

Amino acids 149 and 294 of human lecithin:cholesterol acyltransferase affect fatty acyl specificity

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Abstract We identified two regions of human LCAT (hLCAT) that when mutated separately to the corresponding rat sequence (E149A and Y292H/W294F) and transiently expressed in COS-1 cells increased phospholipase A₂ (PLA₂) activity by 5.5- and 2.8-fold, respectively, and increased cholesteryl ester (CE) formation by 2.9- and 1.4-fold, respectively, relative to hLCAT using substrate particles containing 1-16:0,2-20:4-*sn*-glycero-3-phosphocholine (PAPC). In contrast, both activities with 1-16:0,2-18:1-*sn*-glycero-3-phosphocholine (POPC) substrate were similar among the three LCAT proteins. The triple mutant (E149A/Y292H/W294F) had increased PLA₂ activity with PAPC similar to that observed with the E149A mutation alone; however, unlike E149A, the triple mutant demonstrated a 50% decrease in activity with POPC for both PLA₂ activity and CE formation, suggesting an interaction between the two regions of LCAT. Additional mutagenesis studies demonstrated that W294F, but not Y292H, increased PLA₂ activity by 3-fold with PAPC without affecting activity with POPC. The E149A/W294F double mutation mimicked the LCAT activity phenotype of the triple mutant (more activity with PAPC, less with POPC). In conclusion, separate mutation of two amino acids in hLCAT to the corresponding rat sequence increases activity with PAPC, whereas the combined mutations increase PAPC and decrease POPC activity, suggesting that these amino acids participate in the LCAT PC binding site and affect fatty acyl specificity.—Zhao, Y., A. K. Gebre, and J. S. Parks. Amino acids 149 and 294 of human lecithin:cholesterol acyltransferase affect fatty acyl specificity. *J. Lipid Res.* 2004. 45: 2310–2316.

Supplementary key words cholesteryl ester formation • mutagenesis • substrate specificity • recombinant high density lipoprotein • recombinant lecithin:cholesterol acyltransferase • *sn*-2 fatty acid

Lecithin:cholesterol acyltransferase (EC 2.3.1.43), a water-soluble glycoprotein secreted by the liver, is responsible for cholesteryl ester (CE) formation on the surface of plasma lipoproteins (1). The preferred lipoprotein substrate of LCAT is HDL, which contains the main activator of LCAT, apolipoprotein A-I (2). During the reaction, LCAT displays two enzymatic activities (3). The first is a phospholipase A₂

(PLA₂) step, in which the fatty acyl chain from the *sn*-2 position of phosphatidylcholine (PC) is cleaved to form an acyl-enzyme intermediate with the active site serine and the release of lyso-PC into solution. The subsequent step is an acyltransferase activity, in which the fatty acyl chain is transferred to the 3 β -hydroxyl group of free cholesterol (FC) to generate CE (3). The CE product is extremely hydrophobic and partitions into the core of the HDL particle (4). LCAT plays a key role in the maturation of nascent HDL and in the reverse cholesterol transport pathway, a process in which excess FC in peripheral tissues is taken up by HDL particles, esterified by LCAT, and transported to the liver for uptake by the scavenger receptor class B type I (5).

The fatty acyl composition of PC is the primary regulator of LCAT activity in plasma (1). It has been known for more than 30 years that human LCAT (hLCAT) prefers PC substrates containing 18:1 and 18:2 in the *sn*-2 position for CE synthesis, whereas rat and mouse LCAT prefer PC containing 20:4 in the *sn*-2 position (6–9). This fatty acyl preference was apparent even when 90% of the recombinant HDL (rHDL) particle surface was replaced with enzymatically unreactive matrix lipid (PC ether), which forms a uniform substrate interface (10). PC specificity of LCAT activity has also been observed at the PLA₂ step of the reaction, measured as release of free fatty acid from the *sn*-2 position of the PC substrate when cholesterol is not included in the rHDL substrate particles (11).

The molecular basis for the difference in PC substrate specificity between the human and rodent LCAT enzymes was unknown until Subbaiah et al. (12) found that the middle region of LCAT (amino acids 130–306) was responsible for determining fatty acyl substrate specificity by analyzing the activity profile of human and mouse chimeric LCAT cDNA constructs. Subsequent to that study, we used

Abbreviations: CE, cholesteryl ester; FC, free cholesterol; hLCAT, human LCAT; PAPC, 1-16:0,2-20:4-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PLA₂, phospholipase A₂; POPC, 1-16:0,2-18:1-*sn*-glycero-3-phosphocholine; rHDL, recombinant HDL; rLCAT, rat LCAT.

