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Co-administration of a Liver X Receptor Agonist and a Proxisome Proliferator Activator Receptor a Agonist in Mice—Effects of Nuclear Receptor Interplay on High-Density Lipoprotein and Triglyceride Metabolism *In Vivo*

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Running title: Activation of LXR and PPARα on lipid homeostasis in vivo

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Abbreviations: HDL, high-density lipoproteins. LXR, liver X receptor. PPAR, peroxisome proliferator activator receptor. PLTP, phospholipid transfer protein. SREBP, sterol regulatory element binding protein. LPL, lipoprotein lipase. ABCA1, ATP-binding cassette transporter protein A1.

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Abstract

LXRs are master transcription factors regulating cholesterol and fatty acid metabolism. Treatment of C57B6 mice with a specific synthetic LXR agonist, T0901317, resulted in elevated HDL cholesterol as well as plasma and liver triglycerides. PPARα agonists are known to induce peroxisomal fatty acid β-oxidation and also mediate HDL cholesterol metabolism. We have explored the hypothesis that simultaneous activation of PPARα and LXR may lead to additive effects on HDL cholesterol elevation as well as attenuation of triglyceride accumulation. Co-administration of T0901317 and the specific PPARα agonist Wy14643 in mice led to synergistic elevation of HDL cholesterol that was primarily associated with enlarged HDL particles enriched with ApoE and ApoAI. Liver PLTP mRNA and plasma PLTP activity were additively elevated, suggesting a role of PLTP in the observed HDL cholesterol elevation. Moderate increases in plasma triglyceride levels induced by LXR activation was reduced while the accumulation of triglyceride in the liver was not altered upon co-administration of the PPARα agonist. Peroxisomal fatty acid β-oxidation in the liver was dramatically elevated upon PPARα activation as expected. Interestingly, activation of LXRs via T0901317 also led to a significant increase in peroxisomal fatty acid β-oxidation. SREBP1c expression was dramatically upregulated by the LXR agonist but was not changed with PPARα agonist treatment. Liver lipoprotein lipase expression was additively increased upon LXR agonist and PPARα agonist co-administration. Our studies mark the first exploration of nuclear receptor interplay on lipid homeostasis in vivo.

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Introduction

Plasma high-density lipoproteins (HDL) are inversely correlated with coronary artery events and play a major role in mediating reverse cholesterol transport (RCT) and anti-inflammatory actions. It is thus desirable to modulate HDL cholesterol level pharmacologically to achieve cardiovascular benefits. Recent studies have suggested that one potential way to elevate HDL cholesterol is through nuclear receptor modulation.

Nuclear receptors are a distinct class of transcription factors that control a multitude of cellular processes. A pair of nuclear receptors named liver X receptors (LXRα and LXRβ) are instrumental in regulating RCT (Repa and Mangelsdorf, 2002). LXRα and LXRβ were originally isolated as orphan nuclear receptors with distinct tissue distributions (Lu et al., 2001). Certain oxysterols were subsequently identified as LXR native ligands suggesting their role in cholesterol homeostasis (Janowski et al., 1996; Janowski et al., 1999; Fu et al., 2001). The spectrum of target genes identified to date indicates that these receptors are master transcription factors mediating cholesterol catabolism. First, LXRs regulate ABCA1(Costet et al., 2000; Repa et al., 2000b) that is both essential and rate limiting in mediating peripheral cholesterol efflux. LXRs also regulate several apolipoproteins that can mediate cholesterol efflux (Laffitte et al., 2001; Mak et al., 2002b). Second, LXRs regulate HDL modifying enzymes, including CETP and PLTP (Luo and Tall, 2000; Cao et al., 2002; Mak et al., 2002a; Laffitte et al., 2003b). Third, LXRs control cholesterol excretion into the bile in the liver and intestinal cholesterol absorption through regulating ABCG5 and ABCG8 (Repa et al., 2002; Yu et al., 2003). In addition, LXRs also play critical roles in mediating glucose

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homeostasis(Cao et al., 2003; Laffitte et al., 2003a). Thus, LXRs have emerged as prime targets through which to modulate cholesterol catabolism for metabolic diseases and artherosclerosis.

The pursuit of LXRs as therapeutic targets, however, has been hampered by the fact that LXRs regulate hepatic fatty acid biosynthesis pathway via the transcription factor SREBP1c (Repa et al., 2000a; Schultz et al., 2000). Studies performed in mice with specific synthetic LXR agonists produced marked liver triglyceride accumulation and hypertriglyceridemia (Grefhorst et al., 2002). Accumulation of triglycerides in the liver significantly compromises liver function and thus is highly undesirable. Hypertriglyceridemia is associated with metabolic diseases and also regarded as an independent risk factor for cardiovascular disease (Ginsberg, 2002). These observations thus present major hurdles in developing therapies through LXR modulation.

Proxisome Proliferator Activator Receptor α (PPARα) belongs to a family of nuclear receptors that also plays critical roles in multiple physiopathological conditions. The fibrate class of drugs that are effective clinically in reducing plasma triglyceride levels exert efficacy through PPARα activation (Staels et al., 1998). In rats, diet induced liver triglyceride accumulation is effectively dissipated through the addition of a synthetic PPARα ligand (Foxworthy and Eacho, 1991). PPARα upregulates apoAV (Vu-Dac et al., 2003) and downregulates apoCIII (Staels et al., 1995) and the combined effects lead to plasma triglycerides reduction. PPARα also mediates liver fatty acid β-oxidation to dissipate fat accumulation (Staels et al., 1998). Fibrates also modestly elevate HDL

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cholesterol in humans (Staels et al., 1998). It has been reported that PPARα agonists can upregulate human apoAI, which may be responsible for the observed HDL effect in humans (Berthou et al., 1996). Treatment of human apoAI transgenic mice with a PPARα agonist fenofibrate resulted in a dramatic HDL cholesterol increase (Berthou et al., 1996). PPARα also regulates PLTP, which is essential for HDL particle size increase (Bouly et al., 2001).

Our hypothesis was that liver triglyceride accumulation and hypertriglyceridemia mediated by LXR activation could be prevented through activation of PPARα via dissipation of triglycerides in the liver and plasma. We have also noted recent reports of cross talk between LXRs and PPARα that could impact HDL metabolism (Tobin et al., 2000; Chinetti et al., 2001). In this report, we have investigated the effects of simultaneous activation of both LXRs and PPARα through co-administration of a specific LXR agonist, T0901317, and the specific PPARα agonist Wy14643 or fenofibrate on both HDL cholesterol metabolism and triglyceride metabolism.

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Methods

Animals. Eight-week old C57/BL6 mice (six per group) were purchased from Harlan (Indianapolis, Indiana) and acclimated for one week before the experiments. The mice were provided Purina 5001 food ad lib throughout the experiments. Wy14643 (10 mg/kg) and fenofibrate (10, 75 and 300 mg/kg respectively) were purchased from Calbiochem and Sigma respectively. T0901317 (50 mg/kg) was available from Cayman Chemical (Ann Arbor, Michigan). Compounds were formulated in wet granulation vehicle (212.5 mg Povidone, 3.77 g Lactose Anhydrous (granular) and 64.8 µl Polysorbate 80 (Tween 80) in 250 ml water). Animals were treated through oral gavage with either vehicle or various doses of compounds daily for seven days and sacrificed by CO_2 euthanasia two hours after the last dose. Plasma and liver tissue were prepared for various analyses. Use of mice was approved by the Institutional Animal Care and Use Committees of the American Association for Accreditation of Laboratory Animal Care-accredited institutions and Lilly Research Laboratories in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. One-way ANOVA or Student t-test was used for statistical analysis.

