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

Seong Hwan Hwahng,* Sung Hwan Ki,* Eun Ju Bae, Hyun Eun Kim, and Sang Geon Kim

Dithiolethiones, a novel class of adenosine monophosphate-activated protein kinase (AMPK) activators, prevent insulin resistance through AMPK-dependent p70 ribosomal S6 kinase-1 (S6K1) inhibition. There is no known effect of S6K1 for liver X receptor-alpha (LXRα)-mediated lipogenic gene expression and steatosis, a cause of chronic liver disease. This study investigated the role of S6K1 in LXRα activation and the effects of oltipraz (prototype) and other dithiolethiones on LXRα-dependent lipogenesis in hepatocytes and high-fat diet animal model. Oltipraz prevented the ability of LXRα agonist (T0901317) to activate sterol regulatory element binding protein-1c (SREBP-1c), inhibiting its own mRNA and protein induction. Impaired SREBP-1c activity by oltipraz caused inhibition of LXRα-induced transcription of the fatty acid synthase, LXRα, acetyl-CoA carboxylase, stearoyl-CoA desaturase-1, and adenosine triphosphate-binding cassette transporter A1 genes. S6K1 activation antagonized the inhibitory effect of oltipraz on SREBP-1c activation, whereas dominant negative (DN) mutant S6K1 and rapamycin inhibited the T0901317-induced SREBP-1c expression. Oltipraz impaired LXRα DNA binding activity and LXR agonist-induced CYP7A1-LXRE-luciferase (CYP7A1) transactivation. Moreover, in vitro S6K1 directly phosphorylated LXRα at serine residues for gene transactivation, which was antagonized by its DN mutant. S6K1 inhibition antagonized CYP7A1 induction promoted by AMPK inhibition, whereas AMPK activation abrogated S6K1-dependent CYP7A1 induction, supporting the opposing role of S6K1 and AMPK in LXR activity. Finally, oltipraz was found to inhibit hepatic triglyceride accumulation and lipogenic gene induction in mice fed a high-fat diet. Other dithiolethiones also inhibited SREBP-1c induction by T0901317. Conclusion: Our findings showing the role of AMPK-S6K1 pathway in LXR activity and S6K1-dependent inhibition of LXRα-induced lipogenic gene transactivation by a novel class of dithiolethiones led to the identification of S6K1 as a particularly attractive target for intervention in hepatic steatosis. (HEPATOLOGY 2009;49:1913-1925.)

The metabolic syndrome is defined as a constellation of impaired glucose metabolism, dyslipidemia, hypertension, and central obesity, and may lead to type 2 diabetes mellitus and cardiovascular diseases. Because nonalcoholic fatty liver disease is closely associated with metabolic syndrome and insulin resistance, this disease may represent the hepatic component of metabolic syndrome. Among the conditions grouped within metabolic syndrome, hyperinsulinemia results in the elevated lipogenesis of fatty acids and triglycerides (TGs) in the liver. In the regulation of lipogenesis, the nuclear hormone receptor liver X receptor-alpha (LXRα)
serves as a lipid sensor that enhances fatty acid synthesis and hypertriglyceridemia\(^1\,\,^2\) and that mediates insulin-stimulated lipogenesis.\(^3\)

LXR\(\alpha\) promotes both fatty acid and cholesterol metabolism because this receptor regulates the expression of lipogenic genes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), and the expression of cholesterol transporter genes.\(^4\) An important target gene of LXR\(\alpha\) is the sterol regulatory element binding protein-1c (SREBP-1c).\(^5\) LXR\(\alpha\)-activation of SREBP-1c gene expression may enhance the conversion of glucose to fatty acids, thus reducing hyperglycemia.\(^1\) However, administration to mice of a chemical agonist of LXR results in increases in fatty acid synthesis and steatosis in the liver, leading to the increased secretion of low-density lipoproteins and to hypertriglyceridemia.\(^6\) Apparently, the deleterious effects of chemical agonism of LXR result from the induction of the SREBP-1c gene.\(^5\) Moreover, the chronic activation of LXR\(\alpha/\beta\) can lead to beta-cell apoptosis, which might be mediated by the hyperactivation of lipogenesis.\(^7\) To avoid these undesired effects, methods to inhibit LXR\(\alpha\) activity show promise for the treatment of hepatic steatosis.

