Reactive Oxygen Species (ROS) Mediate p300-dependent STAT1 Protein Interaction with Peroxisome Proliferator-activated Receptor (PPAR)-γ in CD36 Protein Expression and Foam Cell Formation*

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Background: CD36 plays a role in lipid uptake, foam cell formation, and atherogenesis.

Results: 15(S)-HETE induces CD36 expression and foam cell formation by triggering p300-mediated STAT1 acetylation and its interaction with PPARγ.

Conclusion: STAT1 and PPARγ interact with each other in CD36 expression and foam cell formation.

Significance: 15(S)-HETE appears to play an important role in foam cell formation, a critical event in atherogenesis.

Atherosclerosis, which is a chronic inflammatory disease of blood vessels, is one of the major causes of death and morbidity in the Western world (1, 2). Oxidation of LDL and its uptake by macrophages via scavenger receptors such as CD36 and thereby becoming foam cells and accumulation of these foam cells in the artery are the essential factors underlying the pathogenesis of atherosclerosis.

Previously, we have demonstrated that 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) induces CD36 expression involving STAT1. Many studies have shown that peroxisome proliferator-activated receptor (PPAR)-γ mediates CD36 expression. Therefore, we asked the question whether these transcriptional factors interact with each other in the regulation of CD36 expression by 15(S)-HETE. Here, we show that STAT1 interacts with PPARγ in the induction of CD36 expression and foam cell formation by 15(S)-HETE. In addition, using molecular biological approaches such as EMSA, supershift EMSA, ChIP, re-ChIP, and promoter-reporter gene assays, we demonstrate that the STAT1 and PPARγ complex binds to the STAT-binding site at −107 nucleotides in the CD36 promoter and enhances its activity. Furthermore, the interaction of STAT1 with PPARγ depends on STAT1 acetylation, which is mediated by p300. In addition, our findings show that reactive oxygen species-dependent Syk and Pyk2 stimulation is required for p300 tyrosine phosphorylation and activation. Together, these results demonstrate that an interaction between STAT1, p300, and peroxisome proliferator-activated receptor-γ is required for 15(S)-HETE-induced CD36 expression, oxidized low density lipoprotein uptake, and foam cell formation, critical events underlying the pathogenesis of atherosclerosis.

Atherosclerosis, which is a chronic inflammatory disease of blood vessels, is one of the major causes of death and morbidity in the Western world (1, 2). Oxidation of LDL and its uptake by macrophages via scavenger receptors such as CD36 and thereby becoming foam cells and accumulation of these foam cells in the artery are the essential factors underlying atherogenesis (2, 3). Toward elucidating the mechanisms of LDL oxidation, it was discovered that the epitopes of oxidized low density lipoprotein (OxLDL) were co-localized with 15-lipoxygenase (15-LO) mRNA and protein, the findings that led to the idea that 15-LO might be involved in the pathogenesis of this vascular lesion (4–7). Indeed, many studies using both genetic and pharmacological approaches have demonstrated that lipoxygenases (LOs), particularly 5- and 15-LOs, mediate LDL oxidation and thus play a role in atherogenesis (8–13). Two 15-LOs, namely 15-LO1 and 15-LO2, have been characterized in humans, and both convert arachidonic acid (AA) to 15(S)-HETE (14–17). Interestingly, some studies have shown that atherosclerotic arteries possess increased capacity of converting arachidonic acid to 15-HETE compared with normal arteries (18, 19). In addition, many cardiovascular disease risk factors such as hypercholesterolemia, diabetes, obesity, and smoking have also been shown to be associated with increased expression and/or activity of 12/15-LO, the murine ortholog of human 15-LO1 (20–23). Because many studies have shown that cardiovascular risk factors influence 12/15-LO activity (20–23) and atherosclerotic arteries produce 15(HETE) (18, 19), a major 15-LO1/2 metabolite of AA, we asked the question whether this eicosanoid has any role in the pathogenesis of atherosclerosis. Toward this end, we have previously reported that 15(S)-HETE induces CD36 expression and foam cell formation (24). However, many studies have shown that PPARγ mediates CD36 expression (25, 26). Based on these observations, we asked the question whether both STAT1 and PPARγ interact with each other in mediating CD36 expression and foam cell formation. In this study, we report that 15(S)-HETE-induced CD36 expression requires STAT1 interaction with PPARγ. We also show that STAT1 acetylation is essential for its...
interaction with PPARγ. Surprisingly, STAT1 acetylation requires 
\(\text{XO}\) and NADPH oxidase-dependent ROS production 
leading to Syk- and Pyk2-targeted p300 tyrosine phosphorylation 
and its activation.

