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## The Induction of Atherogenic Dyslipidemia In Poloxamer 407-treated Mice Is Not Mediated Through PPAR $\alpha$

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### Abstract

The copolymer surfactant poloxamer 407 (P-407) has been used to induce a dose-controlled dyslipidemia in both mice and rats. Human macrophages cultured with P-407 exhibit a concentration-dependent reduction in cholesterol efflux to apolipoprotein A1 (apoA1) due to down-regulation of the ATP-binding cassette transporter A1 (ABCA1). Peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) can increase expression of liver X receptor alpha (LXR $\alpha$ ) in macrophages and thereby promote the expression of ABCA1, which, in turn, mediates cholesterol efflux to apoA1. The present study investigated point(s) along this signaling pathway at which P-407 might act to inhibit cholesterol efflux from macrophages. A transactivation assay was used to evaluate whether P-407 could either a) activate PPAR $\alpha$ , or b) block the activation of PPAR $\alpha$  by an established PPAR $\alpha$  agonist. P-407 was also evaluated for its potential to alter plasma lipid concentrations following its administration to both normal C57BL/6 and PPAR $\alpha$ -deficient mice. P-407 was unable to modulate PPAR $\alpha$  activity, as determined in cell-based transactivation assays. Moreover, P-407-induced dyslipidemia occurred at the same rate and to the same extent in PPAR $\alpha$ -deficient mice as was observed in C57BL/6 mice, suggesting no role for PPAR $\alpha$  in P-407-mediated dyslipidemia. Although PPARs are known to mediate the transcriptional regulation of the two major apolipoproteins associated with HDL (apoA1 and apoA2), P-407 treatment resulted in a similar decrease (~30%) in the plasma concentration of apoA1 in both control and PPAR $\alpha$ -deficient mice. Since our previous work demonstrated that P-407 was unable to abrogate the capacity of a known LXR $\alpha$  agonist to increase cholesterol efflux from macrophages, P-407 is likely to exert its effect, either directly or indirectly, on ABCA1, rather than on LXR $\alpha$ . On the basis of these findings it is concluded that PPAR $\alpha$  does not mediate the P-407-dependent reduction in apoA1-facilitated cholesterol efflux from macrophages.

### Keywords

Apolipoprotein; Dyslipidemia; Lipid; Peroxisome proliferator-activated receptor (PPAR); Transactivation assay

### INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that, upon heterodimerization with retinoid X receptor (RXR), function as ligand-activated transcriptional

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regulators of genes controlling lipid and glucose metabolism (Pineda et al 1999). PPAR $\alpha$ , which is activated by fibrates, fatty acids, and eicosanoids (Chinetti et al 2000a), is highly expressed in liver, heart, muscle, and kidney, but is also present in cells of the arterial wall, including monocytes, macrophages, macrophage foam cells, smooth muscle, and endothelial cells (Chinetti et al 2000a). PPAR $\alpha$  has a role in macrophage lipid homeostasis and cholesterol efflux, which is the first step in the reverse cholesterol transport pathway. In macrophages, PPAR $\alpha$  activators induce the expression of the scavenger receptor CLA-1/SR-BI, which binds HDL with high affinity (Chinetti et al 2000b). In addition, in differentiated macrophages and macrophage-derived foam cells, PPAR $\alpha$  activators induce ATP-binding cassette transporter A1 (ABCA1) gene expression through their inductive effects on the expression of liver X receptor alpha (LXR $\alpha$ ) and promote cholesterol efflux to apolipoprotein (apo) A1 (Chinetti et al 2001).

Recently, we reported how a polymeric chemical compound known as poloxamer 407 (P-407) affected cholesterol homeostasis in primary human monocyte-derived macrophages (Johnston et al 2006). P-407 is a triblock copolymer comprised of repeating poly(oxyethylene) and poly(oxypropylene) units and is normally used as a surfactant. However, our laboratory has utilized P-407 to develop a murine model of dose-controlled atherogenic dyslipidemia [elevated plasma triglyceride (TG) with a simultaneous reduction in high-density lipoprotein (HDL) cholesterol] (Leon et al 2006; Johnston 2004). Administration of P-407 to mice for 16 weeks results in the formation of aortic fibrofatty lesions comparable in size and number to those formed using classic diet-induced and gene-knockout mouse models of atherogenesis (Leon et al 2006; Palmer et al 1998). Because this model is routinely utilized by numerous laboratories throughout the world, we continue to explore the mechanisms responsible for the atherogenic dyslipidemia produced in this model, as well as any changes that may occur in cell lipid, insulin, and glucose homeostasis. Examples of how researchers use this model include a) determining how the pharmacokinetics of specific drugs are altered in the context of atherogenic dyslipidemia (Brocks et al 2006), b) determining the immune response to oxidized LDL-cholesterol produced in this model (Johnston & Zhou 2007), c) determining how the dyslipidemic state affects key enzymes of lipid metabolism (*e.g.*, endothelial lipase) (T Ishida et al 2007; Personal Communication), and d) determining the hepatic production rate of triglycerides (Millar et al 2005). Thus, it is critical to develop a better understanding of the mechanisms behind the cell lipid, enzyme, and gene signaling pathways altered by P-407 in this mouse model of atherogenic dyslipidemia.