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a cluster mutagenesis approach to mutate six regions over the middle third of hLCAT to the corresponding rat sequence (11). The mutant LCAT enzymes were tested for their ability to react with PC substrate molecules containing 1-16:0,2-18:1-*sn*-glycero-3-phosphocholine (POPC) or 1-16:0,2-20:4-*sn*-glycero-3-phosphocholine (PAPC). We discovered two regions of hLCAT that were able to convert the PC fatty acyl specificity of hLCAT to that of rat LCAT (rLCAT) for both PLA₂ and cholesterol esterification activity, resulting in increased activity with PAPC compared with POPC. In one region, located in a flexible loop region close to the active site serine 181 of hLCAT, we found that substitution of the rat for human sequence at a single amino acid at position 149 of hLCAT (glutamic acid replaced by alanine; E149A) increased activity with PAPC substrate to a level observed for rLCAT. In the second region, similar substitutions of rat for human sequence at positions 292 and 294 (Y292H/W294F) resulted in a more modest activation of enzyme activity with PAPC compared with that observed with hE149A. In the present study, we wished to determine whether one or both amino acids in the Y292/W294 cluster were involved in determining PC fatty acyl specificity and whether substitutions in this cluster were additive with that of hE149A with regard to increased activity with PAPC.

EXPERIMENTAL PROCEDURES

PCR site-directed mutagenesis

The mutant hE149A and the mutant hY292H/W294F of hLCAT were individually generated by a megaprimer PCR site-directed mutagenesis procedure using the hLCAT cDNA in a pCMV5 expression vector as the template (11). The rest of the mutants were generated by the overlapping extension site-directed mutagenesis method using PCR (13). PCR procedures were carried out using *pfu* polymerase. The hE149A/Y292H/W294F was generated using the hY292H/W294F cDNA construct as a template. Two fragments were amplified in two separated PCR procedures. The first fragment with a mutation site at the 3' end was generated by a 5' sense primer to the vector (5'-GGAGGTCTATATAG-CAGAG-3') flanking the 5' end of inserted LCAT cDNA and a 3' mutagenic antisense primer (5'-TACTCCTCCTGCTGGCCGG-GCGCCAGCCGCCAGTCATA-3'). The second fragment with a 5' end mutation site was generated by the complementary 5' mutagenic sense primer (5'-TATGACTGGCCGGCTGGCCCGCC-CAGCAGGAGGAGTA-3') and a 3' antisense primer to the vector flanking the 3' end of the LCAT cDNA (5'-GGGTCACAGGGAT-GCCAC-3'). The short overlapping regions at the ends of the fragments allowed the two fragments to anneal and be extended to form a template for PCR amplification using the two flanking vector primers. The full-length mutant LCAT cDNA was restriction digested with *Bss*HII and *Pst*I to generate the fragment including all three mutations, then ligated into *Bss*HII- and *Pst*I-digested pCMV His-tagged hLCAT cDNA (14). By a similar mutagenic approach, the hY292H and hW294F mutants were generated using wild-type hLCAT cDNA as a template, whereas hE149A/Y292H and hE149A/W294F mutants were generated using hE149A mutant cDNA as a template. The sequences of the mutagenic sense primers were as follows: Y292H, 5'-TTTGAGGAAGGCTGGCA-CATGTGGCTGCAGTCA-3'; W294F, 5'-GAAGGCTGGTACATG-TTCCTGCAGTCACGTGAC-3'. The correct sequences of the mu-

tant constructs between the two restriction sites were confirmed by dideoxy sequencing.

In vitro expression of hLCAT and mutants

The wild-type and mutant pCMV hLCAT cDNAs were transiently transfected, using FuGENE™ 6 (Roche), into COS-1 cells grown in 35 mm tissue culture dishes. After the transfection, the cells were washed three times with Hanks' balanced salt solution (Mediatech) and switched to serum-free DMEM/F12 for an additional 72 h at 37°C. The medium was then collected, centrifuged at 500 g for 10 min, and immediately frozen at -70°C until assays were performed.

