

MOL #72769

**Rimonabant, a CB1 inverse agonist, inhibits hepatocyte lipogenesis by  
activating LKB1 and AMPK axis downstream of  $G\alpha_{i/o}$  inhibition**

Hong Min Wu, Yoon Mee Yang, and Sang Geon Kim

*Innovative Drug Research Center for Metabolic and Inflammatory Diseases, College of Pharmacy and*

*Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea*

**MOL #72769**

**Running title:** LKB1-AMPK Activation by Rimonabant

**To whom corresponding should be addressed:**

Sang Geon Kim, Ph.D., College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, Korea Tel. +82-2-880-7840; Fax. +82-2-872-1795; E-mail: [sgk@snu.ac.kr](mailto:sgk@snu.ac.kr)

**The information of manuscript statistics:**

Number of Text Pages: 29

Number of Tables: 0

Number of Figures: 7

Number of References: 48

Words in Abstract: 250

Words in Introduction: 713

Words in Discussion: 1289

**The abbreviations used are:**

ABCA1, ATP-binding cassette transporter A1; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CAB39, calcium binding protein 39; CB1, cannabinoid receptor type 1; FAS, fatty acid synthase; LKB1, liver kinase B1; LXR $\alpha$ , liver X receptor- $\alpha$ ; LXRE, LXR $\alpha$ -response element; NAFLD, nonalcoholic fatty liver disease; PKA, cAMP-dependent protein kinase A; SCD-1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; T090, T0901317

**MOL #72769**

### **Abstract**

Liver X receptor- $\alpha$  (LXR $\alpha$ ) and its target sterol regulatory element protein-1c (SREBP-1c) play key roles in hepatic lipogenesis. Rimonabant, an inverse agonist of cannabinoid receptor type-1 (CB1), has been studied as an anti-obesity drug. In view of the link between CB1 and energy metabolism, this study investigated the effect of rimonabant on LXR $\alpha$ -mediated lipogenesis in hepatocytes, and the underlying basis. Rimonabant treatment inhibited CYP7A1-LXRE gene transactivation and an increase in LXR $\alpha$  mRNA level by LXR $\alpha$  agonist (T0901317) in HepG2 cells. Consistently, rimonabant attenuated the activation of SREBP-1c and its target gene induction. The reversal by CB1 agonists on rimonabant's repression of SREBP-1c supported the role of CB1 in this effect. Rimonabant inhibited the activation of SREBP-1c presumably via  $G\alpha_{i/o}$  inhibition, as did pertussis toxin. Adenylyl cyclase activator forskolin or 8-Br-cAMP treatment mimicked the action of rimonabant, suggesting that  $G\alpha_{i/o}$  inhibition causes the repression of SREBP-1c by increasing cAMP level. Knockdown or chemical inhibition of protein kinase A (PKA) prevented the inhibition of LXR $\alpha$  by rimonabant, supporting that an increase in cAMP content and PKA activation, which catalyzes LXR $\alpha$  inhibitory phosphorylation, might be responsible for the anti-lipogenic effect. In addition, rimonabant activated liver kinase B1 (LKB1), resulting in the activation of AMP-activated protein kinase responsible for LXR $\alpha$  repression. Moreover, PKA inhibition prevented the activation of LKB1, supporting that PKA regulates LKB1. In conclusion, rimonabant has an anti-lipogenic effect in hepatocytes by inhibiting LXR $\alpha$ -dependent SREBP-1c induction, as mediated by increase in PKA activity and PKA-mediated LKB1 activation downstream of CB1-coupled  $G\alpha_{i/o}$  inhibition.

## Introduction

The metabolic syndrome is made up of a series of obesity and heart disease-related risk factors including hypertension, hyperglycemia, and dyslipidemia (Cornier et al., 2008). The increasing rate of obese subjects in the world results in the growing incidence of metabolic syndrome (Alexander et al., 2003). Mechanisms of the pathogenesis involved in the disorders are not yet fully understood. Nonalcoholic fatty liver disease (NAFLD), a hepatic component of metabolic syndrome, is a common liver disease that refers to liver dysfunction and/or injury, disturbing the overall metabolic function of the liver (Kotronen and Yki-Järvinen, 2008). NAFLD ranges from steatosis to steatohepatitis, fibrosis, and cirrhosis (Marra et al., 2008).

Liver X receptor- $\alpha$  (LXR $\alpha$ ), a lipid sensor, promotes lipid and cholesterol metabolism in the cell. In regulating lipid metabolism, LXR $\alpha$  stimulates fatty acid synthesis and triglyceride accumulation in hepatocytes. Upon activation with LXR ligand (e.g., endogenous ligand, oxysterol; and synthetic ligand, T0901317 (T090)), LXR $\alpha$  and retinoid X receptor dimerize and bind to DNA responsive elements (LXRE). Sterol regulatory element binding protein-1c (SREBP-1c) is a major target gene of LXR $\alpha$  (Repa et al., 2000), and plays a key role in the induction of lipogenic genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1) and ATP-binding cassette transporter A1 (ABCA1) (Stoeckman and Towle, 2002; Ntambi, 1999). Thus, SREBP-1c activation by LXR $\alpha$  may facilitate progression to steatosis and steatohepatitis (Repa et al., 2000). Therefore, methods to inhibit LXR $\alpha$  activity represent a potential way for the treatment of hepatic steatosis.

Cannabinoid receptor type-1 (CB1) has a modulating effect on the fat metabolism at peripheral organs such as liver, adipose tissue, and skeletal muscle (Osei-Hyiaman et al., 2005; Yan et al., 2007; Liu et al., 2005). In several animal models, CB1 blockade decreases *de novo* lipogenesis (Jeong et al., 2008; Jourdan et al., 2010). Rimonabant (SR141716A) is the first-in-class CB1 blocker for obesity treatment (Van Gaal et al., 2005). The data obtained from clinical trials suggest that rimonabant may have a beneficial effect against disrupted lipid metabolism in

**MOL #72769**

the liver (Després et al., 2005; Scheen et al., 2006; Hollander et al., 2010). However, the anti-steatotic effect of rimonabant in hepatocytes and the molecular basis responsible for this action remain to be elucidated.

Protein kinase A (PKA), recognized as a fasting signal that is sensitive to increased cAMP content, plays a role in lipid metabolism (McKnight et al., 1998). In animal models, PKA activation by adrenergic stimulation resulted in lean phenotypes, and improves insulin sensitivity (Cummings et al., 1996; Schreyer et al., 2001). In addition, PKA directly phosphorylates LXR $\alpha$  and thereby hinders LXR $\alpha$  dimerization with retinoid X receptor (Yamamoto et al., 2007). Thus, PKA activation attenuates SREBP-1 activity and SREBP-1-mediated lipogenesis (Yamamoto et al., 2007; Lu and Shyy, 2006). Since CB1 is coupled to G proteins, chemical modulation of this receptor may affect the adenylyl cyclase-cAMP-dependent PKA axis (Bayewitch et al., 1995).

AMP-activated protein kinase (AMPK) serves as an intracellular sensor for energy homeostasis. The AMPK pathway regulates lipid and glucose metabolism; its activation inhibits not only hepatic lipogenesis mainly through inhibitory phosphorylation of ACC, a rate-limiting step of fatty acid synthesis, but hepatic cholesterol production through suppression of 3-hydroxy-3-methylglutaryl-CoA reductase (Motoshima et al., 2006). In addition, an increase in AMPK activity reduces glucose production in the liver, whereas it enhances glucose uptake in the skeletal muscle (Sakoda et al., 2002). A series of findings indicate that AMPK contributes to antisteatotic and antidiabetic action (Hwahng et al., 2009; Bae et al., 2007). In hepatic and adipose tissues, CB1 agonist treatment has been shown to inhibit AMPK activity (Kola et al., 2005). So, it is plausible that the antagonism of CB1 may result in the activation of AMPK.