Recently, we demonstrated both a significant reduction in the expression of ABCA1, and, as a result, apoA1-mediated cholesterol efflux, when macrophages were cultured with P-407 (Johnston et al 2006). Interestingly, although PPAR $\alpha$  activation reduces the cholesteryl ester:free cholesterol (CE:FC) ratio in macrophages by inhibiting acyl-CoA:cholesterol acyltransferase (ACAT), which, in turn, inhibits cellular CE formation (Chinetti et al 2003), we observed an increase in the values of the CE:FC ratio in liver and in eight peripheral tissues in P-407-treated rats (Johnston et al 2006). Even more intriguing is our finding that neither hepatic microsomal ACAT activity, nor hepatic ACAT2 protein expression is altered in P-407-treated mice (Leon et al 2006). Therefore, it is important to determine whether P-407, either directly or indirectly, perturbs PPAR $\alpha$  activity in a manner that could help explain the increased CE levels seen in tissues of P-407-treated rats (Johnston et al 2006), and whether any such modulation of PPAR $\alpha$  activity might contribute to the atherogenic dyslipidemia observed in P-407-treated mice.

Previous key findings include: a concentration-dependent decrease in a) ABCA1 gene expression, and b) cholesterol efflux in macrophages treated with P-407 (Johnston et al 2006), c) an increase in the CE:FC ratio determined for liver and eight peripheral tissues in P-407-treated rats (Johnston et al 2006), and d) no effect of P-407 on ACAT protein and activity

levels (Leon et al 2006). On the basis of these earlier findings, we sought to investigate the potential for P-407 to modulate PPAR $\alpha$  activity, as determined in a cell-based transactivation assay. Additionally, we evaluated whether the atherogenic dyslipidemia in mice treated with P-407 was dependent on PPAR $\alpha$ , as determined using a PPAR $\alpha$ -deficient mouse model. Lastly, in addition to their role in peroxisome proliferation in rodents, PPARs are also involved in the control of HDL cholesterol levels based, in part, on PPAR-mediated transcriptional regulation of the major apolipoproteins, apoA1 and apoA2 (Schoonjans et al 1996). Since it is well established that apoA1, rather than apoA2, confers HDL's anti-atherogenic effect, we also assessed whether there was any change in the concentration of apoA1, relative to apoE, in plasma obtained from P-407-treated mice.

## METHODS

### Materials

Wy-14,643 was obtained from Sigma Chemical Co. (St. Louis, MO). Male, PPAR $\alpha$ -knockout mice (~18 g) were purchased from the Jackson Laboratory (strain name = 129S4/SvJae-Ppara<sup>tm1Gonz/J</sup>). Plasmids were obtained from the same sources as previously reported (Maloney & Waxman 1999; Shipley et al 2004a; Shipley & Waxman 2004b).

### Transactivation Assay

The transactivation assay described previously (Maloney & Waxman 1999; Shipley et al 2004a; Shipley & Waxman 2004b) was used to assess the effect of P-407 on PPAR $\alpha$  transcriptional activity. Briefly, COS-1 cells (American Type Culture Collection, Rockville, MD) were passaged in 100-mm tissue culture dishes (Greiner Labortechnik, Germany) in DMEM supplemented with 10% FBS (Gibco, Grand Island, NY) and 50 U/mL penicillin/streptomycin (Gibco). Cells were cultured overnight at 37 °C and then reseeded at 2000 to 4000 cells/well in a 96-well tissue culture plate (Greiner Labortechnik) in DMEM containing 10% FBS. The cells were grown for 24 h and then transfected as described previously (Chang & Waxman 2005), using FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN). Previous studies indicated little or no significant endogenous PPAR $\alpha$  activity in these cells (Maloney & Waxman 1999). Twenty-four h after P-407 treatment, cells were washed once in cold phosphate-buffered saline (pH 7.4), and then lysed by incubation at 4 °C in passive cell lysis buffer for 15-30 min (Promega). Firefly and Renilla luciferase activities were measured in the cell lysate using the Dual Luciferase Activity Kit (Promega).

### In Vivo Experiments

Three groups of mice ( $n = 6$  mice per group) were used to assess whether PPAR $\alpha$  played a role in P-407-mediated dyslipidemia in mice. Group 1 consisted of B6 mice treated with P-407 (0.5 g/kg), while Groups 2 and 3 consisted of PPAR $\alpha$ -knockout mice treated with either normal saline or P-407 (0.5 g/kg), respectively. To reduce animal numbers, we utilized our previously-reported literature values of plasma lipid concentrations obtained following the administration of saline to B6 mice (Group 4 mice). After intraperitoneal administration of 0.5 mL of either normal saline or P-407 (dissolved in normal saline), blood samples (50  $\mu$ L) were obtained from each mouse by tail vein sampling at 0, 2, 4, 8, 16, and 24 h post-dosing. All blood samples were collected into heparinized tubes, the plasma obtained, and the samples stored at -80 °C until the time of lipid analysis. All procedures for P-407 administration and subsequent blood collection were in accordance with the institution's guide for the care and use of laboratory animals, and the treatment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Missouri-Kansas City.