ELISA for quantification of LCAT mass

Media samples were assayed in four replicates for LCAT mass using a sandwich ELISA procedure. Purified carboxy-terminal histidine-tagged human recombinant LCAT protein (14) was used as a standard for the assay. The wells of microtiter plates were coated with affinity-purified anti-hLCATh6 rabbit antibody (2 µg/ml; Novus Biologicals, Inc.) in coating buffer (0.1 M Na₂CO₃ and 0.1 M NaHCO₃) at 4°C overnight (0.4 µg/well) and then incubated with blocking buffer (10 mM Tris-base, 200 mM NaCl, 0.1% Tween 20, and 0.1% BSA) at 37°C for 2 h. The media samples were diluted into 200 µl of blocking buffer containing 5 mM β-mercaptoethanol, incubated at 37°C for 1 h, and then applied to the wells for incubation at 37°C for 3 h. After incubation, the wells were washed three times with TBS-Tween buffer (10 mM Tris-base, 200 mM NaCl, and 0.1% Tween 20), a 1:1,000 dilution of biotin-labeled anti-hLCAT rabbit antibody in blocking buffer was applied to each well, and the plate was incubated at 37°C for 2 h. The plate was then washed and incubated with streptavidin-HRP (1:100,000 dilution; Pierce) at 37°C for 1 h. After the plate wells were washed, the plate was developed with one-step Turbo TMB ELISA (Pierce) and read at 450 nm. The log-transformed standard curve (log LCAT mass) was linear from 0.03 to 2 ng/well.

Recombinant HDL synthesis

rHDLs, used as substrate particles for CE formation and PLA₂ activity assays of LCAT, were synthesized by a cholate dialysis procedure as detailed in a previous publication (15). Two PC species were used for rHDL synthesis: POPC and PAPC (Sigma). For measurement of CE formation, rHDLs were made with purified human plasma apolipoprotein A-I, cholesterol, and PC in a starting molar ratio of 1:5:80, with a trace amount of [³H]cholesterol (NEN; 50,000 dpm/µg cholesterol). For measurement of PLA₂ activity, rHDLs were made with purified human apolipoprotein A-I and PC in a starting molar ratio of 1:80, with [¹⁴C]PC in the *sn*-2 position. 1-Palmitoyl-2-[¹⁴C]oleoyl PC was purchased from Amersham Biosciences, and 1-palmitoyl-2-[¹⁴C]arachidonoyl PC was purchased from NEN Life Science Products.

LCAT CE formation and PLA₂ activity assays

LCAT assays were performed in duplicate in 0.5 ml of buffer containing saturating amounts of rHDL substrate (1.2 µg of cholesterol for CE formation and 38 µg of PC for PLA₂ activity) using 50–100 µl of medium as an enzyme source as described previously (15, 16). The incubation time was adjusted to keep the CE formation or free FA release under 20% to prevent product inhibition. After incubation, the lipids were extracted by the Bligh-Dyer method (17). The radiolabeled FC and CE or free FA and PC were separated by TLC using a neutral solvent system (hexane-ether-acetic acid, 70:30:2) and quantified by scintillation counting. LCAT activity was expressed as nanomoles of CE formed per microgram of LCAT per hour for CE formation or as

nanomoles of FA released per microgram of LCAT per hour for PLA₂ activity.

Statistical analyses

Statistical analyses of LCAT activity data for different LCAT proteins were performed using one-way ANOVA with post hoc pairwise comparisons performed with Tukey's method.

RESULTS

We combined the E149A and Y292H/W294F mutations to generate a triple mutation construct (E149A/Y292H/W294F) and then transiently expressed the E149A, Y292H/W294F, and E149A/Y292H/W294F constructs in COS cells along with hLCAT and rLCAT cDNA as controls. The media were assayed for PLA₂ activity using rHDL containing [¹⁴C]POPC or [¹⁴C]PAPC with no cholesterol and for LCAT acyltransferase activity measured as cholesterol esterification activity using POPC or PAPC rHDL with [³H]cholesterol. The PAPC/POPC activity ratios, representing the fatty acyl preference for PAPC relative to POPC, for both PLA₂ and cholesterol esterification activity are summarized in **Fig. 1**. For PLA₂ activity, hLCAT had an activity ratio of 0.4 ± 0.02 , whereas rLCAT had a ratio of 2.14 ± 0.01 . The activity ratios of E149A (1.59 ± 0.05) and Y292H/W294F (1.26 ± 0.1) were higher than that of hLCAT but still less than that of rLCAT. The E149A/Y292H/W294F