In order to find ways to alter LXR\(\alpha\) activity, the interacting regulatory mechanisms influencing LXR\(\alpha\) must be better understood. Pharmacological treatments of fatty liver disease have identified two kinases involved in the process of insulin-sensitization: adenosine monophosphate-activated protein kinase (AMPK) and p70 ribosomal S6 kinase-1 (S6K1). First, the biochemical actions of metformin and glitazones converge on AMPK, a metabolic energy sensor that may contribute to insulin sensitivity enhancement.\(^8\) Because AMPK is involved in the degradation of SREBP-1c,\(^8\) AMPK may negatively regulate the LXR\(\alpha\)-SREBP-1c pathway. Second, S6K1 is implicated because the mammalian target of rapamycin (mTOR)-S6K1 pathway underlies the emergence of insulin resistance. Moreover, the regulatory pathways for these kinases may not be independent: the activation of AMPK inhibits the mTOR-S6K1 pathway.\(^10\) Thus, the possible inhibitory effect of AMPK on the LXR\(\alpha\)-SREBP-1c pathway may be opposed by the action of S6K1. Presently, no therapy targeting S6K1 with the concurrent ability to activate AMPK is currently available for liver steatosis.

One potential candidate is 4-methyl-5-(2-pyrazinyl)-1,2-dithiol-3-thione (oltipraz), an investigational drug for the treatment of liver cirrhosis as well as cancer chemoprevention.\(^11\) Previous work on an S6K1 knockout model suggested a crucial role of S6K1 in insulin resistance\(^12\). Our laboratory found that the modulation of S6K1 by oltipraz inhibited the development of insulin resistance and hyperglycemia through the AMPK-S6K1 pathway.\(^13\)

In addition, the inhibition of S6K1 in combination with the activation of AMPK was feasible \textit{in vivo} using dithiolethiones, a new class of AMPK activators. Although S6K1 has been identified as an attractive target for treating insulin resistance, the functional role of AMPK-S6K1 pathway in LXR\(\alpha\) activity and LXR\(\alpha\)-dependent lipogenesis in the liver has not been explored. Furthermore, because LXR\(\alpha\)-mediated increases in SREBP-1c promote the expression of lipogenic genes and enhance fatty acid synthesis, this study investigates the effects of dithiolethiones, a novel class of AMPK activators, on LXR\(\alpha\)-dependent lipogenesis.

### Materials and Methods

#### Materials

Information on the materials used in this study is given in the Supporting Information.

#### Cell Culture

H4IIE and HepG2 cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA). Primary hepatocytes were isolated from male Sprague-Dawley rats.\(^11\) Cells were plated at \(1 \times 10^5\) per well in six-well plates, and wells with 70%-80% confluency were used. All cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Total cell lysates and nuclear extracts were prepared as previously described.\(^13\,\,^14\)

#### Animal Treatments

Animal studies were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Male C57BL/6 mice were obtained from Charles River Orient (Seoul, Korea) and were acclimatized for 1 week in a clean room at the Ani-
mal Center for Pharmaceutical Research, Seoul National University. At 6 weeks of age, C57BL/6 mice were started on either a normal diet or a high-fat diet (HFD) (Dyets, Bethlehem, PA) for 10 weeks. The HFD mice then were distributed into three treatment groups. Oltipraz (10 or 30 mg/kg) dissolved in 40% polyethyleneglycol 400 was orally administered to mice three times per week during the last 4 weeks of diet feeding. Control animals (n = 8) received vehicle only.

**Descriptions of Laboratory Assays and Procedures.** Detailed descriptions of all laboratory assays and procedures are contained in the Supporting Information.