In view of the association between endocannabinoid physiology and lipid metabolism, and the inhibitory regulation of AMPK by CB1, we were interested in the effect of rimonabant, a selective CB1 inverse agonist, on LXR $\alpha$ -SREBP-1c-mediated lipogenesis in hepatocytes and the underlying basis. Here, we report the beneficial effect of rimonabant's antagonism of CB1

**MOL #72769**

on LXR $\alpha$ -SREBP-1c-mediated lipogenesis. Moreover, this study identified the regulatory role of PKA in activating liver kinase B1 (LKB1) and AMPK downstream of CB1 inverse agonism. An important finding of this study is the link between CB1-G $\alpha_{i/o}$  inhibition and LKB1-AMPK activation that leads to the inhibition of LXR $\alpha$ -mediated-SREBP-1c activity.

**MOL #72769**

## **Materials and Methods**

**Materials.** Rimonabant HCl (99%) was provided from AK Scientific (Mountain View, CA). Hu-210 and arachidonyl-2'-chloroethylamide (ACEA) were purchased from Tocris (Ellisville, Missouri). Anti-phospho-AMPK, anti-AMPK, anti-phospho-ACC, anti-ACC, anti-acetylated-lysine, and anti-phospho-LKB1 (Ser428) antibodies were obtained from Cell Signaling (Beverly, MA). PKI (PKA inhibitor) and the antibodies directed against SREBP-1,  $G\alpha_i$ , PKA, LKB1, c-myc and CaMKK $\beta$  were supplied from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- $\beta$ -actin and anti-phosphorylated serine antibody, GW3965, 8-Br-cAMP, and forskolin were obtained from Sigma-Aldrich Co. (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit, and goat anti-mouse IgGs were purchased from Zymed Laboratories (San Francisco, CA). Pertussis toxin, H89, T0901317, and compound C were provided from Calbiochem (San Diego, CA).

**Cell Culture.** HepG2 (human hepatocyte-derived cell line), AML-12 (mouse hepatocyte-derived cell line), and HeLa cells were supplied from ATCC (Manassas, VA). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin at 37°C in humidified atmosphere with 5% CO<sub>2</sub>. Cells were plated in a six-well dish at a density of  $1 \times 10^6$  cells per well, and wells with 70-80% confluency were used. The cells were incubated with either vehicle or LXR $\alpha$  agonist (i.e., T090 and GW3965) in combination with rimonabant for 12 h.

**Transient Transfection and Reporter Gene Assays.** The cells were transiently transfected with pGL2-FAS (1  $\mu$ g) or TK-CYP7A1-LXR $\alpha$  response element (LXRE)x3-LUC (0.5  $\mu$ g) for 3 h in the presence of Lipofectamine® reagent. The activity of luciferase was measured by adding luciferase assay reagent (Promega, Madison, WI). The FAS reporter plasmid (pGL2-FAS) was a gift from Dr. T. F. Osborne (University of California, Irvine, CA). LXR luciferase reporter, TK-

## MOL #72769

CYP7a-LXREx3-LUC that contains three tandem copies of the sequence (5'-gcttGGTCActcaAGTTCAagtta-3') from the rat CYP7A1 gene, was provided from Dr. D. J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX).

**RNA Isolation and Real-time PCR Assays.** Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The complementary DNA was obtained by reverse transcription using an oligo(dT)<sub>16</sub> primer to obtain cDNA. It was amplified by polymerase chain reaction (PCR). Real-time PCR was conducted with a Light Cycler 1.5 apparatus (Roche, Mannheim, Germany) using a Light Cycler DNA master SYBR green-I kit according to the manufacturer's instructions. The levels of target mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The following primer sequences were used: human SREBP-1, 5'-CGACATCGAAGACATGCTTCAG-3' (sense) and 5'-GGAAGGCTTCAAGAGAGGAGC-3' (antisense); human LXR $\alpha$ , 5'-GATCGAGGTGATGCTTCTGGAG-3' (sense) and 5'-CCCTGCTTTGGCAAAGTCTTC-3' (antisense); human FAS, 5'-GACATCGTCCATTCGTTTGTG-3' (sense) and 5'-CGGATCACCTTCTTGAGCTCC-3' (antisense); human ACC1, 5'-GCTGCTCGGATCACTAGTGAA-3' (sense) and 5'-TTCTGCTATCAGTCTGTCCAG-3' (antisense); human SCD-1, 5'-CCTCTACTTGGAAGACGACATTCGC-3' (sense) and 5'-GCAGCCGAGCTTTGTAAGAGCGGT-3' (antisense); human ABCA1, 5'-TGTCCAGTCCAGTAATGGTTCTGT-3' (sense) and 5'-AAGCGAGATATGGTCCGGATT-3' (antisense); and human GAPDH, 5'-GAAGATGGTGATGGGATTTC-3' (sense) and 5'-GAAGGTGAAGGTCGGAGTC-3' (antisense).

**Immunoprecipitation and Immunoblot Assays.** Cell lysates (500  $\mu$ g/each aliquot) were immunoprecipitated with anti-LXR $\alpha$  antibody or anti-LKB1 antibody overnight at 4°C. The antigen-antibody complex was immunoprecipitated after incubation with protein G-agarose for



**MOL #72769**

2 h. Immune complexes were solubilized in  $2 \times$  Laemmli buffer and were boiled for 5 min. Protein samples were resolved and were immunoblotted with anti-phosphorylated-serine, anti-phosphorylated-threonine or anti-acetylated-lysine antibody.

**siRNA Knockdown.** To knockdown target molecules, cells were transfected with either a siRNA directed against human  $G\alpha_i$ , PKA or LKB1 (Santa Cruz Biotechnology, Santa Cruz, CA), or a nontargeting control siRNA (100 pmol/ml) using Lipofectamine 2000 according to the manufacturer's instructions. After transfection for 48 h, the cells were treated with vehicle or LXR $\alpha$  agonist in the presence or absence of rimonabant for 12 h. Immunoblottings verified the knockdown effects of  $G\alpha_i$ , PKA and LKB1.

**PKA Activity Assay.** PKA assay was performed using a commercially available solid phase ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA) according to the manufacturer's instruction. Briefly, samples (30  $\mu$ g each) were loaded in the appropriate wells of the provided PKA substrate microtiter plate, followed by addition of 10  $\mu$ g ATP. After termination of the kinase reaction, the antibody that recognizes the substrate phosphorylation was added to the wells. The phosphospecific antibody was then bound by a peroxidase-conjugated secondary antibody. The color of assay was developed in proportion to PKA phosphotransferase activity using tetramethylbenzidine substrate. After stopping color development with acid stop solution, the intensity of the color was measured in a microplate reader (TECAN) at 450 nm.

**Data Analysis.** One way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant treatment effect, the Newman-Keuls test was used to compare multiple group means.

**MOL #72769**

## **Results**

### **Inhibition of LXR $\alpha$ -dependent SREBP-1c induction**

The *in vitro* effect of rimonabant on lipogenic gene induction by T090 was examined in HepG2 cells: T090, a synthetic LXR $\alpha$  agonist, was employed to induce LXR $\alpha$  and SREBP-1c activation. Treatment of cells with T090 increased LXR-dependent gene transactivation, as shown by an increase in CYP7A1-LXRE-luciferase (CYP7A1) activity, which was significantly attenuated by simultaneous treatment of cells with rimonabant (Fig. 1A, left). T090 may also increase transactivation of the PXR target gene (CYP3A23-PXRE-luciferase gene) (Mitro et al., 2007). PXRE reporter activity was unaffected by rimonabant (data not shown), confirming that rimonabant treatment specifically inhibits the induction of LXR $\alpha$  target gene. Since the LXR $\alpha$  gene itself contains an LXRE (Laffitte et al., 2001), the activation of LXR $\alpha$  by T090 treatment for 12 h caused its own gene induction. This increase was prevented by concomitant treatment of cells with rimonabant (Fig. 1A, right).

