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Pro- and anti-atherogenic effects of a dominant negative P465L mutation of PPARγ in apolipoprotein E-null mice

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Abstract

Objective—The dominant-negative mutation, P467L, in Peroxisome Proliferator Activated Receptor gamma (PPARγ) affects adipose tissue distribution, insulin sensitivity and blood pressure in heterozygous humans. We hypothesized that the equivalent mutation, PPARγ-P465L, in mice will worsen atherosclerosis.

Methods and Results—ApolipoproteinE-null mice with and without PPARγ-P465L mutation were bred in 129S6 inbred genetic background. Mild hypertension and lipodystrophy of PPARγ-P465L persisted in the apoE-null background. Glucose homeostasis was normal, but plasma adiponectin was significantly lower and resistin was higher in PPARγ-P465L mice. Plasma cholesterol and lipoprotein distribution were not different, but plasma triglycerides tended to be reduced. Surprisingly, there were no overall changes in the atherosclerotic plaque size or composition. PPARγ-P465L macrophages had a small decrease in CD-36 mRNA and a small yet significant reduction in VLDL uptake in culture. In unloaded apoE-null macrophages with PPARγ-P465L, cholesterol uptake was reduced while apoAI-mediated efflux was increased. However, when cells were cholesterol loaded in presence of acetylated LDL, no genotype difference in uptake or efflux was apparent. A reduction of VCAM1 expression in aorta suggests a relatively anti-atherogenic vascular environment in mice with PPARγ-P465L.

Conclusions—Small, competing pro- and anti-atherogenic effects of PPARγ-P465L mutation result in unchanged plaque development in apoE-deficient mice.

Keywords

mouse model; macrophage; bone marrow transfer; PPAR gamma; atherosclerosis

Introduction

Most individuals with cardiovascular disease (CVD) have multiple risk factors including dyslipidemia, hypertension, abdominal obesity, insulin resistance, and pro-inflammatory and pro-thrombotic state, which together constitute the Metabolic Syndrome (MetS).[1] Although insulin resistance is a significant component of MetS, diabetes by itself can make patients

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more susceptible to atherosclerotic disease and also increases the severity of pre-existing cardiovascular disease. Many diabetic patients are being treated with Peroxisome Proliferator Activated Receptor gamma (PPARγ) agonist Thiazolidinedione drugs, which act as insulin sensitizers. Multiple studies have demonstrated the anti-atherosclerotic effects of PPARγ agonists using mouse models of atherosclerosis lacking apolipoprotein E (apoE) or low-density lipoprotein receptor (LDLR).[2–4] PPARγ is a nuclear receptor, and amongst its myriad functions, is necessary for adipocyte differentiation and triglyceride deposition.[5] The major site of expression of PPARγ is in adipocytes, where two isoforms, PPARγ1 and PPARγ2, are transcribed. While PPARγ2 is adipocyte specific and is the major form expressed in adipocytes, PPARγ1 is expressed at lower levels in multiple cell types and has been detected in macrophage derived foam cells in atherosclerotic plaques.[6] PPARγ has emerged as an important regulator of scavenger receptor CD-36, which can affect lipid accumulation in the macrophages.[7] On the other hand, PPARγ has also been implicated in lipid efflux from macrophages through the activation of ABCA1 (ATP-Binding Cassette A1).[8] Consistent with its role in efflux, high fat fed LDLR-deficient mice reconstituted with bone marrow from mice with macrophage-specific deletion of PPARγ had increased atherosclerosis compared to mice reconstituted with wild type bone marrow.[8,9] In the vasculature, PPARγ1 isoform is expressed in endothelial cells and vascular smooth muscle cells, and plays a role in blood pressure regulation.[10] Also in high-fat fed LDLR-deficient mice, endothelial cell-specific deletion of PPARγ accelerated atherosclerosis[11], and smooth-muscle cell specific PPARγ disruption enhanced angiotensin II induced atherosclerosis in males.[12]

While global lack of PPARγ is incompatible with development and not observed in humans, there are polymorphisms that subtly affect its function. Among them are various point mutations that affect adipose tissue distribution and insulin sensitivity. For example, mutations in the ligand binding domain at the C-terminus of the protein increase its binding to co-repressors but impair binding to co-activators.[13] Since these mutant proteins retain DNA binding ability, they cause dominant-negative reduction of PPARγ activity in heterozygotes. One of these mutations, Pro to Leu substitution at position 467, was originally identified in patients with severe insulin resistance, hyperglycemia, lipodystrophy and hypertension.[14] Mice heterozygous for the corresponding P465L mutation also have ~8 mmHg higher blood pressure than normal and have mild hyperinsulinemia with an increased pancreatic islet mass particularly on high-fat diet.[15] However, they exhibit normal plasma glucose and insulin sensitivity, unless they are stressed by severe obesity[16] or by suppression of insulin production.[17] In the present study, we evaluated the effects of the P465L mutation on the development of atherosclerosis in mice lacking apolipoprotein E (apoE). Detrimental effects of the mutation on body fat distribution and blood pressure are present in mice with apoE deficiency. Conversely, small, potentially athero-protective changes were also detectable in macrophage lipid metabolism and vascular VCAM1 expression. Overall, however, the P465L mutation in PPARγ exhibited no detectable effects on the atherosclerotic plaque development in the apoE-null mice.

Methods

Generation of Mutant Mice and Diet

Pparg$^{P465L/+}$ heterozygous mice[15] were crossed with *Apoe*$^{-/-}$ mice[18], both on the inbred 129S6/SvEvTac background. *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice were crossed with *Apoe*$^{-/-}$ mice to generate littermates with P465L mutation (L/+) or without (+/+) for experiments. Genotypes of mice were determined as previously described.[15] The mice were fed normal rodent chow (NC, Lab Diet 5P76; PMI Nutrition International), or a high fat western type diet containing 21% (w/w) fat and 0.15% (w/w) cholesterol (HFW, TD88137; Harlan Teklad). Animals

were handled following procedures approved by the Institutional Animal Care and Use Committee at The University of North Carolina at Chapel Hill.