**Gel Shift Assay.** LXRα-retinoid X receptor-α (RXRα)-DNA complex formation was assessed by gel shift assay using a DNA probe for the LXRα response element (LXRE) of the SREBP-1c gene and sterol regulatory element (SRE) of the FAS gene.

**In Vitro Kinase Assay.** Recombinant AMPK or S6K1, LXRα protein, and [32P]ATP were mixed and were incubated in a kinase buffer. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, the phosphorylated proteins were visualized by autoradiography.

**In Cell Kinase Assay.** HepG2 cells were transfected with a control or an LXRα overexpression vector in combination with constitutively active (CA)-S6K1, dominant negative (DN)-S6K1, CA-AMPK, or DN-AMPK. LXRα phosphorylation by S6K1 or AMPK was visualized by autoradiography.

**Immunoprecipitation and Immunoblot Analysis.** Immunoprecipitation and immunoblotting procedures are given in the Supporting Information. For the assessment of serine or threonine phosphorylation of LXRα, cells were transfected with plasmids encoding LXRα and CA-S6K1, DN-S6K1, CA-AMPK, or DN-AMPK. LXRα phosphorylation by S6K1 or AMPK was visualized by autoradiography.

**Chromatin Immunoprecipitation (ChIP) Assay.** ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY).

**RNA Isolation and Real-Time Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis.** Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and was reverse-transcribed. The resulting cDNA was amplified by PCR. Real-time RT-PCR was performed with the Light Cycler 1.5 (Roche, Mannheim, Germany) using a Light CyclerDNA master SYBR green-I kit.

**Immunocytochemistry of SREBP-1.** To confirm the nuclear translocation of SREBP-1 protein, standard immunocytochemistry procedures were followed by counterstaining with propidium iodide.

**Transient Transfection and Reporter Gene Assay.** The sources of the vectors and the procedures used in this study for transient transfections and reporter gene assays are described in the Supporting Information.

**TG Measurement.** TG content was measured in mouse liver homogenates.

**Oil Red O Staining.** Oil Red O staining was used to visualize neutral TG and lipids in frozen sections of the left lateral lobe of the liver.

**Results**

**Inhibition of the T090-Induced Activation of SREBP-1c.** SREBP-1c is tethered to the membranes of the endoplasmic reticulum and nuclear envelope and is released as a transcription factor upon stimulation. Because elevation in the nuclear active form of SREBP-1c accounts for the up-regulation of lipogenic enzyme expression, the effect of oltipraz on nuclear SREBP-1c activation was examined. Combinatorial treatment with oltipraz prevented LXRα agonist T0901317 (T090)-associated increase in nuclear SREBP-1c content (Fig. 1A). Although anti-SREBP-1 antibody binds to both SREBP-1a and -1c, the bands represented mostly SREBP-1c, the major form expressed in hepatocytes. Immunocytochemistry verified the inhibitory effect of oltipraz on T090-induced nuclear localization of SREBP-1c (Fig. 1B).

The effect of oltipraz on T090-dependent SREBP-1c induction in hepatocyte cell lines and primary rat hepatocytes was investigated because SREBP-1c induction and maturation are markers of hepatic lipogenesis. Oltipraz pretreatment markedly inhibited T090-induced increases in SREBP-1c mRNA (Fig. 1C). A similar pattern of oltipraz action was observed in the SREBP-1c protein levels measured in HepG2 and H4IIE cells and in primary hepatocytes (Fig. 1D). Moreover, pretreatment with oltipraz (1 hour) prevented the abilities of other LXR agonists, 22(R)-hydroxycholesterol and GW3965, to induce SREBP-1c expression (Fig. 1E).

The central role of LXRα in the insulin-mediated activation of both SREBP-1c transcription and fatty acid synthesis in the liver was previously established. Because SREBP-1c is an important downstream regulator of insulin signaling and lipogenic enzyme gene induction, the effect of oltipraz treatment on insulin-mediated LXRe-dependent transactivation was studied. Although insulin treatment increased SREBP-1c levels in cell lysates, oltipraz treatment inhibited SREBP-1c activation by insulin (Fig. 1F).