Plasma samples were analyzed for total cholesterol (Allain et al 1974) and triglycerides (Bucolo & David 1973) using standard enzymatic, colorimetric assay kits. The concentration of HDL-

cholesterol was determined by first precipitating the VLDL and LDL fractions with a phosphotungstic acid/magnesium solution (Bachorik & Albers 1986) and then analyzing the supernatant obtained after centrifugation at  $10,000 \times g$  for total cholesterol. Non-HDL-cholesterol was simply calculated as total cholesterol minus HDL-cholesterol.

### Apolipoprotein Analysis

ApoA1 in plasma was measured by an apoA1 ELISA assay (Dansky et al 1999). Rabbit anti-mouse apoA1 (Biosdesign, Saco, ME) was purified on protein A affinity columns (Pierce) and an aliquot was biotinylated (Amersham). Purified antibody, diluted 1:400 in bicarbonate buffer, was applied to wells of Nunc Maxisorb ELISA plates and incubated overnight at 4 °C. All subsequent incubations were performed at room temperature with gentle agitation. Wells were washed with PBS and blocked with casein blocker (Pierce) for 1 h, followed by addition of plasma and serum standards for 2 h. Serum standards were calibrated by comparing with purified mouse apoA1. Plates were washed with PBS/Tween (0.5%) and biotinylated antibodies, diluted 1:250 in PBS/Tween, were applied for 2 h. After washing, streptavidin/HRP (Pierce) was applied for 1 h. HRP enzyme was detected by incubation with TurboTMB substrate (Pierce). The reaction was terminated with 1 mol/L sulfuric acid, and the absorbance was measured at 450 nm on a model 450 microplate reader (Bio-Rad, Richmond, CA).

A similar ELISA assay was used to quantify the plasma concentrations of apoE (Wahrle et al 2004). Briefly, rabbit anti-mouse apoE (Biosdesign, Saco, ME) was purified on protein A affinity columns (Pierce) and then applied (0.5 µg/well) to 96-well plates overnight. Plates were then washed with PBS, blocked with 1% milk in PBS, and then washed again. Plasma samples were diluted directly into 0.1% bovine serum albumin, 0.025% Tween in PBS. Standards were based on pooled plasma from B6 mice containing approximately 70 µg/mL apoE. Following a 2-hour sample incubation, the plate was washed and 3 µg/well of biotinylated goat anti-apoE [Calbiochem (catalog no. 178479)] was added. The antibody was biotinylated using a biotin-maleimide reagent (Vector Laboratories, Burlingame, CA). After incubation of the secondary antibody (2 h), the plate was washed and poly-horseradish peroxidase streptavidin (Pierce) was added at 1:6000 and incubated for 1 h. The plate was washed, developed with tetramethylbenzidine (Sigma), and read at 650 nm.

### Data Analysis

Luciferase activity values were normalized for transfection efficiency by dividing the measured Firefly luciferase activity values by the Renilla luciferase activity obtained for the same cell extract, *i.e.*, (Firefly/Renilla) X 1000. Data is presented as the mean  $\pm$  standard deviation luciferase activity for  $n = 3$  separate determinations. Data obtained from the transactivation assays was assessed for statistical significance, relative to vehicle controls, by using a one-way analysis-of-variance (ANOVA). *Post hoc* analysis of individual differences between treatment groups was assessed using the Method of Scheffe, with  $p < 0.05$  deemed significant.

Data acquired from the *in vivo* experiments were processed in the following manner. The increased plasma total cholesterol and HDL-cholesterol seen in PPAR $\alpha$ -deficient mice (Peters et al 1997) was taken into account before comparing the plasma lipid vs. time profiles for PPAR $\alpha$ -deficient mice to the corresponding profiles for B6 mice. Using plasma total cholesterol concentrations for P-407-treated, PPAR $\alpha$ -deficient mice and P-407-treated, B6 mice as an example, the average increase in the plasma concentration of total cholesterol for saline-treated, PPAR $\alpha$ -deficient mice, relative to saline-treated, B6 mice (~106 mg/dl; see lower two curves in Fig. 3A, below), was subtracted from each plasma total cholesterol concentration for the P-407-treated, PPAR $\alpha$ -deficient mice. The resulting values were then compared to the cholesterol concentrations at each time point for the P-407-treated, B6 mice. A similar procedure was followed for comparing plasma concentrations of HDL-cholesterol.

The unpaired Student's *t*-test was used for comparing plasma lipid concentrations at each time point between genotypes (PPAR $\alpha$ -deficient vs. B6) for a given treatment (P-407 or saline), as well as for comparing corresponding plasma lipid concentrations at each time point between treatments (P-407 vs. saline) within a given genotype (PPAR $\alpha$ -deficient or B6). A value of  $p < 0.05$  was deemed statistically significant. Values used for the lipid and lipoprotein plasma concentrations for saline-treated B6 mice (Group 4) were those reported previously (Palmer et al 1998; Johnston et al 1999). Mean values of the plasma concentrations of apoA1 and apoE were processed in the same manner as plasma total and HDL-cholesterol concentrations described above.

## RESULTS

### Transactivation Assay

A cell-based transactivation assay was used to investigate whether P-407 directly modulates PPAR $\alpha$  activity. Treatment of cells with P-407 at concentrations ranging from 50 nm to 200  $\mu$ M did not increase the transcriptional activity of either mouse PPAR $\alpha$  or human PPAR $\alpha$  relative to vehicle (Figs. 1A and 1B, respectively). Moreover, P-407 did not inhibit human PPAR $\alpha$  transcriptional activity stimulated by the PPAR $\alpha$  agonist Wy-14,643 (Fig. 2).

