

## **A Functional Crosstalk Between LXR $\alpha$ And CAR Links Lipogenesis And Xenobiotic Responses**

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**Running Title:** LXR $\alpha$  and CAR crosstalk links lipogenesis and xenobiotic responses

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**Abbreviations:** CAR, constitutive androstane receptor; LXR, liver X receptor; LXRE, LXR responsive element; PXR, pregnane X receptor; PBRE, phenobarbital response element; *Cyp*, cytochrome P450; *Acc-1*, acetyl CoA carboxylase 1; *Fas*, fatty acid synthase; *Scd-1*, stearyl CoA desaturase-1; SRC1, steroid receptor co-activator 1; *Srebp-1c*, sterol regulatory element-binding protein 1c; FABP, fatty acid binding protein; TO1317 (TO901317), N-methyl-n-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl)-phenyl]-benzenesulfonamide; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; VP, viral protein 16

## ABSTRACT

The liver X receptor (LXR) and constitutive androstane receptor (CAR) are two nuclear receptors postulated to have distinct functions. LXR is a sterol sensor that promotes lipogenesis, whereas CAR is a xenosensor that controls xenobiotic responses. Here we show that LXR $\alpha$  and CAR are functionally related *in vivo*. Loss of CAR increased the expression of lipogenic LXR target genes, leading to increased hepatic triglyceride accumulation; whereas activation of CAR inhibited the expression of LXR target genes and LXR ligand-induced lipogenesis. Conversely, a combined loss of LXR  $\alpha$  and  $\beta$  increased the basal expression of xenobiotic CAR target genes; whereas activation of LXR inhibited the expression of CAR target genes and sensitized mice to xenobiotic toxicants. The mutual suppression between LXR $\alpha$  and CAR was also observed in cell culture and reporter gene assays. LXR $\alpha$ , like CAR, exhibited constitutive activity in the absence of an exogenously added ligand by recruiting nuclear receptor co-activators. Interestingly, although CAR competed with LXR $\alpha$  for co-activators, the constitutive activity and recruitment of co-activators was not required for CAR to suppress the activity of LXR $\alpha$ . *In vivo* chromatin immunoprecipitation (ChIP) assay showed that co-treatment of a CAR agonist compromised the LXR agonist responsive recruitment of LXR $\alpha$  to *Srebp-1c*, whereas a LXR agonist inhibited the CAR agonist responsive recruitment of CAR to *Cyp2b10*. In conclusion, our results have revealed dual functions of LXR $\alpha$  and CAR in lipogenesis and xenobiotic responses, establishing a unique role of these two receptors in integrating xenobiotic and endobiotic homeostasis.

## INTRODUCTION

Metabolic homeostasis, including those of the endogenous chemicals (endobiotics) and foreign substances (xenobiotics), are essential for the survival of mammals. Nuclear hormone receptors play an important role in metabolic homeostasis. These include the sterol sensors liver X receptors (LXRs) that control lipid homeostasis (for a review, see Tontonoz and Mangelsdorf, 2003), as well as the xenosensor constitutive androstane receptor (CAR) (Honkakoski et al., 1998; Wei et al, 2000) that regulates the expression of drug metabolizing enzymes and transporters.

LXRs, both the  $\alpha$  and  $\beta$  isoforms, were defined as sterol sensors. LXR $\alpha$  is highly expressed in the liver, whereas LXR $\beta$  is ubiquitously expressed. In addition to being activated by endogenous oxysterols, LXRs are also activated by synthetic agonists, such as T0901317 (TO1317) (Schultz et al., 2000) and GW3965 (Collins et al., 2002). In rodents, LXRs increase hepatic cholesterol catabolism and formation of bile acids by inducing cholesterol 7 $\alpha$ -hydroxylase (Peet et al., 1998). LXRs were later found to promote hepatic lipogenesis by activating SREBP-1c (Repa et al., 2000), a transcriptional factor that regulates the expression of lipogenic enzymes ACC-1, FAS, and SCD-1. ACC-1, FAS and SCD-1 can also be directly regulated by LXR (Chu et al., 2006; Talukdar et al., 2006; Joseph et al., 2002). Loss of both LXR isoforms in mice resulted in an increased expression of *Cyp3a11* and *2b10* (Gnerre et al., 2005), two drug metabolizing enzymes and primary target genes of CAR and pregnane X receptor (PXR) (Kliwer et al., 1998; Blumberg et al., 1998). However, the mechanism by which LXRs affect the expression of drug metabolizing enzymes remains unknown.

CAR, along with its sister receptor PXR, has been shown to function as a master

xenosensor by its coordinated transcriptional regulation of drug metabolizing enzymes and transporters (for a review, see Swales and Negishi, 2004).  $CAR^{-/-}$  mice showed defective basal and inducible expression of xenobiotic enzymes and altered responses to drugs (Wei et al., 2000). CAR has recently been suggested to play a role in energy metabolism, ranging from thyroid hormone metabolism (Maglich et al., 2004; Qatanani et al., 2005) to lipogenesis (Roth et al., 2008a; 2008b; Maglitch et al., 2009; Gao et al., 2009), gluconeogenesis (Ueda et al., 2002; Kodama et al., 2004; Miao et al., 2006; Gao et al., 2009; Wada et al., 2009), and obesity and diabetes (Gao et al., 2009; Dong et al., 2009). It is unclear whether the effect of CAR on lipogenesis involves the crosstalk with LXRs.

In this report, we found  $LXR\alpha$  and CAR are mutually suppressive in their target gene regulation, which could be translated into their effects on lipogenesis and xenobiotic responses. Our results suggest dual and unique roles of  $LXR\alpha$  and CAR in integrating xenobiotic and endobiotic homeostasis.

## MATERIALS AND METHODS

**Animals and drug treatment.** The creation of PXR<sup>-/-</sup> (Xie et al., 2000a), CAR<sup>-/-</sup> (Wei et al., 2000), PC DKO (Saini et al., 2004), LXR DKO (Peet et al., 1998), VP-CAR transgenic (Saini et al., 2004), and FABP-VP-LXR $\alpha$  transgenic (Uppal et al., 2007) mice has been described. All transgenic and their wild type control mice were maintained in a mixed background of C57BL/6J and 129/SvJ, except the wild type mice used in Figure 2 which were C57BL/6J mice purchased from the Jackson Laboratory. TO1317 (50 mg/kg) and GW3965 (20 mg/kg) were given by gavage (Joseph et al., 2002; Laffitte et al., 2003). TCPOBOP (3 mg/kg) was given by i.p. injection (Wei et al., 2000). The drug treatment lasted for three days. Tribromoethanol tolerance experiment was performed as we have previously described (Xie et al., 2000a). The use of mice in this study has complied with all relevant federal guidelines and institutional policies.

**Measurement of liver and circulating lipids.** To measure circulating lipid levels, mice were fasted for 16 hrs prior to sacrificing and blood collection. Lipid tissue lipids were extracted as we have previously described (Zhou et al., 2006). Triglyceride and cholesterol levels were measured by assay kits from Stanbio (Boerne, TX).

**Real-time RT-PCR.** Total RNA was isolated using TRIZOL reagent from Invitrogen (Carlsbad, CA). SYBR Green-based real-time RT-PCR was performed with the ABI 7300 real-time PCR System. The gene expression was normalized against the expression of cyclophilin. PCR primer sequences are listed in Supplementary Table 1.

**DNA constructs, transient transfection, and GST pull-down assay.** tk-Scd1/LXRE (Chu et al., 2006), tk-MRP2 (Mu et al., 2005), pGL-Scd1 (Chu et al., 2006), pGL-Cyp2b10 (Xie et al., 2000b), and Gal-SRC1 (Saini et al., 2005) constructs were previously described.

pCMX-Flag-hLXR $\alpha$  and pCMX-HA-mCAR were cloned by PCR. Transfection of HepG2 or CV-1 cells on 48-well plates was performed as previously described (Uppal et al., 2007).