**Materials and Methods**

**Reagents**—Anti-phosphoserine/threonine antibodies (ab17464) 
were obtained from Abcam (Cambridge, MA). 15(S)-HETE 
(34720) was purchased from Cayman Chemicals (Ann Arbor, 
MI). Anti-pSTAT1 antibodies (7649) were bought from Cell 
Signaling Technology (Beverly, MA). Anti-peroxisome prolif-
erator-activated receptor δ antibodies (NBPIP-39684) were pur-
chased from Novus Biologicals (Littleton, CO). Anti-acetyl-ly-
sine (SC-32268), anti-CD36 (SC-9154), anti-CBP (SC-369), 
anti-p300 (SC-584), anti-PPARα (SC-9000), anti-PPARβ 
(SC-7197), anti-PPARγ (SC-7196), anti-STAT1 (SC464), 
anti-STAT3 (SC-482), anti-STAT5 (SC-835), and anti-β-tubulin 
(SC-9104) antibodies, normal mouse serum (SC-45051), and 
normal rabbit serum (SC-3888) were obtained from Santa Cruz 
Biotechnology (Santa Cruz, CA). Acetyl coenzyme A (A2056), 
apocynin (A10809), allopurinol (A8003), and Oil-red-O 
culture medium overnight before using for experiments.

**Cell Culture**—THP1 cells, a human leukemia monocyte cell 
line, were purchased from American Type Culture Collection 
(Manassas, VA) and cultured in RPMI 1640 medium supple-
mented with 10% heat-inactivated fetal bovine serum (FBS), 50 
units/ml penicillin, 50 μg/ml streptomycin, and 50 μM μ-mer-
captoethanol. Cultures were maintained in a humidified 95% 
air and 5% CO₂ atmosphere at 37°C.

**Western Blotting**—Cell extracts containing an equal amount 
of protein were resolved by electrophoresis on 0.1% (w/v) SDS 
and 10% (w/v) polyacrylamide gels. The proteins were trans-
ferred electrophoretically to a nitrocellulose membrane. 
After blocking in either 5% (w/v) nonfat dry milk or 5% (w/v) BSA, 
the membrane was probed with appropriate primary antibodies 
followed by incubation with horseradish peroxidase-conju-
gated secondary antibodies. The antigen-antibody complexes 
were detected using enhanced chemiluminescence detection 
reagent kit (GE Healthcare).

**Acetyltransferase Assay**—The acetyltransferase activity of 
p300 was measured using a p300/CBP immunoprecipitation 
HAT assay kit from Millipore.

**ROS Measurement**—ROS production was measured as 
described by us previously (24).

**Transfections**—Transfections were performed as described 
previously (24). Briefly, cells were transfected with the indi-
caled ASOs at 100 nM concentration using Lipofectin transfection 
reagent for 6 h following the manufacturer’s instructions. 
After transfection, cells were maintained in complete RPMI 
1640 medium for 36 h followed by growth arrest in serum-free 
medium overnight before using for experiments.

**Cloning and Mutagenesis**—Human CD36 promoter cloning 
was described previously (24). Site-directed mutations of 
STAT1 K410R/K413R were introduced using the QuikChange 
site-directed mutagenesis kit according to manufacturer’s 
instructions and using the following primers: forward, 5'-
CCATGTCAGAAGACCGAATTCGTCGCCAC-3', and 
reverse, 5'-GTGGCGCATCTGGTTCGCGAATTCG- 
GCC-3', to yield STAT1 K410R/K413R mutant. The boldface 
letters indicate the mutated bases. The clones were verified by 
DNA sequencing using vector specific primers.