Blood pressure (BP) measurements

BP and pulse rate were measured by tail-cuff method and were calculated from the mean of 30 daily measurements for 6 consecutive days.[19]

Glucose and lipid metabolism

Animals were fasted for 4 hours prior to the blood collection. Plasma glucose and cholesterol were measured by colorimetric assay (Wako Chemicals USA Inc). Plasma triglycerides were measured with reagents from Stanbio Lab. Glucose tolerance, liver TG secretion and fat tolerance were determined on 4–5 months old male mice of each genotype after 4h fast.[15,20] Plasma insulin, resistin and leptin were measured by multiplex immunoassays (Luminex), and adiponectin was measured by ELISA.[21] Males on HFW were treated with heparin (100U/kg body weight) and plasma lipase activity was measured with 4-methylumbelliferyl heptanoate (RPI Corp) as described.[23]

Peritoneal macrophage lipoprotein uptake and cholesterol efflux

Mice were given intraperitoneal injections of sterile 4% thioglycollate (BD Biosciences) in PBS. Four days later, macrophages were collected by peritoneal lavage into PBS. Macrophages were cultured in F-10 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Very low density lipoproteins (VLDL) were isolated from *Apoe*$^{-/-}$ mice, labeled with 1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine perchlorate (DiI C_{18}; Molecular Probes) and macrophage uptake of VLDL was measured as described.[25] For cholesterol efflux, peritoneal macrophages were cultured in a serum-free F-10 medium for 24 hours prior to incubation with 2μCi/ml H^3-cholesterol (Perkin-Elmer) with or without acetylated human LDL for 24 hours. Cells were washed and incubated in F-10 medium with or without 10 μg/ml of apolipoprotein A1 (apoA1, Sigma) for 24 hours. Efflux was calculated as a ratio of radioactivity in the medium divided by the total in cells and medium.[23]

Bone Marrow Transfer

Bone marrow cells were collected from femurs and tibias of donor male mice by flushing with F-10 medium. *Apoe*$^{-/-}$ female recipients were lethally irradiated (9.5 grays) at 10 weeks of age, and injected with 2×10^6 bone marrow cells in 0.2 ml of medium through tail veins.[23]

Atherosclerotic Lesion

Mice were sacrificed using an overdose of 2,2,2-bromophenol and perfused with heparinized PBS and 4% paraformaldehyde. The aortic root was serially sectioned and stained with Sudan IVB and Hematoxylin. Atherosclerosis was scored as described.[24,18]

Gene expression

Total RNA was purified from tissues using Automated Nucleic Acid Workstation ABI 6700, and real-time PCR was performed in ABI PRISM 7700 Sequence Detector (Applied Biosystems). β-Actin mRNA was used for normalization. Primers and probe sets used are available online at http://atvb.ahajournals.org.

Data analysis

Values are reported as mean±SEM. Two-way ANOVA with genotype and sex as two factors was used for analyses. Student's t test was used for comparisons between genotypes.

Results

General Phenotypes and Glucose Metabolism

Pparg$^{P465L/+}$*Apoe*$^{-/-}$ mice and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice were born in the expected Mendelian ratio, appeared healthy and gained weight normally. General phenotypes of mice fed normal chow (NC) and high fat diet (HFW) are summarized in Supplemental Tables S1 and S2, respectively. Blood pressure of the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice was significantly elevated compared to *Pparg*$^{+/+}$*Apoe*$^{-/-}$ littermates by about 8 mmHg regardless of diet (Figure 1A, P456L effect P<0.03 in NC and HFW). Thus, the hypertensive effects of the PPARγ-P465L mutation are retained on the apoE-deficient background.

Previously we identified that PPARγ-P465L causes altered body fat distribution in mice,[17] and this effect was also present in mice on apoE-null background. Although animals had similar body weights and total body fat content, *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice had a smaller visceral (perigonadal) adipose tissue mass but larger subcutaneous (inguinal) adipose tissue mass when compared to the corresponding fat depots in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 1B). Consequently, *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice had a significantly higher inguinal to perigonadal ratio than the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ littermate controls (Table S1, S2). Individual inguinal fat cells were larger (Figure 1C, median area 1222μm^2 vs 760μm^2), suggesting that the cell number is not increased. Increased inguinal fat cell size is associated with increased expressions of lipoprotein lipase (LPL) and resistin, and with decreased uncoupling protein 1 (UCP1). Expressions of adipocyte fatty acid binding protein (aP2) and adiponectin were not different (Figure 1D). HFW increased the total amount of adipose tissue in both genotypes. However, fat distribution (IF/GF) ratio remained similar to that on NC diet (Tables S1 and S2).

Fasting plasma glucose levels were higher in male than female mice and higher in mice fed a HFW (Figure 2A). The levels were slightly lower in *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice than in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice, but the overall genotype effect was not significant. *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice also handled a bolus of glucose equally well (Figure 2B). The fasted plasma insulin levels were not significantly different between the two genotypes (Figure 2C). PPARγ regulates the expression of adipokines. While plasma leptin levels were the same, resistin levels in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice were two-fold higher while adiponectin levels were two-fold lower than in the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice (Figure 2D–F).

Together, these data demonstrate that the effects of PPARγ-P465L on body fat distribution, blood pressure regulation, and glucose metabolism persist in apoE-deficient mice. These phenotypes are well-known risk factors for atherosclerosis.

Lipid Metabolism

ApoE deficiency causes plasma accumulation of cholesterol-rich remnants of chylomicrons and very low-density lipoproteins (VLDL). Plasma cholesterol was not different between the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice on normal chow diet (Table S1). Cholesterol levels increased four-fold in both genotypes with HFW; however, there was no genotype difference (Table S2). The overall distribution of plasma cholesterol among lipoproteins as assessed by fast protein liquid chromatography was also not different (Figure 3A, 3B).

Fasted plasma TG levels tended to be slightly lower in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice than in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice on NC, although the difference was not statistically significant. While plasma TG levels increased in both genotypes in response to HFW, the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice had a significantly smaller increase than the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ controls, particularly in males (Figure 3C). Plasma TG values are a function of lipid absorption, rate of clearance and liver TG synthesis and secretion. Despite the difference in plasma TG, the post-prandial lipid handling as assessed by a lipid tolerance test (Figure 3D) and the secretion of TG from the livers measured under the inhibition of lipolysis by Tyloxapol injection (Figure 3E, 3F) were not different between the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice regardless of diet. The difference in plasma TG may be explained by the higher LPL activity as *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ inguinal fat tissues express 2.3 times higher LPL (Figure 1F), but the total lipase activities in heparinized plasma were not different (data not shown).

Taken together, these data demonstrate that the hypercholesterolemia of apoE-null mice is not affected by the PPARγ-P465L mutation. However, a trend of reduced plasma TG levels in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice could protect them from atherosclerosis.