When necessary, cells were treated with drugs for 24 hrs prior to luciferase assay.

Transfection efficiency was normalized against the  $\beta$ -gal activities from a co-transfected

CMX- $\beta$  gal vector. GST pull-down using bacteria expressed GST-SRC1 and *in vitro*

translated and [<sup>35</sup>S]-labeled receptor proteins were performed as we have previously described (Saini et al., 2005).

**Chromatin immunoprecipitation (ChIP) assay.** Eight weeks old wild type female mice were pre-treated with an i.p. injection of DMSO or TCPOBOP (3 mg/kg) and/or a gavage of vehicle or GW3965 (20 mg/kg) 1 hr before being liver transfected with pCMX-Flag-hLXR $\alpha$  and pCMX-HA-mCAR plasmids by a hydrodynamic gene delivery method (Zhou et al., 2006). Mice were sacrificed 24 hrs after transfection and the liver tissues were harvested for ChIP assay and Western blot analysis. The ChIP procedures followed the Upstate protocol (Cat No: 17-371) and were essentially as described (Zhou et al., 2006). Antibodies used for immunoprecipitation include an anti-HA antibody (Cat # C29F4) from Cell Signaling, an anti-Flag antibody (Cat # F1804) from Sigma, and a normal mouse IgG antibody (Cat # 12-371B) from Upstate. PCR was carried out with *Cyp2b10*-specific primers

encompassing the PBRE (5' - CTCCAGTGACTTAGGAGGAAG-3' ;

5'-AAGTATTGTGCCAGTTGCTG -3'), and *Srebp-1c*-specific primers encompassing the

DR-4 site (5'-TCCAGGCAAGTTCTGGGTGTGTGCG-3' ;

5-CGGGTTTCTCCCGGTGCTCTGAATG-3'). The sequences for Cyp2b10/PBRE and

*Srebp-1c*/LXRE are

5'-TCTGTACTTTCCCTGACCTTGGCACAGTGCCACCATCAACTTGCTGACACC-

3' (Sueyoshi et al., 1999) and 5'-ACAGTGACCGCCAGTAACCCAGC-3' (Yoshikawa et

al., 2001), respectively.



## RESULTS

### Reciprocal activation of target gene expression in mice deficient of CAR and LXR

We have recently reported that PXR<sup>-/-</sup> mice had increased basal expression of the LXR target genes *Scd-1* (Zhou et al., 2006). This observation prompted us to examine the effect of loss CAR on LXR target gene expression, as compared to PXR<sup>-/-</sup> mice and PXR/CAR double knockout (PC DKO) mice. Loss of PXR induced the expression of *Scd-1*, but had little effect on the expression of *Srebp-1c*, *Acc-1*, *Fas*, *Abcg5* and *Abcg8* (Fig. 1A), consistent with our previous finding (Zhou et al., 2006). In contrast, CAR<sup>-/-</sup> mice showed significantly increased expression of all these LXR target genes (Fig. 1A). Combined loss of CAR and PXR (PC DKO) had a synergistic effect in inducing *Scd-1*, *Srebp-1c*, and *Abcg8*. Interestingly, the synergistic effect of PC DKO appeared to be gene specific. Compared to CAR<sup>-/-</sup> mice, the induction of *Abcg5* remained unchanged and the induction of *Acc-1* and *Fas* was actually decreased in PC DKO mice. The mechanism for this gene specific effect remains to be determined. Loss of PXR and/or CAR had little effect on the expression of LXR $\alpha$  or LXR $\beta$  (Fig. 1A). Despite their higher basal expression, LXR target genes remained inducible by TO1317 in CAR<sup>-/-</sup> mice (Fig. 1B, left panel). Compared to their wild type counterparts (Fig. 1B, right panel), the TO1317-responsive induction of *Fas*, *Scd-1* and *Abcg5* was more dramatic in CAR<sup>-/-</sup> mice. TO1317 at 50 mg/kg has been reported to activate PXR *in vivo* (Mitro et al., 2007). We showed that PC DKO mice responded similarly to TO1317 as the CAR<sup>-/-</sup> mice (Supplementary Fig. 1), suggesting that the TO1317 effect on the expression of LXR target genes in CAR<sup>-/-</sup> mice can be PXR independent. Consistent with the patterns of gene expression, we found the triglyceride content in the liver of CAR<sup>-/-</sup> and PC DKO mice was

nearly three times that of the wild type and PXR<sup>-/-</sup> mice (Fig. 1C). No significant changes in the hepatic cholesterol levels were observed (Fig. 1C). The circulating levels of triglycerides increased in CAR<sup>-/-</sup>, but not PC DKO, mice (Fig. 1D).

The reciprocal effect of loss of LXR on CAR target gene expression was evaluated in LXR  $\alpha$  and  $\beta$  double knockout mice (LXR DKO). The expression of *Cyp2b10* and *Cyp3a11*, two CAR target genes, was induced in LXR DKO mice (Fig. 1E) as expected (Gnerre et al., 2005). Loss of LXRs had little effect on the expression of PXR or CAR (Fig. 1E). Despite their high basal expression, *Cyp2b10* and *Cyp3a11* remained inducible by TCPOBOP in LXR DKO mice (Fig. 1F).

### **Mutual repression of ligand-dependent target gene expression by pharmacological activation of LXR and CAR**

This was evaluated in wild type C57BL/6J mice treated with the LXR agonist GW3965 and CAR agonist TCPOBOP individually or in combination. GW3965 alone induced the hepatic expression of *Srebp-1c*, *Acc-1*, *Fas*, *Scd-1*, *Abcg5* and *Abcg8* as expected (Fig. 2A). TCPOBOP alone, on the other hand, suppressed the basal expression of *Srebp-1c*, *Acc-1*, *Fas*, *Scd-1* and *Abcg5* (Fig. 2A). The most notable phenotype, however, is that the GW3965-induced LXR target gene activation was largely abolished in mice treated with both drugs (Fig. 2A). Consistent with the pattern of gene expression, the hepatic content of triglycerides in dual treated mice was lower than mice treated with GW3965 alone (Fig. 2B). Interestingly, TCPOBOP alone caused a modest but significantly increased triglyceride level (Fig. 2B) despite the suppression of lipogenic enzymes in this group (Fig. 2A). This mild

steatosis might be secondary to TCPOBOP-induced hepatomegaly (Wei et al., 2000).

Treatment with GW3965 increased serum concentration of triglycerides, but this effect was abolished in dual treated mice (Fig. 2C). This regimen of drug treatment had little effect on cholesterol levels (Fig. 2B and 2C). When the expression of CAR target genes was analyzed, we found that GW3965 suppressed the basal expression of both *Cyp2b10* and *Cyp3a11* (Fig. 2D). The expression of *Cyp2b10* and *Cyp3a11* was induced by TCPOBOP as expected, and the TCPOBOP effect was largely intact in dual treated mice (Fig. 2D), suggesting that the CAR agonist plays a dominant role in regulating xenobiotic enzymes when ligands for both CAR and LXR are present.