**EMSA**—Nuclear extracts of THP1 cells with and without 
appropriate treatments were prepared as described previously 
(24). The protein content of the nuclear extracts was 
determined using a micro-BCA method (Pierce). Biotinylated 
double-stranded oligonucleotides encompassing the STAT- 
binding element at −107 nt forward (5'-ATTTTTTTTTTTT-
TTCAATTTCTCTAGGAACAAAACCCACACTG-3') and 
reverse (5'-CAGTGTGGTGTGTGTTCTCAGAAGATT-
GAAATGAAAAGAAAAAAT-3') were used as a WT probe and 
forward (5'-ATTTTTTTTTTTTTCAATGGTCTAGGAA-
AACAAAACCCACACTG-3') and reverse (5'-CAGTGTGGTGT-
GTGTTCACATTCAATGGAAAAAAGAAAAAT-3') were used as a mutant probe. Briefly, 5 μg of nuclear extract was 
incubated in a binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM 
KCl, 1 mM DTT, 15% glycerol) with 2.5 μM biotin-labeled probe 
and 2 μg of poly(dI-dC) for 30 min at room temperature in 
a total volume of 20 μl on ice, and the DNA-protein complexes 
were analyzed by electrophoresis on 6% polyacrylamide gels 
and visualized by chemiluminescence imaging. To perform a
supershift EMSA, the complete reaction mix was incubated with 2 μg of the indicated antibody for 1 h on ice before separating it by electrophoresis. Normal serum was used as a negative control.

**Luciferase Assay**—THP1 cells were co-transfected with pGL3 empty vector, pGL3-hCD36, or pGL3-hCD36m promoter along with the indicated plasmids using Lipofectamine transfection reagent. After growth-arrest in serum-free medium for 12 h, cells were treated with and without 0.1 μm 15(S)-HETE for 6 h, washed with cold PBS, and lysed in 200 μl of lysis buffer. The cell extracts were cleared by centrifugation at 12,000 rpm for 2 min at 4 °C. The supernatants were assayed for luciferase activity using luciferase assay system (Promega) and a single tube luminometer (TD20/20; Turner Designs, Sunnyvale, CA) and expressed as relative luciferase units.

**Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assays**—ChIP assay was performed on THP1 cells with and without the indicated treatments using a kit and following the supplier’s protocol (Upstate Biotechnology, Inc., Lake Placid, NY). STAT1, PPARγ, and p300-DNA complexes were immunoprecipitated using anti-STAT1, anti-PPARγ, and anti-p300 antibodies, respectively. Pre-immune mouse serum or rabbit serum was used as negative control. For chromatin re-immunoprecipitation (re-ChIP) assay, the chromatin complexes were eluted from the first ChIP with 10 mM DTT at 37 °C for 30 min and diluted 20 times with ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl) and immunoprecipitated with the indicated antibodies. In the case of re-ChIP, chromatin was immunoprecipitated in a sequential manner with the indicated antibodies. The immunoprecipitated DNA was uncross-linked, subjected to proteinase K digestion, and purified using QIAquick columns (catalog no. 28104, Qiagen, Valenica, CA). The ChIP and re-ChIP samples were analyzed by QPCR by using the following primers: forward, 5’-GGGGAAACTCAGCAAGTCAG-3’, and reverse, 5’-AGTGTCAATCCTCCAGTG-3’ that would amplify the 228-bp fragment encompassing the STAT-binding site at −107 nt. QPCR was performed on 7300 real time PCR systems (Applied Biosystems) using SYBR Green PCR master mix. The reactions were carried out with following conditions: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s with extension at 72 °C for 1 min followed by 1 cycle at 95 °C for 10 min. Ct values were normalized to input DNA Ct values to obtain ΔCt values. The ΔΔCt values were calculated by subtracting the ΔCt values of control ΔCt from the experimental ΔCt values. The fold enrichment of STAT1, PPARγ, and p300-bound CD36 promoter is calculated by using 2−ΔΔCt.

**Dil-OxLDL Uptake Assay**—THP1 cells with and without the indicated treatments were incubated with Dil-OxLDL (10 μg/ml) for 6 h at 37 °C. After incubation with Dil-OxLDL, cells were washed with PBS, resuspended in PBS, and analyzed by FACSCalibur flow cytometer (BD Biosciences) with an acquired capacity of 10,000 cells. The data were analyzed using CellQuest software, and Dil-OxLDL uptake is presented as a percentage of the total cells.