### PPAR $\alpha$ -deficient Mouse Experiments

In B6 mice, plasma total cholesterol increased from a baseline value of 78 mg/dl to 693 mg/dl by 24 h after administration of P-407 (Fig. 3A). The P-407-induced increase was evident as early as 4 hr ( $p < 0.05$ ). Administration of P-407 to PPAR $\alpha$ -deficient mice produced a similar time-course in the plasma concentration of total cholesterol, although the profile was situated above the concentration-time profile obtained with the P-407-treated B6 mice. This reflects the increase in plasma concentrations of HDL-cholesterol and total cholesterol that characterize PPAR $\alpha$ -deficient mice (Peters et al 1997) (also see two lower curves in Fig. 3A). When this increase in the plasma total cholesterol was taken into account, no significant difference in the plasma total cholesterol profile was observed in P-407-treated, PPAR $\alpha$ -deficient mice when compared to P-407-treated, B6 mice.

The plasma concentration-time profiles for non-HDL cholesterol (calculated) and plasma TG are depicted in Figures 3B and 3C, respectively. There was no significant difference between the non-HDL cholesterol concentration-time profiles for P-407-treated B6 and P-407-treated PPAR $\alpha$ -deficient mice (Fig. 3B). However, as with the plasma total cholesterol concentration-time profiles, there was a significant ( $p < 0.05$ ) difference between the profiles of the P-407-treated B6 mice and P-407-treated PPAR $\alpha$ -deficient mice when each profile was individually compared to its respective saline control. These same trends can be observed in Figure 3C with regard to the plasma TG concentration-time profiles.

Figure 3D illustrates the plasma HDL concentration-time profiles resulting from the administration of either P-407 or saline to B6 and PPAR $\alpha$ -deficient mice. Again, disruption of the gene for PPAR $\alpha$  causes a greater baseline plasma HDL-cholesterol concentration (Peters et al 1997) in saline-treated PPAR $\alpha$ -deficient mice than for saline-treated B6 mice. Baseline plasma HDL concentrations were  $\sim 100$  mg/dl for PPAR $\alpha$ -deficient mice, compared to  $\sim 60$  mg/dl for B6 mice. Administration of P-407 to both B6 and PPAR $\alpha$ -deficient mice resulted in a gradual, but steady, increase in plasma HDL concentrations throughout the duration of the experiment (Fig. 3D). With the P-407-induced mouse model of atherogenic dyslipidemia, plasma HDL concentrations increase slightly following P-407 administration, but most of the cholesterol is found in the LDL and VLDL fractions, rather than in the HDL fraction. The slight, but gradual rise in the plasma HDL-cholesterol concentration for 24 hours post-dosing reflects increased cholesterol synthesis (Leon et al 2006; Johnston 2004; Johnston & Palmer

1997), although the ratio of plasma HDL-cholesterol:non-HDL-cholesterol concentrations in P-407-treated mice typically range from 3.5 at time 0 h (prior to injection) to 0.2 at 24 h (*i.e.*, the time point at which maximum plasma lipid concentrations are attained in this model following a single injection of P-407) (Johnston 2004).

As with the other plasma lipid concentration *vs.* time profiles, all plasma HDL cholesterol concentrations for P-407-treated mice (both B6 and PPAR $\alpha$ -deficient) were significantly greater ( $p < 0.05$ ) than the corresponding HDL cholesterol concentrations for their respective saline-injected controls beginning at 4 h post-dosing. However, there was no significant difference in plasma HDL cholesterol concentrations between B6 and PPAR $\alpha$ -deficient mice when each group was administered P-407 (Fig. 3D). The same trend was observed when both B6 and PPAR $\alpha$ -deficient mice were administered saline (Fig. 3D).

### Plasma Apolipoprotein Analysis

Because atherogenesis is well-documented in apoE-deficient mice and both apoA1 and apoE have been shown to function as cholesterol acceptors and thereby facilitate cholesterol efflux from macrophages (Joyce et al 2002), we analyzed plasma for both apoA1 and apoE. P-407 decreased the plasma concentration of apoA1 to a similar extent (30 to 32%;  $p < 0.05$ ) in both B6 and PPAR $\alpha$ -deficient mice (Table 1). In contrast, the plasma concentration of apoE increased slightly, but, not significantly ( $p > 0.05$ ) in both B6 and PPAR $\alpha$ -deficient mice administered P-407.

## DISCUSSION

Previous work had demonstrated that P-407 treatment significantly decreased cholesterol efflux from macrophages by down-regulating ABCA1 gene expression and that P-407 did not abrogate the action of a known LXR $\alpha$  agonist to stimulate apoA1-mediated cholesterol efflux from macrophages (Johnston et al 2006). In the present study, we investigated whether P-407 modulated PPAR $\alpha$  activity with the goal of extending our work to the PPAR-LXR-ABCA1 signaling pathway and to further elucidate the mechanism(s) responsible for reduced cellular cholesterol efflux along this pathway, as well as the dyslipidemia observed in this murine model of atherogenesis (Johnston 2004).