Plasma lipid analysis. Plasma total cholesterol and triglyceride were measured utilizing a Monarch Plus clinical chemistry analyzer. Lipoproteins were separated with Fast Protein Liquid Chromatography (FPLC) and cholesterol quantified with an in-line detection system based on that described by Kieft, et al (Kieft et al., 1991). Briefly 50 µl of pooled sample was applied to a Superose® 6 HR 10/30 size exclusion column (Amersham Pharmacia Biotech) and eluted with phosphate buffered saline pH 7.4 (GibcoBRL 70011-044, dilution 1:10), containing 5 mM EDTA, at 0.5 ml/min. Cholesterol reagent (Roche

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Diagnostics Chol/HP 704036) at 0.16 ml/min mixed with the column effluent. The colored product produced in the presence of cholesterol was monitored in the flow stream at 505 nm and the analog voltage from the monitor was converted to a digital signal for collection and analysis.

Western blot analysis. Designated FPLC fraction samples (10 μl each) were separated on Tris-Glycine gels (Novex) under denaturing conditions. Protein was transferred to nitrocellulose membranes and then blotted with antibodies specific for apolipoproteins AI or E (Biodesign) or apolipoprotein B48/100 (US Biological). Blots were developed with ECL western blotting detection reagents (Amersham) and documented using X-OMAT film (Kodak).

mRNA measurement. All mRNAs were measured by RNase protection assay (RPA). Total RNAs were prepared from frozen tissue samples TRIzol reagent (Invitrogen). Mouse PLTP and ABCA1 probes were described previously (Cao et al., 2002). The primer sets to amplify probes of mouse SREBP1c and lipoprotein lipase (LPL) were: 5'-ATC GGC GCG GAA GCT GTC GGG GTA GCG TC-3', 5'-ACT GTC TTG GTT GTT GAT GAG CTG GAG CAT-3' and 5'-GGA AGC CTT TGA GAA AGG-3', 5'-GGT TGT GTT GCT TGC CAT TC-3' respectively. The resulting PCR fragments were cloned into pGEM-T Easy (Promega) and sequenced. The resulting construct was linearized and RNA probe was synthesized using Promega's T7/SP6 Transcription kit. Specific activity was $>10^8$ dpm/ug. After column purification, the probe was used for RPA analysis by using a kit from Ambion. A probe for 28S RNA was used to normalize the RNA samples used in the experiments. The signal was quantified with a Molecular Dynamics Phosphoimager Model 51.

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Determination of liver triglycerides. Liver samples were frozen in liquid nitrogen and homogenized by polytron on ice. Triglycerides in liver homogenates were determined enzymatically using a commercially available enzymatic Kit (Wako) according to the manufacturer's recommendations.

Peroxisomal beta oxidation measurement. Animals were sacrificed by carbon dioxide asphyxiation and a portion of liver homogenized in nine volumes of cold 0.25M sucrose. Samples were centrifuged at 600x g for 10 minutes and the supernatants were decanted. Triton X-100 was added to an aliquot of the supernatant (final concentration 1.0%). The samples were then assayed for peroxisomal ß-oxidation in the presence of KCN, which inhibits mitochondrial ß-oxidation. The oxidation of palmitoyl CoA was quantified by spectrophotometrically measuring the reduction of NAD^+ at 340 nm. The rate of NAD^+ reduction is directly related to the rate of fatty acid oxidation (Lazarow, 1981).

PLTP Activity measurement. PLTP activity was measured with an assay kit according to the manufacturer's instructions. (Cardiovascular Target Inc., New York).

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Results

Previously it was shown that treatment of C57B6 mice with a specific synthetic LXR agonist, T0901317, at 50 mg/kg increased HDL cholesterol and particle size (Schultz et al., 2000; Cao et al., 2002). It was also known that treatment of mice with a specific PPARα agonist, Wy14643, would induce peroxisomal fatty acid beta oxidation and reduce plasma triglycerides levels. We treated C57B6 mice orally with the vehicle, 10 mg/kg Wy14643, 50 mg/kg T0901317 alone or in combination daily for seven days. Plasma samples from different groups were pooled and subjected to lipoprotein analysis by fast protein liquid chromatography (FPLC). Similar to what we reported previously, T0901317 treatment resulted in a significant increase in plasma HDL cholesterol and particle size. Wy14643 led to some moderate increase in HDL particle size. Combined treatment, however, resulted in a synergistic elevation of HDL cholesterol that largely resided in the enlarged HDL portion (Fig.1A). These were confirmed through western blot analysis to show that from fractions 28-36 where enlarged HDL was located, there was a dramatic increase in apoE and apoAI content while apoB level was not significantly changed (Fig.1B). To further confirm our findings, we carried out dose-response studies with another PPARα ligand, fenofibrate, in combination with T0901317 in C57B6 mice (Fig.2). We observed an increase in HDL size with increased doses of fenofibrate treatment. Compared to T0901317 alone, combined treatment of T0901317 with various doses of fenofibrate resulted in dose-dependent increases in HDL cholesterol levels without change in HDL particle size. Thus, simultaneous activation of LXR and PPARα in mice led to synergistic elevation of HDL cholesterol that is largely residing in enlarged, apoE and apoAI enriched HDL particles.

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To explore the molecular mechanisms responsible for the effects observed on HDL we examined the expression of two important target genes regulated by PPARα and LXRs-PLTP and ABCA1 in the liver that are intimately involved in HDL cholesterol metabolism. Wy14643 treatment resulted in a moderate increase in ABCA1 (43%) whereas T0901317 treatment led to a 270% increase in ABCA1 mRNA levels. The combined treatment, however, did not result in further elevation of ABCA1 mRNA (Fig.3A). PLTP mRNA was significantly elevated upon activation of either PPARα or LXRs. The combination of two ligands in mice appeared to lead to a synergistic effect on PLTP mRNA levels (Fig.3B). Consistent with liver mRNA data, plasma PLTP activity was increased 2.0 and 3.4 fold with Wy14643 and T0901317 treatment respectively. The combined treatment elevated PLTP activity further to 4.4 fold ($P<0.001$) (Fig. 3C). Thus the synergistic elevation of HDL cholesterol was closely associated with the increases in hepatic PLTP mRNA and serum PLTP activity.

Previously Schultz et al. reported the induction of plasma triglycerides upon LXR activation (Schultz et al., 2000). Joseph et al. reported the transient elevation of plasma triglycerides after three days of dosing with T0901317 treatment at 50 mg/kg and minimal plasma triglyceride increases after seven days of dosing (Joseph et al., 2002). We have observed a moderate plasma triglyceride elevation after daily doses of T0901317 for seven days (about 60% at 50 mg/kg vs vehicle, $p=0.01$ by t-test and about 25% at 100 mg/kg, not significant) (Fig.4A). Liver triglyceride accumulation, however, was more pronounced at the higher doses used (Fig.4B). Treatment of mice with

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Wy14643 at 10 mg/kg for seven days trended towards a slight reduction of plasma triglycerides relative to vehicle treated animals (p=0.09). Wy14643 co-administration with LXR agonist significantly reduced plasma triglycerides when compared to T0901317 alone treated animals (Fig.5A, p=0.002 of T+Wy vs T). Surprisingly, Wy14643 had no effect on liver triglyceride at basal level compared to vehicle control and appeared to only minimally reduce liver triglyceride accumulation induced by T0901317 treatment (Fig. 5B, no significance of T+Wy vs T). Similar results were obtained when fenofibrate was used in comparable studies (data not shown).

To investigate the molecular mechanisms of the above-mentioned observations, we first examined SREBP1c regulation. SREBP1c is the master transcription factor that controls the entire fatty acid biosynthetic pathway. T0901317 significantly upregulated SREBP1c, which was consistent with the previous report (Schultz et al., 2000). Treatment of mice with Wy14643 did not significantly alter SREBP1c expression either at basal level or in the presence of LXR agonist (Fig. 6A). We then examined the peroxisome fatty acid β-oxidation in these mice. As expected, Wy14643 treatment resulted in a 6.8 fold induction of peroxisome fatty acid β-oxidation. Interestingly, T0901317 also resulted in a significant increase in β-oxidation (2.7 fold). The combined treatment of two agonists did not lead to any significant change in β-oxidation compared to Wy14643 treatment alone (Fig. 6B).