**Foam Cell Assay**—THP1 cells that were treated with and without the indicated doses of 15(S)-HETE for 4 h were incubated with OxLDL (10 μg/ml) for 6 h at 37 °C. Cells were then fixed with 4% paraformaldehyde for 30 min, stained with Oil-red-O for 10 min, and counterstained with hematoxylin. Cell staining was observed under a Nikon Eclipse 50i microscope with ×40/0.65 magnification, and the images were captured with a Nikon Digital Slight DS-L1 camera. After capturing the images, the Oil-red-O stain was eluted by incubating the slides with isopropanol alcohol for 15 min at room temperature, and the optical density was measured at 500 nm in a SpectraMax 190 spectrophotometer (Molecular Devices).

**Statistics**—All the experiments were repeated three times, and the data are presented as mean ± S.D. The treatment effects were analyzed by Student’s t test, and the p values < 0.05 were considered statistically significant. In the case of EMSA, supershift-EMSA, histochemistry, and Western blotting, one representative set of data is shown.

**Results**

Previously, we have demonstrated that 15(S)-HETE induces CD36 expression and foam cell formation involving STAT1 (24). However, a convincing body of evidence shows that PPARγ mediates CD36 expression in response to its ligands, including 15(S)-HETE (25, 26, 27). To understand how 15(S)-HETE induces CD36 expression by recruiting two unrelated transcriptional factors, we asked the question whether there is any interaction between these two transcriptional factors. Co-immunoprecipitation experiment revealed that STAT1 exists in complex with PPARα, PPARβ, and PPARδ constitutively, and in response to 15(S)-HETE, its association with PPARγ increases in a time-dependent manner with maximum effect at

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**Figure 1.** 15(S)-HETE stimulates STAT1 association with PPARγ in the induction of CD36 expression. A, quiescent THP1 cells were treated with vehicle or 15(S)-HETE (0.1 μM) for the indicated time periods, and cell extracts were prepared. Cell extracts containing equal amounts of protein from control and each treatment were immunoprecipitated (IP) with anti-STAT1 antibodies, anti-PPARγ antibodies, or normal IgG and the immunocomplexes were analyzed by Western blotting for the indicated proteins using their specific antibodies and normalized for STAT1 or PPARγ. B, cells were transfected with vector (pDNA3.1) or the dominant negative STAT1 PFS1YF mutant and quiesced were treated with and without 15(S)-HETE (0.1 μM) for 1 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed either by Western blotting for STAT1 tyrosine phosphorylation using its phospho-specific antibodies and the blot was reprobed for STAT1 to show the overexpression of dominant negative STAT1 PFS1YF or immunoprecipitated with anti-STAT1 antibodies or normal IgG, and the immunocomplexes were analyzed by Western blotting for PPARγ using its specific antibodies and normalized for STAT1. C, upper panel, equal amounts of proteins from control and the indicated time periods of 15(S)-HETE (0.1 μM)-treated cells were immunoprecipitated with anti-STAT1 antibodies or normal IgG, and the immunocomplexes were analyzed by Western blotting using anti-acetyl-lysine antibodies followed by normalization for STAT1. Lower panel, cells were transfected with vector or STAT1 