Atherosclerosis

We next measured the average size of atherosclerotic plaques in the aortic roots of 5-month old NC fed mice and 4-month old mice fed HFW diet for 2 months. Contrary to our expectation, the lesion size in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ and *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice did not differ in either dietary condition (Figure 4A, 4B). Since our mice are 129S6 inbred background, they developed significant amount of plaques in the aortic arch,[18] but without detectable differences in the plaque covered areas between *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Tables S1, S2). The plaques in *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice equally increased in size and complexity with age (Figure 4C–F), and plaque compositions based on histological assessment of the occurrence of fibrous cap, necrotic core cholesterol clefts and calcifications were similar (Supplemental Table S3). Immunohistochemical staining of similar sized plaques for macrophages, T-cells and smooth muscle cells was not remarkably different (Supplemental Figure S1). Taken together, we conclude that the dominant negative PPARγ-P465L mutation has no effect on overall atherosclerotic plaque development in apoE-null mice.

Macrophage Function

Peritoneal macrophages from *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice had a small but significant reduction (11%) in DiI labeled VLDL uptake, compared to the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ controls (Figure 5A). Furthermore, when macrophages were incubated with ^{3}H-labeled cholesterol for 24 hours (unloaded), cholesterol incorporation into the cells was significantly lower at about 75% (Figure 5B), and apolipoprotein A1 stimulated cholesterol efflux by *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ macrophages during the following 24 hours was significantly higher than *Pparg*$^{+/+}$*Apoe*$^{-/-}$ macrophages (Figure 5C). However, when the macrophages were loaded with cholesterol in the presence of acetylated human LDL to model foam cells (AcLDL-loaded), cholesterol incorporation and efflux were not different and apolipoprotein A1 equally stimulated cholesterol efflux from *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ macrophages.

To test the functions of *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ macrophages in the atherosclerotic lesion development in vivo, we next harvested bone marrows from *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ male mice and transplanted them into 10 week-old *Pparg*$^{+/+}$*Apoe*$^{-/-}$ female recipients. Two months after the transfer, the size of the foam cell lesions in the aortic sinus of the mice that received *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ bone marrow were not significantly different from those that received the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ marrow (Figure 5D).

Plaques were mostly of early stages of foam cell lesions in the recipients of bone marrows from either genotype (not shown). Taken together, the presence of PPARγ-P465L mutation reduced macrophage lipid accumulation and enhanced cholesterol efflux from unloaded macrophage. However, PPARγ-P465L appears to have no effects on cholesterol efflux once foam cells are formed, and has little effects on atherosclerosis development in the hyperlipidemic apoE-null animals.

Macrophage and vascular gene expressions

Since macrophages lacking PPARγ are reported to have impaired cholesterol efflux with reduced ABCA-1 expression, we sought the effects of dominant-negative P465L mutation on the expression of genes important for cholesterol metabolism in macrophage (Figure 6A). We found a small increase in ABCA-1 and a small decrease in CD36 in $Pparg^{P465L/+}Apoe^{-/-}$ macrophages compared to $Pparg^{+/+}Apoe^{-/-}$ littermates, but the differences were not significant. Although DiI-VLDL uptake was reduced, genes that may influence VLDL uptake and cellular cholesterol contents in macrophage including SRB1 and LDLR were not different. VLDLR expression tended to be higher in $Pparg^{P465L/+}Apoe^{-/-}$ macrophage, although again the difference was not statistically significant.

Inflammatory response genes such as TGFb1, IL6 and iNOS differed widely in individual macrophage isolates but were not significantly different between the two genotypes. Notably, at an individual animal basis, the level of CD36 expression is strongly and positively correlated with PPARγ mRNA levels (r=0.72), while it is negatively correlated with ABCA1 mRNA levels (r=− 0.4, Figure 6B). ABCA1 mRNA levels are in turn positively correlated with the iNOS gene expression (r=0.68), suggesting that the inflammatory state of the macrophage as well as PPARγ influence the expression of genes for lipid metabolism in macrophage.

To assess macrophage infiltration and vessel response, we next measured mRNA levels of genes in the aortic arches of $Pparg^{P465L/+}Apoe^{-/-}$ and $Pparg^{+/+}Apoe^{-/-}$ mice at 3 months of age when foam cells just begin to accumulate in aortic arch (Figure 6C). No difference was found in the expression of a macrophage marker CD68, ATP-binding cassette transporter G1 (ABCG1) that mediates free cholesterol efflux, or LPL, consistent with the histological observations. Expression of osteopontin (SPP1), ABCA1 and endothelial nitric oxide synthase (eNOS) in $Pparg^{P465L/+}Apoe^{-/-}$ aortas trended higher than in $Pparg^{+/+}Apoe^{-/-}$ aortas. In contrast, the basal expression of vascular cell adhesion molecule 1 (VCAM1) was significantly reduced to 40% in the $Pparg^{P465L/+}Apoe^{-/-}$ compared to in the $Pparg^{+/+}Apoe^{-/-}$ vessels. These findings suggest that the PPARγ-P465L could be athero-protective in aorta, but that macrophages accumulate in the vessels with a similar rate in $Pparg^{P465L/+}Apoe^{-/-}$ and $Pparg^{+/+}Apoe^{-/-}$ aortas in the very early stage of plaque development.

Discussion

PPARγ has been implicated in the pathogenesis of atherosclerosis through its regulation of circulating lipid and glucose levels and also by modulating vascular cell functions. All the characteristics of the PPARγ-P465L mutation previously observed in mice with wild type apoE were present in $Pparg^{P465L/+}Apoe^{-/-}$ mice, including increased blood pressure, moderate hyperinsulinemia and altered body fat distribution. Hypercholesterolemia of the apoE-null mice was not affected by the PPARγ-P465L mutation. Overall, however, we observed no detectable differences in the size or complexities of the aortic root plaques in $Pparg^{P465L/+}Apoe^{-/-}$ mice fed NC or 2 months of HFW, or in $Apoe^{-/-}$ mice replaced with $Pparg^{P465L/+}Apoe^{-/-}$ bone marrow. Our data unequivocally demonstrate that the dominant-

negative PPARγP465L mutation alone has no effect on plaque development in apoE-deficient mice.