The effect of rimonabant on T090-dependent induction of SREBP-1c, a marker of hepatic lipogenesis, was determined in HepG2 cells. As expected, the level of SREBP-1c protein was increased 12 h after T090 treatment, and rimonabant attenuated the T090 induction of SREBP-1c protein (Fig. 1B): SREBP-1c transcript level was also repressed by 1  $\mu$ M or 10  $\mu$ M rimonabant pretreatment (Fig. 1C). Moreover, treatment of cells with rimonabant prevented the ability of GW3965, another LXR $\alpha$  agonist, to induce SREBP-1c expression (Fig. 1D). AM251, a CB1 antagonist, blocked SREBP-1c induction by T090, as did rimonabant (Fig. 1E). In addition, treatment of cells with Hu-210 or ACEA, CB1 agonists, reversed the ability of rimonabant to inhibit SREBP-1c induction by T090 (Fig. 1F). Our results indicate that rimonabant antagonizes the activation of LXR $\alpha$  and SREBP-1c by T090, which may be mediated by CB1 antagonism.

### **Inhibition of SREBP-1c-mediated lipogenic gene induction**

## MOL #72769

To assess the transcriptional activity of SREBP-1c, FAS reporter assays were conducted in HepG2 cells transfected with the construct containing the sterol response element, but not the LXRE, in the –150 bp FAS promoter region (Fig. 2A, upper). Incubation of the cells with T090 increased luciferase activity from the construct, which was diminished by rimonabant (Fig. 2A, lower). Because SREBP-1c controls the transcription of genes encoding for lipogenic enzymes, the effects of rimonabant on their gene expression were monitored by measuring relative changes in FAS, ACC, SCD-1, and ABCA1 transcript levels (Fig. 2B). Rimonabant attenuated the ability of LXR $\alpha$  agonist to induce the target genes of SREBP-1c. Our results demonstrate that rimonabant indeed has the ability to prevent SREBP-1c-mediated lipogenic gene induction by LXR $\alpha$  agonist. In subsequent experiments, 10  $\mu$ M of rimonabant was used to ensure the optimal effect.

### SREBP-1c repression by cAMP-PKA activation downstream of G $\alpha_{i/o}$ inhibition

GPCRs that activate the G $\alpha_i$  class of G $\alpha$  subunits inhibit cAMP production, whereas other GPCRs that activate the G $\alpha_s$  class of G $\alpha$  subunits facilitate it (Neves et al., 2001). CB1 may be coupled with G proteins of the G $\alpha_{i/o}$  family (i.e., G $\alpha_i$  1, 2 and 3, and G $\alpha_o$  1 and 2), triggering signaling pathways mainly by inhibiting the cAMP-dependent PKA pathway. As an effort to identify the downstream component of CB1 antagonism, we examined the role of G $\alpha_{i/o}$  inhibition in the repression of lipogenesis by rimonabant. Either knockdown of G $\alpha_i$  or treatment of cells with pertussis toxin (a G $\alpha_{i/o}$  inhibitor) inhibited T090-mediated induction of SREBP-1c (Fig. 3A). G $\alpha_{i/o}$  inhibition causes an increase in cellular cAMP content through adenylyl cyclase (Neves et al., 2001). Treatment of cells with forskolin, an agent that increases cAMP by activating adenylyl cyclase, also inhibited the induction of SREBP-1c by T090 (Fig. 3B, left). Similarly, 8-Br-cAMP treatment diminished SREBP-1c induction by T090 (Fig. 3B, right). Since cAMP activates PKA, we next assessed the effect of rimonabant on the activity of PKA.

## MOL #72769

As expected, rimonabant elevated PKA activity, which was abolished by treatment of cells with either H89 (a PKA/PKG/PKC inhibitor) or PKI (a PKA-specific inhibitor) (Fig. 3C).

### **Role of PKA in the inhibition of LXR $\alpha$ and SREBP-1c activation**

PKA may directly phosphorylate LXR $\alpha$  at serine residue, which inactivates LXR $\alpha$  (Yamamoto et al., 2007). Consistent with this report, rimonabant facilitated the phosphorylation of LXR $\alpha$  at serine residue, which was reversed by either PKA knockdown (siPKA) or H89 treatment (Fig. 4A). Furthermore, H89 attenuated the ability of rimonabant to prevent LXRE reporter induction by T090 (Fig. 4B). Moreover, either transfection of specific siRNA directly against PKA  $\alpha$  catalytic subunit or H89 treatment reversed the repression of SREBP-1c by rimonabant (Fig. 4C). Hence, cAMP-dependent PKA activation by rimonabant may contribute to SREBP-1c repression by rimonabant via LXR $\alpha$  inactivation.

### **PKA-dependent activation of LKB1 by rimonabant**

PKA may activate LKB1 through direct phosphorylation (Collins et al., 2000). Given the regulatory effect of PKA on LKB1, we next examined whether rimonabant is capable of activating LKB1. Rimonabant promoted the activation of LKB1 in HepG2 or AML-12 cells (a normal mouse hepatocyte cell line, known to synthesize fatty acids/lipids), as shown by increased phosphorylation of LKB1 on Ser428 residue (Fig. 5A). LKB1 activation was further supported by its deacetylation and binding with calcium binding protein 39 (CAB39) (Fig. 5B). To link the increased PKA activity and LKB1 activation by rimonabant, we determined whether PKA inhibition affected LKB1 activation by rimonabant. We found that siRNA knockdown of PKA completely prevented the ability of rimonabant to promote LKB1 phosphorylation (Fig. 5C). Similarly, treatment of cells with H89 abolished the activation of LKB1 by rimonabant (Fig. 5D). These data provide evidence that increase in PKA activity by rimonabant may lead to the activation of LKB1.

**MOL #72769**

### **LKB1-dependent AMPK activation for the inhibition of LXR $\alpha$**

LKB1 is an upstream kinase of AMPK. As expected, rimonabant activated AMPK in a time-dependent manner, as evidenced by phosphorylations of AMPK and ACC (a substrate of AMPK) in HepG2 or AML12 cells (Fig. 6A). Consistently, siRNA knockdown of LKB1 eliminated the ability of rimonabant to activate AMPK (Fig. 6B, left). In addition, rimonabant did not activate AMPK in HeLa cells deficient of LKB1 (Fig. 6B, right). Calcium/calmodulin-dependent kinase kinase  $\beta$  (CaMKK $\beta$ ) is a member of the serine/threonine protein kinase family. Treatment of cells with STO-609 (1  $\mu$ g/ml), an inhibitor of CaMKK, failed to antagonize AMPK activation by rimonabant (Fig. 6C). Inhibition of CaMKK $\beta$  autophosphorylation at serine/threonine by STO-609 confirmed the effectiveness of STO-609. All of these results show that rimonabant activates AMPK through LKB1, but not CaMKK $\beta$ .

Activated AMPK inhibits LXR $\alpha$  through phosphorylation at threonine residue (Hwahng et al., 2009). To verify the role of AMPK in repressing LXR $\alpha$  and SREBP-1c by rimonabant, we assessed the effect of AMPK inhibition on the ability of rimonabant to repress T090 activation of LXR $\alpha$  and SREBP-1c induction. As expected, overexpression of a dominant negative mutant of AMPK $\alpha$  (DN-AMPK $\alpha$ ) reversed LXR $\alpha$  phosphorylation at threonine residue increased by rimonabant (Fig. 6D, left), whereas it did not affect LXR $\alpha$  phosphorylation at serine residue (Fig. 6D, middle). Since PKA is the upstream kinase of LKB1-AMPK axis, PKA activation also leads to the phosphorylation of LXR $\alpha$  at threonine through AMPK (Fig., 6D, right). Because the antibodies directed against phospho-serine or phospho-threonine of LXR $\alpha$  were not available, LXR $\alpha$  immunoprecipitates were immunoblotted for phospho-serine or phospho-threonine. Consistently, AMPK inhibition antagonized the ability of rimonabant to repress SREBP-1c (Fig. 6E, left). Similarly, treatment of cells with compound C, a chemical inhibitor of AMPK, reversed the SREBP-1c-repressing effect of rimonabant (Fig. 6E, right). Our results showed that

**MOL #72769**

AMPK activation by rimonabant contributes to repressing SREBP-1c presumably via LXR $\alpha$  inhibition (i.e., threonine phosphorylation). Collectively, rimonabant has an anti-lipogenic effect in hepatocytes not only by promoting PKA activation, which directly inhibits LXR $\alpha$ , but also PKA-mediated activation of AMPK responsible for LXR $\alpha$ -SREBP-1c repression (Fig. 7).