**Oltipraz Attenuation of SREBP-1c Target Gene Induction.** To more completely understand SREBP-1c activity in cells treated with T090 and oltipraz, FAS reporter gene assays were performed using HepG2 cells transfected with the construct containing the SRE, but
not the LXRE, in the −150 bp FAS promoter region (Fig. 2A). Exposure of the transfected cells to T090 resulted in a three-fold increase in luciferase activity, which was substantially decreased in a concentration-dependent manner by simultaneous treatment with oltipraz (Fig. 2A, left). Also, the effect of oltipraz on pGL2-FAS luciferase gene induction by ectopic SREBP-1c expression was examined. Treatment of cells with oltipraz did not significantly change the luciferase induction by SREBP-1c (Fig. 2A, right). T090 treatment of SREBP-1c-transfected HepG2 cells led to greater induction of luciferase as compared to SREBP-1c transfection alone, which was prevented by concomitant oltipraz treatment. In addition, gel shift analyses were carried out and the results corroborated those of the reporter gene assays (Fig. S1). Our data verified that oltipraz inhibition of pGL2-FAS luciferase gene induction depends on LXR activity.

As the promoter region of the LXRα gene itself contains an LXRE, induction of LXRe expression results in further LXRe induction. Therefore we next analyzed the expression of LXRe by quantitative real-time PCR assays (24 hours). Consistent with other assay results, the expression of LXRe mRNA was increased three-fold after treatment with T090 for 24 hours; this induction was
significantly inhibited by concomitant treatment of oltipraz (Fig. 2B).

Because SREBP-1c controls the transcription of genes encoding lipogenic enzymes, the inhibitory effects of oltipraz on the expression of lipogenic genes were assessed by measuring relative changes in FAS, ACC, stearoyl-CoA desaturase-1 (SCD-1), and ATP-binding cassette transporter A1 (ABCA1) mRNA levels (Fig. 2B). Oltipraz treatment attenuated the ability of T090 to stimulate each of these SREBP-1c and/or LXR target genes. As expected, oltipraz inhibited the induction of FAS and ACC proteins by T090 (Fig. 2C).

**Repression of T090-Induced SREBP-1c by S6K1 Inhibition.** Because LXRα agonist induction influences hepatic fatty acid synthesis and S6K1 activation causes insulin resistance,12 we determined whether oltipraz’s inhibition of T090-stimulated SREBP-1c activation was mediated by the inhibitory effect of oltipraz on the S6K1 pathway. When CA-S6K1 was expressed in HepG2 cells, the inhibitory effect of oltipraz on SREBP-1c activation was blocked (Fig. 3A, left). Transfection with DN-S6K1 reduced the induction of SREBP-1c expression by T090 (Fig. 3A, right). Rapamycin inhibits mTOR-S6K1 activity by binding to
FKBP12 and inducing the dissociation of mTOR-raptor complex; thus, the S6K1 pathway is rapamycin-sensitive.\textsuperscript{17-20} In this study the inhibition of S6K1 by rapamycin was confirmed by a decrease in S6 phosphorylation: these data were in parallel with the rapamycin inhibition of the T090-induced SREBP-1c expression in HepG2 cells and primary rat hepatocytes (Fig. 3B). These results indicate that oltipraz treatment antagonizes SREBP-1c activation, which is mediated at least in part by S6K1 inhibition.

**Role of AMPK in the Inhibition of T090-Stimulated SREBP-1c Induction.** Certain types of AMPK activation (e.g., oltipraz treatment) inhibit the mTOR-S6K1 pathway,\textsuperscript{10,13} whereas others do not.\textsuperscript{21} Because S6K1 is an activator of LXRα-dependent SREBP-1c induction and AMPK is an inhibitor of mTOR-mediated S6K1 action, the effect of AMPK activation by oltipraz on SREBP-1c levels in HepG2 cells was examined. The ability of oltipraz to inhibit the T090-mediated SREBP-1c induction was antagonized by DN-AMPK transfection (Fig. 4A, left). Treatment with Compound C also reversed the inhibitory effect of oltipraz on SREBP-1c activation as did the DN (Fig. 4A, middle). In addition, the introduction of CA-AMPK reduced the T090-mediated induction of SREBP-1c expression (Fig. 4A, right). Finally, to further elucidate the role of AMPK activation in SREBP-1c expression, we determined the effect of AMPK activators on SREBP-1c induction in H4IIE cells and primary rat hepatocytes (Fig. 4B). Treatment of cells with AICAR or metformin attenuated SREBP-1c induction by T090. Our results demonstrate that AMPK activation contributes to inhibition of the T090-mediated SREBP-1c activation.