Using a transactivation assay, we determined that P-407 was unable to activate the transcriptional activity of either mouse or human PPAR $\alpha$ . Furthermore, P-407 did not inhibit a known PPAR $\alpha$  agonist from activating human PPAR $\alpha$ . To ascertain whether PPAR $\alpha$  played a role in P-407-mediated dyslipidemia, we compared the effects of P-407 treatment in wild-type (B6) and PPAR $\alpha$ -deficient mice. It was critical to evaluate P-407 for its potential to mediate dyslipidemia in PPAR $\alpha$ -deficient mice, since a compound's ability to modulate PPAR $\alpha$  is not always predicted from the results of an *in vitro* transactivation assay (Peters et al 1996). After taking into consideration the increase in plasma HDL-cholesterol and, consequently, total cholesterol, seen in PPAR $\alpha$ -deficient mice, there were no significant differences in P-407-induced plasma concentration-time profiles of total cholesterol, HDL-cholesterol, non-HDL-cholesterol (calculated), and TG. Therefore, these data strongly support the conclusion that neither P-407, nor some intermediate which results from the administration of P-407, is functioning as a PPAR $\alpha$  agonist *in vivo*, similar to the action of fibrate drugs described below.

Assessment of anti-atherogenic activity of PPAR $\alpha$  agonists in rodent models of atherosclerosis is hampered by the fact that a) rodents develop a proinflammatory peroxisome proliferative response in the liver, and b) classical animal models, such as the LDL-receptor or apoE-deficient mice, display an aberrant hyperlipidemic response to the fibrate class of hypolipidemic drugs (Marx et al 2004). As an example of the latter, several studies have

evaluated the use of fibrates (PPAR $\alpha$  agonists) in several rodent models. Duez *et al.* reported that even though administration of fenofibrate to apoE<sup>-/-</sup> mice at a dose of 100 mg/kg/d for 8 weeks resulted in a significant increase in both plasma total cholesterol and triglycerides, there was a reduction in atherosclerosis (Duez et al 2002). Similarly, Fu *et al.* (Fu et al 2003) reported that ciprofibrate markedly increased the plasma levels of atherogenic lipoproteins in apoE<sup>-/-</sup> mice; however, in contrast to Duez *et al.*, this investigator reported a significant enhancement in the development of atherosclerosis. Clearly, the ability of fibrate drugs to increase plasma lipids in mice is well established, although their capacity to inhibit atherosclerosis is still controversial. Notably, the effects of fibrates in rodents are just opposite to that observed clinically. Activation of PPAR $\alpha$  with fibrate drugs in humans increases fatty acid oxidation, decreases very-low-density lipoprotein (VLDL), increases lipolysis, and increases HDL-cholesterol and reverse cholesterol transport. These actions result in a decrease in plasma TG, a decrease in plasma small, dense, atherogenic LDL-cholesterol, and an increase in HDL-cholesterol (Marx et al 2004).

Finally, if PPAR $\alpha$  had been involved in the dyslipidemic response induced by administration of P-407, then plasma lipid concentration-time profiles for mice in Group 3 (PPAR $\alpha$ -knockouts administered P-407), after correcting for increases in HDL, and, in turn, total cholesterol (due to the gene knockout), would not have been similar to corresponding profiles determined for mice in Group 1 (B6 mice administered P-407). Furthermore, as described above, P-407, or some intermediate formed following its administration, is not functioning as a PPAR $\alpha$  agonist, similar to the fibrate drugs. If P-407 had functioned as a PPAR $\alpha$  agonist, then its administration to B6 mice would have been expected to increase plasma lipid concentrations similar to the studies cited above for fibrates; however, P-407 would have no capacity to increase plasma lipids to the same extent in PPAR $\alpha$ -deficient mice (Group 3 mice). Therefore, we conclude that P-407 does not modulate PPAR $\alpha$  activity (*e.g.*, by functioning as either a PPAR $\alpha$  agonist or antagonist) following its administration to B6 mice. Additionally, our data support the conclusion that the dyslipidemic response to P-407 in mice is not mediated by its effects at the level of PPAR $\alpha$ , which strongly corroborates our findings using the transactivation assay. Instead, acute P-407 administration to mice (*i.e.*, a single injection) causes hypertriglyceridemia, in part, by inhibiting the activity of capillary-bound lipoprotein lipase (Johnston & Palmer 1993) and hypercholesterolemia, in part, by inhibiting the activity of cholesterol 7 $\alpha$ -hydroxylase (the rate-limiting enzyme for elimination of cholesterol into bile) (Johnston et al 2001), while simultaneously increasing both the activity and protein expression of 3-hydroxy-3-methylglutaryl-CoA reductase and down-regulating low-density lipoprotein receptor expression (Leon et al 2006; Johnston & Palmer 1997).