## Discussion

Rimonabant is the pioneering CB1 inverse agonist that had been used in clinical settings based on its body weight loss effect as well as metabolic syndrome correction (Després et al., 2005). Unfortunately, it was recently withdrawn from the market due to neuropsychiatric adverse events (Nathan et al., 2010). Nevertheless, the endocannabinoid system still has an important researching value in new drug development as peripheral CB1 antagonism shows a potential beneficial effect in regulating metabolic syndrome. Since drugs with no psychiatric disturbances appear particularly interesting, one possible future aim could be the development of endocannabinoid modulators that have only peripheral effects.

LXR $\alpha$  facilitates fatty acid synthesis mainly through transcription of SREBP-1c, a master regulator of lipogenic genes (Repa et al., 2000). The role of LXR $\alpha$  in lipogenesis was proven by the result of *in vivo* experiment showing that administration of LXR agonist (T090) to mice increased hepatic and plasma triglyceride levels and switched on LXR-dependent lipogenic genes in the liver, whereas this effect was abolished in LXR-null mice (Schultz et al., 2000). In the present study, we explored the mechanistic basis of rimonabant's anti-lipogenic properties in a hepatocyte model using LXR $\alpha$  ligand. Our finding that the pharmacological interference on CB1 suppressed T090 induction of SREBP-1c expression is in agreement with the observation that treatment of HU210, a CB1 agonist, enhanced SREBP-1c level in mice, and which was abolished by rimonabant pretreatment (Osei-Hyiaman et al., 2005). All of these results support the notion that rimonabant may act peripherally in the liver to antagonize CB1 and decrease LXR $\alpha$ -dependent SREBP-1c induction, leading to reduced fatty acid synthesis. The lack of increase in SREBP-1c level by HU210 or ACEA treatment alone may be due to weak activity of endogenous LXR $\alpha$  ligand (De Gottardi et al., 2010). Without LXR $\alpha$  ligand, CB1-mediated signal transduction might not be sufficient to activate LXR $\alpha$ . Also, the CB1 agonist treatment did not potentiate the lipogenic effect of either 0.1  $\mu$ M or 1  $\mu$ M T090: this might result from limiting activity of downstream molecule(s).

## MOL #72769

CB1 belongs to the superfamily of GPCRs. Ligand activation of CB1 transmits signals to the G proteins of the  $G\alpha_{i/o}$  family (Howlett et al., 1986). Agonist-stimulated CB1 signal transduction pathway results in the inhibition of adenylyl cyclase activity through  $G\alpha_{i/o}$  proteins (Howlett et al., 1986). An important finding of the current study is the identification of  $G\alpha_{i/o}$  inhibition, especially  $G\alpha_i$  inhibition, as a modulating step that prevents LXR $\alpha$ -dependent steatosis. This hypothesis was strengthened by not only the repression of SREBP-1c after either  $G\alpha_i$  knockdown or chemical inhibition of  $G\alpha_{i/o}$ , but the reversal of rimonabant's effect by CB1 agonist that stimulates  $G\alpha_{i/o}$  (Glass and Northup, 1999).

Inhibition of adenylyl cyclase by  $G\alpha_{i/o}$  activation decreases cellular cAMP content in most tissues and cells (Neves et al., 2001). On the contrary, an increase in cAMP content by  $G\alpha_{i/o}$  inhibition contributed to the anti-lipogenic effect of rimonabant in the present study, as supported by SREBP-1c repression after forskolin or 8-Br-cAMP treatment. It is well recognized that cAMP activates PKA, a major protein kinase involved in energy-metabolic pathways (e.g. lipid metabolism). As expected, our data shown here demonstrated the *bona fide* activation of PKA after rimonabant treatment, which agrees with the reports shown in the experiments using muscle or endothelial cells (Esposito et al., 2008; Huang et al., 2010). PKA directly phosphorylates LXR $\alpha$  at serine residue, and impairs LXR $\alpha$ -DNA binding by inhibiting LXR $\alpha$  and retinoid X receptor dimerization. Thus, PKA activation suppresses SREBP-1c induction by LXR $\alpha$  both *in vitro* and *in vivo* (Yamamoto et al., 2007). An important aspect of our finding is the identification of rimonabant's activation of PKA for the inhibition of LXR $\alpha$ -mediated SREBP-1c induction. This concept was also strengthened by our observation that rimonabant enhanced LXR $\alpha$  serine phosphorylation through PKA.

LKB1 activates several kinases in the cell (Hawley et al., 2003). Another novel finding of our study is the activation of LKB1 by rimonabant, as supported by our finding that rimonabant treatment decreased lysine acetylation of LKB1 with an increase in its CAB39 binding. SIRT1



## MOL #72769

regulates LKB1 deacetylation, and thereby promotes its translocation to the cytoplasm and binding with STRAD and CAB39 (Boudeau et al., 2003; Lan et al., 2008). This complex formation then enhances the catalytic activity of LKB1. PKA is known as an upstream kinase of LKB1 (Sapkota et al., 2001). Our results also provided evidence that a deficiency in PKA prevented LKB1 activation by rimonabant, corroborating the regulatory effect of PKA on LKB1. Poly(ADP-ribose) polymerase is another possible regulator of LKB1 (Huang et al., 2009). In an additional experiment, we found that rimonabant failed to change the level of poly(ADP-ribose) polymerase (data not shown). Hence, it is highly likely that PKA activation by rimonabant contributes to LKB1 activation.

LKB1 and CaMKK are the two main upstream kinases of AMPK (Hawley et al., 2003; Hawley et al., 2005). LKB1-dependent AMPK activation by a natural product, ajoene, contributed to treating high fat diet-induced hepatic steatosis (Han et al., 2011). The present finding also showed that LKB1 was necessary for the activation of AMPK by rimonabant, which was verified by the reversal of this effect by LKB1 knockdown. AMPK plays a role in lipid metabolism; it reduces fatty acid synthesis by down-regulating SREBP-1c and its target genes (Zhou et al., 2001). Cannabinoids that activate CB1 inhibited the activity of AMPK in the liver and stimulated lipogenic and diabetogenic effects (Kola et al., 2005). In an ob/ob mouse model, rimonabant increased hepatic AMPK activity, and enhanced the synthesis of adiponectin, a hormone that possibly activates AMPK (Watanabe et al., 2009). Hwang et al. showed an opposite role of AMPK and p70 ribosomal S6 kinase-1 (S6K1) in regulating LXR $\alpha$  activity: the inhibitory and activating phosphorylations of LXR $\alpha$  were mediated by AMPK and S6K1, respectively (Hwang et al., 2009). The role of AMPK in SREBP-1c repression by rimonabant was confirmed by our finding that AMPK inhibition reversed the repressing effect of rimonabant on LXR $\alpha$  and SREBP-1c. However, overexpression of activated mutant of S6K1 unaffected it (data not shown), suggesting that the anti-lipogenic effect of rimonabant may stem from LKB1-dependent AMPK activation, but not S6K1 inhibition.

## MOL #72769

In our study, PKA was activated at an early time (i.e., 30 min), which may also be directly responsible for rimonabant's anti-lipogenic effect via the inhibitory phosphorylation of LXR $\alpha$  at serine (i.e., two consensus PKA target sites at serine 195/196 and serine 290/291). PKA activation by isoproterenol leads to AMPK phosphorylation at Ser173 at early phase (0.5~2 h), and delayed AMPK phosphorylation at Thr172 (Djouder et al., 2010). The phosphorylation of AMPK at Ser173 by PKA precludes AMPK phosphorylation at Thr172 by LKB1 at the early phase. We found that AMPK phosphorylation occurred at later times (3~12 h) following activating phosphorylation of LKB1 (0.5~12 h) after rimonabant treatment. AMPK directly phosphorylates LXR $\alpha$  at threonine residue and thereby inhibits LXR $\alpha$  activity (Hwahng et al., 2009). So, rimonabant's inhibition of lipogenesis at an early phase may be directly controlled by PKA, whereas that at the later phase may be done by AMPK downstream of PKA-LKB1 axis. Overall, our finding identifies that the anti-lipogenic effect of rimonabant in hepatocytes may rely on the activation of PKA, a kinase that on one hand directly inhibits LXR $\alpha$  via serine phosphorylation, and on the other hand stimulates LKB1 for AMPK activation; the activated AMPK would then inhibit LXR $\alpha$  for a prolonged period of time through threonine phosphorylation.