PFS1YF and quiesced before subjecting them to treatment with and without 15(S)-HETE (0.1 μM) for 1 h and analyzing for STAT1 acetylation as described in the upper panel. D and E, all the conditions were the same as in C, lower panel, except that cells were transfected with vector or STAT1 K410R/K413R mutant and quiesced before subjecting them to treatment with and without 15(S)-HETE (0.1 μM) for 1 h for STAT1 acetylation, tyrosine phosphorylation, and its association with PPARγ. The blots were reprobed for STAT1 to show the overexpression of STAT1 K410R/K413R. In the case of PPARγ-antagonist GW-9662, it was added to cells to a final concentration of 5 μM 30 min before the indicated treatment. The bar graphs represent mean ± S.D. of three experiments *, p < 0.01 versus vehicle control or vector; **, p < 0.01 versus 15(S)-HETE or vector + 15(S)-HETE.
1 h (Fig. 1A). Conversely, PPARγ formed a complex only with STAT1 but not with STAT3 or STAT5 in response to 15(S)-HETE (Fig. 1A). To find the role of tyrosine phosphorylation of STAT1 for its interaction with PPARγ, we tested the effect of PFS1YF, a dominant negative STAT1 Tyr-701 mutant (28), on STAT1 and PPARγ interactions. Transfection of cells with
STAT1 PFS1YF completely inhibited 15(S)-HETE-induced STAT1 tyrosine phosphorylation (Fig. 1B). However, it did not affect 15(S)-HETE-induced interaction between STAT1 and PPARγ (Fig. 1B). This result suggests that tyrosine phosphorylation of STAT1 has no influence on its association with PPARγ. Many studies have shown that besides phosphorylation, acetylation also plays a role in the activation of STATs, particularly STAT1 and STAT3 (29–31). Therefore, to explore the underlying mechanisms of STAT1 interaction with PPARγ, we next tested the role of STAT1 acetylation. 15(S)-HETE induced STAT1 acetylation in a time-dependent manner (Fig. 1C). Forced expression of STAT1 PFS1YF mutant had no effect on the acetylation of STAT1 (Fig. 1C).
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on 15(S)-HETE-induced STAT1 acetylation (Fig. 1C). This observation indicates that STAT1 acetylation by 15(S)-HETE occurs independent of its tyrosine phosphorylation. Previous studies from other laboratories have shown that STAT1 gets acetylated at Lys-410/413 (30). To identify the potential lysine residues of STAT1 that are acetylated by 15(S)-HETE, we mutated Lys-410/413 to 410/413R and tested its effect on 15(S)-HETE-induced STAT1 acetylation. Transfection of cells with STAT1 K410R/K413R plasmid blocked 15(S)-HETE-induced STAT1 acetylation (Fig. 1D). This result infers that STAT1 is acetylated at Lys-410/413 in response to 15(S)-HETE. To find whether STAT1 acetylation has any role in STAT1 phosphorylation, we tested the effect of STAT1 K410R/K413R mutant on 15(S)-HETE-induced STAT1 tyrosine phosphorylation. STAT1 K410R/K413R mutant had no effect on 15(S)-HETE-induced STAT1 tyrosine phosphorylation (Fig. 1D). In contrast, STAT1 K410R/K413R mutant blocked 15(S)-HETE-induced STAT1 association with PPARγ (Fig. 1E). This finding indicates that acetylation of STAT1 at Lys-410/413 is required for its association with PPARγ. To find any role for PPARγ in STAT1 acetylation, we tested the effect of PPARγ antagonist GW-9662 (32) on 15(S)-HETE-induced STAT1 acetylation. GW-9662 had no effect on 15(S)-HETE-induced STAT1 acetylation (Fig. 1E).

