Effect of a synthetic liver X receptor agonist TO901317 on cholesterol concentration in goose primary hepatocytes

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Abstract

In this study, we investigated the role of liver X receptor (LXR) in regulating cholesterol concentration in goose primary hepatocytes. Intracellular and extracellular cholesterol concentration, mRNA expression levels of genes related to phosphatidylinositol 3-kinase (PI3K) pathway, sterol regulatory element-binding protein 2 (SREBP-2), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) were measured in primary goose hepatocytes. We found that the intracellular cholesterol concentration showed an up trend manner when the TO901317 concentration was under 1 μmol/L; compared with 1 μmol/L TO901317, 10 μmol/L TO901317 had an inhibiting effect (P<0.05). The regulation mode of SREBP-2 and HMGR gene expression is similar with that of intracellular cholesterol concentration. These data suggested that LXR activation may stimulate the cholesterol biosynthesis by activating expression of SREBP-2 and HMGR. Interestingly, the mRNA levels of genes related with PI3K pathway increased in the presence of TO901317, which suggested that LXR activation could promote PI3K signalling activity.

Introduction

Cholesterol is a central component of lipid rafts, specialised microdomains of the plasma membrane that serve as organising centers for the assembly of signalling molecules (Zhao and Dahlman-Wright, 2009; Guo et al., 2011). Sterol regulatory element-binding protein 2 (SREBP-2) is a transcription factor that regulates many genes involved in cholesterol synthesis and uptake in response to cellular cholesterol levels (Shimano et al., 1997), whose nuclear fragments are released from membranes by controlling proteolysis. When cells are overloaded with sterols, SREBP-2 remains bound to the endoplasmic reticulum membranes, and consequently, transcription of its target genes declines (Horton et al., 2002). Many studies in recent years have significantly enhanced our understanding of the molecular mechanisms of liver X receptor (LXR) signalling as an important global regulator of cholesterol homeostasis involved in the efflux, transport, and excretion of cholesterol (Peet et al., 1998; Repa and Mangelsdorf, 2000; Tontonoz and Mangelsdorf, 2003). One recent study showed that SREBP-2 positively regulates transcription of the cholesterol efflux gene ABCA1, by generating oxysterol ligands for LXR (Wang et al., 2006).

Cholesterol synthesis is normally tightly regulated by a feedback mechanism to maintain the appropriate level. Cholesterol synthesis is catalysed by a group of microsomal enzymes, including rate-limiting enzymes HMG-CoA synthase and reductase. Studies in vivo and in vitro have shown that 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is highly regulated at the transcriptional level by SREBP-2 (Goldstein et al., 2006; Wong et al., 2006). Activation of SREBP-2 is dependent on the cholesterol status of the cell, and when cholesterol concentration drops in the endoplasmic reticulum (ER), the SREBP-2 transcription factor is released and binds to a sterol-response element (SRE) located on the HMGR promoter. This leads to an increased transcription of the HMGR gene, stimulating the cholesterol biosynthesis and safeguarding the adequate cholesterol concentration within the cell (Goldstein et al., 2006). A common link between SREBP-2 and LXR-mediated processes is their regulatory response to certain oxidised cholesterol derivatives (oxysterols). Certain oxysterols inhibit SREBP-2 activation, but serve as ligands for LXR (Venkateswaran et al., 2000; Janowski et al., 2001; Abildayeva et al., 2006). A role for SREBP-2 in the positive regulation of LXR-target genes has been implied in two previous studies (Forman et al., 1997; DeBose-Boyd et al., 2001). A recent study showed that LXR activation up-regulates the expression of SREBP-2 and its regulatory genes in astrocytes (Abildayeva et al., 2006). However, Wang et al. demonstrated that LXR knock-down would result in an increased cholesterol content in HepG2 cells by regulating the expression of SREBP-2 and its target genes (Wang et al., 2008). All these data showed that both LXR and SREBP-2 play an important role in regulating the cholesterol metabolism, and in different cell types can exhibit different effects. Interestingly, recent studies have well established that the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is involved in the regulation of cholesterol metabolism (Emerling and Akcakanat, 2011; Laplante et al., 2012). The PI3K-AKT downstream target gene mTOR, to a large extent, acts through SREBP-2 transcription factor that controls the expression of genes involved in cholesterol synthesis. Mammalian target of rapamycin appears to regulate SREBP-2 function through several mechanisms, including 4E-BP1 and S6K (Poirsmann et al., 2008; Li et al., 2011; Wang et al., 2011). In some cell types (such as hepatocellular and carcinoma cells), S6K was a key regulator of cholesterol biosynthesis (Düwel et al., 2010). These findings suggested that mTOR inhibition would reduce mRNA expression level of SREBP-2.