We then examined the expression of hepatic lipoprotein lipase, another critical gene involved in triglyceride metabolism. Adult liver expresses virtually no LPL. Wy14643

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treatment induced liver LPL almost four fold. T0901317 treatment led to a nearly five-fold elevation of LPL mRNA level, possibly through direct and indirect LXR regulation of LPL. Combined treatment resulted in an additive ten-fold increase in LPL mRNA levels (Fig. 6C). As LPL plays a critical role in hydrolyzing triglyceride-rich lipoproteins, the dramatic regulation of LPL in the liver of mice treatment with PPARα and/or LXR ligands may play a significant role in mediating plasma and liver triglyceride metabolism that is induced via PPARα and/or LXR activation.

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Discussion

Nuclear receptors play prominent roles in regulating lipid homeostasis. Fibrates as a drug class effectively reduce plasma triglyceride levels and moderately elevate HDL cholesterol. These effects have been largely attributed to their activation of PPARα (Staels et al., 1998). LXRs on the other hand, are novel receptors that have been regarded as the master transcription factors mediating cholesterol catabolism (Repa and Mangelsdorf, 2002). In this report, we have investigated the effects of nuclear receptor interplay on lipid homeostasis in vivo. We have shown that simultaneous activation of PPARα and LXRs led to significant accumulation of enlarged HDL cholesterol that is enriched in apoE and apoAI. The enlarged HDL cholesterol is closely associated with liver PLTP mRNA induction and the elevation of plasma PLTP activity. Co-administration of PPARα and LXR agonists also reduced plasma triglycerides and produced virtually no changes in liver triglyceride levels through intricate modulation of fatty acid biosynthesis, fatty acid β-oxidation and uptake. Our results are physiologically relevant as fatty acids and cholesterol are both components of diet, the former are native ligands of PPARα (Staels et al., 1998) and the derivatives of the latter are native ligands of LXRs (Janowski et al., 1996). Whether these compounds might affect the pharmacokinetics of the co-administered compound(s) needs to be further explored.

It has been shown that PPARα activation leads to increased apoAI transcription through a PPARα responsive element in human apoAI promoter (Berthou et al., 1996), thus increasing human plasma apoAI and HDL cholesterol level. In mice, however, ApoAI is reciprocally regulated (Berthou et al., 1996). Dispite the downregulation of apoAI in

mice, PPARα agonists appear to slightly increase HDL cholesterol and particle size. These effects may be largely due to PPARα upregulation of liver PLTP mRNA and plasma PLTP activity (Bouly et al., 2001) while downregulation of SR-BI, the HDL receptor that slows the HDL particle catabolism (Mardones et al., 2003). The PLTP regulation by PPARα results in HDL particle size increases (Bouly et al., 2001). LXR activation leads to increased HDL cholesterol as well as HDL particle size (Cao et al., 2002). The observed effects of LXR agonists on HDL metabolism have been attributed to primarily ABCA1 activation and liver PLTP regulation (Schultz et al., 2000; Cao et al., 2002). Previously, it was shown that in rats activation of PPARα led to increased LXRα expression in the liver (Tobin et al., 2000). It was also reported that in macrophage cells PPARα regulates LXRα and thus indirectly ABCA1 expression (Chinetti et al., 2001). In our studies, we have observed synergistic elevation of HDL cholesterol upon simultaneous activation of both receptors. The cholesterol largely resides in enlarged HDL particles as analyzed by FPLC and western blot analysis. We did not observe additive regulation of liver ABCA1 mRNA, rather PLTP mRNA was upregulated in an additive manner. In addition, plasma PLTP activity was further augmented with both PPAR and LXR activation with their cognate agonists. As PLTP plays critical roles in HDL biogenesis and remodeling, and its expression level is closely associated with the HDL cholesterol increase, we believe induction of PLTP mRNA in the liver and PLTP activity in the plasma was largely responsible for the observed HDL effect upon activation of both receptors.

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LXR agonists regulate SREBP1c that controls the entire fatty acid biosynthetic pathway (Shimomura et al., 1998; Repa et al., 2000a; Schultz et al., 2000). Activation of LXRs thus leads to increased fatty acid synthesis and VLDL secretion (Grefhorst et al., 2002). Schultz et al. suggested a significant increase in plasma triglycerides in mice and hamsters with LXR activation (Schultz et al., 2000). Joseph et al. reported that the induction of plasma triglycerides in mice was transient by LXR agonists (Joseph et al., 2002). This transient induction of plasma triglycerides could be a result of increased lipid uptake as in apoE deficient mice or LDL receptor deficient mice with human apoB transgene expression, dramatically elevated plasma triglycerides persisted after one week oral treatment of T0901317 (unpublished data). We observed moderate plasma triglyceride induction in mice upon LXR agonist T0901317 treatment for seven days (Fig. 4A). We have also observed the reduction of plasma triglycerides with LXR activation upon PPARα agonist treatment in these mice (Fig. 4A). The exact molecular mechanism for these observations needs to be further defined. PPARα activation has been shown to reduce apoCIII levels in humans, thus increasing LPL activity and triglyceride hydrolysis (Staels et al., 1995). It was also reported recently that activation of PPARα increased the expression of apoAV, the newly identified secreted protein that plays a critical role in determining plasma triglyceride levels in humans (Vu-Dac et al., 2003). We observed no change in apoCIII mRNA in the liver when 10 mg/kg Wy 14643 was administered to mice and paradoxical downregulation of apoAV mRNA in mice by both LXR and PPAR activation (unpublished data). The difference of apoAV regulation by PPARα in mice compared to that in humans is reminiscent of reciprocal apoAI regulation by PPARα in humans and in mice (Berthou et al., 1996).

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While plasma triglyceride induction by LXR agonists was moderate, the liver triglycerides accumulation was dramatic and persistent (Grefhorst et al., 2002). We explored the hypothesis that increased fatty acid β-oxidation through PPARα activation may help dissipate triglycerides accumulation in the liver that was induced by LXR agonists. To our surprise, PPARα activation with Wy14643 alone did not change liver triglyceride levels significantly even though it induced peroxisomal fatty acid β-oxidation (Figure 5A and 6B). Examination of liver LPL mRNA indicated that this molecule, which is normally not expressed in adult tissue, was significantly induced. We hypothesize that increased LPL expression in the liver could have resulted in increased triglyceride-rich lipoprotein hydrolysis and liver fatty acid uptake. LXR agonist treatment of mice also led to significant increase in LPL mRNA in the liver, consistent with a previous report (Zhang et al., 2001). Thus, LXR activation could have generated a futile cycle by increasing VLDL production and secretion while also increasing triglyceride-rich lipoprotein hydrolysis and liver fatty acid uptake. Combined treatment of mice with both PPARα and LXR agonists maintained a constant level of fatty acid β-oxidation and SREBP1c level, but augmented liver LPL mRNA level. Thus, intricate balance of fatty acid biosynthesis, β-oxidation and fatty acid uptake may have contributed to the overall steady-state triglyceride level in the liver.

Through combined treatment with a specific PPARα agonist and a specific LXR agonist, we have shown that simultaneous activation of both receptors can lead to synergistic HDL cholesterol elevation and attenuation of plasma triglycerides but not liver

triglyceride accumulation. Our studies suggest the potential utility of a PPARα/LXR dual agonist in modulating lipid homeostasis. Sparrow et al. reported a potent small molecule LXR agonist that is relatively big in size suggesting a relative big binding pocket of the ligand binding domain of LXRs (Sparrow et al., 2002). This was recently comfirmed with the crystal structure of LXRβ ligand binding domain (LBD) (Farnegardh et al., 2003; Williams et al., 2003). Menke et al. also reported an LXR agonist with significant PPARα activity suggesting the possibility of developing LXR-PPAR dual agonists (Menke et al., 2002). The implication of our studies in humans remains to be explored.

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Figure Legends.