### **Reciprocal repression of target gene expression by genetic activation of LXR and CAR in transgenic mice**

We have recently created transgenic mice expressing the activated LXR $\alpha$  (VP-LXR $\alpha$ ) in the liver (Uppal et al., 2007; Gong et al., 2008; Lee et al., 2008). VP-LXR $\alpha$  was created by fusing the VP16 activation domain of the herpes simplex virus to the amino-terminal of mouse LXR $\alpha$ . In VP-LXR $\alpha$  transgenic mice, in addition to the expected activation of LXR target genes, we observed the suppression of *Cyp2b10* and *Cyp3a11* (Fig. 3A). The expression of *Cyp2b10* and *Cyp3a11* remained inducible by TCPOBOP in VP-LXR $\alpha$  transgenic mice (Fig. 3B), but the magnitude of *Cyp2b10* induction was markedly lower than that observed in TCPOBOP-treated LXR DKO (Fig. 1F) or wild type (Fig. 2D) mice. VP-LXR $\alpha$  transgenic mice were more sensitive to the anesthetic effect of tribromoethanol, consistent with the notion that *Cyp2b10* and *Cyp3a11* play a role in the detoxification of this drug (Xie et al., 2000a; Xie

et al., 2001). Loss of PXR or CAR sensitized mice to tribromoethanol-induced sleep (Fig. 3C). When the LXR effect was evaluated, we found that wild type mice slept for an average of 20 min, whereas the VP-LXR $\alpha$  transgenic mice slept for nearly 50 min (Fig. 3D). The tribromoethanol sensitizing effect was also observed in wild type mice pre-treated with GW3965 (Fig. 3D).

We have also reported the creation and characterization of transgenic mice that bear the expression of activated CAR (VP-CAR) in the liver (Saini et al., 2004; 2005). VP-CAR transgenic mice showed decreased expression of LXR target genes *Srebp-1c*, *Acc-1*, *Fas*, *Scd-1*, *Abcg5* and *Abcg8* (Fig. 3E). VP-LXR $\alpha$  transgenic mice had a spontaneous hepatic accumulation of triglycerides, whereas the VP-CAR transgene had little effect (Fig. 3F)

### **Mutual suppression between LXR $\alpha$ and CAR in promoter reporter gene assays**

tk-*Scd1*/LXRE-Luc and tk-PBRE-Luc reporter genes contain a DR-4 (direct repeat spaced by 4 nucleotides) type LXRE from the *Scd-1* gene promoter and phenobarbital response element (PBRE) from the *Cyp2b10* gene promoter, respectively. The tk-*Scd1* report had a 4-fold activation by LXR $\alpha$  in the absence of an exogenously added ligand (Fig. 4A). The activity of LXR $\alpha$  increased in the presence of TO1317 or GW3965, but not TCPOBOP. Co-transfection of CAR inhibited both the constitutive and TO1317/GW3965-inducible activities of LXR $\alpha$  and this inhibition was enhanced by TCPOBOP (Fig. 4A). CAR itself had little effect on the activity of tk-*Scd1* (Fig. 4A). Conversely, when tk-PBRE reporter was used, the mouse CAR exhibited constitutive activity, which can be further activated by TCPOBOP (Fig. 4B). Both the constitutive and TCPOBOP-dependent CAR activities were inhibited by

the co-transfection of LXR $\alpha$ , and this inhibition was enhanced by TO1317 or GW3965 (Fig. 4B). LXR $\alpha$  itself had little effect on the activity of tk-PBRE (Fig. 4B). When the *Scd-1* natural gene promoter reporter pGL-Scd1 was transfected, both the constitutive and GW3965-dependent activities of LXR $\alpha$  were inhibited by the co-transfected CAR in a dose-dependent manner (Fig. 4C). Conversely, the constitutive and TCPOBOP-dependent activities of CAR on the *Cyp2b10* natural promoter reporter pGL-Cyp2b10 were inhibited by the co-transfected LXR $\alpha$  (Fig. 4D). The LXR $\alpha$ -CAR mutual suppression was also observed when the Gal-LXR $\alpha$  and Gal-CAR chimeric receptors and the Gal4-responsive tk-UAS report were used. The constitutive and GW3965-dependent activities of Gal-LXR $\alpha$  were inhibited by the co-transfection of wild type CAR (Fig. 4E), whereas the constitutive and TCPOBOP-dependent activities of Gal-CAR were inhibited by the co-transfection of wild type LXR $\alpha$  (Fig. 4F).

### **Mechanistic studies for the mutual suppression between LXR $\alpha$ and CAR in cell cultures**

CAR exhibits constitutive activity due to its ligand-independent recruitment of nuclear receptor co-activators (Forman et al., 1998). The high basal activity of LXR $\alpha$  prompted us to examine whether this receptor can also recruit co-activators in the absence of a ligand. We first used a mammalian two-hybrid assay to examine the recruitment of steroid receptor co-activator 1 (SRC1) by LXR $\alpha$ . Cells were transfected with tk-UAS reporter together with the expression vectors for Gal-hSRC1 and VP-LXR $\alpha$ . The activation of reporter in the absence of an exogenously added ligand suggested the constitutive recruitment of SRC1 by LXR $\alpha$  (Fig. 5A). The LXR $\alpha$ -SRC1 interaction was enhanced by TO1317 but not by

TCPOBOP (Fig. 5A). Co-transfection of CAR inhibited the constitutive recruitment of SRC1 by LXR $\alpha$ . The inhibitory effect of CAR was relieved by the treatment of TO1317, but exacerbated by TCPOBOP (Fig. 5A). The inhibition of the LXR $\alpha$ -SRC1 interaction appeared to be CAR specific, because co-transfection of PPAR $\gamma$  had little effect in the absence of a PPAR $\gamma$  agonist (Fig. 5A). Addition of the PPAR $\gamma$  agonist BRL49653 decreased the LXR $\alpha$ -SRC1 interaction (Fig. 5A). The ligand-independent recruitment of SRC1 by LXR $\alpha$  was confirmed by GST pull-down assays. As shown in Fig. 5B, GST-SRC1 interacted with both [<sup>35</sup>S]-LXR $\alpha$  and [<sup>35</sup>S]-CAR, but not [<sup>35</sup>S]-PPAR $\gamma$ , in the absence of an exogenously added ligand. The PPAR $\gamma$ -SRC1 interaction was induced by BRL49653. Consistent with their patterns of co-activator recruitment in the absence of exogenously added ligands, CAR was more efficient than PPAR $\gamma$  in suppressing the constitutive and GW3965-dependent activities of LXR $\alpha$  in reporter gene assays (data not shown). These results suggest that competition for co-activators is a plausible mechanism for the mutual suppression between LXR $\alpha$  and CAR. Indeed, the increasing concentration of Gal-SRC1 was able to titrate the inhibitory effect of CAR in the mammalian two-hybrid assay (Fig 5C). Reciprocally, the constitutive and TCPOBOP-dependent SRC1-CAR interaction was inhibited by the co-transfection of LXR $\alpha$  (Fig. 5D).

We then used two CAR mutants CAR $\Delta$ 8 and CAR $\Delta$ 37 to determine whether the recruitment of co-activators was necessary for the inhibitory effect of CAR on LXR $\alpha$ . CAR $\Delta$ 8 and CAR $\Delta$ 37 lack the C-terminal 8 and 37 amino acids, respectively (Choi et al., 1997). CAR $\Delta$ 8 has the disruption of the AF-2 region, and thus fails to bind to co-activators (Choi et al., 1997; Min et al., 2002). CAR $\Delta$ 37 also lacks the C terminus of the helix 10 that is

important for heterodimerization with RXR, and consequently fails to bind to DNA (Choi et al., 1997). As expected, both CAR $\Delta$ 8 and CAR $\Delta$ 37 cannot activate tk-PBRE reporter (Fig. 5E). Both CAR $\Delta$ 8 and CAR $\Delta$ 37 failed to interact with GST-SRC1 in a GST pull-down assay (Fig. 5F). Interestingly and surprisingly, CAR $\Delta$ 8 was effective in suppressing the constitutive and GW3965-dependent LXR $\alpha$  activities on the tk-Scd1 reporter (Fig. 5G), whereas CAR $\Delta$ 37 completely lost its inhibitory effect. The intact suppression by CAR $\Delta$ 8 suggests that the recruitment of co-activator is not required for the inhibitory effect of CAR. Since RXR is a shared heterodimerization partner for CAR and LXR, we also evaluated whether the inhibition of LXR $\alpha$  activity by CAR can be relieved by the overexpression of RXR. As shown in Fig. 5H, co-transfection of RXR increased the basal activity of LXR $\alpha$ , but did not abolish the suppressive effect of CAR.