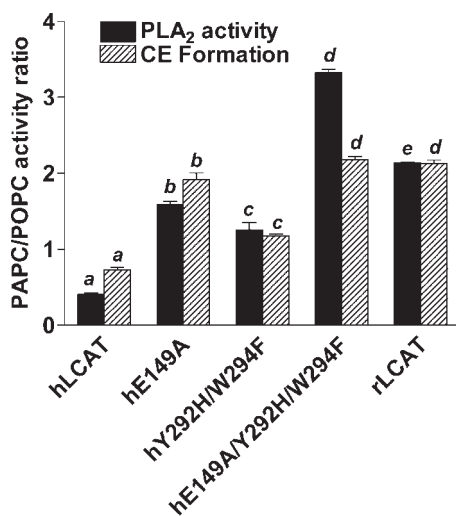


Fig. 1. 1-16:0,2-20:4-*sn*-glycero-3-phosphocholine/1-16:0, 2-18:1-*sn*-glycero-3-phosphocholine (PAPC/POPC) activity ratios for phospholipase A₂ (PLA₂) activity (solid bars) and cholesterol esterification (striped bars). COS cells were transiently transfected with human LCAT (hLCAT), rat LCAT (rLCAT), or mutant cDNA constructs (hE149A, hY292H/W294F, hE149A/Y292H/W294F). The media were assayed for cholesterol esterification and PLA₂ activity using saturating concentrations of rHDLs containing POPC or PAPC in duplicate as described in Experimental Procedures (1.2 μg of cholesterol for cholesterol esterification or 38 μg of phosphatidylcholine for PLA₂ activity). Activity ratios are expressed as means ± SEM of three separate transfections. Unlike letters denote statistically significant differences ($P < 0.05$) in activity ratios among the five LCAT constructs for either PLA₂ activity or cholesterol ester (CE) formation.

triple mutation, combining mutations from these two regions, resulted in a dramatic increase of the activity ratio to 3.33 ± 0.05 , which is much higher than that of rLCAT. Thus, there was an additive influence of Y292H/W294F with the E149A mutation on determining fatty acyl specificity of LCAT PLA₂ activity. A similar trend was observed for cholesterol esterification activity, except that the PAPC/POPC activity ratio of the E149A/Y292H/W294F mutation (2.18 ± 0.04) was only slightly, but significantly, higher than that of the E149A mutation (1.92 ± 0.09). The additive effect of Y292H/W294F and E149A was less for cholesterol esterification activity compared with that for PLA₂ activity.

After the LCAT mass in media was measured by ELISA, the specific activity was calculated for both the PLA₂ and cholesterol esterification assays; the data are shown in **Fig. 2A, B**, respectively. When POPC rHDL was used as a substrate, the PLA₂ activity of the E149A mutant (36.9 ± 1.2 nmol FA/h/μg LCAT) was ~40% higher than that of hLCAT (26.3 ± 1.4 nmol FA/h/μg LCAT), whereas the PLA₂ activity of the Y292H/W294F mutant (23.1 ± 1.3 nmol FA/h/μg LCAT) showed no change from hLCAT. When PAPC rHDL was used as a substrate, the PLA₂ activities of the E149A (58.9 ± 2.0 nmol FA/h/μg LCAT) and Y292H/W294F (29.2 ± 3.5 nmol FA/h/μg LCAT) mutants were 6- and 3-fold higher than that of hLCAT (10.6 ± 1.0 nmol FA/h/μg LCAT), respectively. Adding the Y292H/W294F mutation to the E149A mutant construct (E149A/Y292H/W294F) did not lead to a further increase of PLA₂ activity on PAPC rHDL (52.0 ± 2.6 nmol FA/h/μg LCAT) but rather to a 40% reduction in activity with POPC rHDL (15.7 ± 1.0 nmol FA/h/μg LCAT) compared with hLCAT.

Compared with the results for PLA₂ activity, differences between mutants and the human wild-type LCAT control were not as great for cholesterol esterification activity with rHDL containing PAPC (**Fig. 2B**). The activity of E149A (142.1 ± 4.1 nmol CE/h/μg LCAT) for PAPC became approximately three times that of hLCAT (49.8 ± 3.6 nmol CE/h/μg LCAT), and the PAPC activities of Y292H/W294F and E149A/Y292H/W294F (71.1 ± 6.6 and 73.2 ± 5.4 nmol CE/h/μg LCAT, respectively) were only 40% higher than that of the hLCAT control. When assayed with POPC rHDL, the E149A and Y292H/W294F mutant constructs showed similar activity as hLCAT; only E149A/Y292H/W294F had a 50% reduced cholesterol esterification activity (33.2 ± 2.8 nmol CE/h/μg LCAT) compared with hLCAT (67.9 ± 2.3 nmol CE/h/μg LCAT). Overall, compared with hLCAT, the E149A/Y292H/W294F mutation led to an increase in both PLA₂ and cholesterol esterification activity when PAPC rHDL was used as a substrate and to a decrease in both activities when POPC rHDL was used as a substrate.