In conclusion, we found that rimonabant has the ability to inhibit lipogenesis in hepatocytes, which may result from the inhibition of LXR $\alpha$ -dependent SREBP-1c induction, as mediated by LKB1-AMPK activation downstream of CB1-coupled G $\alpha_{i/o}$ -PKA axis (Fig. 7). Collectively, the current study brings additional information on the pharmacology and mechanistic basis of CB1 inverse agonist, providing insight in determining the use of CB1 antagonism for steatosis in hepatocytes.

**MOL #72769**

### **Authorship Contributions**

***Participated in research design:*** Hong Min Wu, Yoon Mee Yang and Sang Geon Kim.

***Conducted experiments:*** Hong Min Wu and Yoon Mee Yang.

***Performed data analysis:*** Hong Min Wu, Yoon Mee Yang and Sang Geon Kim.

***Wrote or contributed to the writing of the manuscript:*** Hong Min Wu, Yoon Mee Yang and Sang Geon Kim.

**MOL #72769**

## References

- Alexander CM, Landsman PB, Teutsch SM, and Haffner SM (2003) Third National Health and Nutrition Examination Survey (NHANES III); National Cholesterol Education Program (NCEP). NCEP-defined metabolic syndrome, diabetes, and prevalence of coronary heart disease among NHANES III participants age 50 years and older. *Diabetes* **52**:1210-1214.
- Bae EJ, Yang YM, Kim JW, and Kim SG (2007) Identification of a novel class of dithiolethiones that prevent hepatic insulin resistance via the adenosine monophosphate-activated protein kinase-p70 ribosomal S6 kinase-1 pathway. *Hepatology* **46**:730-739.
- Bayewitch M, Avidor-Reiss T, Levy R, Barg J, Mechoulam R, and Vogel Z (1995) The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett* **375**:143-147.
- Boudeau J, Baas AF, Deak M, Morrice NA, Kieloch A, Schutkowski M, Prescott AR, Clevers HC, and Alessi DR (2003) MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *EMBO J* **22**:5102-5114.
- Collins SP, Reoma JL, Gamm DM, and Uhler MD (2000) LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo. *Biochem J* **345**:673-680.
- Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stbo NR, Van Pelt RE, Wang H, and Eckel RH (2008) The metabolic syndrome. *Endocr Rev* **29**:777-822.
- Cummings DE, Brandon EP, Planas JV, Motamed K, Idzerda RL, and McKnight GS (1996) Genetically lean mice result from targeted disruption of the RII  $\beta$  subunit of protein kinase A. *Nature* **382**:622-626.
- De Gottardi A, Spahr L, Ravier-Dall'Antonia F, and Hadengue A (2010) Cannabinoid receptor 1 and 2 agonists increase lipid accumulation in hepatocytes. *Liver Int* **30**:1482-1489.
- Després JP, Golay A, and Sjöström L (2005) Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* **353**:2121-2134.
- Djouder N, Tuerk RD, Suter M, Salvioni P, Thali RF, Scholz R, Vaahtomeri K, Auchli Y, Rechsteiner H, Brunisholz RA, et al. (2010) PKA phosphorylates and inactivates AMPKalpha to promote efficient lipolysis. *EMBO J* **29**:469-481.

**MOL #72769**

- Esposito I, Proto MC, Gazzero P, Laezza C, Miele C, Alberobello AT, D'Esposito V, Beguinot F, Formisano P, and Bifulco M (2008) The cannabinoid CB1 receptor antagonist rimonabant stimulates 2-deoxyglucose uptake in skeletal muscle cells by regulating the expression of phosphatidylinositol-3-kinase. *Mol Pharmacol* **74**:1678-1686.
- Glass M and Northup JK (1999) Agonist selective regulation of G proteins by cannabinoid CB(1) and CB(2) receptors. *Mol Pharmacol* **56**:1362-1369.
- Han CY, Ki SH, Kim YW, Noh K, Lee da Y, Kang B, Ryu JH, Jeon R, Kim EH, Hwang SJ, et al. (2011) Ajoene, a stable garlic by-product, inhibits high fat diet-induced hepatic steatosis and oxidative injury through LKB1-dependent AMPK activation. *Antioxid Redox Signal* **14**:187-202.
- Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR, and Hardie DG (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol* **2**:28.
- Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, and Hardie DG (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* **2**:9-19.
- Hollander PA, Amod A, Litwak LE, and Chaudhari U (2010) Effect of rimonabant on glycemic control in insulin-treated type 2 diabetes: the ARPEGGIO trial. *Diabetes Care* **33**:605-607.
- Howlett AC, Qualy JM, and Khachatrian LL (1986) Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* **29**:307-313.
- Huang NL, Juang JM, Wang YH, Hsueh CH, Liang YJ, Lin JL, Tsai CT, and Lai LP (2010) Rimonabant inhibits TNF- $\alpha$ -induced endothelial IL-6 secretion via CB1 receptor and cAMP-dependent protein kinase pathway. *Acta Pharmacol Sin* **31**:1447-1453.
- Huang Q, Wu YT, Tan HL, Ong CN, and Shen HM (2009) A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress. *Cell Death Differ* **16**:264-277.
- Hwahng SH, Ki SH, Bae EJ, Kim HE, and Kim SG (2009) Role of adenosine monophosphate-activated protein kinase-p70 ribosomal S6 kinase-1 pathway in repression of liver X receptor-alpha-dependent lipogenic gene induction and hepatic steatosis by a novel class of dithiolethiones. *Hepatology* **49**:1913-1925.

**MOL #72769**

- Jeong WI, Osei-Hyiaman D, Park O, Liu J, Bátkai S, Mukhopadhyay P, Horiguchi N, Harvey-White J, Marsicano G, Lutz B, et al. (2008) Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. *Cell Metab* **7**:227-235.
- Jourdan T, Djaouti L, Demizieux L, Gresti J, Vergès B, and Degrace P (2010) CB1 antagonism exerts specific molecular effects on visceral and subcutaneous fat and reverses liver steatosis in diet-induced obese mice. *Diabetes* **59**:926-934.
- Kola B, Hubina E, Tucci SA, Kirkham TC, Garcia EA, Mitchell SE, Williams LM, Hawley SA, Hardie DG, Grossman AB, et al. (2005) Cannabinoids and ghrelin have both central and peripheral metabolic and cardiac effects via AMP-activated protein kinase. *J Biol Chem* **280**:25196-25201.
- Kotronen A and Yki-Järvinen H (2008) Fatty liver: A novel component of the metabolic syndrome. *Arterioscler Thromb Vasc Biol* **28**:27-38.
- Laffitte BA, Joseph SB, Walczak R, Pei L, Wilpitz DC, Collins JL, and Tontonoz P (2001) Autoregulation of the human liver X receptor alpha promoter. *Mol Cell Biol* **21**:7558-7568.
- Lan F, Cacicedo JM, Ruderman N, and Ido Y (2008) SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. *J Biol Chem* **283**:27628-27635.
- Liu YL, Connoley IP, Wilson CA, and Stock MJ (2005) Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obes (Lond)* **29**:183-187
- Lu M and Shyy JY (2006) Sterol regulatory element-binding protein 1 is negatively modulated by PKA phosphorylation. *Am J Physiol Cell Physiol* **290**:C1477-C1486.
- Marra F, Gastaldelli A, Svegliati Baroni G, Tell G, and Tiribelli C (2008) Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis. *Trends Mol Med* **14**:72-81.
- McKnight GS, Cummings DE, Amieux PS, Sikorski MA, Brandon EP, Planas JV, Motamed K, and Idzerda RL (1998) Cyclic AMP, PKA, and the physiological regulation of adiposity. *Recent Prog Horm Res* **53**:139-159.
- Mitro N, Vargas L, Romeo R, Koder A, and Saez E (2007) T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS Lett* **581**:1721-1726.