### **Effects of CAR and LXR agonists on receptor recruitment to target gene promoters *in vivo***

To understand the *in vivo* mechanism for the mutual suppression between LXR $\alpha$  and CAR, we performed ChIP assay to determine the effects of individual and combined treatment of GW3965 and TCPOBOP on the respective recruitment of LXR $\alpha$  to *Srebp-1c* gene promoter and CAR to *Cyp2b10* gene promoter. In this experiment, mouse livers were transfected with both Flag-tagged LXR $\alpha$  (Flag-LXR $\alpha$ ) and HA-tagged CAR (HA-CAR). Anti-Flag and anti-HA antibodies were used for chromatin immunoprecipitation. As shown in Fig. 6A, Flag-LXR $\alpha$  was specifically recruited onto the *Srebp-1c* gene promoter in response to GW3965, but the recruitment was largely abolished in mice co-administered with TCPOBOP.

When the recruitment of HA-CAR onto the *Cyp2b10* gene promoter was evaluated, we found that TCPOBOP enhanced the recruitment of HA-CAR, which was modestly inhibited by the co-treatment of GW3965 (Fig. 6B). We noted that little receptor occupancy of the promoter, especially for LXR $\alpha$ , was detected in the absence of ligands. The lack of more obvious basal occupancy in ChIP assay may be due to the experimental conditions as well as the limitation of sensitivity. The expression of the transfected receptors was confirmed by Western blot analysis (Fig. 6C).



## DISCUSSION

In this study, we have uncovered a mutual suppression between LXR $\alpha$  and CAR that links these two seemingly distinct pathways of lipogenesis and xenobiotic response. Based on our results and as summarized in Fig. 7, we propose a model of LXR $\alpha$ -CAR crosstalk, in which the activation of LXR suppresses CAR-mediated xenobiotic response, leading to sensitization of animals to xenotoxicants. In contrast, activation of CAR may suppress the LXR-mediated lipogenesis.

We showed that LXR $\alpha$  exhibited constitutive activity by interacting with co-activators in the absence of an exogenously added agonist. The competition for co-activators has been proposed to be a mechanism for the mutual inhibition between CAR and the estrogen receptor (Min et al., 2002), CAR and PXR (Saini et al., 2005), CAR and HNF-4 (Miao et al., 2006), as well as LXR and ROR $\alpha$  (Wada et al., 2008). In the current study, we showed that although CAR can compete with LXR $\alpha$  for co-activators, the constitutive activity and recruitment of co-activators did not appear to be required for CAR to suppress the activity of LXR $\alpha$ . However, CAR $\Delta$ 8, a CAR mutant that bears the disruption of the AF-2 region and thus fails to bind to co-activators (Choi et al., 1997; Min et al., 2002), was efficient to suppress the constitutive and ligand-inducible activity of LXR $\alpha$  (Fig. 5G). Moreover, down-regulation of SRC1 by siRNA also did not enhance the inhibitory effect of CAR on LXR $\alpha$  (data not shown). Although the CAR $\Delta$ 8 results cannot eliminate the possibility that the LXR suppression of CAR involves co-activator competition, our results suggest that competition for co-activators is unlikely the primary mechanism for the mutual suppression between LXR $\alpha$  and CAR. The mechanism for the inhibitory effect of CAR $\Delta$ 8 on LXR remains to be clearly defined. Since

CAR $\Delta$ 8 is transcriptionally inactive (Fig. 5E), our results suggested that it is unlikely that unknown CAR target gene(s) are responsible for the inhibitory effect of CAR. Most, if not all, nuclear receptors have two activation functions (AFs), the C-terminal AF2 and the N-terminal AF1. Although AF2 is not required, as suggested by the CAR $\Delta$ 8 results, it remains to be determined whether AF1 of CAR is necessary for the inhibitory effect of CAR on LXR. The AF-1 of PPAR $\alpha$  has been reported to be important for the bidirectional inhibitory crosstalk between PPAR $\alpha$  and signal transducer and activator of transcription 5b (STAT5b) (Shipley and Waxman, 2004).

The loss of inhibitory effect of CAR $\Delta$ 37 is particularly intriguing. CAR $\Delta$ 37 failed to heterodimerize with RXR and bind to DNA (Choi et al. 1997). However, because CAR/RXR heterodimers cannot bind to LXRE and a forced expression of RXR failed to abolish the inhibitory effect of CAR, we cannot conclude that the loss of RXR binding is responsible for the lack of inhibition by CAR $\Delta$ 37. The lack of RXR rescue was in contrast to the reported mutual suppression between LXR $\alpha$  and PPAR $\alpha$ , in which the inhibitory effect of PPAR $\alpha$  on LXR $\alpha$  was completely abolished by a forced expression of RXR (Yoshikawa et al., 2003). Among other potential mechanisms, several reports suggested that CAR or LXR can share or compete for DNA binding sites with other nuclear receptors (Xie et al., 2000a; Handschin et al., 2002). Both LXR and CAR have been reported to bind to DR4 type nuclear receptor binding sites; however, our EMSA results showed that LXR cannot bind to PBRE, and CAR had little affinity toward Srebp-1c/DR-4 (data not shown). Interestingly and despite the lack of share of DNA binding motifs, our ChIP results showed that co-treatment of a CAR agonist compromised the LXR agonist responsive recruitment of LXR $\alpha$  to *Srebp-1c*, whereas a LXR

agonist inhibited the CAR agonist responsive recruitment of CAR to *Cyp2b10* (Fig. 6).

The high basal activity of LXR $\alpha$  is an interesting observation. The biological significance of the constitutive activity of CAR is obvious. As a xenobiotic receptor, CAR is essential in mammals' coping with obnoxious substances (Wei et al., 2000; Zhang et al., 2002). On the other hand, xenobiotic enzymes are mostly produced or induced as needed. As such, sustained over-activation of xenobiotic responses could be harmful, as evidenced by the sensitization to caffeine and acetaminophen toxicity in CAR-activated mice (Wei et al., 2000; Zhang et al., 2002). In this regard, the constitutive activity of LXR $\alpha$  and consequent suppression of CAR activity may have offered a mechanism of "checks and balances" to maintain a proper level of xenobiotic clearance. Reciprocally, lipogenesis is an essential function of the liver, in which LXR $\alpha$  plays an important role. However, over-activation of lipogenesis is potentially harmful, leading to both local and systemic metabolic disorders. It remains to be determined whether CAR represents a cellular factor that helps to keep the lipogenic activity of LXR $\alpha$  in check.

Our results have also shown that the balance between LXR and CAR activities can be shifted by activation of the receptors. This functional interplay between sterol receptor and xenobiotic receptor may have its implications in drug metabolism and lipogenesis. It is conceivable that CAR agonists may be used to limit the intensity and duration of LXR-mediated lipogenesis, thus alleviating the lipogenic side effect of LXR agonists. Indeed, we have recently reported that activation of CAR was beneficial in preventing obesity and relieving type 2 diabetes, in which the CAR-mediated suppression of hepatic lipogenesis played an important role (Gao et al., 2009). Reciprocally, since sustained activation of LXR

may compromise drug metabolism, cautions to avoid drug accumulation and toxicity should be applied when LXRs are being explored as therapeutic targets.