Y292H/W294F alone conferred an increase in the activity with PAPC, but the combination of Y292H/W294F with E149A resulted in decreased activity with POPC. To define the minimal mutation necessary to change fatty acyl specificity, we generated Y292H and W294F mutant LCAT constructs. As shown in **Fig. 3A**, the PAPC/POPC activity ratios of PLA₂ and cholesterol esterification activity for the

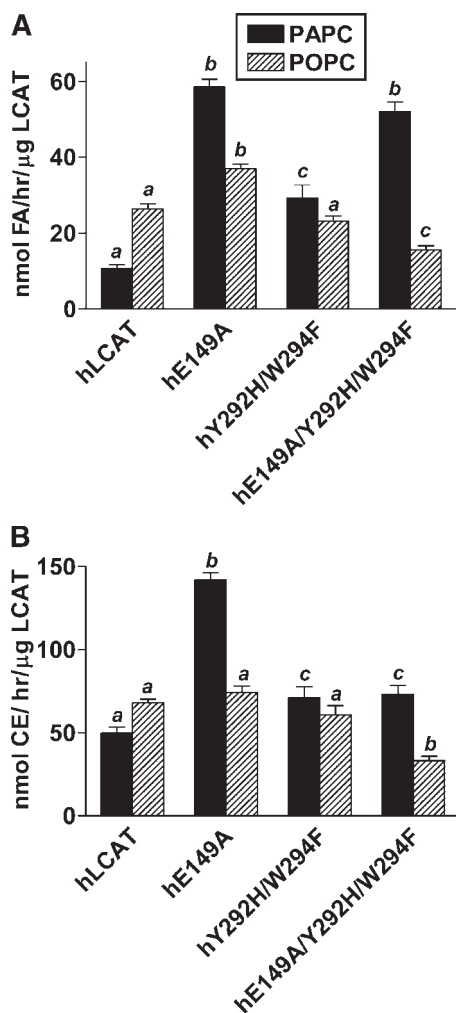


Fig. 2. Specific activity of PLA₂ (A) and cholesterol esterification (B) assays for hLCAT, hE149A, hY292H/W294F, and hE149A/Y292H/W294F LCAT constructs. The experimental procedures were the same as described for Fig. 1. PLA₂ and cholesterol esterification activities were normalized for the amount of LCAT protein in the media, as determined by ELISA. Values are expressed as means \pm SEM for three separate transfections. Unlike letters denote statistically significant differences ($P < 0.05$) in specific activity among the four LCAT constructs for rHDL containing either PAPC or POPC. FA, Fatty acid.

W294F mutant agreed closely with the values for Y292H/W294F, whereas the activity ratios of the Y292H mutant agreed closely with that of hLCAT. PLA₂ specific activity for the W294F and Y292H/W294F mutants was 3-fold higher with PAPC rHDL compared with hLCAT, whereas the Y292H mutant and hLCAT had similar activities (Fig. 3B). PLA₂ activity was similar among all four constructs with POPC rHDL (Fig. 3B). A similar, but less striking, trend was observed for cholesterol esterification (Fig. 3C). These results suggested that mutation of amino acid 294 was responsible for the increase in reactivity with PAPC rHDL, whereas mutation of amino acid 292 had no effect on activity.

Because mutation of amino acid 294 was responsible for the increased activity with PAPC, we generated the E149A/