**MOL #72769**

- Motoshima H, Goldstein BJ, Igata M, and Araki E (2006) AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol* **574**:63-71.
- Nathan PJ, O'Neill BV, Napolitano A, and Bullmore ET (2010) Neuropsychiatric adverse effects of centrally acting antiobesity drugs. *CNS Neurosci Ther* in press.
- Neves SR, Ram PT, and Iyengar R (2001) G protein pathways. *Science* **296**:1636-1639.
- Ntambi JM (1999) Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J Lipid Res* **40**:1549-1558.
- Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, Harvey-White J, Mackie K, Offertáler L, Wang L, et al. (2005) Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* **115**:1298-1305.
- Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, and Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14**:2819-2830.
- Sakoda H, Ogihara T, Anai M, Fujishiro M, Ono H, Onishi Y, Katagiri H, Abe M, Fukushima Y, Shojima N, et al. (2002) Activation of AMPK is essential for AICAR-induced glucose uptake by skeletal muscle but not adipocytes. *Am J Physiol Endocrinol Metab* **282**:E1239-E1244.
- Sapkota GP, Kieloch A, Lizcano JM, Lain S, Arthur JS, Williams MR, Morrice N, Deak M, and Alessi DR (2001) Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at ser431 by p90RSK and cAMP-dependent protein kinase, but not its farnesylation at Cys433, is essential for LKB1 to suppress cell growth. *J Biol Chem* **276**:19469-19482.
- Scheen AJ, Finer N, Hollander P, Jensen MD, and Van Gaal LF (2006) Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomized controlled study. *Lancet* **368**:1660-1672.
- Schreyer SA, Cummings DE, McKnight GS, and LeBoeuf RC (2001) Mutation of the RII subunit of protein kinase A prevents diet-induced insulin resistance and dyslipidemia in mice. *Diabetes* **50**:2555-2562.
- Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, et al. (2000) Role of LXRs in control of lipogenesis. *Genes Dev* **14**:2831-2838.

**MOL #72769**

- Stoeckman AK and Towle HC (2002) The role of SREBP-1c in nutritional regulation of lipogenic enzyme gene expression. *J Biol Chem* **277**:27029-27035.
- Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, and Rössner S; RIO-Europe Study Group (2005) Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. *Lancet* **365**:1389-1397.
- Watanabe T, Kubota N, Ohsugi M, Kubota T, Takamoto I, Iwabu M, Awazawa M, Katsuyama H, Hasegawa C, Tokuyama K, et al. (2009) Rimonabant ameliorates insulin resistance via both adiponectin-dependent and adiponectin-independent pathways. *J Biol Chem* **284**:1803-1812.
- Yamamoto T, Shimano H, Inoue N, Nakagawa Y, Matsuzaka T, Takahashi A, Yahagi N, Sone H, Suzuki H, Toyoshima H, et al. (2007) Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of liver X receptor in the liver. *J Biol Chem* **282**:11687-11695.
- Yan ZC, Liu DY, Zhang LL, Shen CY, Ma QL, Cao TB, Wang LJ, Nie H, Zidek W, Tepel M, et al. (2007) Exercise reduces adipose tissue via cannabinoid receptor type 1 which is regulated by peroxisome proliferator-activated receptor-delta. *Biochem Biophys Res Commun* **354**:427-433.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**:1167-1174.



**MOL #72769**

### **Footnotes**

This work was supported by the National Research Foundation of Korea grant funded by the Korea government (Ministry of Education, Science and Technology Development) [Grant 2011-0001204].

<sup>1</sup> Wu HM and Yang YM contributed equally to this work.

MOL #72769

### Legends for Figures

**Fig. 1.** Effect of rimonabant on LXR $\alpha$  and SREBP-1c activation. A, Inhibition of LXR $\alpha$  activity. The relative LXRE luciferase activity (left) was measured on the lysates of HepG2 cells transfected with an LXRE-luciferase construct and treated with different treatment combinations (vehicle, or 1 or 10  $\mu$ M rimonabant for 13 h; 1  $\mu$ M T090 for 12 h). LXR $\alpha$  transcript levels were analyzed using real-time PCR assays (right). B, SREBP-1c repression by rimonabant. SREBP-1c was assessed by immunoblottings. C, Real-time PCR assays for SREBP-1c mRNA. D, Rimonabant inhibition of SREBP-1c induction by GW3965. SREBP-1c was immunoblotted in the lysates of cells treated with 10  $\mu$ M rimonabant for 1 h and continuously exposed to 5  $\mu$ M GW3965 for 12 h. E, SREBP-1c repression by AM251. Cells were treated with 1  $\mu$ M rimonabant or 1  $\mu$ M AM251 for 1 h, and continuously exposed to 1  $\mu$ M T090 for 12 h. F, Reversal by CB1 agonists of rimonabant's repression of SREBP-1c. HepG2 cells were treated with 100 nM Hu-210 or 10 nM ACEA for 1 h, and subjected to different treatment combinations. Data represent the mean  $\pm$  SE for at least three separate experiments. For LXRE luciferase activity assay and all immunoblots, the statistical significance of differences between each treatment group and control (\*\*p<0.01) or T090 or GW3965 alone (<sup>#</sup>p<0.05, <sup>##</sup>p<0.01) was determined. For all real-time PCR assays, the mRNA level of GAPDH was used as a reference for data normalization. Fold changes were calculated by correlation coefficients of crossing point (Cp) for triplicate PCR results. Statistical significance of differences between each treatment group and the control (\*\*p<0.01) or T090 alone (<sup>#</sup>p<0.05, <sup>##</sup>p<0.01) was determined.

Rimo, rimonabant

**Fig. 2.** Inhibition of SREBP-1c target gene induction by rimonabant. A, Rimonabant inhibition of FAS gene transactivation. FAS luciferase assays were performed on the lysates of HepG2 cells subjected to various treatment combinations (vehicle, 1  $\mu$ M or 10  $\mu$ M rimonabant for 13 h

## MOL #72769

and/or 1  $\mu$ M T090 for 12 h). B, Real-time PCR assays. The transcripts of lipogenic genes were analyzed by real-time PCR assays, with the mRNA level of GAPDH used as a reference for data normalization. Data represent the means  $\pm$  SE of four separate experiments. For both A and B, statistical significance of differences between each treatment group and the control (\*\* $p < 0.01$ ) or T090 alone ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ) was determined. Rimo, rimonabant.

**Fig. 3.** Role of  $G\alpha_{i/o}$ -cAMP-PKA axis in SREBP-1c repression by rimonabant. A, Effect of  $G\alpha_{i/o}$  inhibition on SREBP-1c expression. HepG2 cells were treated with 1  $\mu$ M T090 for 12 h after  $G\alpha_i$  siRNA transfection (100 pmol/ml) for 48 h (left) or 100 ng/ml pertussis toxin (PTX) treatment for 1 h (right). SREBP-1c was immunoblotted on the cell lysates. B, SREBP-1c repression by cAMP. Cells were treated with 1  $\mu$ M T090 after incubation with forskolin or 8-Br-cAMP for 1 h. C, The effect of rimonabant on PKA activity. PKA activity was determined 30 min after treatment of cells with 10  $\mu$ M rimonabant, rimonabant plus H89 (1  $\mu$ M, 30 min pretreatment) or rimonabant plus PKI (10  $\mu$ M, 30 min pretreatment). Data represent the mean  $\pm$  SE for at least three separate experiments. For both A and B, statistical significance of differences between each treatment group and the control (\*\* $p < 0.01$ ) or T090 alone ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ) was determined. For PKA activity assay, statistical significance of differences between each treatment group and the control (\*\* $p < 0.01$ ) or rimonabant alone ( $^{\#\#}p < 0.01$ ) was determined. Rimo, rimonabant; siCON, control siRNA; and si $G\alpha_i$ ,  $G\alpha_i$  siRNA