ApoA1, the major cholesterol acceptor of HDL, is induced by fibrates in humans, but is decreased by fibrates in rodents (Duez et al 2005). This is because HDL metabolism is regulated in an opposite fashion by fibrates in rodents (decrease) and man (increase) due to sequence divergences in the respective apoA1 promoters (Ngoc et al 1998). Consequently, in rats, fibrates, such as fenofibrate and clofibrate, significantly decrease plasma apoA1, apoA2, and apoE concentrations (Staels et al 1992), whereas, in mice, fibrates cause a decrease in the plasma concentration of apoA1, but an increase in apoA2 (Duez et al 2005). In the present study, P-407 caused a reduction in the plasma concentration of apoA1 in treated mice. Since it is primarily apoA1 that confers the atheroprotective effects of HDL, this may help explain why mice develop aortic atherosclerotic lesions after 16 weeks of P-407 administration (Johnston 2004; Palmer et al 1998). Simultaneously, there was a slight increase in the plasma concentration of apoE. Interestingly, we previously demonstrated that HDL, isolated from the plasma of P-407-treated mice, contained more apoE, relative to apoA1 (Johnston et al 1999).

The decrease in plasma apoA1 following P-407 administration is not likely to be mediated by PPAR $\alpha$ , since a) P-407 was unable to directly modulate the activity of mouse PPAR $\alpha$  using a

transactivation assay, and b) the atherogenic dyslipidemia that occurs following administration of P-407 to mice was not mediated through PPAR $\alpha$ . Therefore, as it relates to apolipoproteins, even though P-407 treatment caused a reduction in the plasma concentration of apoA1, similar to the fibrates, we conclude that P-407 did not function as a PPAR $\alpha$  agonist. Instead, we suggest that P-407, by significantly reducing ABCA1 gene expression, contributes, in part, to induction of an atherogenic plasma lipid profile. Indeed, Joyce *et al.* (Joyce et al 2002) demonstrated that overexpression of ABCA1 in C57BL/6 mice resulted in a unique anti-atherogenic profile characterized by decreased plasma cholesterol, cholesteryl esters, free cholesterol, non-HDL cholesterol, and apoB, but markedly increased HDL, apoA1, and apoE. In contrast, administration of P-407 to C57BL/6 mice results in an atherogenic profile characterized by increased plasma total cholesterol, triglycerides, and non-HDL cholesterol, but markedly decreased HDL-cholesterol and apoA1. The fact that P-407 induced this same type of atherogenic profile in PPAR $\alpha$ -deficient mice provides further evidence that the observed reduction in plasma apoA1 levels, which resulted from the administration of P-407, was not mediated through PPAR $\alpha$ , and that P-407 was not functioning as a PPAR $\alpha$  agonist.

## CONCLUSION

In conclusion, as it pertains to the PPAR-LXR-ABCA1 signaling pathway, our findings suggest that acute administration of P-407 to mice does not modulate the activity of PPAR $\alpha$ , and, hence, the reduced cholesterol efflux we previously observed when macrophages were treated with P-407 (Johnston et al 2006) most likely results from P-407's direct inhibitory effect on ABCA1 gene expression. Additionally, the atherogenic dyslipidemia that occurs following P-407 administration to mice, as well as the decrease in the plasma concentration of apoA1, appear not to be regulated by P-407's effect(s) at the level of PPAR $\alpha$ . Future research will focus on whether the protein expression of ABCA1 is also modulated following P-407 administration.