Because LXR $\alpha$  and CAR have functions outside lipogenesis and drug metabolism, the LXR $\alpha$ -CAR crosstalk might be implicated in other physiological and pathophysiological conditions. For example, cholesterol and bile acids homeostasis is tightly controlled by the functions of liver and intestine, in which both LXR $\alpha$  and CAR are highly expressed. Treatment of WT mice with LXR agonists increased high-density lipoprotein (HDL) cholesterol level (Jiang et al., 2003), whereas HDL cholesterol level was elevated in CAR<sup>-/-</sup> mice (Stedman et al., 2005). It is interesting to know whether the presumed increased activity of LXR in CAR<sup>-/-</sup> mice may have contributed to the elevated HDL cholesterol level in this genotype. In another example, the serum bile acid level after bile duct ligation in CAR<sup>-/-</sup> mice was significantly lower than that in WT mice (Stedman et al., 2005), whereas our previous study showed that LXR DKO mice had increased level of circulating bile acids upon bile duct ligation (Uppal et al., 2007). The opposite effect of loss of CAR and LXRs on serum bile acid level also suggested that the LXR $\alpha$ -CAR crosstalk might also play a role in the homeostasis of bile acids.

A recent report suggested that activation of CAR can suppress lipid metabolism by reducing the protein level of the active form of SREBP-1 (Roth et al., 2008a), which was reasoned to be due to the CAR-mediated induction of Insig-1, an anti-lipogenic protein that blocks proteolytic activation of SREBPs (McPherson and Gauthier, 2004). In a subsequent study, the same group showed that activation of SREBP-1 by insulin or cholesterol inhibited the activity of CAR, in which SREBP-1 may function as a non-DNA binding inhibitor that

blocks the interaction of CAR with co-activators (Roth et al., 2008b). These results suggest another possible but not mutually exclusive mechanism by which LXR and CAR might crosstalk. The relative contribution of Insig-1 induction by CAR and LXR inhibition by CAR in the overall effect of CAR on lipogenesis remains to be determined.

In summary, the current study has revealed a mutual repression between LXR $\alpha$  and CAR that links hepatic lipogenesis and xenobiotic responses. The *in vivo* significance of this crosstalk was strongly supported by recent reports that treatment with the CAR agonist TCPOBOP inhibited hepatic steatosis in high-fat diet treated wild type mice and *ob/ob* mice (Gao et al., 2009; Dong et al., 2009; Maglitch et al., 2009).

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## FIGURE LEGENDS

### **Fig. 1. Reciprocal activation of target gene expression in mice deficient of CAR and**

**LXR.** (A) Real-time PCR analysis on the hepatic expression of LXR target genes in wild type,  $PXR^{-/-}$ ,  $CAR^{-/-}$ , and double knockout (PC DKO) mice. (B) Real-time PCR analysis on the hepatic expression of LXR target genes in  $CAR^{-/-}$  mice (left panel) or WT mice (right panel) in the presence or absence of TO1317. (C and D) Measurements of triglycerides and cholesterol in the liver (C) and plasma (D). (E) Real-time PCR analysis on the hepatic expression of CAR target genes in wild type and LXR DKO mice. (F) Real-time PCR analysis on the hepatic expression of CAR target genes in LXR DKO mice in the presence or absence of TCPOBOP. The fold inductions in (F) are labeled. All mice shown are males. Results represent the averages and standard deviation from 4-6 mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , compare to the WT (A, C-E) or DMSO control (B).

### **Fig. 2. Mutual repression of target gene expression by pharmacological activation of**

**LXR and CAR.** (A) Real-time PCR analysis on the hepatic expression of LXR target genes in wild type C57BL/6J male mice treated with GW3965 and TCPOBOP individually or in combination. The expression of individual genes in vehicle (DMSO) treated mice is arbitrarily set as 1. (B and C) Measurements of triglycerides and cholesterol in the liver (B) and plasma (C). (D) Real-time PCR analysis on the hepatic expression of CAR target genes. All mice shown are males. Results represent the averages and standard deviation from 4-6 mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , compare to the DMSO control.

**Fig. 3. Mutual repression of target gene expression by genetic activation of LXR $\alpha$  and CAR in transgenic mice.** (A) Real-time PCR analysis on the hepatic expression of CAR target genes in the wild type (WT) and VP-LXR $\alpha$  transgenic mice. (B) Real-time PCR analysis on the hepatic expression of CAR target genes in VP-LXR $\alpha$  transgenic mice in the presence or absence of TCPOBOP. The fold inductions are labeled. (C) Wild type and CAR<sup>-/-</sup> mice were subjected to the tribromoethanol anesthesia test. (D) The VP-LXR $\alpha$  transgene or treatment with GW3965 sensitized mice to the tribromoethanol anesthesia test. (E) Real-time PCR analysis on the hepatic expression of LXR target genes in WT and VP-CAR transgenic mice. (F) Measurements of triglycerides and cholesterol in the liver of WT and VP-CAR transgenic mice. Mice are males if not specified. Results represent the averages and standard deviation from 4-6 mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , all compare to the WT.

**Fig. 4. Mutual suppression between LXR $\alpha$  and CAR in reporter gene assays.** The tk-LXRE (A), tk-PBRE (B), pGL-Scd1 (C), pGL-Cyp2b10 (D), and tk-UAS (E and F) luciferase reporter genes were transiently transfected into cells in the presence of expression vectors for indicated receptors or their combinations. Where applicable, transfected cells were treated with indicated drugs for 24 hrs before luciferase assay. The transfection efficiency was normalized against the  $\beta$ -gal activity from the co-transfected CMX- $\beta$  gal vector. Results shown are fold induction over vector control and represent the averages and standard deviation from triplicate assays. Drug concentrations are: androstenol, 5  $\mu$ M; TCPOBOP, 250 nM; TO1317, 10  $\mu$ M; GW3965, 10  $\mu$ M.

**Fig. 5. Mechanistic studies for the mutual suppression between LXR $\alpha$  and CAR in cell**

**cultures.** (A) Mammalian two-hybrid assay to demonstrate the SRC1-LXR $\alpha$  interaction and the effect of CAR co-transfection. HepG2 cells were transfected with Gal-SRC1 and the tk-UAS-Luc reporter gene in the presence of indicated receptors. Transfected cells were treated with indicated drugs for 24 hrs before luciferase assay. Results shown are fold induction over vector control and represent the averages and standard deviation from triplicate assays. (B) GST pull-down assay to demonstrate the SRC1-LXR $\alpha$ , SRC1-CAR, and SRC1-PPAR $\gamma$  interactions. Equal volumes of  $^{35}$ S-labeled proteins were loaded to demonstrate the efficiency of protein translation. (C) The inhibition of SRC1-LXR $\alpha$  interaction by CAR was relieved by increased concentration of SRC1 in a mammalian two-hybrid assay. (D) The inhibition of SRC1-CAR interaction by LXR $\alpha$ . (E) CAR $\Delta$ 8 and CAR $\Delta$ 37 lacked transcriptional activity on the tk-PBRE report gene. (F) CAR $\Delta$ 37 failed to interact with GST-SRC1 in the GST pull-down assay. (G) CAR $\Delta$ 37, but not CAR $\Delta$ 8, failed to suppress the constitutive and GW3965-inducible activities of LXR $\alpha$  on the tk-LXRE reporter gene. (H) A forced expression of RXR did not abolish the inhibitory effect of CAR on LXR $\alpha$ . Drug concentrations are: TO1317, 10  $\mu$ M; TCPOBOP, 250 nM; BRL49653, 5  $\mu$ M; GW3965, 10  $\mu$ M.