To test the role of STAT1 and PPARγ interactions on CD36 expression, we first studied the effect of STAT1 PFS1YF mutant. STAT1 PFS1YF did not affect 15(S)-HETE-induced CD36 expression, OxLDL uptake, or foam cell formation as control ASO or p300WT or vector; **, p < 0.01 versus control ASO or p300WT or vector; ††, p < 0.001 versus control ASO or p300WT or vector + 15(S)-HETE. To confirm the role of p300 in STAT1 acetylation, STAT1 interaction with PPARγ, CD36 expression, OxLDL uptake, and foam cell formation, we tested the effect of p300ΔHAT mutant, which lacks acetyltransferase activity (33) on these events. The p300ΔHAT mutant that lacks acetyltransferase activity failed to cause STAT1 acetylation, STAT1 interaction with PPARγ, CD36 expression, OxLDL uptake, and foam cell formation (Fig. 3, C and D). To find the specificity of p300 in 15(S)-HETE-induced CD36 expression, we tested the role of its homologue CBP. ASO-mediated down-regulation of CBP levels had no effect on 15(S)-HETE-induced STAT1 acetylation, STAT1-PPARγ interaction, CD36 expression, and OxLDL uptake (Fig. 3E).

To find the mechanism(s) of p300 activation by 15(S)-HETE, we studied its effect on p300 phosphorylation. 15(S)-HETE, while having no effect on serine/threonine phosphorylation, induced tyrosine phosphorylation of p300 in a time-dependent manner (Fig. 4A). Previously, we have shown that 15(S)-HETE stimulates Syk and Pyk2 in macrophages (24). Therefore, we tested the role of these non-receptor tyrosine kinases in 15(S)-HETE-induced p300 tyrosine phosphorylation. Pharmacological inhibition of either Syk (34) or Pyk2 (35) completely blocked 15(S)-HETE-induced tyrosine phosphorylation of p300, its association with STAT1, STAT1 acetylation, and STAT1 interaction with PPARγ (Fig. 4, B–E). Furthermore, down-regulation of Syk or Pyk2 levels by their respective ASOs inhibited p300 acetyltransferase activity (Fig. 4H). We have reported previously that 15(S)-HETE induces Syk and Pyk2 activation via XO and NADPH oxidase-dependent ROS production (24). Having observed that Syk and Pyk2 play a role in 15(S)-HETE-induced p300 tyrosine phosphorylation and its acetyltransferase activity, we next examined the role of XO and NADPH oxidase in these effects. In line with our previous observations, 15(S)-HETE induced ROS production, and pharmacological inhibitors of XO (36) and NADPH oxidase (37) abrogated this effect (Fig. 5A). In addition, the blockade of XO and NADPH oxidase by their respective inhibitors suppressed 15(S)-HETE-induced p300 tyrosine phosphorylation, its association with STAT1, STAT1 acetylation, and STAT1 interaction with PPARγ (Fig. 5, B–E). To gain additional lines of evidence, we applied the ASO approach as well. ASO-mediated
were analyzed by Western blotting with anti-PY20 antibodies or anti-phosphoserine/threonine antibodies followed by normalization to p300. Equal amounts of protein from cells that were treated with and without 15(S)-HETE (0.1 μM) for 1 h were immunoprecipitated with anti-p300, anti-STAT1, or anti-PPARγ antibodies, and the immunocomplexes were analyzed with anti-PY20, anti-STAT1, or anti-acetyl-lysine antibodies and normalized to p300, STAT1, or PPARγ, respectively. F–H, cells were transfected with control ASO, Pyk2 ASO, or Syk ASO for 1 h, and cell extracts were prepared and analyzed for p300 tyrosine phosphorylation, p300 association with STAT1, STAT1 acetylation, and STAT1 interaction with PPARγ as described in B–E, respectively. F, same cell extracts were also analyzed for Pyk2, Syk, or β-tubulin levels to show the effects of the ASOs on their target and off-target molecule levels. The immunoprecipitation blots in F–H were reprobed for their respective antigen proteins for normalization. I, equal amount of protein from control and the indicated treatment samples of the experiment shown in F were immunoprecipitated with anti-p300 antibodies and the immunocomplexes were analyzed for acetyltransferase activity using a kit. *, p < 0.01 versus control ASO; **, p < 0.01 versus control ASO + 15(S)-HETE.

down-regulation of XO or p47phox levels also inhibited p300 tyrosine phosphorylation, its association with STAT1, STAT1 acetylation, and STAT1 interaction with PPARγ (Fig. 5I). Furthermore, ASO-mediated depletion of XO or p47phox levels attenuated p300 acetyltransferase activity (Fig. 5I).