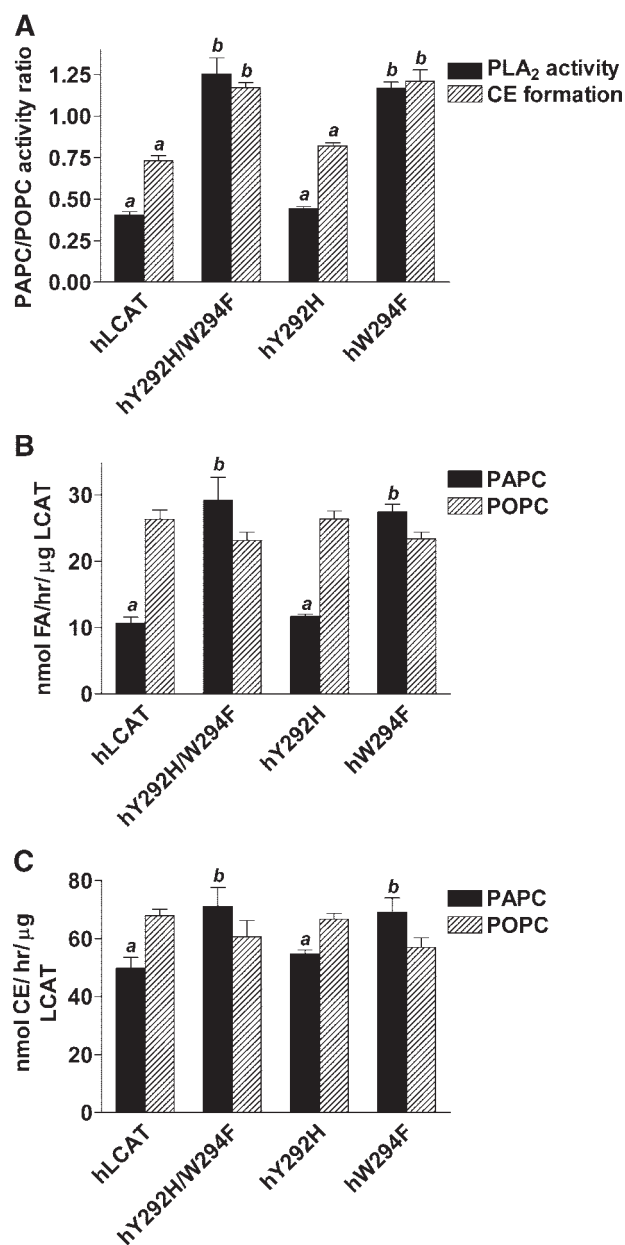


Fig. 3. Effect of Y292H and W294F mutations on LCAT activity. The experimental procedures were the same as those described for Fig. 1. Values are expressed as means \pm SEM for three separate transfections. A: PAPC/POPC activity ratios for PLA₂ activity (solid bars) and cholesterol esterification (striped bars). Unlike letters denote statistically significant differences ($P < 0.05$) in activity ratios among the four LCAT constructs for either PLA₂ or cholesterol esterification. B: Specific activity for the PLA₂ assay. C: Specific activity for the cholesterol esterification assay. In B and C, unlike letters denote statistically significant differences ($P < 0.05$) in specific activities among the four LCAT constructs for rHDL containing PAPC; no significant differences were observed for rHDL containing POPC. FA, Fatty acid.

W294F mutant and tested it for PLA₂ and cholesterol esterification activities (Fig. 4). The E149A/W294F mutant showed a similar PAPC/POPC activity ratio (Fig. 4A) and similar patterns of PLA₂ (Fig. 4B) and cholesterol esterification (Fig. 4C) activities with PAPC or POPC rHDL as the E149A/Y292H/W294F triple mutant. The combination of