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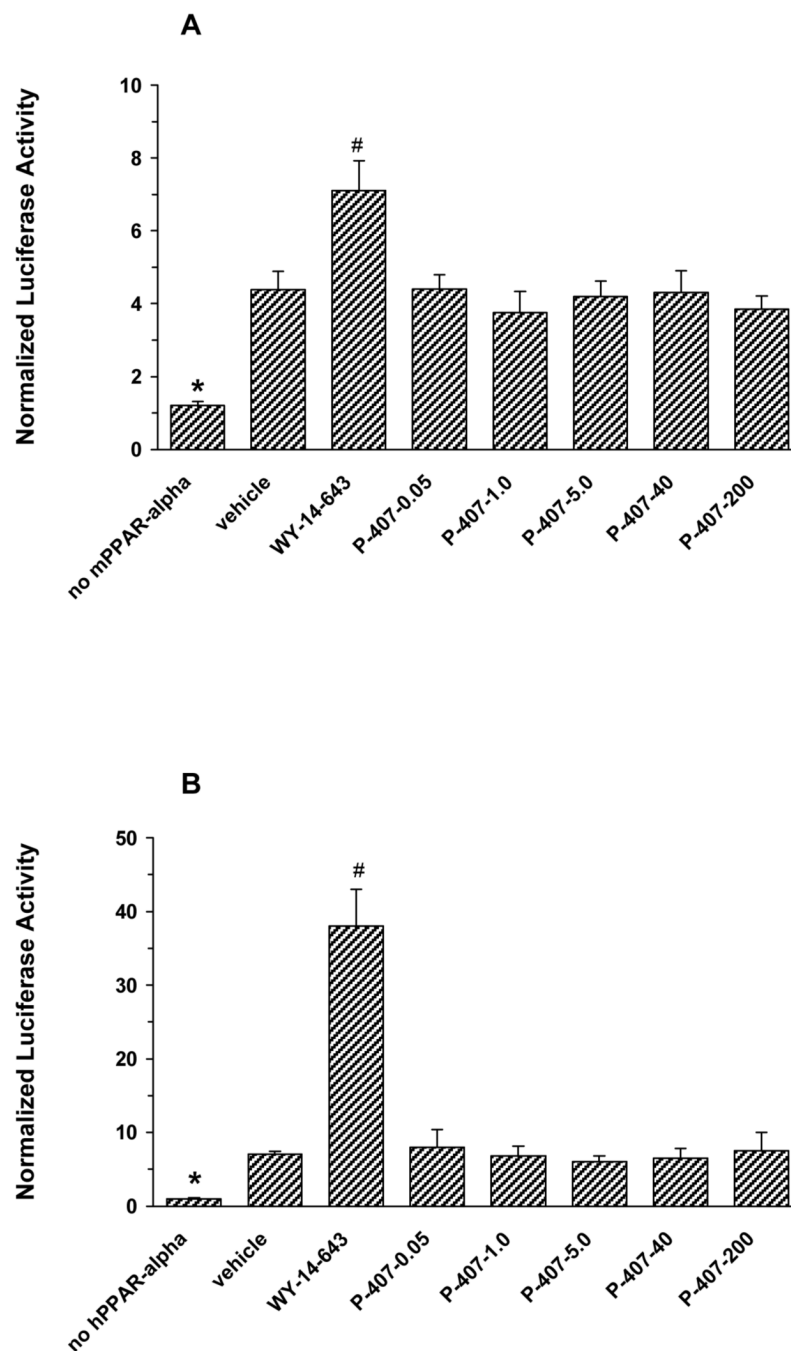
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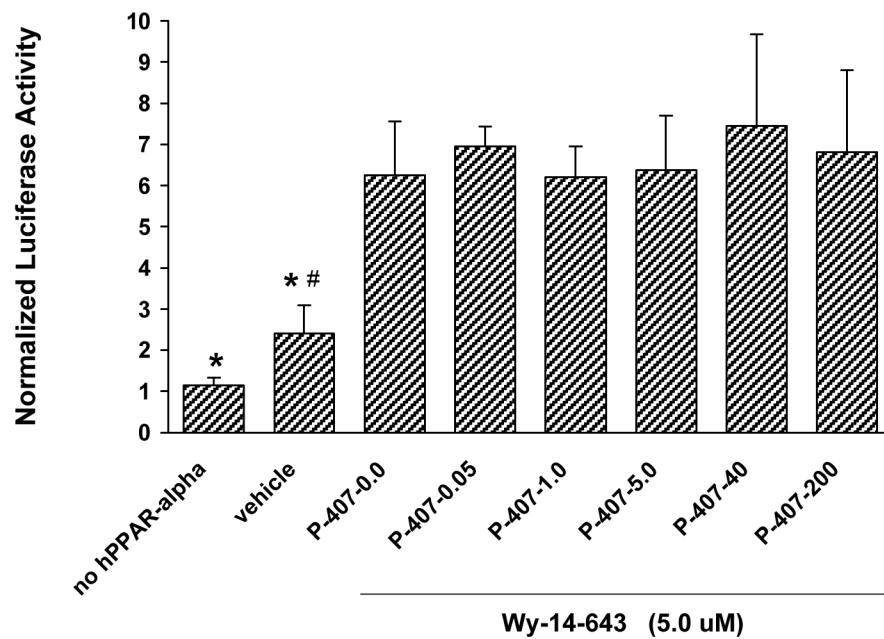
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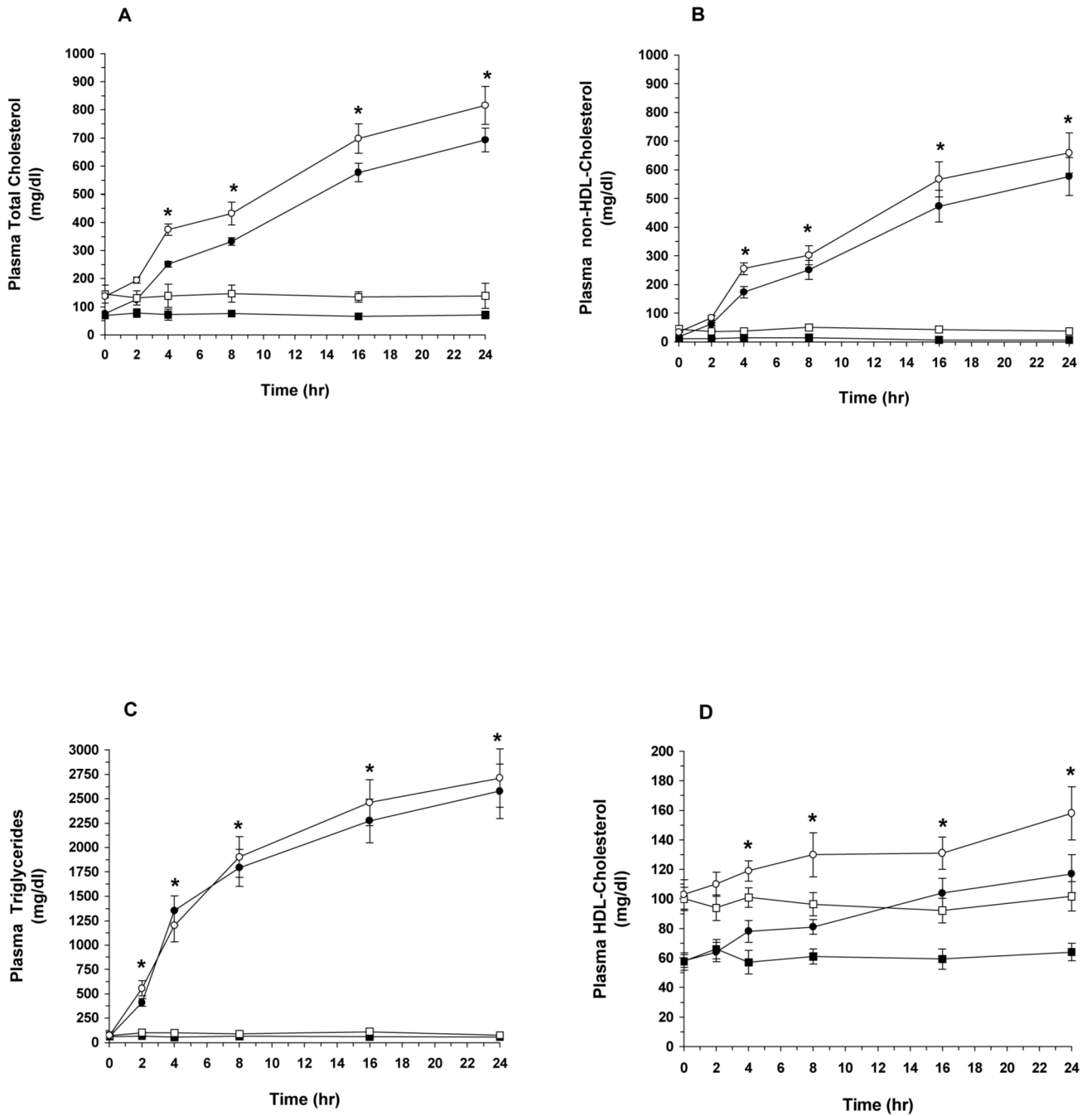
**Figure 1.**

