–0.49	0.63
rs16874967 (A/G)	Intronic	0.06	–0.34	0.74	0.68	0.49
rs1805018 (G/A)	Nonsynonymous coding	0.06	–0.34	0.74	0.68	0.49

CRP SNP (Allele: Minor/Major)†	Type of SNP	MAF	Meta-Analysis (n = 2,026): Z-Score	Meta-Analysis (n = 2,026): p Value
rs1205 (A/G)	3' UTR	0.34	−5.63	1.8×10^{-8}
rs1800947 (G/C)	Synonymous coding	0.07	−5.41	6.4×10^{-8}
rs3091244 (A/G)	5' upstream	0.37	4.40	1.1×10^{-5}
rs12084589 (A/C)	5' upstream	0.07	3.23	1.0×10^{-3}
rs12068753 (A/T)	5' upstream	0.07	3.18	1.0×10^{-3}
rs3093059 (G/A)	5' upstream	0.07	3.14	2.0×10^{-3}
rs3093068 (G/C)	3' downstream	0.06	2.94	3.0×10^{-3}
rs2794521 (G/A)	5' upstream	0.25	0.29	0.77
rs3093066 (A/C)	3' UTR	0.001	1.99	0.05
rs2808634 (A/G)	5' upstream	0.27	0.39	0.69
rs1130864 (A/G)	3' UTR	0.30	2.94	0.003
rs2808631 (G/A)	3' downstream	0.002	–1.49	0.14
rs3093070 (C/A)	3' downstream	0.02	–0.82	0.41
rs3093069 (C/A)	3' downstream	0.001	1.37	0.17
rs3093071 (A/C)	3' downstream	0.01	1.23	0.22

The 19 SNPs in *PLA2G7* fall into 10 LD blocks ($r^2 > 0.8$); therefore, we used Bonferroni multiple testing correction for 10 effective tests, giving a p value threshold of significance of 0.005. The 15 SNPs in *CRP* fall into 15 LD blocks ($r^2 > 0.8$); therefore, we used Bonferroni multiple testing correction for 15 effective tests, giving a p value threshold of significance of 0.0033. *Meta-analysis of data from the SIRCA (Study of Inherited Risk of Coronary Atherosclerosis) and the PDHS (Penn Diabetes Heart Study). †For all analyses, the effect allele corresponds to the minor allele.

LD = linkage disequilibrium; Lp-PLA_2 = lipoprotein-associated phospholipase A_2; MAF = minor allele frequency; SNP = single nucleotide polymorphism; UTR = untranslated region.

sclerotic lesions in humans (10). In this environment, secreted Lp-PLA_2 can hydrolyze oxidized phospholipids and fatty acids on atherogenic lipoproteins, generating reactive lipid mediators thought to promote plaque instability. Inhibition of Lp-PLA_2 suppressed oxidized-LDL–induced macrophage apoptosis (39), a feature of inflammatory plaque. Further, in a porcine model of complex atherosclerosis, suppression of Lp-PLA_2 retarded atherosclerosis progression and decreased plaque inflammation, necrosis, and fibrous cap erosion (7). Compared with placebo, short-term Lp-PLA_2 inhibition in humans also reduced several markers of plaque inflammation in carotid lesions examined ex vivo (5,6). Overall, these data provide indirect evidence for atherogenic actions of Lp-PLA_2 in vascular lesions. Indeed, Lp-PLA_2 inhibition is currently being tested in large phase III clinical trials of CHD in high-risk patients (NCT01000727).

Genetic variation in *PLA2G7* may relate to CHD independent of circulating Lp-PLA_2. Several epidemiological studies revealed an association of higher plasma Lp-PLA_2 mass and

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Table 2 **Association of SNPs in *PLA2G7* But Not in *CRP* With CAC**