**Fig. 4.** Role of PKA in the repression of LXR $\alpha$ -SREBP-1c by rimonabant. A, Effect of PKA on serine phosphorylation of LXR $\alpha$  by rimonabant. HepG2 cells were treated with vehicle or 10  $\mu$ M rimonabant after siPKA transfection (100 pmol/ml) for 48 h or 1  $\mu$ M H89 treatment for 1 h. LXR $\alpha$  immunoprecipitates were immunoblotted with anti-phosphorylated serine antibody. B, Effect of PKA inhibition on LXR $\alpha$  repression by rimonabant. LXRE luciferase activity was

**MOL #72769**

measured on the lysates of HepG2 cells treated with different treatment combinations after 1  $\mu$ M H89 treatment for 30 min. C, Reversal by PKA inhibition of rimonabant repression of SREBP-1c. Immunoblottings were performed on the lysates of cells treated with 1  $\mu$ M T090 or 1  $\mu$ M T090 plus 10  $\mu$ M rimonabant for 12 h following PKA knockdown (100 pmol/ml, 48 h) or H89 treatment. Data represent the mean  $\pm$  SE for at least three separate experiments. The statistical significance of differences between each treatment group and control (\*\*p<0.01) or T090 alone (<sup>##</sup>p<0.01) was determined. Rimo, rimonabant; siCON, control siRNA; and siPKA, PKA siRNA

**Fig. 5.** Role of PKA in the activation of LKB1 by rimonabant. A, LKB1 phosphorylation by rimonabant. Phosphorylated or total form of LKB1 was immunoblotted on the lysates of cells treated with 10  $\mu$ M rimonabant for the indicated time periods. B, LKB1 deacetylation and binding with CAB39 by rimonabant. LKB1 immunoprecipitates were immunoblotted with either anti-acetylated-lysine antibody or anti-CAB39 antibody. C, Effect of PKA knockdown on LKB1 phosphorylation. HepG2 cells were treated with 10  $\mu$ M rimonabant for 3 h following siRNA knockdown (100 pmol/ml, 48 h). D, Effect of H89 on LKB1 phosphorylation. Cells were treated with 1  $\mu$ M H89 for 30 min, and were continuously incubated with 10  $\mu$ M rimonabant for 3 h. For all immunoblottings, data represent the mean  $\pm$  SE for at least three separate experiments (significant compared with control, \*\*p<0.01). Rimo, rimonabant; siCON, control siRNA; and siPKA, PKA siRNA

**Fig. 6.** LKB1 regulation of AMPK for the repression of LXR $\alpha$  and SREBP-1c. A, AMPK activation by rimonabant. B, LKB1-dependent AMPK activation. HepG2 were treated with 10  $\mu$ M rimonabant for 6 h after transfection of control siRNA or siRNA directed against LKB1

## MOL #72769

(100 pmol/ml, 48 h). HeLa cells were treated with 10  $\mu$ M rimonabant for 6 h. C, The effect of CaMKK $\beta$  inhibition on the activation of AMPK. Cells were treated with 1  $\mu$ g/ml STO-609 for 30 min, and were continuously incubated with 10  $\mu$ M rimonabant for 6 h. Inset, immunoblottings of CaMKK immunoprecipitates for phospho-serine or phospho-threonine confirmed the inhibition of CaMKK activity by STO-609. D, Effect of DN-AMPK transfection or PKA knockdown (100 pmol/ml, 48 h) on LXR $\alpha$  phosphorylation by rimonabant. LXR $\alpha$  immunoprecipitates were immunoblotted with anti-phosphorylated threonine or anti-phosphorylated serine antibody. Immunoblottings for c-myc confirmed DN-AMPK overexpression. E, Effects of AMPK inhibition on SREBP-1c repression by rimonabant. Immunoblottings for SREBP-1c were performed on the lysates of HepG2 cells treated with 1  $\mu$ M T090 or 1  $\mu$ M T090 plus 10  $\mu$ M rimonabant for 12 h following DN-AMPK transfection or 5  $\mu$ M compound C treatment. For all immunoblottings, data represent the mean  $\pm$  SE for at least three separate experiments. The statistical significance of differences between each treatment group and control (\* $p$ <0.05, \*\* $p$ <0.01) or T090 alone (<sup>##</sup> $p$ <0.01) was determined. Rimo, rimonabant; siCON, control siRNA; siPKA, PKA siRNA; and siLKB1, LKB1 siRNA

**Fig. 7.** A schematic diagram illustrating the proposed mechanism by which rimonabant inhibits LXR $\alpha$ –SREBP-1c-mediated steatosis in hepatocytes.