We have shown that STAT1 binds to a STAT-binding site in the CD36 promoter and enhances its activity (24). Because STAT1 interacts with PPARγ and p300, we asked the question whether these transcriptional factors and transcriptional co-activator bind to the same site. EMSA and supershift EMSA revealed that STAT1, PPARγ, and p300 bind to a oligonucleotide probe containing the STAT-binding site at −107 nt in the CD36 promoter (Fig. 6A and B). To confirm the binding of STAT1, PPARγ, and p300 to the STAT-binding site at −107 nt in the CD36 promoter, we mutated this site in the oligonucleotide and used it as a probe for EMSA. No protein-DNA complex was observed with nuclear extracts of either control or 15(S)-treated THP1 cells using this mutant probe (Fig. 6C). Next, we tested the role of STAT1 tyrosine phosphorylation and acetylation in 15(S)-HETE-induced STAT-DNA complex formation. Although the STAT1 PFS1YF mutant had no effect, its acetyl mutant STAT1 K410R/K413R inhibited the 15(S)-HETE-induced STAT-DNA binding activity (Fig. 6D). Similarly, p300ΔHAT that lacks acetyltransferase activity reduced 15(S)-HETE-induced STAT-DNA binding activity (Fig. 6D). PPARγ antagonist GW–9662, however, partially blocked 15(S)-
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**FIGURE 5.** 15(S)-HETE-induced p300 tyrosine phosphorylation and its association with STAT1 leading to STAT1 acetylation require XO and NADPH oxidase-mediated ROS production. A, cells were treated with vehicle or 15(S)-HETE (0.1 μM) in the presence and absence of apocyanin (100 μM), DPI (10 μM), or allopurinol (100 μM) for 30 min, and ROS production was measured using CMH2DCFDA dye. B–E, equal amounts of protein from cells that were treated with and without 15(S)-HETE (0.1 μM) in the presence and absence of apocyanin (100 μM), DPI (10 μM), or allopurinol (100 μM), the NADPH oxidase, and XO inhibitors, respectively, for 1 h were immunoprecipitated (IP) with anti-p300 or anti-STAT1 antibodies, and the immunocomplexes were analyzed by Western blotting with anti-PY20, anti-STAT1, anti-acetyl-lysine, or anti-PPARγ antibodies and normalized to p300 or STAT1. IB, immunoblot. F–H, cells were transfected with control ASO, p47phox ASO, or XO ASO, quiesced, and treated with and without 15(S)-HETE (0.1 μM) for 1 h; and cell extracts were prepared and analyzed for p300 tyrosine phosphorylation, p300 association with STAT1, STAT1 acetylation, and STAT1 interaction with PPARγ as described in B–E, respectively. F, same cell extracts were also analyzed for p47phox, XO, or β-tubulin levels to show the effects of the ASOs on their target and off-target molecules levels. The immunoprecipitation blots in F–H were reprobed for their respective antigen proteins for normalization. Equal amount of protein from control and the indicated treatment samples of the experiment shown in F were immunoprecipitated with anti-p300 antibodies, and the immunocomplexes were analyzed for acetyltransferase activity using a kit. *p < 0.01 versus vehicle control or control ASO; **p < 0.01 versus 15(S)-HETE or control ASO + 15(S)-HETE.
HETE-induced STAT-DNA binding activity (Fig. 6D). In addition, ChIP and re-ChIP assays were conducted to determine the binding of STAT1, PPARγ, and p300 to the STAT-binding site at −107 nt in the CD36 promoter. ChIP assay results showed that STAT1, PPARγ, and p300 bind to the same region in the CD36 promoter (Fig. 7A). Re-ChIP analysis of anti-STAT1 chromatin immunocomplexes further showed that PPARγ and p300 bind to the CD36 promoter along with STAT1 (Fig. 7A). Conversely, re-ChIP analysis of anti-PPARγ chromatin immunocomplexes revealed that STAT1 and p300 bind to CD36 promoter along with PPARγ (Fig. 7A). Likewise, re-ChIP analysis of anti-p300 chromatin immunocomplexes showed that STAT1 and PPARγ bind to the CD36 promoter along with p300 (Fig. 7A). Furthermore, although the dominant negative mutant of STAT1, STAT1 PFS1YF, had no effect, its acetyl mutant STAT1 K410R/K413R blocked the recruitment of STAT1, PPARγ, and p300 to the CD36 promoter in response to 15(S)-HETE (Fig. 7B). Similarly, p300ΔHAT that lacks the acetyltransferase activity prevented 15(S)-HETE-induced binding of STAT1, PPARγ, and p300 to the CD36 promoter (Fig. 7C). Next, we tested the effect of the PPARγ antagonist GW-9662 on 15(S)-HETE-induced STAT1, PPARγ, and p300 binding to the CD36 promoter. GW-9662, although blocking PPARγ binding, had no effect on 15(S)-HETE-induced STAT1 or p300 binding to the CD36 promoter (Fig. 7D). Furthermore, the promoter-luciferase reporter gene assays indicated that
15(S)-HETE induces CD36 promoter-luciferase activity in a manner that is greatly dependent on p300 acetyltransferase activity and STAT1 acetylation and partially on PPARγ activation (Fig. 7E).

**Discussion**

The important findings of this study are as follows: 15(S)-HETE induces CD36 expression, OxLDL uptake, and foam cell formation by recruiting both STAT1 and PPARγ as a complex to the CD36 promoter. The capacity of STAT1 to form a complex with PPARγ depends not on its tyrosine phosphorylation but rather on its acetylation. It is interesting to note that STAT1 acetylation requires p300 activation. It is also noteworthy that p300 activation requires tyrosine phosphorylation by XO and NADPH oxidase-mediated ROS production and Syk and Pyk2 stimulation. The balance between uptake and efflux of cholesterol by macrophages is a determinant factor in the formation of foam cell, an important step in the pathogenesis of atherosclerosis (38–41). Among the receptors involved in cholesterol transport, particularly oxidatively modified cholesterol, CD36 plays a major role (40, 41). In understanding the mechanisms, many studies have demonstrated that PPARγ plays a major role.
in CD36 expression and foam cell formation (25, 26), although some reports showed that PPARγ exerts anti-atherogenic effects (42, 43). We have recently shown that CD36 expression in macrophages requires STAT1 (24), and other reports showed that STAT1 plays a role in atherogenesis (44). However, whether there is any unifying mechanism between these two transcriptional factors in the development of foam cell formation is not known.