			SIRCA and PAMSyN (n = 1,279)		PDHS (n = 782)		Meta-Analysis (n = 2,061)	
	Type of SNP	MAF	Beta	p Value	Beta	p Value	Z Score	p Value
PLA2G7 SNP (allele: minor/major)*								
rs1421378 (G/A)	Upstream	0.41	0.31	9.8×10^{-5}	0.05	0.65	3.35	8.1×10^{-4}
rs2216465 (C/G)	Intronic	0.34	0.29	3.9×10^{-4}	0.05	0.67	3.06	0.002
rs12528807 (C/A)	Intronic	0.08	0.39	0.004	0.40	0.07	3.36	7.7×10^{-4}
rs9349373 (A/G)	Upstream	0.20	0.26	0.008	0.22	0.13	3.04	0.002
rs10948300 (T/A)	Intronic	0.21	0.24	0.013	0.32	0.02	3.36	7.8×10^{-4}
rs1805017 (A/G)	Nonsynonymous coding	0.26	0.22	0.014	0.09	0.49	2.37	0.018
rs3799863 (A/T)	Intronic	0.05	0.46	0.016	−0.23	0.39	1.37	0.17
rs16874967 (A/G)	Intronic	0.06	0.39	0.02	−0.14	0.56	1.41	0.16
rs1805018 (G/A)	Nonsynonymous coding	0.06	0.39	0.02	−0.14	0.56	1.41	0.16
rs12195701 (A/G)	Intronic	0.21	0.21	0.03	0.26	0.07	2.85	0.004
rs1421368 (G/A)	Intronic	0.10	−0.27	0.04	−0.17	0.37	−2.16	0.03
rs9472830 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.26	0.02
rs1362931 (A/C)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs1421372 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs3799861 (A/G)	Intronic	0.20	−0.19	0.05	−0.16	0.23	−2.25	0.02
rs7756935 (C/A)	Intronic	0.21	−0.16	0.11	−0.15	0.25	−1.98	0.05
rs1051931 (A/G)	Nonsynonymous coding	0.21	−0.16	0.11	−0.16	0.23	−2.01	0.04
rs16874962 (A/G)	Intronic	0.07	0.12	0.44	0.15	0.46	1.06	0.29
rs17288905 (G/A)	Intronic	0.084	−0.04	0.76	−0.02	0.94	−0.29	0.77
CRP SNP (allele: minor/major)*								
rs3093068 (G/C)	3' downstream	0.06	−0.32	0.04	−0.09	0.70	−1.82	0.07
rs3093059 (G/A)	5' upstream	0.07	−0.31	0.06	−0.11	0.65	−1.78	0.07
rs12068753 (A/T)	5' upstream	0.07	−0.31	0.06	−0.08	0.75	−1.70	0.09
rs12084589 (A/C)	5' upstream	0.07	−0.31	0.06	−0.05	0.83	−1.63	0.10
rs2808631 (G/A)	3' downstream	0.002	−1.52	0.07	0.52	0.64	−1.16	0.25
rs1800947 (G/C)	Synonymous coding	0.07	0.19	0.23	0.29	0.18	1.77	0.08
rs2794521 (G/A)	5' upstream	0.25	−0.11	0.23	0.01	0.93	−0.90	0.37
rs1130864 (A/G)	3' UTR	0.30	0.10	0.27	−0.15	0.21	0.10	0.92
rs2808634 (A/G)	5' upstream	0.27	−0.09	0.30	0.00	0.98	−0.84	0.40
rs3093071 (A/C)	3' downstream	0.01	−0.23	0.54	−0.21	0.67	−0.75	0.45
rs3093066 (A/C)	3' UTR	0.001	−0.57	0.57	−1.52	0.33	−1.04	0.30
rs3093069 (C/A)	3' UTR	0.001	−0.57	0.57	1.54	0.49	−0.01	0.99
rs1205 (A/G)	3' downstream	0.34	0.04	0.65	0.16	0.17	1.20	0.23
rs3093070 (C/A)	5' upstream	0.02	−0.13	0.69	−0.67	0.56	−0.22	0.50
rs3091244 (A/G)	3' downstream	0.37	−0.89	0.99	0.00	0.15	−0.17	0.37

*For all analyses, the effect allele corresponds to the minor allele.
CAC = coronary artery calcification; PAMSyN = Philadelphia Area Metabolic Syndrome Network; PDHS = Penn Diabetes Heart Study; SIRCA = Study of Inherited Risk of Coronary Atherosclerosis; other abbreviations as in Table 1.

activity levels with risk of CHD (9,40–42). Meta-analyses support a modest CHD relationship independent of traditional risk factors and plasma CRP (40,43,44). Published studies, however, may underestimate the degree of confounding because of incomplete measurement and control for all atherogenic lipoproteins (9). In circulation, Lp-PLA$_2$ associates with apolipoprotein B and high-density lipoproteins, with the majority found on LDL particles. Because Lp-PLA$_2$ protein and activity are closely linked to circulating apolipoprotein B lipoproteins (36,45), it is not surprising that genetic factors (e.g., *APOC1*, *PSRC1*, *ZNF259*) that regulate plasma apolipoprotein B lipoproteins are also associated with plasma Lp-PLA$_2$ (46). Parenthetically, we found modest association of lipid-related genes (e.g., *LRP2*, *LPL*, *APOA2*) with plasma Lp-PLA$_2$ likely reflecting this indirect post-translational influence (Online Tables 1A and 1B). Interpretation of studies of plasma Lp-PLA$_2$ in CHD is challenging partly because circulating lipoproteins may grossly confound the association of plasma Lp-PLA$_2$ with CHD (8) and furthermore because lesion macrophage production may be more relevant to the disease than circulating protein.