Hypertension is a well-known risk factor for atherosclerosis. ApoE-null mice with elevated BP caused by genetic lack of eNOS[25], lack of natriuretic peptide receptor A[26], or infusion of angiotensin II in circulation[27], all develop enhanced atherosclerosis, which is attenuated by a reduction in BP. We therefore hypothesized that the elevated BP associated with the PPARγ-P465L would accelerate atherosclerosis in *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice. Although an 8 mmHg increase is modest, hypertension in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice is a potential pro-atherogenic factor. Yet, there was no enhanced atherosclerosis in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice compared to the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice. This suggests that either this degree of BP increase alone is not sufficient to promote atherogenesis, or the mechanism by which PPARγ-P465L cause elevated BP is not directly relevant to plaque development. Cerebral arterioles expressing PPARγ-P465L mutation show increased contraction to serotonin and endothelin 1, and impaired relaxation to acetylcholine.[28] However, response to acetylcholine is not impaired in aorta, implying that PPARγ affects resistance vessels more than larger conduit vessels.[28] Further studies on the role of PPARγ in the interaction between BP regulation and atherosclerosis are warranted.

Our *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice have increased plasma resistin and decreased plasma adiponectin; the profile associated with obesity, insulin resistance and increased inflammatory diseases in subjects with metabolic syndrome.[29] Resistin antagonizes insulin function. Its expression is high in adipose tissues in rodents and we found that its expression in inguinal fat in *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice is elevated. Primary site of resistin expression in humans is mononuclear cells in blood and, although role of resistin in glucose homeostasis in humans is controversial and causative roles are yet to be established, resistin is strongly associated with inflammatory diseases, including atherosclerosis.[30] Adiponectin, in contrast, has insulin sensitizing and anti-inflammatory properties and its expression is regulated by PPARγ. Reduction of plasma adiponectin must be mainly the consequence of reduced production from the perigonadal fat, since adiponectin gene expression in inguinal fat of the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice was not altered. While inguinal fat mass in these mice was increased, individual adipocyte cell size is also increased resulting in unaltered total adipocyte number. Together, plasma adipokine profile of the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice is consistent with reduced PPARγ activity and is suggestive of a pro-inflammatory state. However, the role of adiponectin in rodent atherosclerosis is controversial, since adenovirus mediated adiponectin overproduction in the liver of apoE−/− mice reduced atherosclerosis[31] but neither deficiency nor overproduction altered atherosclerosis in LDLR−/− mice.[32]

Some anti-atherosclerotic factors may be counteracting the pro-atherogenic effects of high BP, lipodystrophy and adipokine profile in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice. For example, we established previously that mice with PPARγ-P465L exhibit normal glucose handling in part because of increased release of postplandial insulin.[15, 17] When global insulin signaling is intact, increased insulin could be anti-atherogenic, since insulin has vasodilatory and anti-inflammatory properties.[33] Consistent with this, loss of insulin signaling in vascular cells accelerated atherosclerosis[34], while chronic oral administration of insulin attenuated atherosclerosis.[35] Reduced plasma TG in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice is also potentially anti-atherogenic.

PPARγ appears to play a complex and multi-faceted role in macrophages, affecting the recruitment and activation of monocytes, cholesterol homeostasis and inflammation. PPARγ activation has been shown to increase the expression of scavenger receptor CD36, which is associated with lipid accumulation in the macrophage.[6,7] For example, while the basal gene expression levels of CD36 were similar between wild type and PPARγ-deficient

macrophages, treatment of wild type mice with rosiglitazone resulted in a robust up-regulation of CD36 expression in a PPARγ-dependent manner.[7] In our *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice, the gene expression of CD36 in thioglycolate-induced macrophages was ~25% lower than in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ macrophages, with the difference approaching statistical significance. This reduction in basal gene expression likely contributes to the reduced lipid accumulation in the unloaded *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ macrophages. In addition, we observed a significant reduction in DiI labeled VLDL uptake compared to macrophages isolated from the *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice.

PPARγ activation also induces macrophage expression of ABCA-1, and the conditional disruption of PPARγ in murine macrophages reduces cholesterol efflux via reduction in ABCA-1 transcription.[8] This mechanism may account for the 50% increase in atherosclerosis in both high-fat fed wild type C57BL/6 and LDL receptor deficient mice reconstituted with bone marrow from mice with a macrophage-specific knockout of PPARγ.[8,9] In our study, the expression of ABCA-1 in macrophage was slightly higher, if any, and unloaded *Ppar*$^{P465L/+}$*Apoe*$^{-/-}$ macrophage had a two-fold higher rates of cholesterol efflux. However, once the PPARγ-P465L cells were loaded with acetylated cholesterol and became foam cell like, the enhanced ability of PPARγ-P465L macrophages to efficiently efflux cholesterol compared to *Pparg*$^{+/+}$*Apoe*$^{-/-}$ controls disappeared. Although the reduced uptake and increased cholesterol efflux may point to an athero-protective phenotype, the loss of advantage in efflux under excess cholesterol load may explain the lack of a reduction in the lesions in our bone marrow transfer experiments. While the expression of CD36 in peritoneal macrophages was highly correlated with the levels of PPARγ gene expression at individual isolate levels, we found that ABCA1 expression was not directly correlated with the PPARγ gene expression. It was, instead, positively correlated with iNOS gene expression, suggesting the role of the inflammatory states in the regulation of ABC-A1 gene in macrophages. This effect was, however, independent of PPARγ-P467L mutation.

Consistent with histological observations, our analysis of gene expression in the aortic arch demonstrated that the recruitment and activation of macrophage in the vessel wall is not significantly different between *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice at an early stage of atherosclerosis. An exception is that the expression of VCAM-1 was significantly lower in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ vessels than in *Pparg*$^{+/+}$*Apoe*$^{-/-}$ vessels. VCAM-1 binds to α4β1 integrin, which is expressed on leukocytes, and mediates rolling and adhesion to endothelium.[36] The reduced expression of VCAM-1 could be anti-atherogenic, since hypomorphic mutant form of VCAM-l has been shown to protect LDLR-null mice from atherosclerosis.[36] In human atherosclerotic plaques, VCAM-1 is not expressed in normal vessels but is focally expressed in endothelial cells and strongly in “spindle shaped cells of macrophage type” in lipid-containing plaques.[37] However, since overall expression of macrophage markers including CD68 and SPP1 genes are not different between the two groups, the significance of a reduced VCAM1 expression in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ mice is not clear, and requires further study.