The effect of P-407 on both mouse (A) and human (B) PPAR $\alpha$  activity *in vitro*. All P-407 concentrations are in  $\mu$ M. Data represents the mean  $\pm$  standard deviation ( $n = 3$ ). First bar, no PPAR $\alpha$  control; second bar, cells transfected with either mouse (A) or human (B) PPAR $\alpha$  expression plasmid and then treated with vehicle. The increase in activity of this sample compared to bar 1 reflects activation of PPAR $\alpha$  by cellular fatty acids and other endogenous activators. Bars 3-8 are the same as bar 2, except that the cells were stimulated for 24 hr with either Wy-14,643 or P-407 at the micromolar concentrations indicated. \* indicates that the mean value of bar 1 (no PPAR $\alpha$ ) was significantly ( $p < 0.05$ ) less than the mean value of all

other bars. # indicates that the mean value of bar 3 (WY-14-643) was significantly ( $p < 0.05$ ) greater than the mean value of all other bars.



**Figure 2.** The effect of P-407 on human PPAR $\alpha$  activity stimulated by the PPAR $\alpha$ -activator, Wy-14-643. All P-407 concentrations are in  $\mu\text{M}$ . Data represents the mean  $\pm$  standard deviation. \* indicates that the mean value of bars 1 (no PPAR $\alpha$ ) and 2 (vehicle) were significantly ( $p < 0.05$ ) less than the mean values of all other bars. # indicates that the mean value of bar 2 (vehicle) was significantly ( $p < 0.05$ ) greater than the mean value of bar 1 (no PPAR $\alpha$ ).



**Figure 3.**

Plasma total cholesterol (A), non-HDL-cholesterol (B), triglycerides (C), and HDL-cholesterol (D) concentrations following administration of normal saline (squares) to either C57BL/6 (■) or PPAR $\alpha$ -deficient (□) mice and P-407 [0.5 g/kg] (circles) to either C57BL/6 (●) or PPAR $\alpha$ -deficient (○) mice. Data represents the mean  $\pm$  standard deviation. \* indicates that beginning at 4 h (2 h for triglycerides), all plasma concentrations for P-407-treated mice (both B6 and PPAR $\alpha$ -deficient) were significantly ( $p < 0.05$ ) greater than the corresponding concentrations for their respective saline-injected controls. However, there was no significant difference in plasma lipid concentrations between P-407-treated B6 and P-407-treated PPAR $\alpha$ -deficient mice when concentrations for the latter group were normalized for changes induced by the

gene knockout as described in *Methods*. Similarly, there was no significant difference in plasma lipid concentrations between saline-treated B6 and saline-treated PPAR $\alpha$ -deficient mice when concentrations for the latter group were normalized for changes induced by the gene knockout as described in *Methods*.

**Table 1**  
**The Effect of Acute P-407 Administration on Plasma Apolipoprotein Concentrations**

Genotype/Treatment	apoA1 (mg/dl)	apoE (mg/dl)
B6/Saline	<sup>a</sup> . 82.0 ± 4.7	2.6 ± 0.3
B6/P-407	<sup>b</sup> . 57.4 ± 3.2	2.9 ± 0.2
PPARα <sup>-/-</sup> /Saline	136 ± 11.9	2.8 ± 0.3
PPARα <sup>-/-</sup> /P-407	<sup>b</sup> . 92.7 ± 8.6	3.2 ± 0.2

<sup>a</sup>. mean value ± standard deviation 24 hr after P-407 administration

<sup>b</sup>. indicates a significant ( $p < 0.05$ ) reduction compared to its saline-injected control