The present work was, thus, undertaken to determine whether SREBP-2 plays a regulatory role in LXR activation-induced cholesterol synthesis, and to investigate how LXR activates PI3K pathway. Primary hepatocytes were isolated from Sichuan white geese (Anser cygnoides) and we investigated the effect of a synthetic LXR agonist, TO901317, on the intracellular cholesterol level and extracellular cholesterol level, and the effect of LXR activation on mRNA level and enzyme

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activity of factors involved in the PI3K/AKT/mTOR pathway. This was the first time to detect a novel role of SREBP-2 and PI3K signalling pathways in response to LXR agonist, and it is significant to understand cholesterol metabolism in the liver.

Materials and methods
Isolation and culture of goose primary hepatocytes
Hepatocytes were isolated from three 30-day-old Sichuan White geese from the Experimental Farm for Waterfowl Breeding at Sichuan Agricultural University using a modification of the two-step procedure described by Seglen (Seglen, 1976). The protocol for bird treatment was in accordance with the Canadian Council on Animal Care guidelines (1994). The method differed from that of Seglen in that the liver was removed before the preperfusion step. Cellular viability was greater than 90%, as assessed by the trypan blue dye exclusion test. Freshly isolated hepatocytes were diluted to 1×10^6 cells/mL. The blue dye exclusion test. Freshly isolated hepato-

Isolation of total RNA and real-time PCR
Total RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the PrimerScriptTM RT system kit for real-time PCR (TaKaRa, Otsu, Japan) using oligo dT primer and random primer according to the manufacturer's instructions. The quantitative real-time PCR reaction contained the newly generated cDNA template, SYBR Premix Ex TaqTM, sterile water, and primers of target genes. Real-time PCR was performed on the Cycler system (one cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 40 s). An 80-cycle melt curve was performed, starting at 55°C and increasing by 0.5 degrees every 10 s, to determine primer

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Isolation of total RNA and real-time polymerase chain reaction
Total RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the PrimerScriptTM RT system kit for real-time PCR (TaKaRa, Otsu, Japan) using oligo dT primer and random primer according to the manufacturer’s instructions. The quantitative real-time PCR reaction contained the newly generated cDNA template, SYBR Premix Ex TaqTM, sterile water, and primers of target genes. Real-time PCR was performed on the Cycler system (one cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 40 s). An 80-cycle melt curve was performed, starting at 55°C and increasing by 0.5 degrees every 10 s, to determine primer specificity. Specific primers are listed in Table 1, and were designed according to the goose gene sequences, PI3K: GenBank No. KFO11500; AKT: GenBank No. KFO11501; mTOR: GenBank No. KC424580; S6K: GenBank No. KC424581; SREBP-2: GenBank No. EF579754; HMGR: GenBank No. EF635218; 18S: GenBank No. D21170; β-actin: GenBank NO: M26111.1.

Table 1. Sequence of polymerase chain reaction primers.

| Gene          | Primer sequence (5’-3’)                  | Product size (bp) |
|---------------|------------------------------------------|-------------------|
| PI3K upstream | ACCCAACCGGAGGATGAGG                        | 241               |
| PI3K downstream | TGGTCCCGGGTTGGAAG                        | 215               |
| AKT upstream  | TGGTGATAAAGGAGGAC                        | 93                |
| AKT downstream | CTTGTTGAGAAGGGAGG                        | 111               |
| mTOR upstream | TCAATTGTACTACTCCCCA                       | 150               |
| mTOR downstream | TTCTAGAGCAGCTTGGGACGAC                 | 183               |
| S6K upstream  | CTCAAATGCTCCTAC                            | 241               |
| S6K downstream | AACTTCTGCAGACCTTC                          | 215               |
| SREBP-2 upstream | GGCAATGCGCAGAC                  | 111               |
| SREBP-2 downstream | GTTTGTGAGGAGAGAGG             | 150               |
| HGMR upstream | TACCGCCCTAGCGAGAACGCGGAGG               | 183               |
| HGMR downstream | GACTCCTCTCCTCTCTAGAC                  | 129               |
| β-actin upstream | CACCGGCTGAGAGGAGGAG                 | 92                |

PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; SREBP-2, sterol regulatory element-binding protein 2; HGMR, 3-hydroxy-3-methylglutaryl-CoA reductase.
were analysed in triplicates. The assay for enzyme activity of PI3K and mTOR enzymes was performed according to manufacturer instructions at 490 nm. All samples were analysed in triplicates.

**Results and discussion**

**Effect of TO901317 on extracellular total protein and albumin accumulation**

To identify the normal cell function after the treatment with TO901317 in goose primary hepatocytes, we measured the extracellular TP and ALB accumulation. The results showed that treatment with the LXR agonist TO901317 induced extracellular TP accumulation in a dose-dependent manner from 0.01 to 10 μmol/L. Compared with the control group, TO901317 at 0.01, 0.1, 1, or 10 μmol/L all had a significant up-regulation effect (P<0.05). Similar with the result of TP, after the treatment with 0.01, 0.1, 1, or 10 μmol/L TO901317, ALB level all existed the significant increase (P<0.05) (Table 2). These data indicated that TO901317 could promote primary hepatocytes protein synthesis, and the cells had the normal function.

**Effect of TO901317 on intracellular and extracellular cholesterol concentration**

To understand the role of LXR in cholesterol homeostasis, we investigated the effect of LXR agonist on the concentration of intracellular and extracellular cholesterol. As shown in Figure 1, there was an up regulation manner of extracellular cholesterol accumulation when culture with 0, 0.01, 0.1, 1, and 10 μmol/L TO901317. The intracellular cholesterol concentration only showed an uptrend manner when the TO901317 concentration was under 1 μmol/L, and it decreased when TO901317 reached 10 μmol/L (P<0.05).

Liver X receptor agonist TO901317 treatment regulated hepatocellular expression of phosphatidylinositol 3-kinase signalling pathway

Figure 2 presented the effect of TO901317 on the mRNA expression of PI3K, AKT, mTOR and S6K by quantitative real-time PCR analysis. TO901317 at 0.01 μmol/L did not have a significant effect on gene expression level of mTOR (P>0.05). However, the treatment with 0.1, 1, or 10 μmol/L TO901317 all had significant effects on the mRNA level of these genes (P<0.05), and the up-regulation of gene expression in response to TO901317 was dose-dependent. To determine whether LXR agonist affects PI3K and mTOR translation, goose hepatocytes were exposed to the LXR agonist TO901317 for 48 h. As shown in Figure 3, After incubation with 0.01, 0.1, 1, or 10 μmol/L TO901317, the PI3K and mTOR enzyme activity were up-regulated by all dose of TO901317 (P<0.05).

**Effect of TO901317 on mRNA expression level of sterol regulatory element-binding protein 2 and 3-hydroxy-3-methylglutaryl-CoA reductase**

To confirm the roles of SREBP-2 in mediating cholesterol biosynthesis induced by TO901317 in hepatocytes, we studied the effect of TO901317 on mRNA expression level of SREBP-2 and HMGR in goose hepatocytes. As shown in Figure 4, the gene expression levels of SREBP-2 and HMGR were regulated by TO901317 at 0.01, 0.1 and 1 μmol/L in an upregulation manner, but when the TO901317 concentration increased, the gene expression levels decreased.

### Table 2. Total protein and albumin accumulation of goose primary hepatocytes after TO901317 treatment.

| TO901317, μmol/L | TP accumulation, g/L | ALB accumulation, μg/mL |
|------------------|----------------------|-------------------------|
| 0                | 8.70±0.37*           | 41.67±2.50*             |
| 0.01             | 10.77±0.33*          | 48.00±2.45*             |
| 0.1              | 12.77±0.54*          | 52.33±2.87*             |
| 1                | 14.20±0.49*          | 56.33±1.25*             |
| 10               | 16.60±0.91*          | 63.00±1.63*             |

TP: total protein; ALB: albumin. Different letters in the same row show significant difference among the treatment of different concentrations (P<0.05), while the same letters display no significant statistical differences.