It is important to acknowledge that PPARγ is identified as a crucial athero-protective molecule primarily with a use of pharmacological drugs, which may exert PPARγ independent effects, or in the LDLR null mice that lack PPARγ in specific tissues while present in the rest of the body. Our genetic model is maintained on inbred 129/SvEv background, which ensures that no polymorphic variations present in different inbred mouse strains are accidentally segregating with the PPARγ-P465L mutation and obscuring its effects. In this model, a reduction of PPARγ function due to the P465L mutation affects the whole body, and therefore cross talks between different cell and organ systems are intact. Consequently, the balance between mild pro-atherogenic and anti-atherogenic effects of the

PPARγ-P465L mutation can mutually dampen the potential deleterious effects in a single cell type or organ, as observed in the experiments of tissue specific knockouts. The lack of demonstrable PPARγ-P465L effects on atherosclerosis of the apoE null mice can also be attributable to altered function of the mutant PPARγ, as dominant -negative mutations are different from full deletion. Thus severe hypercholesterolemia in apoE-deficient mice may not only mask small deleterious effects of PPARγ-P465L, but may even increase the availability of some endogenous PPARγ ligands which allow overcome dominant-negative effects of P465L substitution.[38] Finally, we note that one of the athero-protective roles of PPARγ is an induction of apoE expression in macrophages which helps cholesterol efflux[39,40], and this induction is missing in apoE-null model. The possibility that the PPARγ-P456L mutation may differentially alter the phenotype of some other model of atherosclerosis retaining endogenous apoE expression, such as LDLR-null mice, cannot be eliminated.

In conclusion, we found that the dominant negative PPARγ-P465L mutation has no overall effect on atherosclerotic plaque formation in apoE-null model. While this specific mutation is rare in humans, its effects mimic those of other mutations and polymorphisms present in the human population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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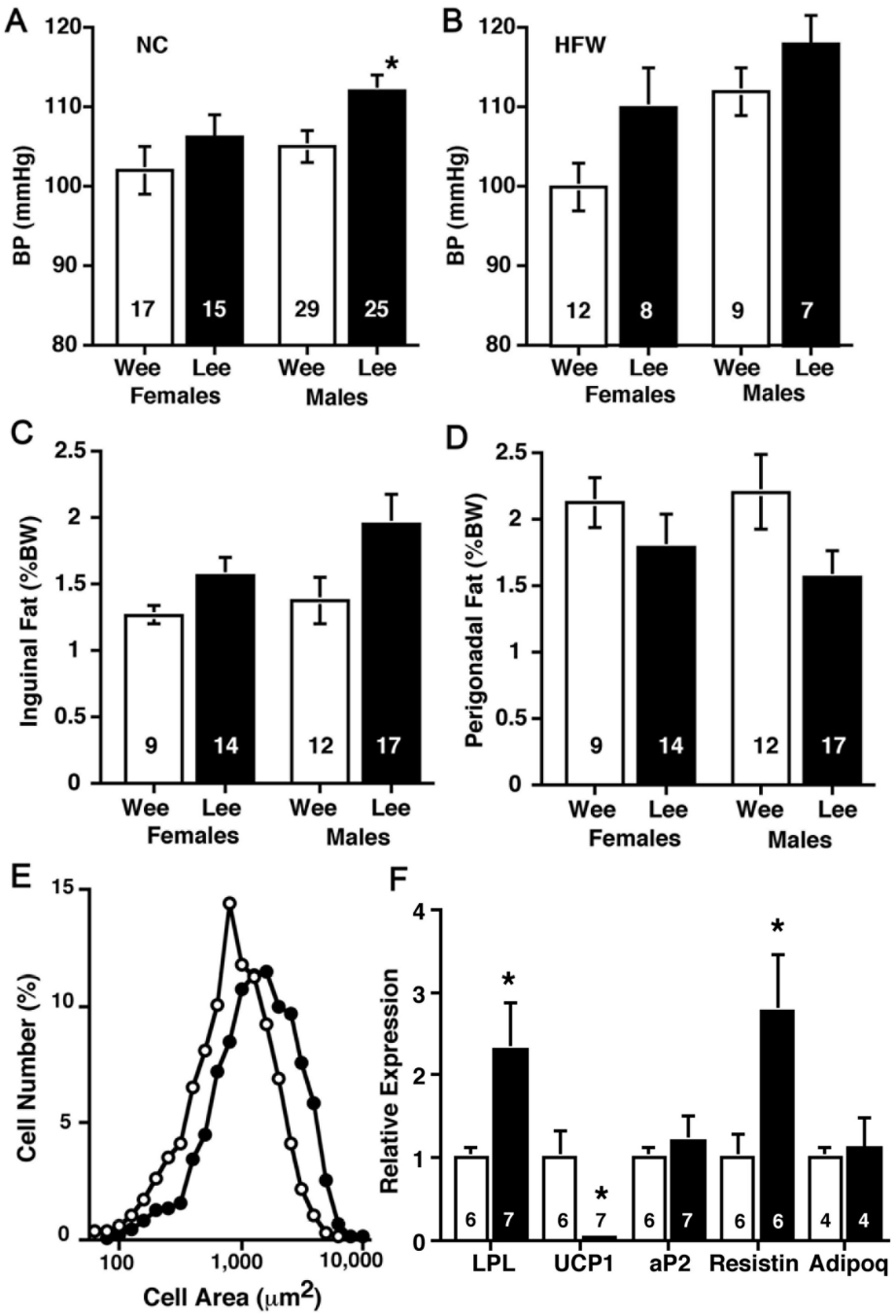

Figure 1.

Elevated blood pressure and altered body fat deposition in apoE-deficient mice with PPARγ P465Lmutation (Lee, black bars) compared to those with wild type PPARγ (Wee, white bars). (A, B), blood pressure in mice on normal chow (NC) and on a western type high fat diet (HFW). Inguinal fat (C) and perigonadal fat weights (D) in NC fed mice are expressed as % of body weights. (E), Distribution of cell size in inguinal adipose tissues. Over 2500 cell were measured from 5 mice in each genotype and expressed as % in each size range. Open circles, Wee; filled circles, Lee. (E), mRNA for PPARg target genes in inguinal adipose tissues. LPL gene expression in inguinal fat of Lee female mice on NC relative to

the mean of mRNA levels in Wee females. Numbers in the bars are the number of mice used. * P<0.01 between the two genotype groups.