**Oltipraz Inhibition of LXRα Activation and LXRα-Dependent CYP7A1 Gene Induction.** In view of the importance of LXRα in SREBP-1 expression and SREBP-1-mediated lipogenesis, we next examined the effect of oltipraz on the T090-mediated LXRα activation and the role of S6K1 and AMPK for the activation of LXRα. First, gel shift analysis of protein binding to the LXRE binding site was performed on nuclear extracts of HepG2 cells using radiolabeled LXRE binding oligonucleotides. Treatment of cells with oltipraz (3 hours) reversed the increases in LXRα-RXRα-LXRE binding that...
were observed in cells treated only with LXRα and RXRα (Fig. 5A, upper). Supershift experiments confirmed that the increase in LXRα-RXRα DNA-binding depended on both LXRα and RXRα (Fig. 5A, lower).

Next, ChIP analysis was carried out to verify recruitment of the LXRα-RXRα heterodimer to the LXRE region of either the SREBP-1c or the ABCA1 promoter. In each case, samples taken from cells treated with oltipraz showed lower levels of PCR product (Fig. 5B), confirming the inhibition of LXRα-RXRα-LXRE binding in both the SREBP-1c and ABCA1 genes. Treatment of HepG2 cells with T090 increased the transactivation of a CYP7A1-LXRE-luciferase (CYP7A1) gene: conversely, simultaneous treatment of oltipraz inhibited this effect in a concentration-dependent manner (Fig. 5C, upper). As expected, treatment of HepG2 cells with T090 also increased the transactivation of the PXR target gene (CYP3A23-PXRE-luciferase gene), which was not affected by oltipraz treatment (Fig. 5C, lower), confirming that oltipraz specifically inhibits the induction of the LXRα target gene. Furthermore, either CA-S6K1 or DN-AMPK transfection eliminated the ability of oltipraz to decrease T090-induced LXRE-luciferase gene induction (Fig. 5D).

**Direct Phosphorylations of LXRα by S6K1 and AMPK, and Their Opposing Action on LXRα Activity.** The outcome of S6K1 activity modulation on LXRα phosphorylation was determined using in vitro and in cell kinase assays. In the in vitro kinase assay, S6K1 directly phosphorylated LXRα (Fig. 6A, upper). In HepG2 cells, transfection with CA-S6K1 magnified LXRα phosphorylation, whereas transfection with DN-S6K1 completely abolished it (Fig. 6A, middle). Moreover, we found that CA-S6K1 transfection enhanced the phosphorylation of LXRα at serine residues, but DN-S6K1 transfection reduced it; modification of S6K1 activity did not affect the

Fig. 4. AMPK, whose activation leads to S6K1 inhibition, contributes to inhibit the induction of SREBP-1c by T090. (A) The role of AMPK in SREBP-1c induction by T090. SREBP-1c was immunoblotted from the lysates of cell populations treated with T090 or T090 plus oltipraz (30 μM) for 12 hours after modulation of AMPK activity. Compound C (3 μM, 1 hour pretreatment). The statistical significance of differences between each treatment group and the control (⁎P < 0.05, **P < 0.01) or T090 alone (⁎⁎P < 0.05, ⁊⁎P < 0.01) was determined.

(B) The effects of chemical activators of AMPK. H4IIE cells or primary rat hepatocytes (control or T090-stimulated) were treated with either AICAR or metformin for 12 hours.
level of phosphorylation at threonine residues (Fig. 6A, lower). Therefore, S6K1 phosphorylated LXRα at serine residues, but not threonine residues.