**Fig. 6. Effects of CAR and LXR agonists on receptor recruitment to target gene**

**promoters *in vivo*.** (A and B) Flag-LXR $\alpha$  and HA-CAR expression vectors were hydrodynamically transfected into the mouse liver. Transfected mice were treated with

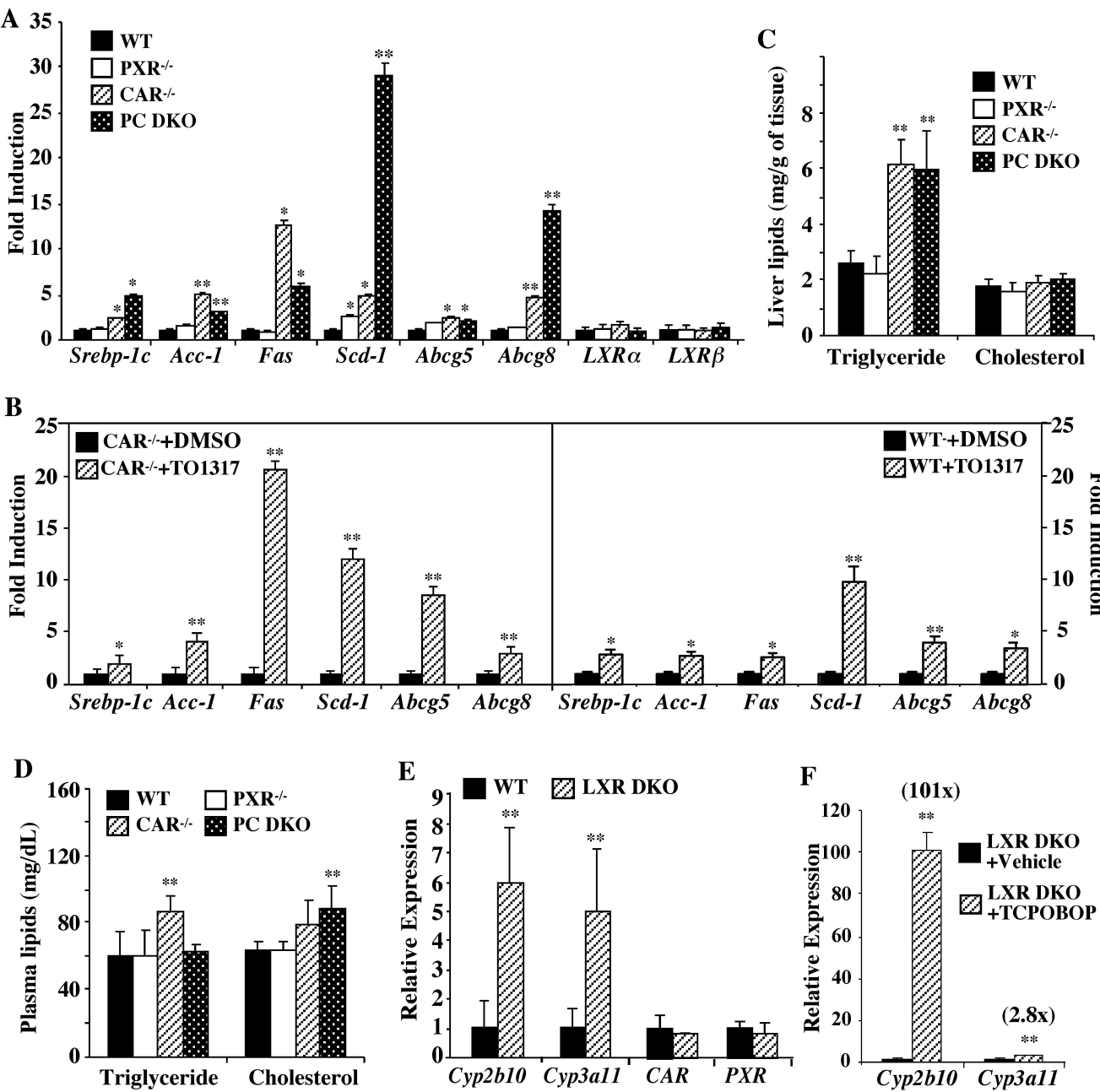
GW3965 and/or TOPOBOP for 8 hrs before sacrificing and ChIP assay using anti-Flag and anti-HA antibodies. PCRs in (A) and (B) encompass the *Srebp-1c*/DR4 LXRE and *Cyp2b10*/PBRE, respectively. ChIP with normal IgG was included as negative controls. Lanes represent individual mice. (C) The expression of Flag-LXR $\alpha$  and HA-CAR proteins in transfected livers was confirmed by Western blot analysis. The  $\beta$ -actin blot was included as a protein loading control.

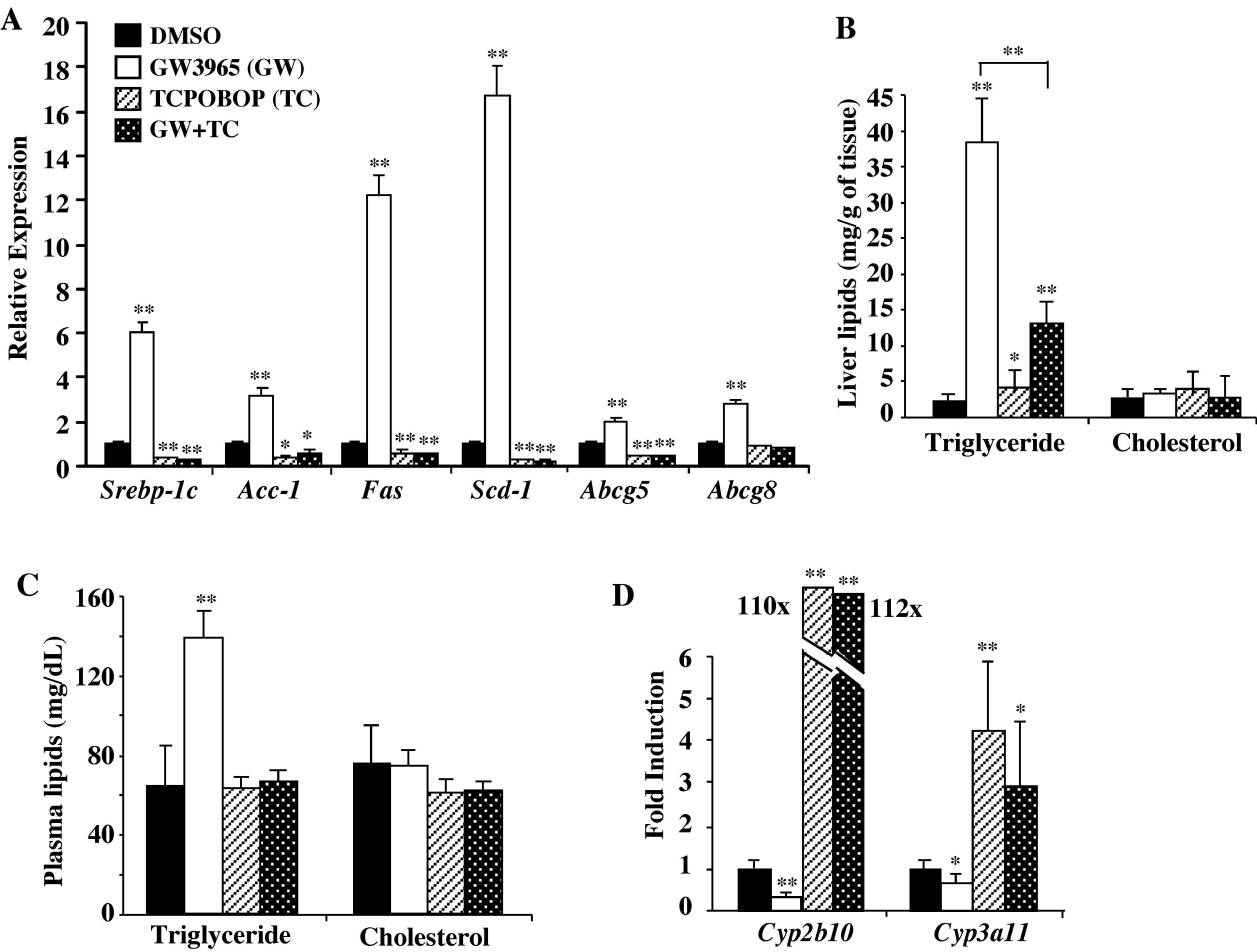
**Fig. 7. A model of functional crosstalk between LXR and CAR in regulating lipogenesis and xenobiotic responses.** The mutual suppression may have linked LXR-mediated endobiotic and CAR-mediated xenobiotic metabolism in the liver and intestine. CAR, constitutive androstane receptor, LXR, liver X receptor; LXRE, LXR response element; PBRE, phenobarbital response element

**Supplementary Fig. 1. Effects of TO1317 on the expression of LXR target genes in PC DKO mice as determined by real-time PCR analysis.** The expression of individual genes in vehicle (DMSO) treated mice is arbitrarily set as 1.