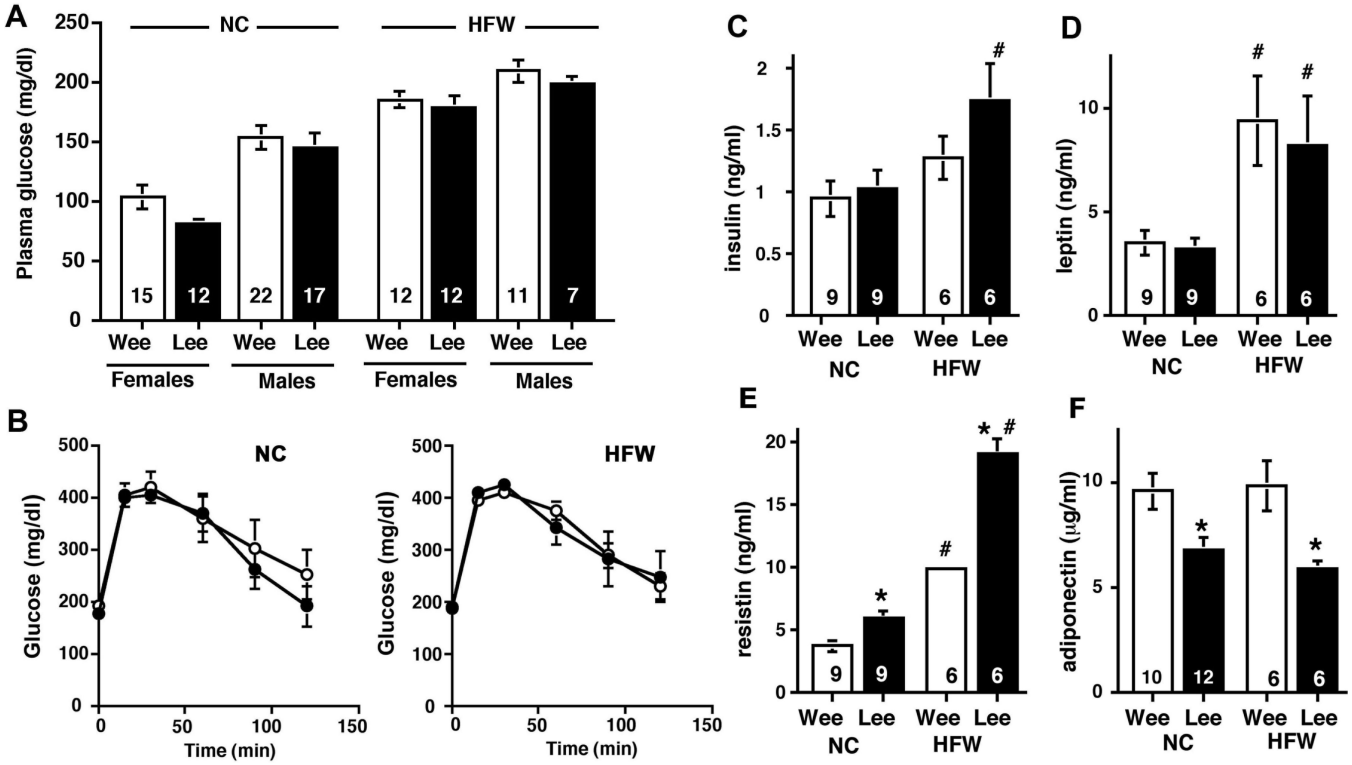

Figure 2.

Glucose metabolism in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ (Lee) and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Wee) mice. (A), Fasting plasma glucose levels. (B), Oral glucose tolerance tests on male mice (n=6) on normal chow (NC) or a high fat western diet (HFW). Plasma levels of insulin (C), leptin (D), resistin (E) and adiponectin (F) in male mice. Black bars and black circles indicate Lee mice and white bars and white circles indicate Wee mice. Numbers in the bars are the number of mice used. * P<0.05 between genotypes, # P<0.05 between the diets.

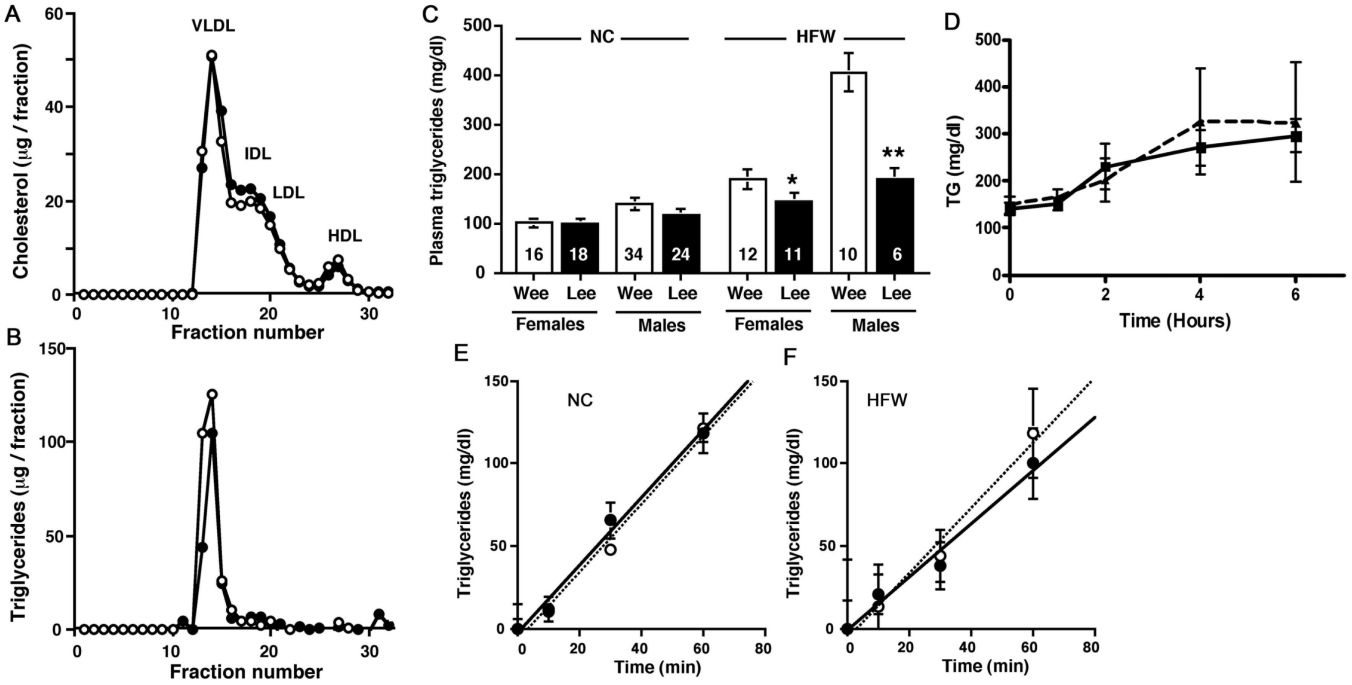

Figure 3.

Lipid metabolism in the *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ (Lee) and *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Wee) mice. Distribution of cholesterol (A) and triglycerides (B) among lipoproteins assessed by the fast protein liquid chromatography of pooled plasma from females on NC (n=3 each). (C), Plasma triglyceride levels. (D), Plasma triglycerides after lipid loading with gavage. (E, F) Liver triglyceride secretion after tyloxapol injection in 4 months old males fed NC or HFW. White dots and white bars represent Wee mice, black dots and black bars represent Lee mice.