While we failed to detect significant association between plasma Lp-PLA$_2$ and common SNPs in *PLA2G7,* the same *PLA2G7* variants were associated with CAC within our study samples. Our preliminary exploration also revealed only nominal associations of *PLA2G7* SNPs with Lp-PLA$_2$ mRNA levels in multiple cells and tissues. These eQTL

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findings should be interpreted cautiously because of limited power, relatively low levels of Lp-PLA_2 expression in tested cells, and (unlike *CRP*) well-characterized *cis*-acting SNPs for *PLA2G7* are lacking. Further, appropriately powered studies are needed to determine whether *PLA2G7* SNPs are related to expression of Lp-PLA_2 in inflammatory macrophages and foam cells, sources that may be most relevant to atherosclerosis. However, our data suggest caution in using circulating leukocyte Lp-PLA_2 mRNA levels as surrogates for effects of *PLA2G7* variation on arterial pathology. Overall, our findings support the concept that *PLA2G7* may relate to atherosclerosis independent of circulating Lp-PLA_2 mRNA and protein.

Published studies of *PLA2G7* in CHD are conflicting. In a meta-analysis of individuals of European ancestry, *PLA2G7* SNPs did not associate with risk of CHD (n ~5,000) (42), although there were relationships between Lp-PLA_2 activity and CHD and between *PLA2G7* SNPs and Lp-PLA_2 activity. However in a meta-analysis of over 13,000 Asians, a common nonsynonymous *PLA2G7* SNP showed evidence of association with CHD (31). Additional nonsynonymous SNPs have been associated with carotid plaque in Japanese (32) and recently a loss-of-function variant in *PLA2G7* was shown to protect against CHD in Koreans (30). Due to the absence in Caucasian samples of the functional *PLA2G7* SNP found in Asians (rs76863441 or V279F), we were not able to evaluate the effect of this functional variant in our samples. However, common variation in *PLA2G7* is well covered on the ITMAT Broad Care array platform (tag SNP-coverage r^2 >0.8 for alleles with minor allele frequency ≥2% in the gene ± 5 kilobases) (21). Therefore, we are confident that we achieved excellent coverage of common variation in this gene region in Caucasians. Whereas ethnic difference in the presence of allelic variation may exist, most published data suggest a relationship of *PLA2G7* with clinical CHD supporting our CAC findings.

Findings for *CRP* in our samples are consistent with published data and contrast with that observed for *PLA2G7*-Lp-PLA_2. Thus, even though a number of SNPs in *CRP* had strong associations with circulating CRP levels, there was no relationship between these same SNPs and CAC. These data are in line with hallmark Mendelian randomization studies of clinical CHD outcomes (33,34) and support a model of confounding or reverse causation for CRP associations with CAC and CHD.

Study limitations. First, our studies are correlative and do not define causality. We have not studied loss-of-function or gain-of-function variants in *PLA2G7* for their relation to CAC or CHD and, therefore, cannot infer Lp-PLA_2 directional actions in atherosclerosis. However, expression data in inflammatory macrophages and foam cells coupled to preliminary studies of Lp-PLA_2 inhibition in human atherosclerosis support an atherogenic role for human *PLA2G7*. Second, recent studies have shown stronger associations of *PLA2G7* with circulating Lp-PLA_2 measures than were found in our sample. This may relate to our smaller sample size, heterogeneity in the SIRCA and PDHS study samples, or differences in Lp-PLA_2 assays used across studies. The *PLA2G7*-Lp-PLA_2 system, however, may be a poor target for Mendelian randomization studies for several reasons including: heterogeneous environmental and genetic influences on circulating levels; *PLA2G7* actions in atherosclerosis are likely to be independent of circulating Lp-PLA_2; and well-characterized *cis*-acting SNPs to use as instrumental variables for *PLA2G7* are lacking. Finally, although not a direct measure of coronary atherosclerosis, studies show that CAC provides a quantitative estimate of coronary atherosclerosis (47) and is a useful predictor of CHD events (48).

Conclusions

We have demonstrated that Lp-PLA_2, in contrast to CRP, is not an acute phase protein in humans. Lp-PLA_2 has limited expression in circulating leukocytes or unstimulated monocytes ex vivo, but it is induced during differentiation to macrophages and in foam cells. Thus, robust biomarkers of Lp-PLA_2 action in atherosclerosis and of its pharmacological modulation in vascular tissues are lacking. Common variation in *PLA2G7*, but not in *CRP*, is related to the burden of CAC, suggesting that *PLA2G7* may indeed modulate human atherosclerosis. Our data provide support for the atherogenicity of Lp-PLA_2 in humans while highlighting the challenges in using plasma Lp-PLA_2 as a biomarker of CHD and in determining drug-dosing and therapeutic efficacy in atherosclerosis.

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Key Words: coronary artery calcification ▪ lipoprotein-associated phospholipase A$_2$ ▪ *PLA2G7*.

APPENDIX

For supplementary methods, please see the online version of this paper.

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