Figure 1. Combined treatment of mice with a PPARa agonist WY14643 and an LXR agonist T0901317 led to synergistic elevation of enlarged HDL cholesterol. A. Male C57B6 mice (6 per group) were orally treated once daily with either vehicle, 10 mg/kg WY14643, 50 mg/kg T0901317 or in combination for 7 days. Plasma samples were pooled for FPLC analysis. Fractions 36-42 represent the original HDL peak. Fractions 27-41 represent the enlarged HDL peak on top of the LDL fraction. B. Lipoprotein analysis of FPLC fractions. Individual fractions were pooled and applied to Tris-Glycine gel. After protein transfer to nitrocellulose membrane, the blots were carried out with either apoE or apoAI antibody (Biodesign) or apoB antibody (US Biological). Significant increase in both apoAI and apoE contents in fractions 28-36 was obvious. No significant change in apoB level was noted. "-" indicates samples from vehicle and "+" denotes samples from combined treatment.

Figure 2. Dose dependent induction of enlarged HDL. Male C57B6 mice were treated with either vehicle or various doses of fenofibrate alone or in combination with T0901317 at 50 mg/kg in a similar scheme as in Figure 1A. Plasma samples were prepared and pooled for FPLC analysis as described in the Methods. Note the dramatic effect on enlarged HDL cholesterol production when T0901317 and fenofibrate were combined.

Figure 3. Liver ABCA1 and PLTP regulation. Animals were treated as described in Figure 1. Liver total RNA was prepared from individual samples and subjected to mRNA measurement as described in Methods. A. Liver ABCA1 mRNA was regulated by both Wy14643 and T0901317. No further increase in liver ABCA1 mRNA abundance was

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observed with the combined treatment. B. Additive elevation of liver PLTP mRNA upon combined treatment of Wy14643 and T0901317. Both Wy14643 and T0901317 upregulated PLTP mRNA significantly. Additive effect was apparent when mice were co-administered with both compounds. C. Plasma PTLP activity measurement. Individual lasma samples were diluted 5 fold before subjecting to the PLTP activity assay as described in the Methods. Both Wy14643 and T0901317 elevated plasma PLTP activity. Further increase in PLTP activity was observed when two compounds were combined (a, $p<0.001$ against vehicle. b, $p<0.001$ with T+Wy against T alone with Student t-test).

Figure 4. Dose dependent effects on plasma and liver triglycerides of T0901317 in C57B6 mice. A. Moderate plasma triglyceridemia induced by T0901317. Male C57B6 mice were treated with various doses of T0901317 once daily for 7 days. Plasma triglycerides were then analyzed as described in the Methods (* $p=0.01$ by t-test). B. Significant liver triglyceride accumulation upon LXR activation. Animals were treated with T0901317 as described in A and liver triglyceride were measured as described in the Methods. Note the dose dependent and dramatic induction of triglyceride accumulation in the liver upon LXR activation (* $p<0.001$ against vehicle, Student t-test).

Figure 5. Effects on plasma and liver triglycerides with combined treatment of T0901317 and Wy14643 in C57B6 mice. C57B6 mice were treated as described in Fig. 1A and plasma and liver triglycerides were measured as described in Methods. A. Mild hypertriglyceridemia induced by LXR agonist was attenuated by a PPARα agonist. Plasma triglyceride measurement indicated attenuation of plasma triglyceride induced by T0901317 with Wy14643. D. Minimal effect of a PPARα agonist on liver triglyceride accumulation induced by LXR activation. No significant liver triglyceride change was

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noted comparing the T0901317 group and the group with combined treatment. *P<0.001 against vehicle.

Figure 6. Additive induction of liver LPL mRNA with combined treatment of Wy14643 and T0901317. A. SREBP1c mRNA was measured by RPA as described in the Methods. Significant induction was noted upon LXR activation and no attenuation was observed with combined treatment. B. Peroxisome fatty acid β-oxidatin measurement. Animals were treated as described in Figure 1. Individual liver samples were subjected to peroxisome fatty acid β-oxidation measurement as described in the Methods. Values represent mean +/- sem of 6 mice. [a]Statisticaly different from vehicle control, p<0.001. [b]Statistically different from Tularik alone, P<0.001. [c]Not statistically different from Wy14643 alone by one-way ANOVA. Note the significant induction of β-oxidation induced by T0901317 (P<0.001 by one-way ANOVA). C. LPL mRNA was regulated by both Wy14643 and T0901317 and additive regulation in LPL mRNA was observed upon co-administration of Wy14643 and T0901317.