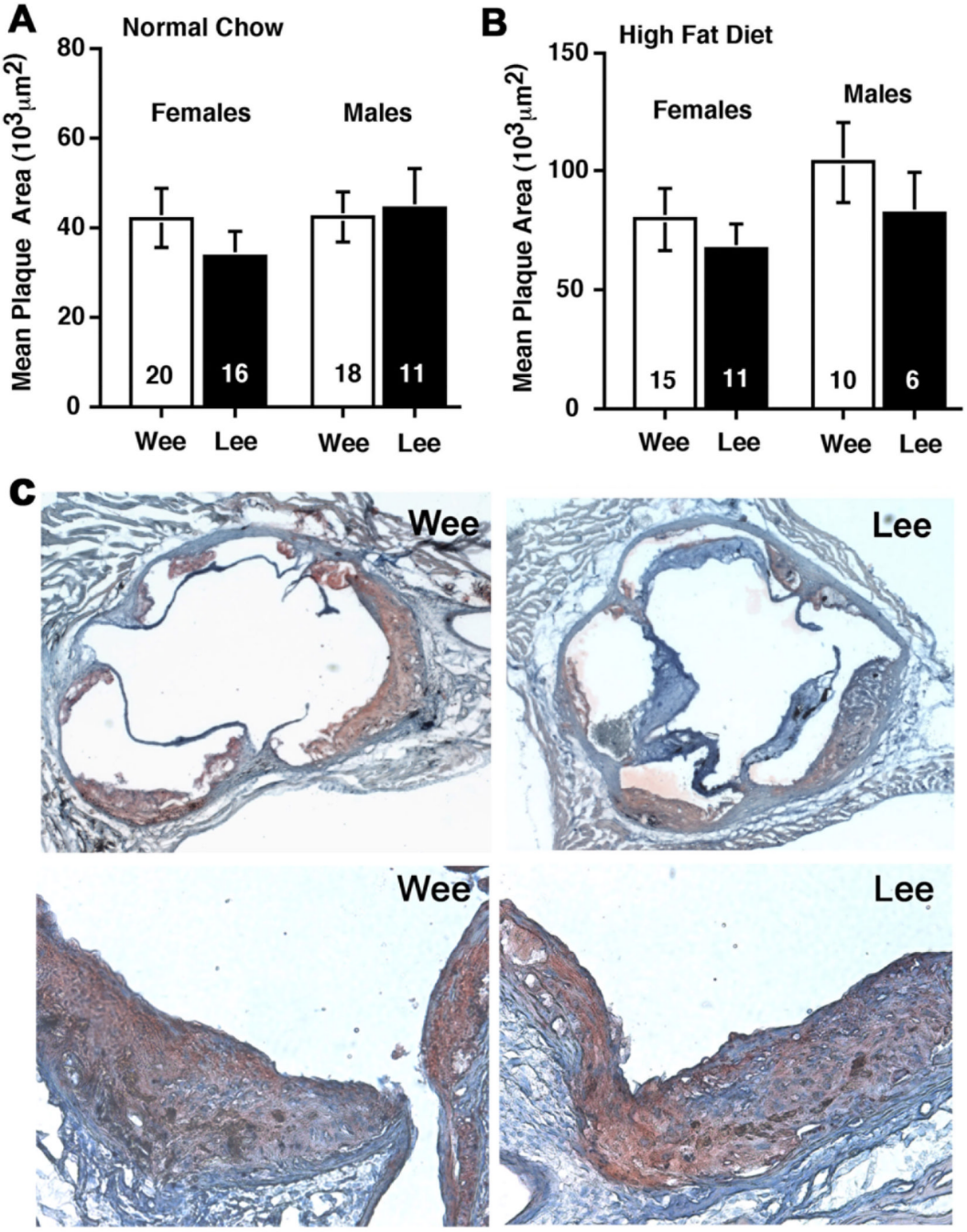

Figure 4.

Atherosclerosis. Average plaque size in the aortic roots of *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Wee, white bars) and *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ (Lee, black bars) mice fed normal chow at 5 months of age (A), or fed high fat diet at 4 months of age (B). The numbers within the bars indicate sample size. (C), Cross sections at the aortic roots of 7 months old Wee and Lee male littermate mice on normal chow. Bottom panels are plaques near the coronary ostiums from another set of littermates.