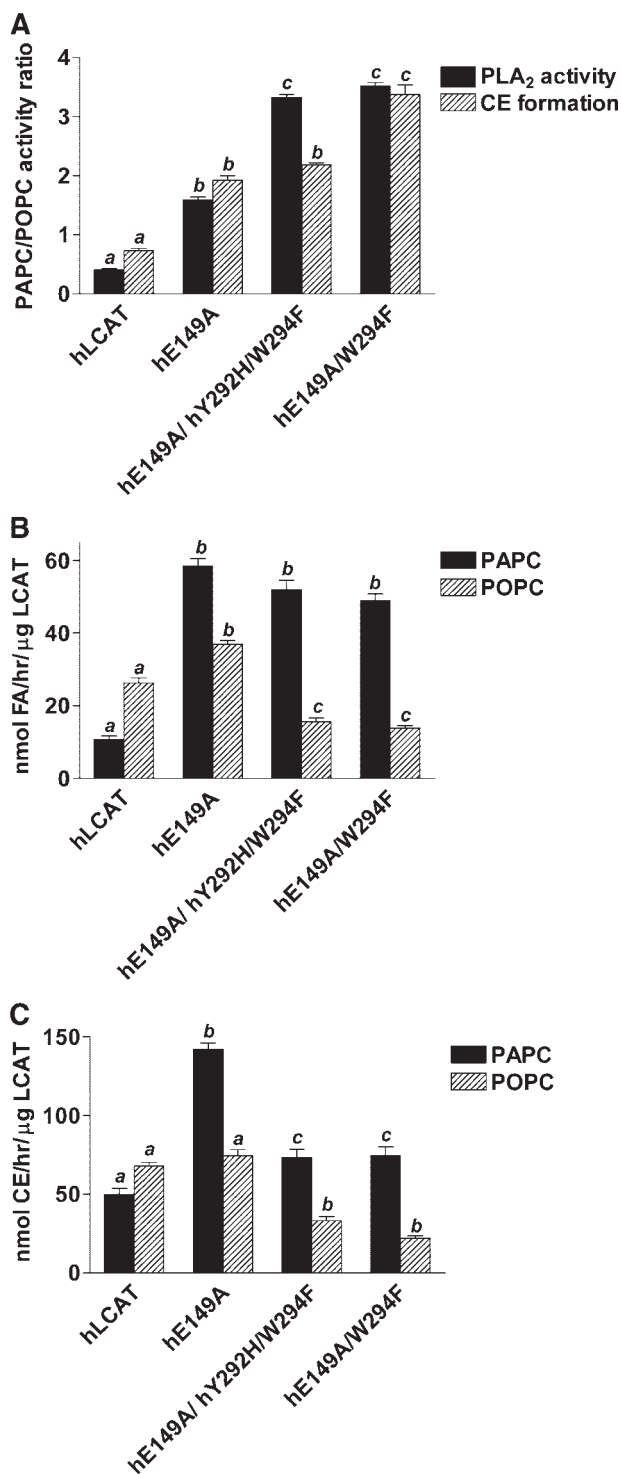


Fig. 4. Effect of E149A/Y292H/W294F and E149A/W294F mutations on hLCAT activity. The experimental procedures were the same as those described for Fig. 1. Values are expressed as means \pm SEM for three separate transfections. A: PAPC/POPC activity ratios for PLA₂ activity (solid bars) and cholesterol esterification (striped bars). Unlike letters denote statistically significant differences ($P < 0.05$) in activity ratios among the four LCAT constructs for either PLA₂ activity or CE formation. B: Specific activity for the PLA₂ assay. C: Specific activity for the cholesterol esterification assay. In B and C, unlike letters denote statistically significant differences ($P < 0.05$) in specific activities among the four LCAT constructs for rHDL containing either PAPC or POPC. FA, Fatty acid.

these two point mutations led to reduced PLA₂ (Fig. 4B) and cholesterol esterification (Fig. 4C) activities with POPC rHDL, similar to the results observed for the triple mutation. Also note that the cholesterol esterification activities with PAPC for the E149A/Y292H/W294F triple mutant and the E149A/W294F mutant were significantly lower compared with hE149A (Fig. 4C), whereas the three proteins in the PLA₂ assay (Fig. 4B) had similar activities.

DISCUSSION

In our previous study, we discovered that changing the glutamic acid (E) at position 149 of hLCAT to alanine (A), the amino acid at the same position of rLCAT, resulted in increased activity with PAPC rHDL compared with hLCAT for both PLA₂ and cholesterol esterification activities (11). In the present study, another amino acid mutation at position 294 was shown to increase activity with PAPC rHDL, although not to the extent observed with the E149A mutation. When the E149A and W294F mutations were combined, the PLA₂ activity with PAPC rHDL was similar to that for the E149A mutation; however, PLA₂ activity with POPC was reduced for the double mutant. In addition, the impact of these mutations was much more striking for PLA₂ activity than it was for cholesterol esterification. These results show that 2 of the 416 amino acids in hLCAT are involved in determining PC fatty acyl substrate specificity and likely lie in regions of the LCAT protein that form the binding pocket for PC. In support of this idea, the mutation of both amino acids results in a decrease in PLA₂ activity with POPC and a general decrease in cholesterol esterification rate compared with the single E149A mutation, suggesting that the W294F mutation interferes with binding of POPC and cholesterol to the active site of the enzyme.