Figure 1A

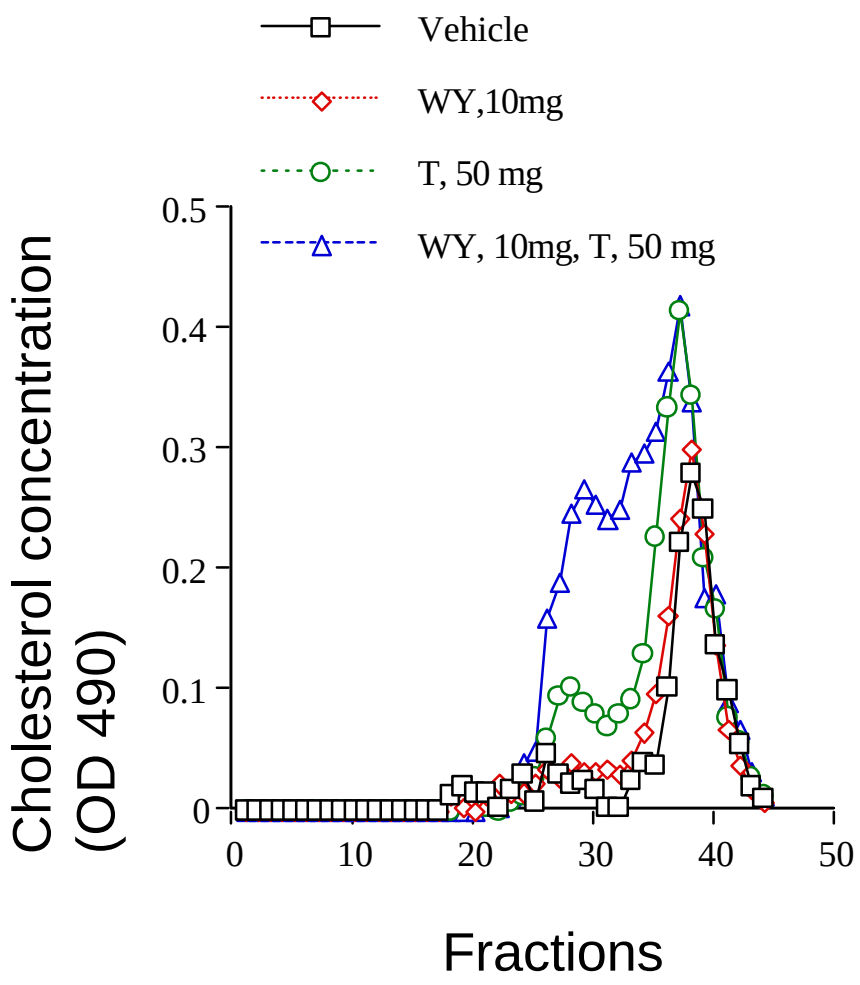


Figure 1B

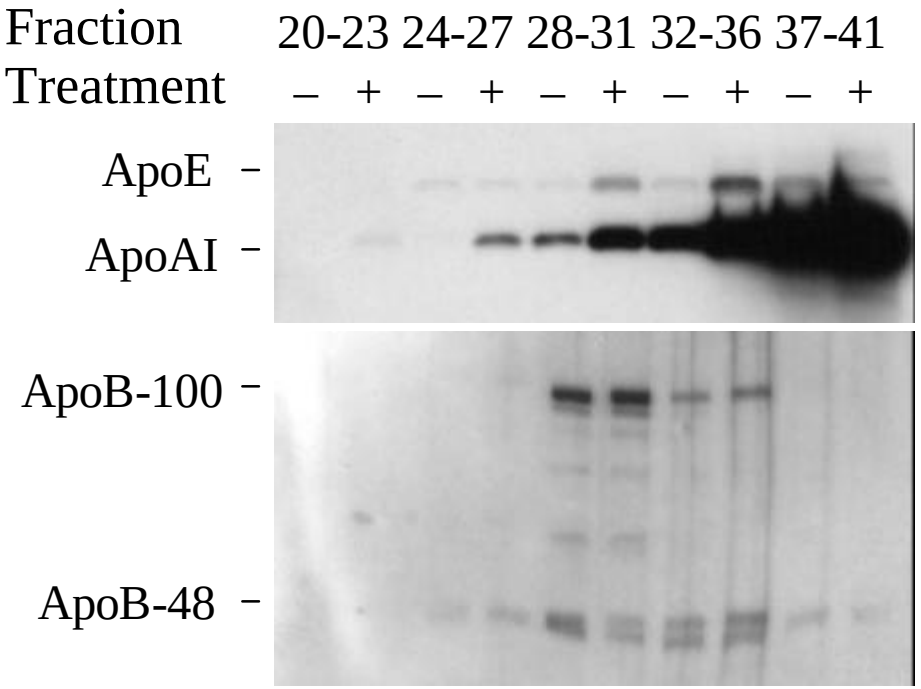


Figure 2

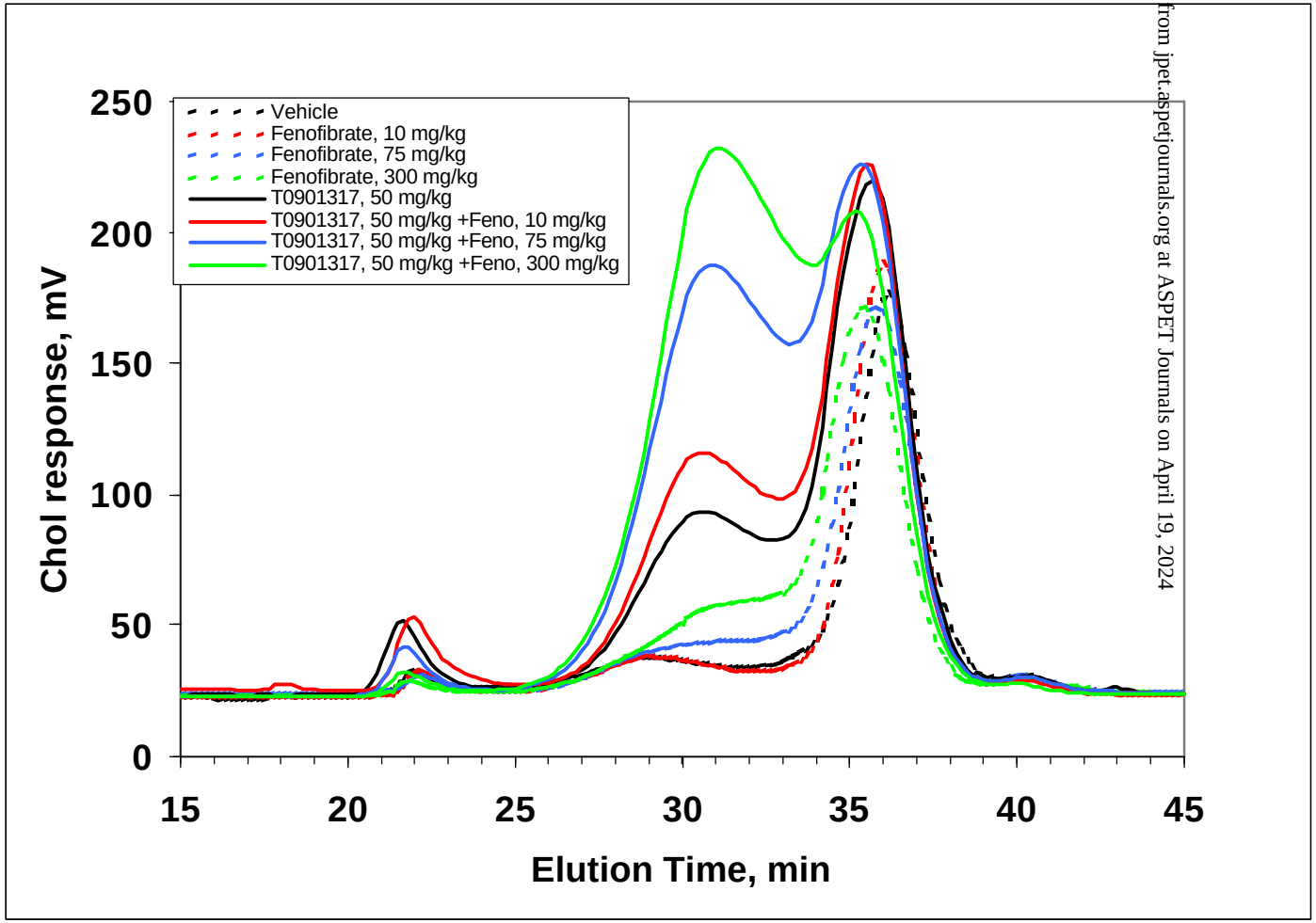
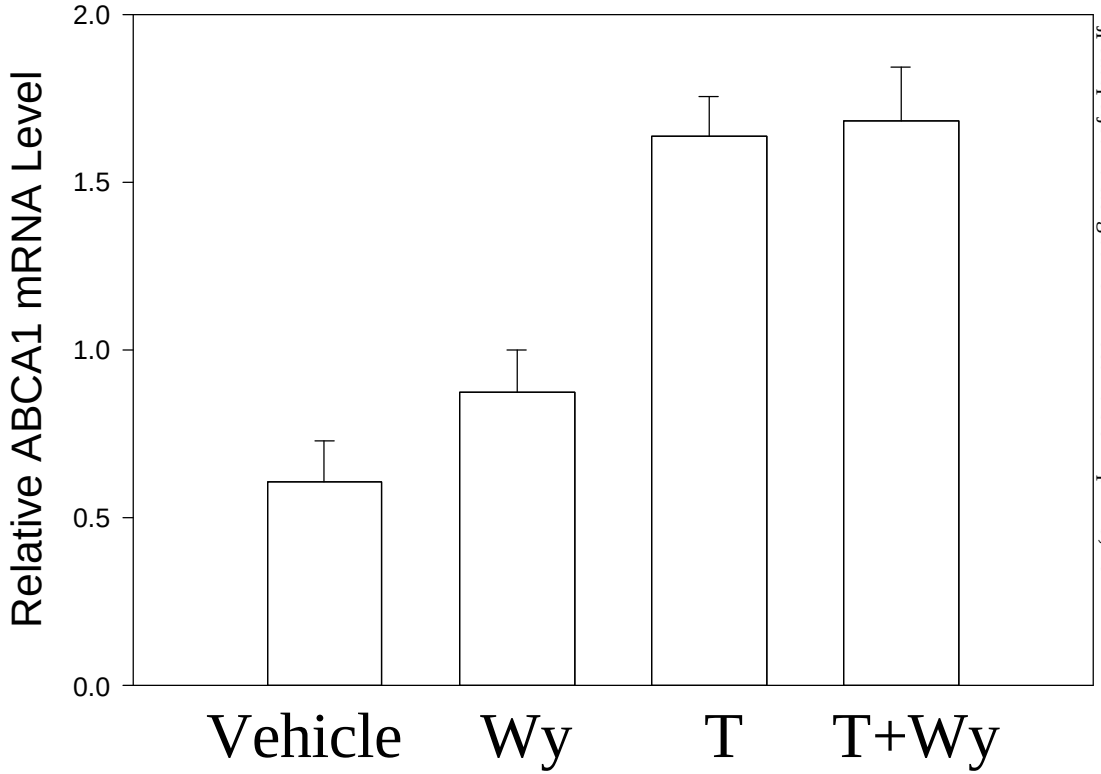