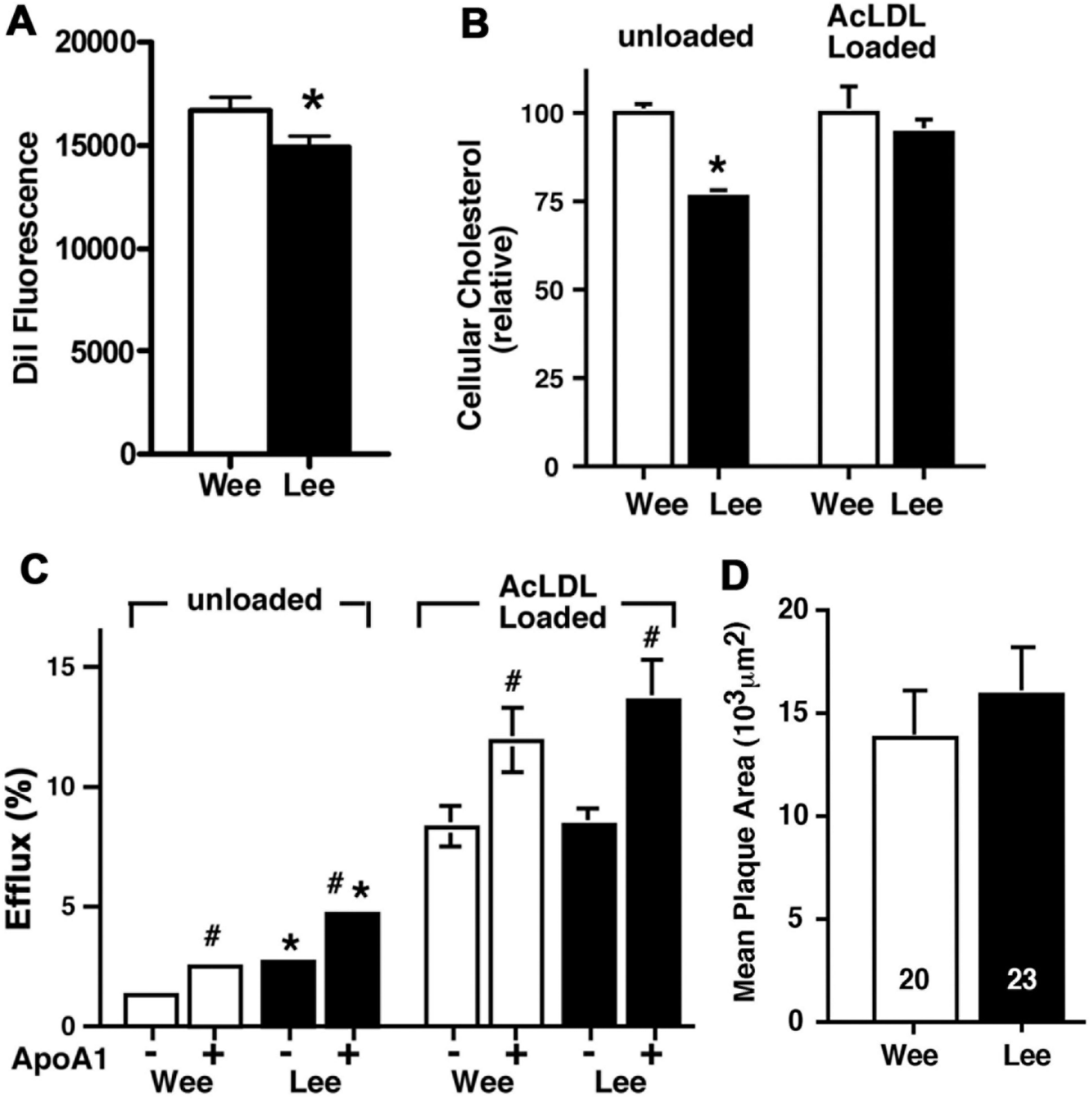

Figure 5.

Function of macrophage from *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Wee, white bars) and *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ (Lee, black bars). (A), Uptake of DiI labeled VLDL from the medium by macrophages. (B), Relative cellular H^3 cholesterol uptake after incubation without (unloaded) and with acetylated human LDL (AcLDL loaded). (C), Cholesterol efflux in the absence or presence of apolipoprotein A1 from peritoneal macrophages labeled with H^3 cholesterol without (unloaded) and with acetylated human LDL (Loaded). At least 6 wells were used for macrophages isolated from each mouse (three mice of each genotype). * P<0.05 between the two genotypes. #P<0.05 for apoA1 effects. (D) Plaque size in the aortic roots of apoE-

deficient females receiving bone marrows from Wee (white bars) or Lee (black bars) male donors.

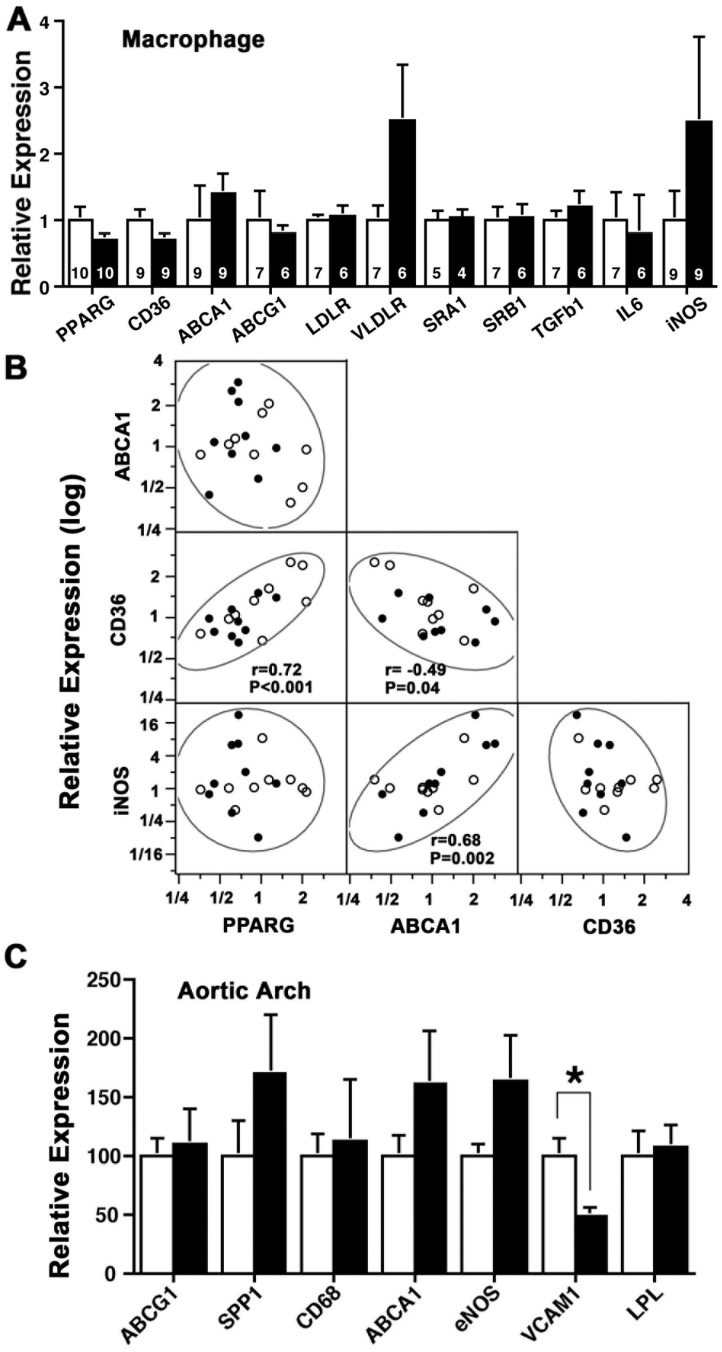

Figure 6.

Gene expression normalized to β-actin in peritoneal macrophages isolated from *Pparg*$^{+/+}$*Apoe*$^{-/-}$ (Wee, white bars) and *Pparg*$^{P465L/+}$*Apoe*$^{-/-}$ (Lee, black bars). Data are mean±SE expressed relative to the mean of Wee. (B), Scatterplot matrix of gene expressions for PPARγ, ABCA1, CD36 and iNOS (in log scale) in individual macrophage isolates. Density ellipses are set at α=0.95. Open circles are Wee and closed circles are Lee. (C), Gene expression in the aortic arch of 3 month old mice on normal chow. Data are expressed relative to the mean mRNA amount of each gene in Wee mice as 100. N>17 except for n=6 each. *, P<0.01.