Several studies (18–21) have suggested that the rate-limiting step in the LCAT reaction is the PLA₂ cleavage of PC substrate. The LCAT reaction appears to share a similar catalytic mechanism with lipases and phospholipases (3, 22). Using human pancreatic lipase and *Candida antarctica* lipase as templates, Peelman et al. (23) identified D345 and H337, together with S181, as the catalytic triad and built a three-dimensional model for the central domain of LCAT, which consisted of seven conserved β strands connected to four α helices by loop regions. Crystallographic study of pancreatic lipase suggests that the PC substrate molecule binds to a hydrophobic pocket before hydrolysis of the *sn*-1 fatty acid (24). Therefore, it is likely that a similar hydrophobic active site in LCAT should also exist for binding the PC molecule, which controls the catalytic rate of the PLA₂ reaction. Helix α 4-5 in the three-dimensional model of LCAT has lipid binding activity (25) and is proposed to be involved in binding of the PC molecule to the active site (26). E149A, residing on the hydrophilic loop N terminal of helix α 4-5, might change the orientation of helix α 4-5, resulting in a different size or shape of the active site pocket that more easily interacts with arachidonic acid (27). The long excursion at residues 210–332 was not

included in the three-dimensional model, because of the lack of an appropriate template. When the hydrophobic apolar W at position 294 was substituted with a less bulky apolar F, the PLA₂ activity of the enzyme increased with PAPC rHDL (Fig. 3B). This outcome suggested that the size of amino acid 294 may play a role in determining fatty acid specificity. We speculate that W294 might be directly involved in forming the PC substrate binding pocket at the active site. Therefore, a small change in amino acid side chain size results in a conformational change that favors the binding of the bulkier and more flexible fatty acyl chain at the active site, perhaps by increasing the size of the PC substrate binding pocket. However, the combination of E149A and W294F did not further increase the catalytic efficiency with PAPC but dramatically reduced the PLA₂ activity with POPC (Fig. 4B) and decreased the cholesterol esterification rate with both POPC and PAPC. The combined conformation change induced by the E149A and W294F mutations might reduce the binding of POPC and cholesterol to the substrate binding pocket or to an unfavorable orientation for reaction within the catalytic triad.

We do not believe that the mutations result in decreased binding of LCAT to the HDL particle surface, because the E149A mutant and hLCAT have been shown to have similar binding to the surface of rHDL particles (27). In addition, the apparent K_m values for hLCAT and the E149A mutant were similar with both PAPC and POPC substrate. In another study, we showed that hLCAT had increased the binding affinity to rHDLs containing PAPC compared with those containing POPC, but LCAT activity remained low with PAPC substrate (28). Bolin and Jonas (29) have shown that the apparent K_m of the LCAT reaction reflects the binding of the enzyme to the substrate particle surface, whereas the apparent V_{max} reflects monomeric substrate preference at the active site of the enzyme. Taken together, these studies suggest that LCAT binding to the substrate lipoprotein particle surface is not the rate-limiting step for enzyme catalysis. Further studies with purified enzyme will be necessary to determine whether the combined E149A/W294F mutation affects rHDL particle surface binding and apparent K_m or whether the differences in activity observed in this study were attributable to the binding of monomer PC molecules at the active site of LCAT and were primarily mediated by effects on apparent V_{max} .

In the PLA₂ assay, cholesterol-free rHDLs are used as substrate particles and the enzyme uses water as the acyl acceptor for the *sn*-2 fatty acyl group in the transacylation step of the reaction. For all of the mutant and control LCAT constructs, PLA₂ activity was relatively low, compared with the cholesterol esterification activity assayed with rHDLs containing cholesterol. This could be attributable to the absence of cholesterol, which is the best acyl acceptor, or may be the result of limited penetration of water into the hydrophobic active site (30). Other explanations are also possible. Different substrate particles and reactions were used to monitor PLA₂ activity and CE formation, and as such, the assay conditions were not identical.

PLA₂ activity was measured as the release of radiolabeled fatty acid from the *sn*-2 position of PC molecules. If reesterification of lyso-PC with the released *sn*-2 fatty acid resulted in regeneration of PC, as described previously (31, 32), then PLA₂ activity would be underestimated. Finally, if hLCAT uses some *sn*-1 fatty acid when it encounters polyunsaturated PC species such as PAPC, PLA₂ activity would also be underestimated (33). Any combination of these possibilities could explain the relatively lower PLA₂ activities.

E149A, W294F, and the combined mutation, E149A/W294F, showed large increases in PLA₂ activity with PAPC rHDL compared with the hLCAT control (Fig. 4B); however, the extent of the increase for the mutants compared with the wild-type control was considerably reduced for acyltransferase activity (cholesterol esterification activity) with PAPC rHDL (Fig. 4C). These results demonstrate that the two point mutations of LCAT in our study affect both the PLA₂ and acyltransferase reactions. There appear to be distinct sites for PC and cholesterol binding, because a PC and CE molecule can simultaneously be present in the active site to form cholesteryl diester (34). Our data support the hypothesis that PC and cholesterol binding sites are close to each other and might even have some overlap in the active site of LCAT. 

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