Figure 3A



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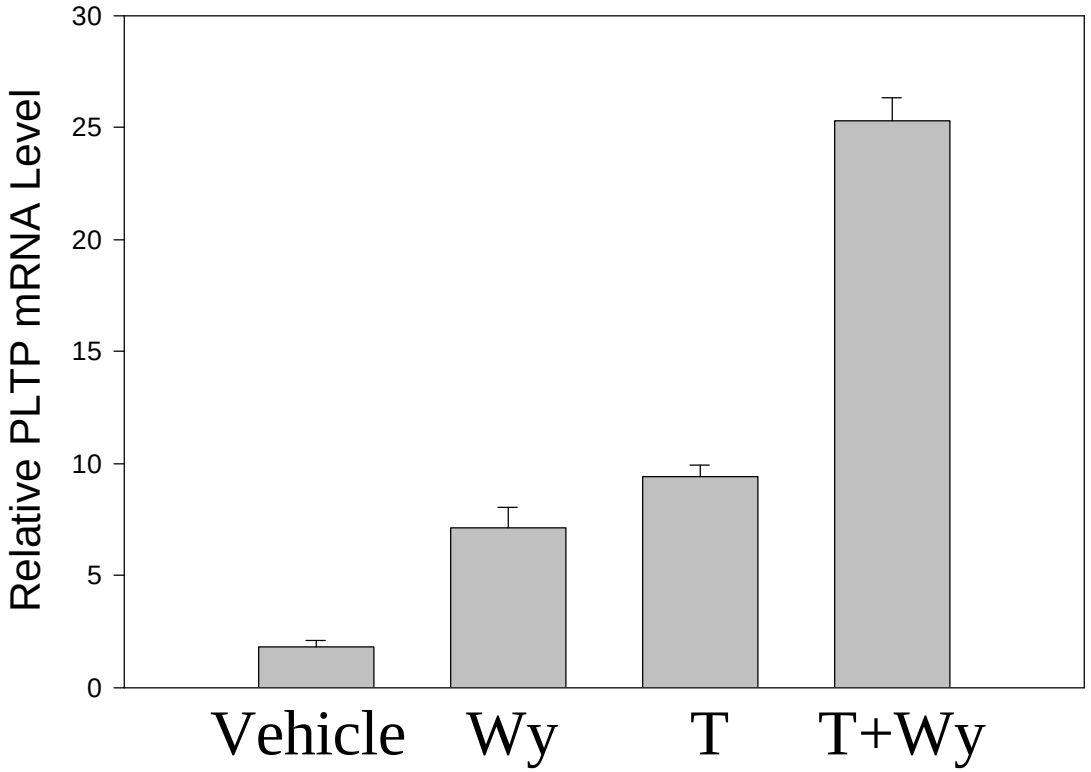
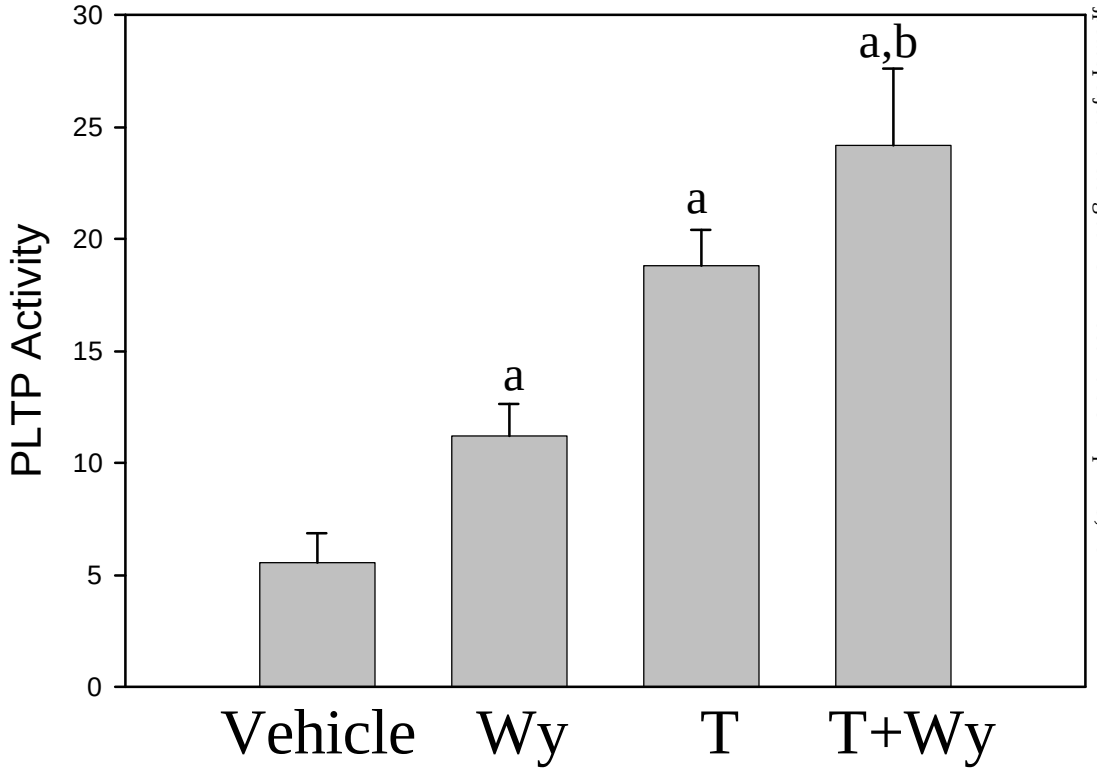


Figure 3C



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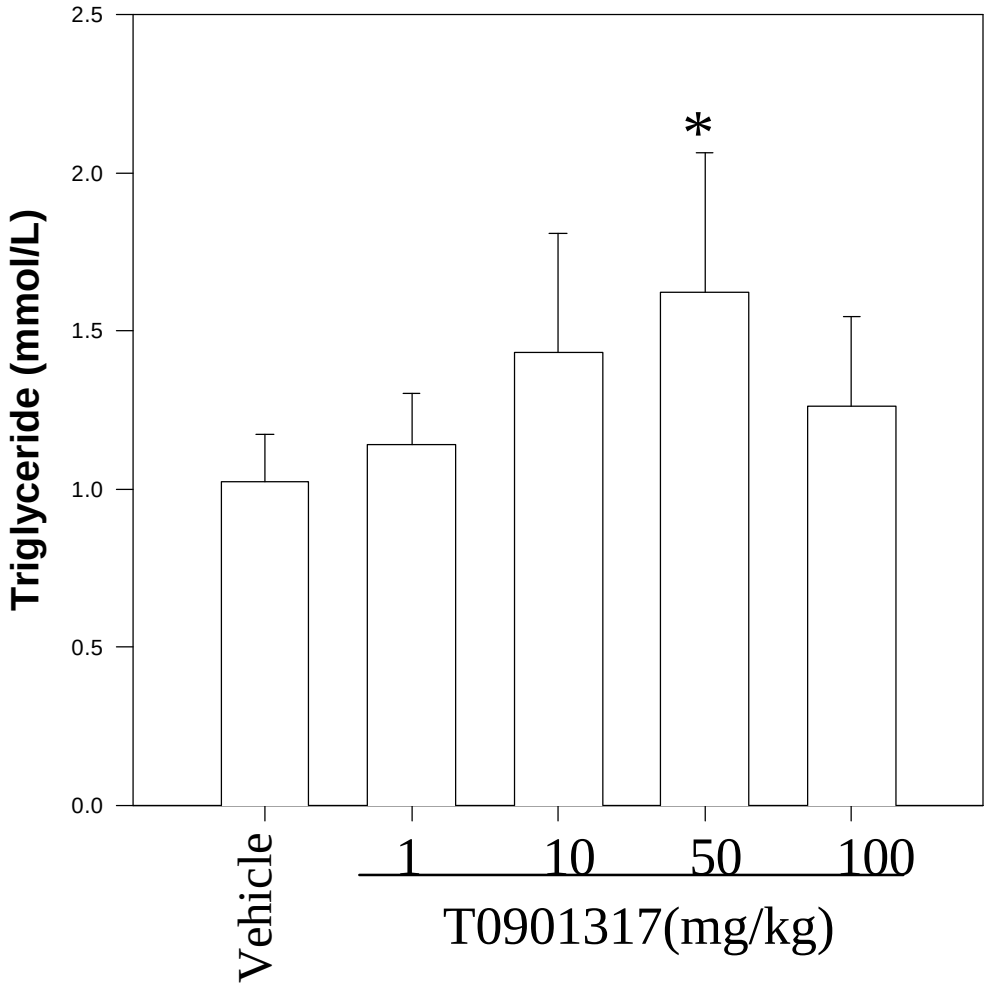