Next, the possible phosphorylation of LXRα by AMPK was also investigated. In an in vitro kinase assay, AMPK directly phosphorylated LXRα (Fig. 6B, upper). In the in cell kinase assay using HepG2 cells, transfection with CA-AMPK increased the level of LXRα phosphorylation, whereas transfection with DN-AMPK slightly reduced it (Fig. 6B, middle). In addition, phosphorylation of LXRα at threonine residues was enhanced by transfection with CA-AMPK, but was reduced by transfection with DN-AMPK. In contrast, modification of AMPK activity did not alter the level of phosphorylation at serine residues (Fig. 6B, lower). Therefore, AMPK phosphorylated LXRα at threonine residues, but not serine residues.

To examine the opposing effects of S6K1 and AMPK on LXRα activation, LXRα-dependent CYP7A1 transactivation was monitored. In tests of LXRα transcriptional activity, CYP7A1 transactivation was substantially higher in cells transfected with CA-S6K1, but was significantly lower in those transfected with DN-S6K1 (Fig. 6C, upper). However, AMPK caused the opposite effect on LXRα activation. In contrast, the LXRα transcriptional activity was not increased by CA-AMPK transfection, but was markedly elevated by DN-AMPK transfection (Fig. 6C, upper). Moreover, transfection with CA-AMPK attenuated in a dose-dependent manner the ability of CA-S6K1 to induce CYP7A1 gene expression (Fig. 6C, lower). Also, transfection with DN-S6K1 completely antagonized the level of CYP7A1 induction observed in cells treated with DN-AMPK alone (Fig. 6C,
lower right). Hence, S6K1 and AMPK had opposite effects on the induction of LXRα-dependent gene expression. Overall, our results demonstrate that S6K1 and AMPK oppositely regulate LXRα activity through their direct phosphorylations at different residues.

**In Vivo Effects of Oltipraz on Fatty Liver and Lipogenic Gene Induction, and the In Vitro Activities of Dithiolethione Congeners.** In animals fed an HFD, fat accumulation in the liver is associated with LXRα-dependent signaling. Here, oltipraz was administered to assess whether oltipraz had an antisteatotic effect on HFD mice. Mice given an HFD for 10 weeks exhibited hepatic fat accumulation (Fig. 7A). Oltipraz treatment during the last 4 weeks of the 10-week HFD period markedly reduced fat accumulation in the liver, indicating that oltipraz exhibited an antisteatotic effect in vivo. In addition, oltipraz reduced the liver to body weight ratio and plasma ALT activity (biomarker of nonalcoholic fatty liver disease) (Table 1). Real-time RT-PCR analysis demonstrated that oltipraz treatment inhibited HFD-induced
LXRα and SREBP-1c gene induction (Fig. 7B). Furthermore, oltipraz abolished the HFD induction of FAS and ACC mRNA in the liver.

Finally, we measured the effects of treatment with a series of dithiolethione analogs, capable of inhibiting S6K1 with AMPK activation, on SREBP-1c activation. Because H4IIE cells are the most responsive cells among the hepatocyte-derived cell lines examined, levels of SREBP-1c protein were measured in H4IIE cells treated with T090 in combination with 30 μM of each analog

*Table 1. Liver to Body Weight Ratio and Serum ALT Activity*

| Treatment                        | Liver/Body Weight (%) | ALT (U/dL) |
|---------------------------------|-----------------------|------------|
| Normal diet mice (n = 8)        | 3.05 ± 0.09           | 28.2 ± 1.8 |
| HFD mice (n = 8)                | 3.80 ± 0.13*          | 111.0 ± 10.9* |
| + Oltipraz 10 mg/kg (n = 8)     | 3.16 ± 0.13†          | 63.2 ± 6.7† |
| + Oltipraz 30 mg/kg (n = 8)     | 3.06 ± 0.09‡          | 70.2 ± 10.2‡ |