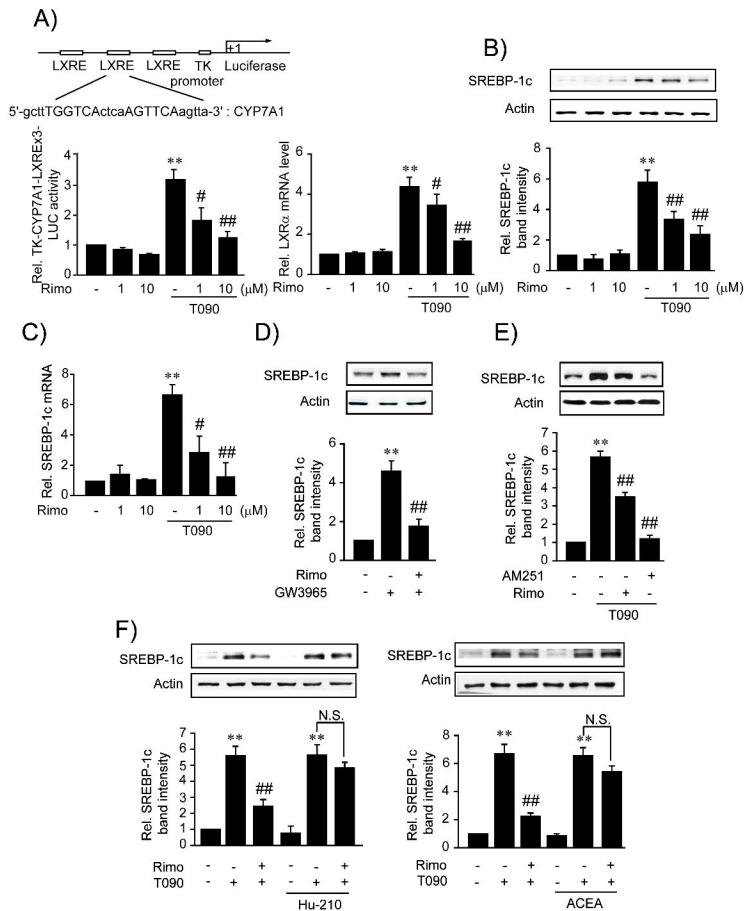


Fig. 1

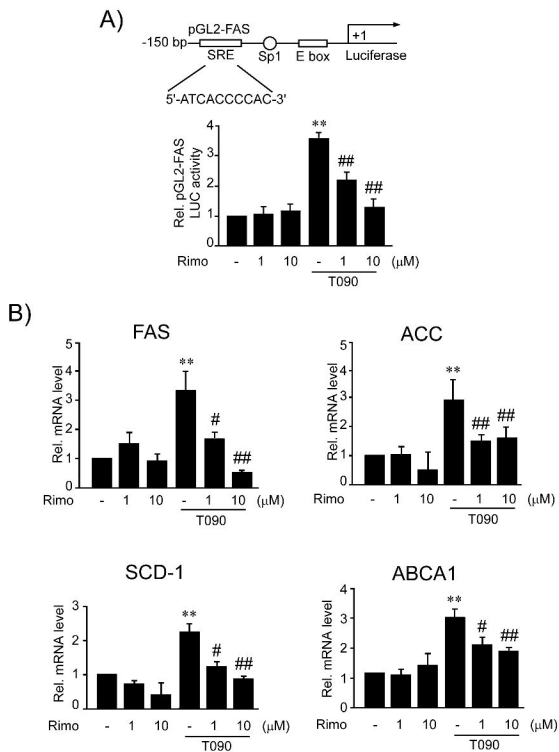


Fig. 2

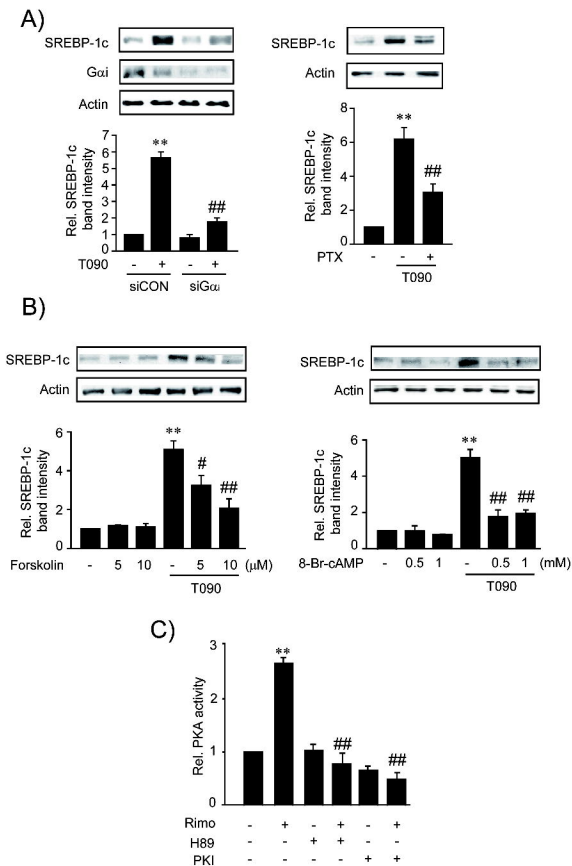


Fig. 3



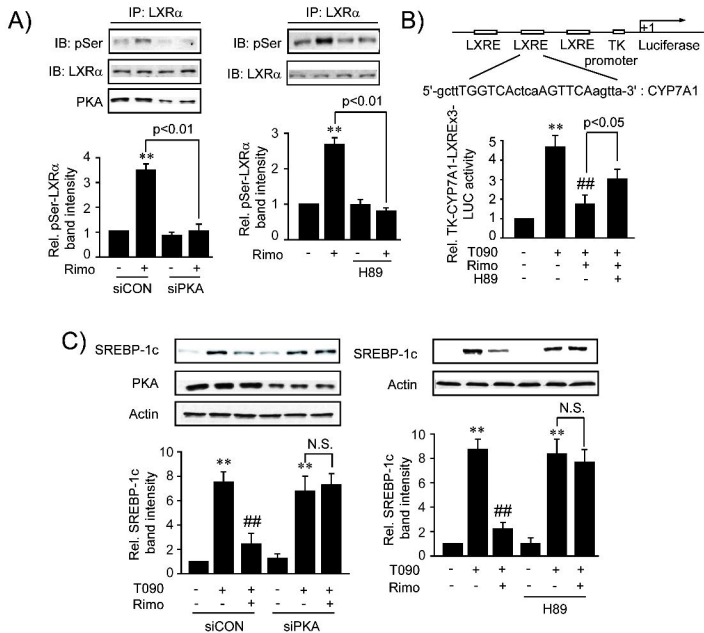


Fig. 4

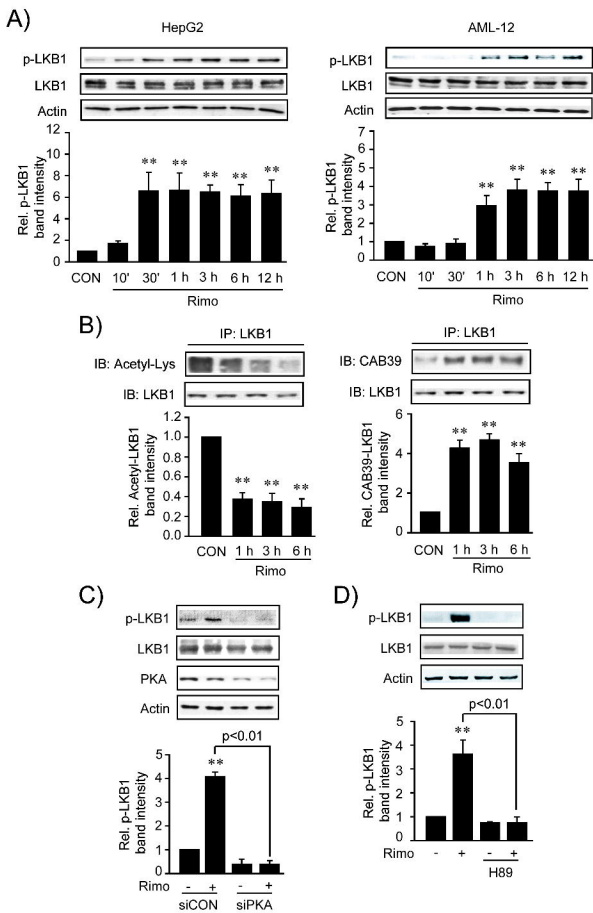


Fig. 5

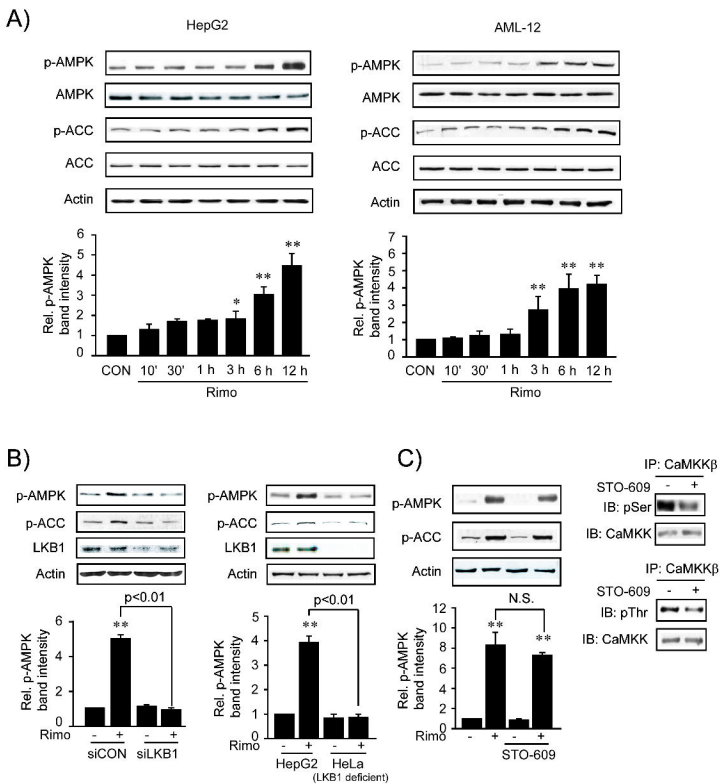
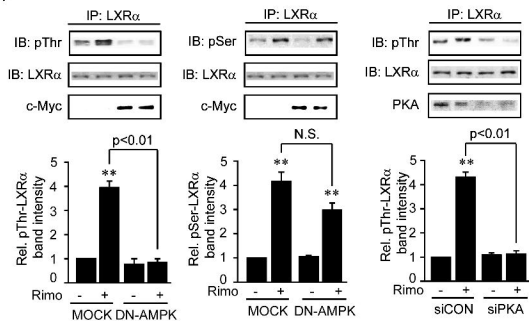


Fig. 6

D)



E)

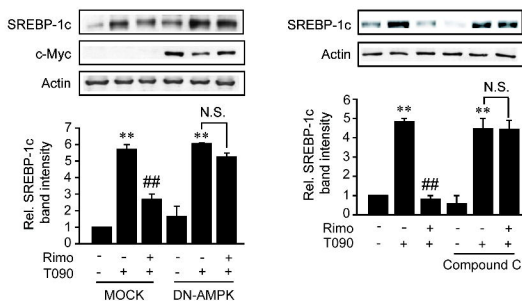


Fig. 6 (Continued)

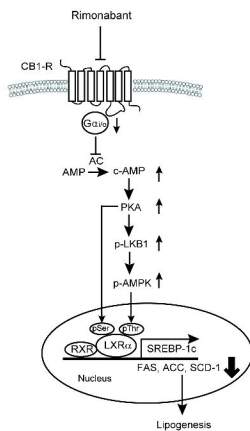


Fig. 7