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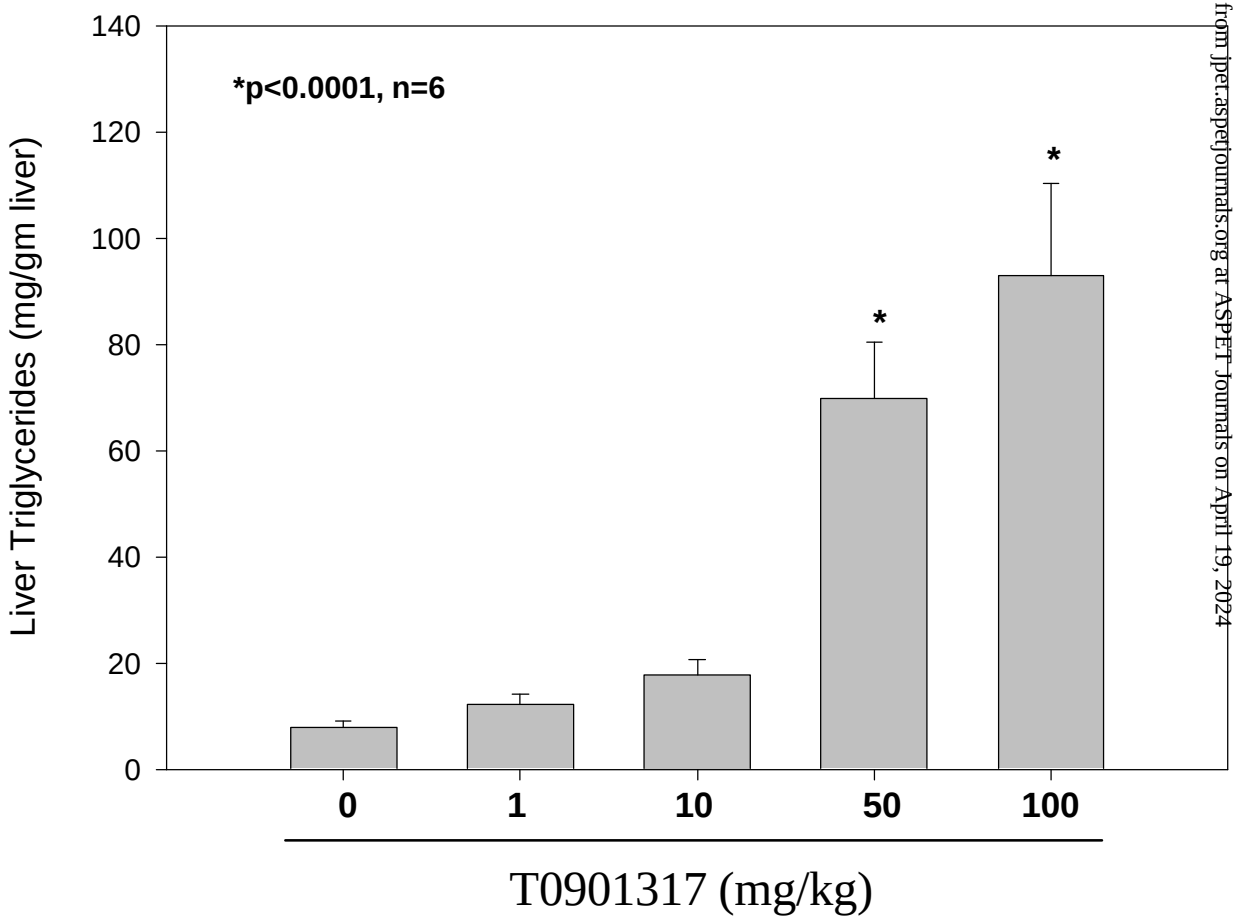