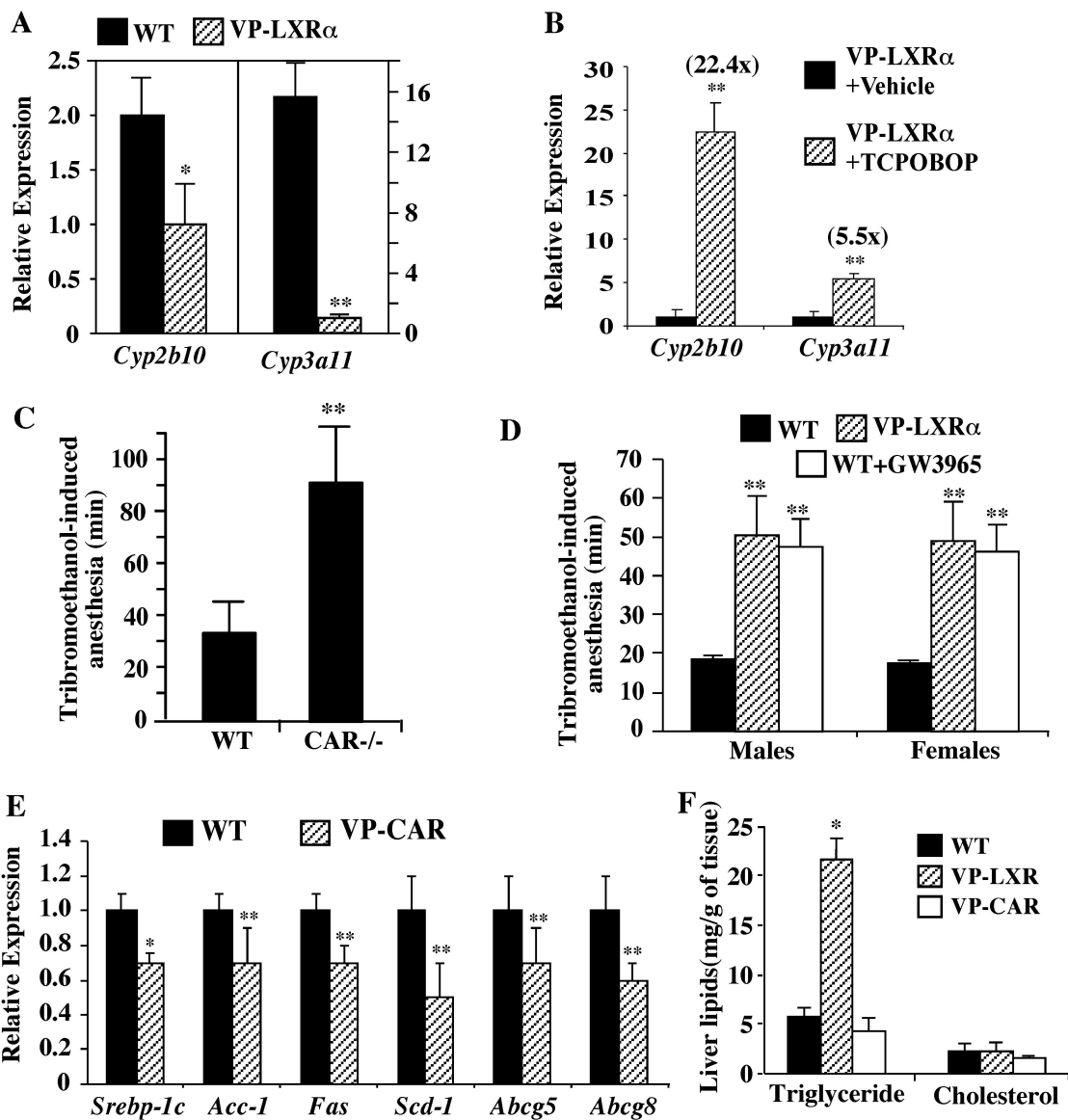


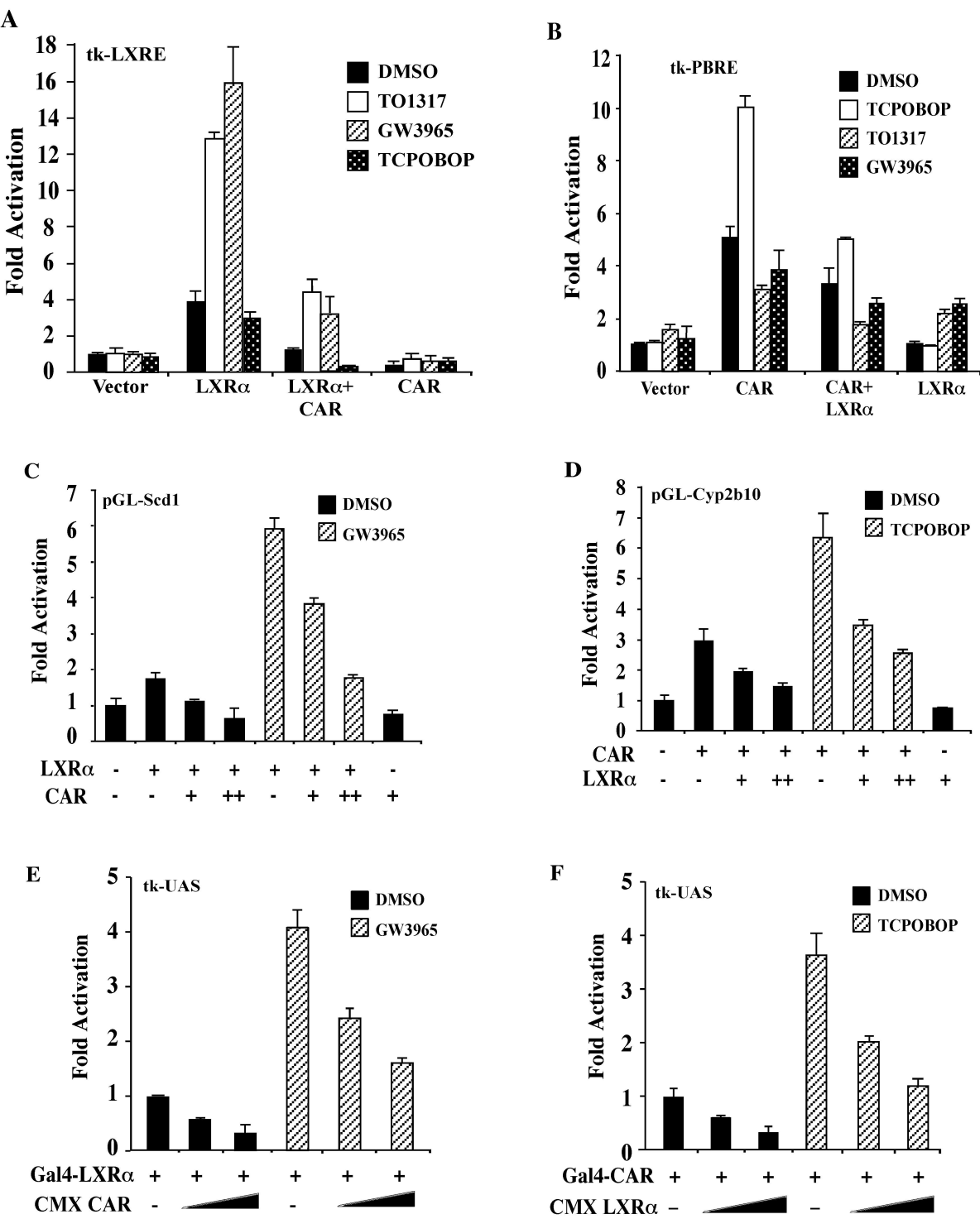
Fig. 1



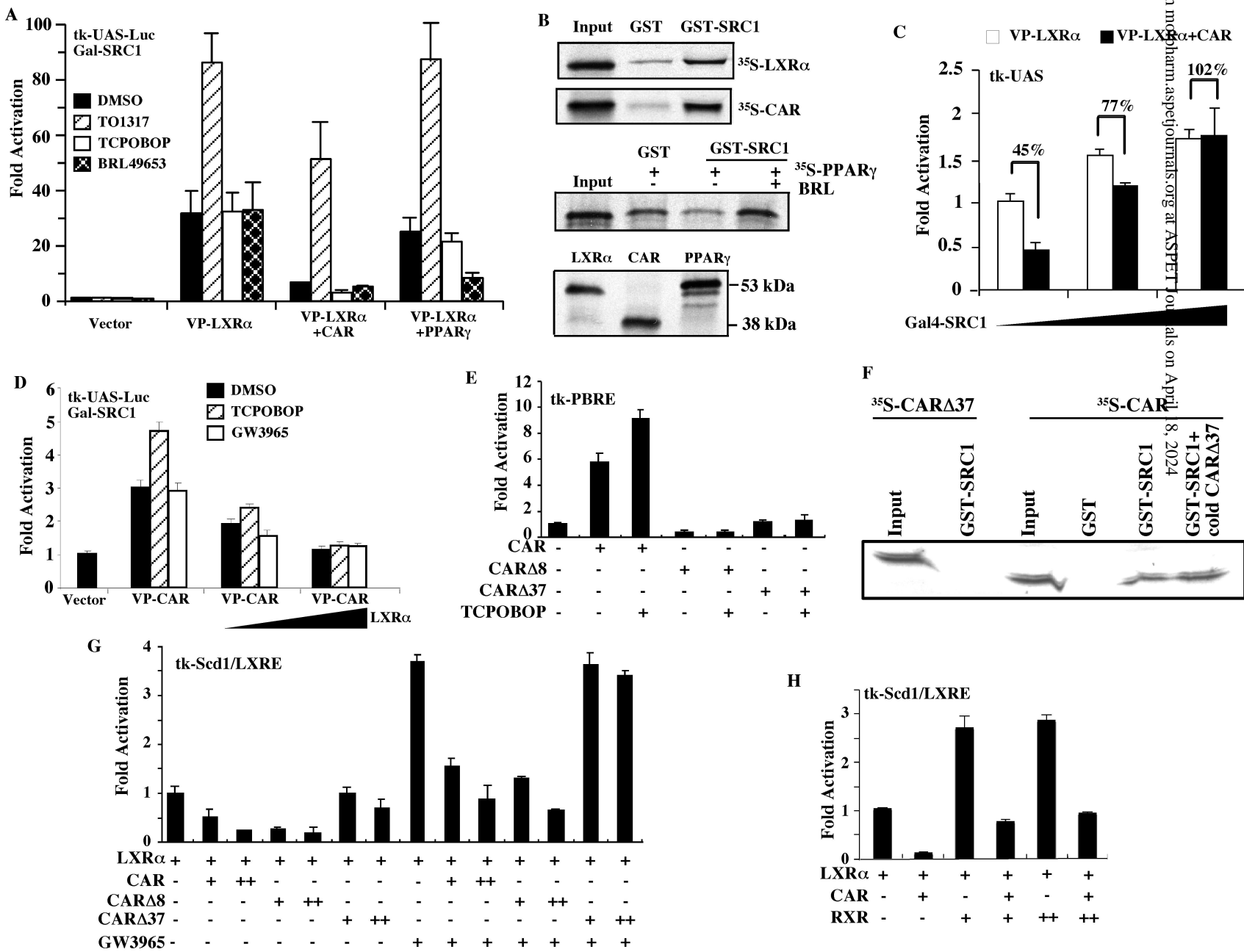