In understanding the mechanisms of CD36 expression by 15(S)-HETE, in this study we show that both STAT1 and PPARγ interact with each other in the expression of CD36 in macrophages and enhance their capacity to take up OxLDL and form foam cells. In addition, it is found that the interaction between STAT1 and PPARγ requires STAT1 acetylation rather than its tyrosine phosphorylation, and this event is mediated by p300. Previous studies have shown that p300 activation depends on its Ser/Thr phosphorylation (45). However, our results show that 15(S)-HETE stimulates tyrosine phosphorylation of p300 via XO and NADPH oxidase–mediated ROS production and Syk and Pyk2 activation. Furthermore, recent studies have demonstrated that both XO and NADPH oxidase play a role in atherogenesis (46, 47). A role for both Syk and Pyk2 in atherogenesis has also been reported (48, 49). Although both Syk and Pyk2 could phosphorylate and activate several signaling molecules, their role in p300 activation has not been known. In this aspect, the present findings for the first time demonstrate that 15(S)-HETE by generating ROS and activating Syk and Pyk2 mediate p300 tyrosine phosphorylation and its activation. In view of all these findings, it is conceivable that XO and NADPH oxidase, Syk, Pyk2, and p300 play a unifying role in the recruitment of STAT1 and PPARγ to the CD36 promoter and enhance its activity in macrophages in response to 15(S)-HETE. However, the re-ChIP analysis of anti-PPARγ chromatin immunocomplexes revealed that activation of PPARγ is not required for binding of STAT1 or p300 to the CD36 promoter. This result infers that p300-mediated STAT1 acetylation rather than PPARγ activation is crucial in the binding of the STAT1-PPARγ complex to the CD36 promoter. Because PPARγ antagonist inhibits PPARγ binding to the CD36 promoter and attenuates the promoter activity partially, it is quite possible that in addition to its involvement in STAT1-mediated promoter activity, PPARγ alone might also be involved in the regulation

![Figure 7](FIGURE 7—continued)
of CD36 promoter activity and its expression at least to some level.

A large body of data suggests that oxidative stress plays a role in atherogenesis (50, 51). Our results reveal that the 12/15-LO-12/15(S)-HETE axis induces ROS production both in vitro and in vivo by activating XO and/or NADPH oxidase (24, 52). In addition, we have reported that 15(S)-HETE via ROS production and activation of non-receptor tyrosine kinases such as Src and Pyk2 mediates TJ protein tyrosine phosphorylation and TJ disruption causing endothelial cell dysfunction (52–54). Many studies have reported that 15-LO mediates LDL oxidation (55, 56) and 15-LO products possess the prooxidant activity (57). Based on all these observations, it appears that 15(S)-HETE by producing ROS triggers a plethora of events, including inflammation via induction of expression of proinflammatory cytokines such as IL-6 (58) and IL-17A (59), chemokines such as MCP1 (60), endothelial cell dysfunction via phosphorylation of TJ proteins and TJ disruptions (52–54), and foam cell formation via LDL oxidation (55, 56) and CD36 expression (24) may play quite an important role in atherogenesis.

In summary, as depicted in Fig. 8, 15(S)-HETE via XO and NADPH oxidase-dependent ROS production and Syk and Pyk2 tyrosine phosphorylation and activation lead to tyrosine phos-
phorylation and activation of p300, which in turn, by acylat-
ing STAT1 facilitates its interaction with PPARγ. Then the
STAT1, p300, and PPARγ complexes by binding to and
enhancing CD36 promoter activity increase CD36 expression,
which by uptaking OxLDL transforms macrophage into foam
cells.

Author Contributions—S. K. performed all the experiments and ana-
alyzed the data; G. N. R. conceived the overall scope of the project,
interpreted the data, and wrote the manuscript.

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