Figure 5A

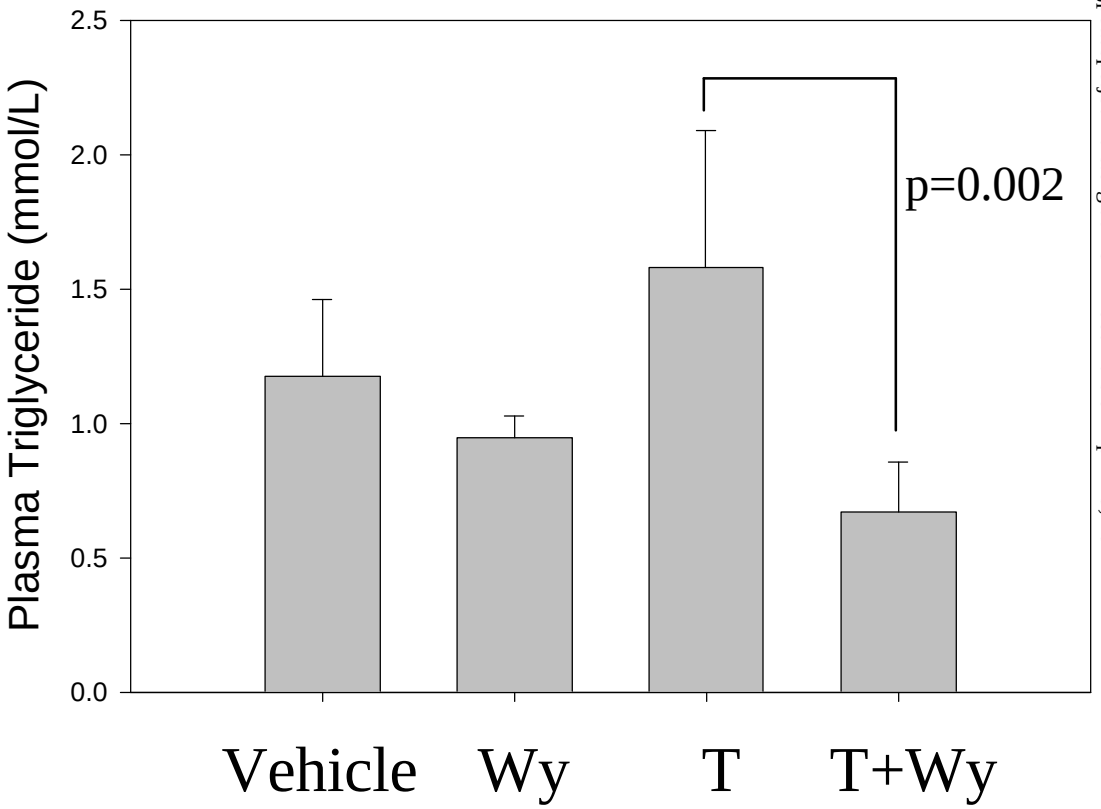

Figure 5B

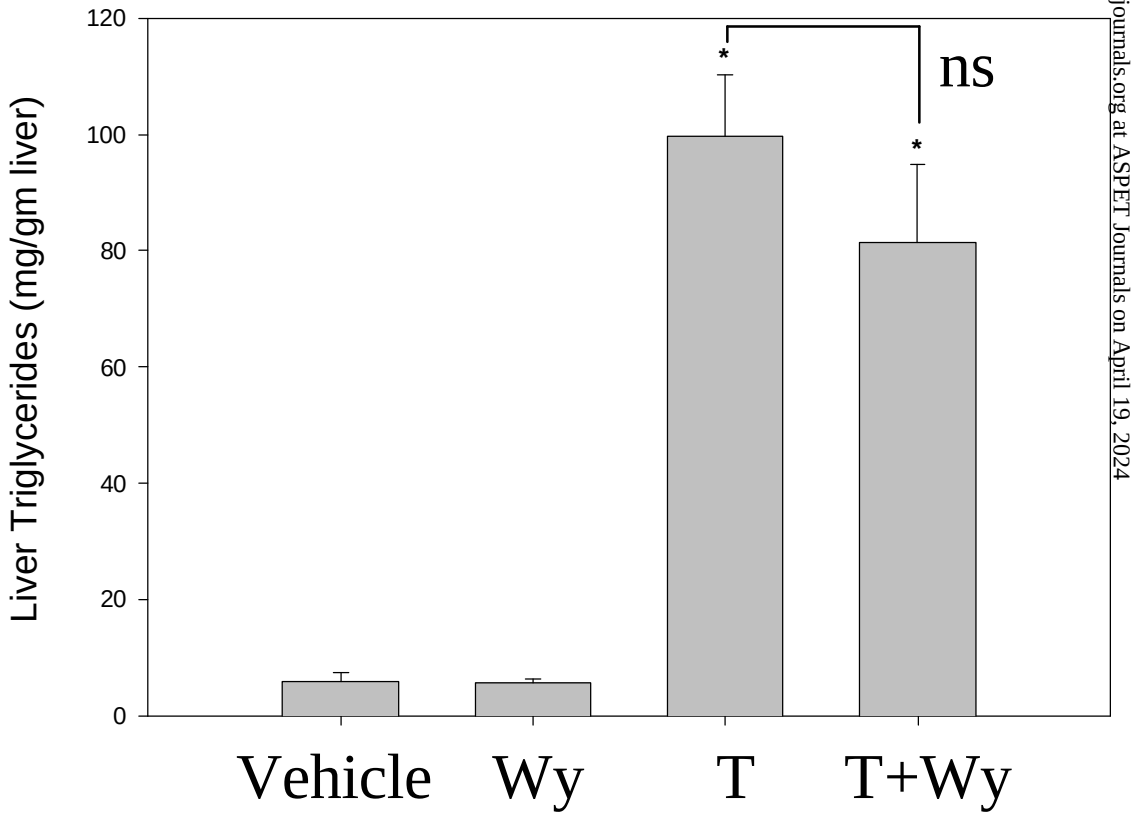

Figure 6A

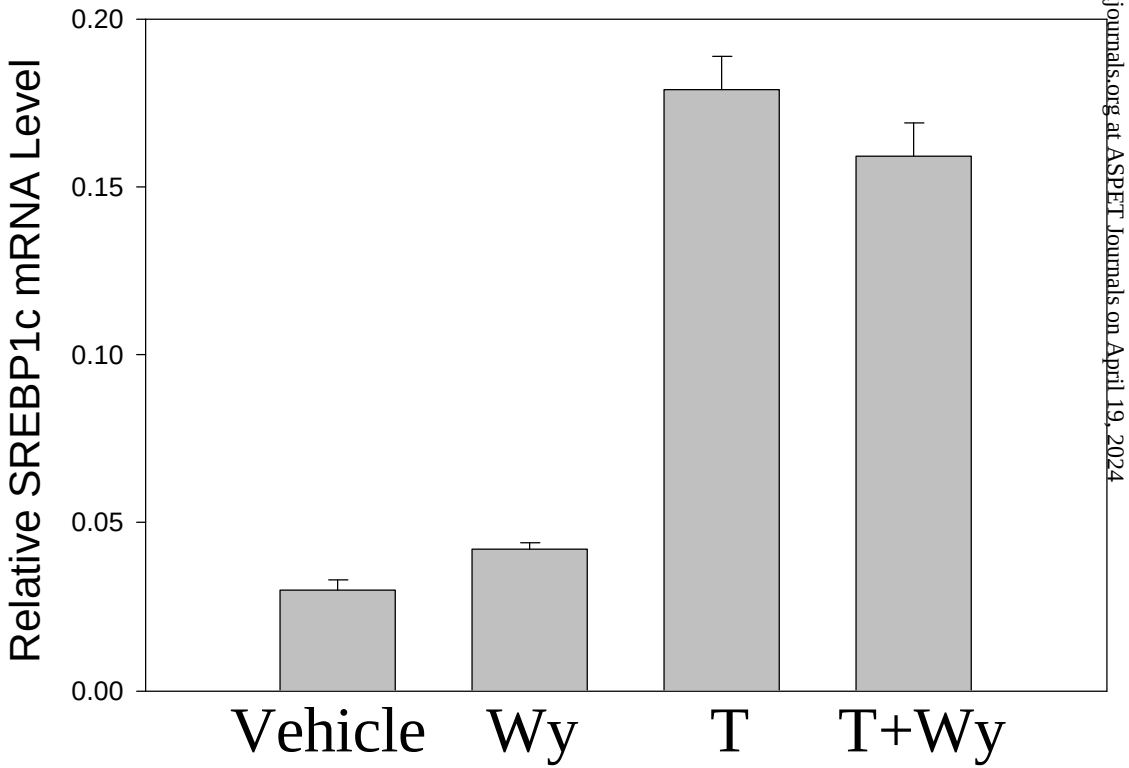

Figure 6B

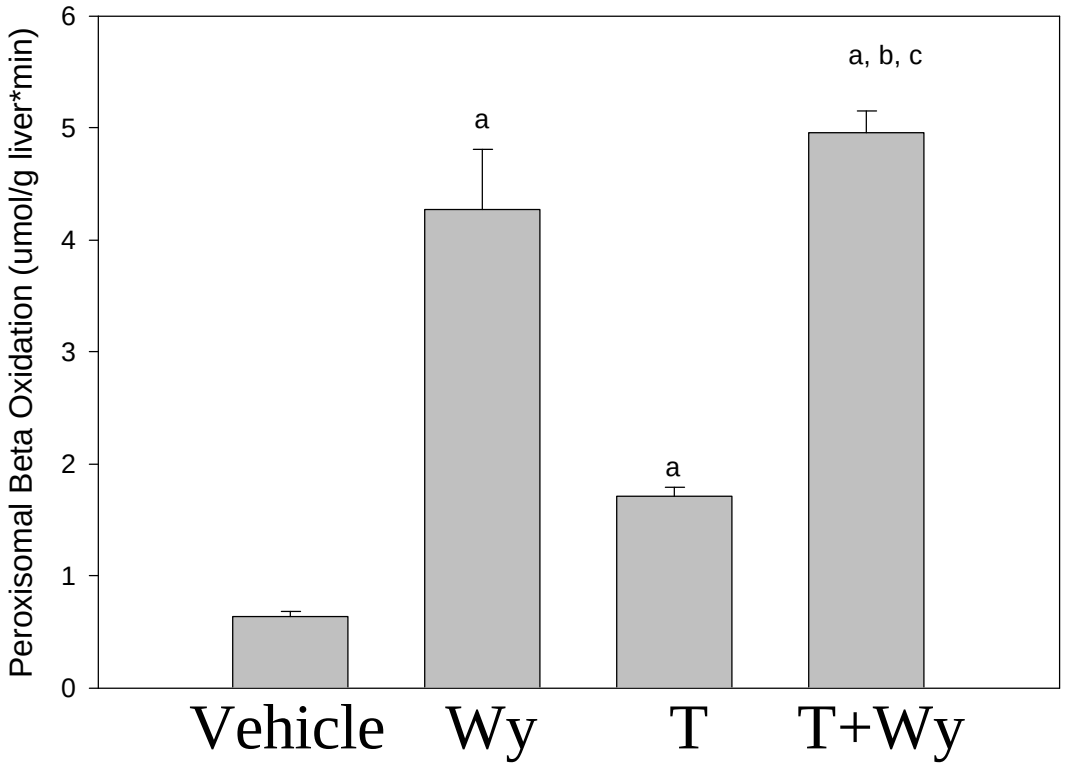

Figure 6C