Oltipraz (10 or 30 mg/kg) was administered to mice three times per week during the last 4 weeks of diet feeding (10 weeks). The statistical significance of differences between each treatment group and normal diet (*P < 0.01) or HFD (†P < 0.05, ‡P < 0.01) was determined.
Discussion

Our work highlights the inhibitory effects of the dithiolethiones, a new class of AMPK activators, on SREBP-1 activation and LXRα-mediated SREBP-1-dependent lipogenesis. Oltipraz antagonizes SREBP-1c-dependent mechanism of LXRα-mediated lipogenic gene induction (Fig. 8). Because overexpression of CA-AMPK has a similar effect on SREBP-1c, a specific form of AMPK may be involved in the process. Flavonoids exert beneficial effects on lipid accumulation through activation of the LKB1-dependent AMPK and reactive oxygen species pathways. In comparison, dithiolethiones have no effect on LKB1 (unpubl. data), but do have antioxidant efficacy. Because AMPK may be phosphorylated by multiple kinases, dithiolethiones may have direct target(s) upstream of AMPK and S6K1. It has also been claimed that carbohydrate response element binding protein (ChREBP) may play a role as a regulator of lipid synthesis in the liver based on the finding that it is necessary for the glucose-induced expression of liver-type pyruvate kinase (L-PK) and FAS. We also examined whether T090 increases ChREBP activity, and found no changes in its expression and nuclear translocation in HepG2 cells (data not shown). Our data are consistent with the previous report and the observation that the induction of ACC and FAS in the fatty liver patients is associated with SREBP-1c, but not ChREBP. Therefore, the inhibition of SREBP-1c activation might be responsible for the repression of LXRα-mediated lipogenesis by dithiolethiones in hepatocytes.

Some of the deleterious effects seen in fatty liver disease result from the promotion of fatty acid synthesis by LXRα activation. The AMPK-S6K1 pathway plays a key role in energy metabolism, yet no study had been conducted to assess how S6K1 and AMPK function in the modulation of LXRα activity and the subsequent induction of LXRα-dependent lipogenesis. Although it has been suggested that multiple kinases might be responsible for phosphorylation of LXRα, only two have been studied in detail: casein kinase-2 and protein kinase A. LXRα is phosphorylated by casein kinase-2 at serine residues and may be phosphorylated by protein kinase A at serine residues and at threonine residues, which might lead to the inactivation of LXRα. Aside from these two examples, no other information has been available on potential LXRα kinases.

Here we provide the first evidence of two additional kinases that act directly on LXRα. First, S6K1 directly phosphorylates LXRα at serine residues, which results in LXRα-dependent gene induction. Second, AMPK also directly phosphorylates LXRα, but at threonine residues; this threonine phosphorylation is associated with LXRα inactivation. The antagonistic action of S6K1 and AMPK is supported by two findings: (1) S6K1 regulates LXRα-dependent CYP7A1 induction, which can be inhibited by the modulation of AMPK activity, and (2) DN-AMPK raises the level of LXRE-luciferase gene induction, whereas DN-S6K1 lowers induction levels. Together, these results confirm that both S6K1 and AMPK directly phosphorylate LXRα and that these two kinases affect LXRα activation in opposite ways. NanoLC-MS/MS is emerging as a tool to identify phosphorylation sites. In an additional experiment, we attempted to find the amino acid residues of LXRα phosphorylated by S6K1 or AMPK using NanoLC-MS/MS and a prediction program of NetPhos2.0 server, and extracted several putative serine and threonine sites (Ser 125 and Ser 293 for S6K1; Thr 144 for AMPK). It has been suggested that the consensus motif phosphorylated by AMPK is PXBXX(S/T)XXXP (P, a hydrophobic residue; B, a basic residue). The exact phosphorylation residues and their functional relationship with LXRα-mediated transcriptional activity should be clar-
ified using mutagenesis and molecular biological experiments in a separate study.