**Fig. 2**

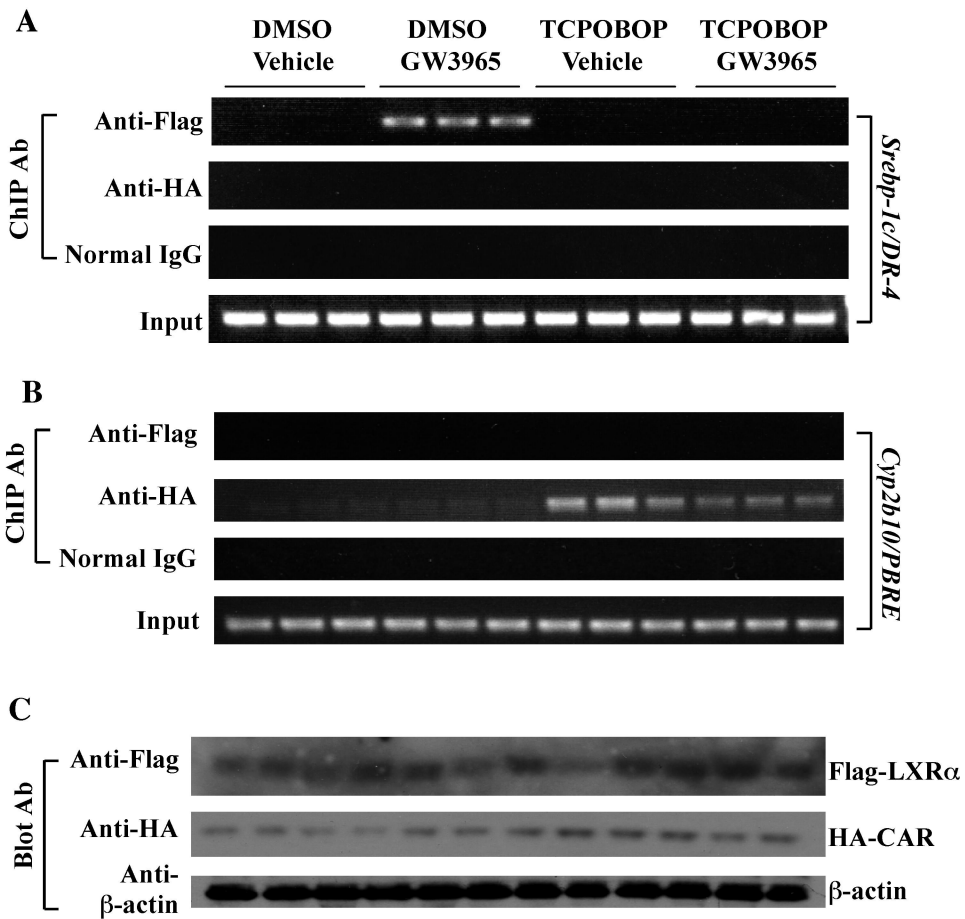
**Fig. 3**



**Fig. 4**

**Fig. 5**



**Fig. 6**

**Fig. 7**