The stimulatory effect of S6K1 on LXRα activity identifies S6K1 as a pharmacological target for the treatment of fatty liver disease. LXRα promotes the expression of lipogenic genes through LXRE activation, as verified by the gel shift, ChIP, and luciferase reporter gene assays employed in this study. LXRXα dominantly induces SREBP-1c, which is a marker of lipogenesis in the liver. Conversely, the inhibition of S6K1 activity negatively affects LXRXα-dependent SREBP-1c regulation and lipogenesis. The regulatory mechanism of S6K1 is highly complex, involving the mTOR-S6K1 pathway. Rapamycin is a known inhibitor of this pathway: treatment of cells with this compound results in the inhibition of LXRXα agonist-induced SREBP-1c activation. Rapamycin inhibits mTOR-S6K1 activity by binding to FKBP12 and inducing the dissociation of mTOR-RICTOR complex. Prolonged treatment of HepG2 cells with rapamycin (24 hours) inhibited the phosphorylation of Akt/PKB through suppression of mTOR-RICTOR assembly, which induces Akt/PKB phosphorylation.33 Hence, mTOR-RICTOR as well as mTOR-RIaptor may have a role in the S6K1-mediated LXRXα activation.

AMPK activation also leads to the suppression of the mTOR-S6K1 pathway, by way of phosphorylation of tuberous sclerosis complex 2, a negative regulator.10,34-36 In contrast, hyperosmotic conditions activate not only AMPK, but also mTOR-S6K1; therefore, this stress condition activates S6K1 in spite of its AMPK activation. Either AICAR or metformin, representative AMPK activator, treatment decreased the expression of SREBP-1c in our hepatocytes models.37,38 The results of this current study concur with previous work and identify AMPK as another pharmacological target for the treatment of fatty liver disease: AMPK exerts inhibitory effects on LXRXα-dependent lipogenesis directly by threonine-phosphorylation of LXRXα and indirectly by regulation of the mTOR-S6K1 pathway. Our conclusion is consistent with the observation that an increase in AMPKα1 phosphorylation by microRNA-122 inhibition reduced hepatic steatosis in a mouse model (Fig. 8).39

The mechanism by which oltipraz inhibits T090-induced LXRXα activation may entail changes in the corepressor recruitment and/or coactivator replacement events that help regulate LXRXα-dependent genes. LXRXα binds to the LXRE in complex with the nuclear receptor corepressor (NCoR)40; in the absence of a ligand, NCoR plays an inhibitory role in regulating LXRXα target genes. Ligand-induced conformational change to LXRXα may facilitate the exchange of the corepressor for a CBP coactivator. Although the LXRE regions of SREBP-1a and SREBP-2 promoters bind to CBP, the LXRE of SREBP-1c does not. For this gene, a decrease in NCoR binding to the DNA leads to induction of lipogenesis. In a supplemental experiment, oltipraz recruited NCoR to the DNA transcription complex on the SREBP-1c gene (data not shown). In addition, oltipraz induces the dissociation of LXRXα from the LXRE (Fig. 5A). Both events help to explain the pharmacological manipulation of LXRXα-dependent gene regulation through oltipraz treatment.

The liver plays a central role in metabolic adaptation to the nutritional environment. Increased concentrations of glucose and insulin create the necessary conditions for the induction of genes involved in the synthesis of fatty acids from glucose, such as L-PK, FAS, ACC, SCD, and glu-cokinase.41,42 Because oltipraz and other dithiolethione compounds can inhibit the lipogenic pathway activated by a LXRXα agonist, these compounds have antisteatotic effects. First, in vivo oltipraz treatment for 4 weeks completely abolishes HFD-induced fat accumulation, confirming the pharmacological effectiveness of oltipraz for the treatment of hepatic steatosis. Second, oltipraz inhibits LXRXα, SREBP-1c, FAS, and ACC transcription in mice fed an HFD. In our experiment, the equivalent daily dose and the antisteatotic efficacy of oltipraz in the dose regimen have clinical relevance.43 Next, oltipraz treatment does not alter serum lipid levels.13 Finally, supplemental experiments indicate that oltipraz inhibits inducible TNFα and nitric oxide production in macrophages (unpublished data), which would be additionally beneficial for treating inflammation in the liver.

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