Figure 1. Effect of different concentrations of TO901317 on intracellular (A) and extracellular (B) cholesterol levels. The data presented are means±standard error of three independent experiments. In the same curve different letters indicate statistically significant differences between different concentrations (P<0.05), while the same letters indicate no statistically significant differences.
concentration reached 10 μmol/L, the effect decreased. Compared with the effects of TO901317 at other concentrations, the effect of 1 μmol/L TO901317 was most evident.

**Effect of TO901317 on extracellular triglyceride concentration**

Based on these data, we demonstrated that SREBP-2 and its target gene HMGR play a regulatory role in LXR-induced cholesterol synthesis. In order to understand whether changes in LXR activity affects TGs content, we tested the TG content of hepatocytes. As shown in Figure 5, the extracellular content of TG had a rising trend with the increase of TO901317.

**General remarks**

In our previous study, we found that overfeeding to geese induced an increase of the plasma cholesterol concentrations and the cholesterol content in their liver, accompanied with the formation of hepatic steatosis (Han et al., 2008). Thus, changes of hepatic cholesterol synthesis may have a close relation with the goose hepatic steatosis. In liver, intracellular cholesterol levels are finely controlled by SREBP-2, a transcription factor whose nuclear fragments are released from membranes by controlled proteolysis. To our knowledge, it has not yet been investigated whether the activation of LXR influences the expression of SREBP-2 and its target genes in liver. During the last several years, one study has provided experimental evidences supporting the notion that the activation of LXR can result in lipogenesis in goose hepatocytes (Han et al., 2009). Aravindhan et al. demonstrates that incubation with synthetic LXR agonists increased cholesterol synthesis in HepG2 cells (Aravindhan et al., 2006).

In this study, we found an up regulation manner of extracellular cholesterol accumulation after treatment with 0, 0.01, 0.1, 1, and 10 μmol/L TO901317, and the intracellular cholesterol concentration only showed an uptrend manner when the TO901317 concentration was under 1 μmol/L, but 10 μmol/L TO901317 had an inhibiting effect (P<0.05) (Figure 1). Compared with the effect of 1 μmol/L TO901317, the mRNA expression levels of SREBP-2 and HMGR in goose hepatocytes decreased after incubation with 10 μmol/L TO901317, which is similar with the change of intracellular cholesterol concentrations. This result clearly demonstrates that SREBP-2 and its target gene HMGR play a regulatory role in LXR-induced cholesterol synthesis. This is similar with a previous report that SREBP-2 and LXR together response to cellular cholesterol status through regulating the mRNA expression of their target genes (Tamehiro et al., 2007).

We also found that treatment with TO901317 at all dose increased evidently the extracellular TP and ALB levels in this study. So it was obvious that the decrease of the intracellular cholesterol level induced-by the high concentration of TO901317 at 10 μmol/L was not because of hepatic dysfunction. On one hand, the treatment of high dose TO901317 (10 μmol/L) could increase the intracellular total TG accumulation (Figure 5).
but decrease the intracellular cholesterol level (Figure 1). We propose the synthesised intracellular cholesterol induced by 10 μmol/L TO901317 could active the de novo lipogenesis. On the other hand, the treatment of high dose TO901317 (10 μmol/L) could increase the extracellular cholesterol accumulation. It was predicted that the LXR activation increases the cholesterol efflux from the hepatocytes, and a similar result reported by Zanotti et al., showed that in vivo stimulation of LXR by TO901317 increase the efflux of cholesterol from peritoneal macrophages (Zanotti et al., 2008). Another previous study showed that LXR agonist TO901317 has been shown to induce the expression of ABCA1 and other LXR-responsive genes. However, in primary human fibroblasts an increase in cellular cholesterol efflux in response to TO901317 was not demonstrated (Sparrow, 2002). We assumed that the nifdeference results may due to the cell types and species differences.

Figure 4. Effect of TO901317 on mRNA expression level of SREBP-2 and HMGR in goose primary hepatocytes by quantitative real-time PCR analysis. The data presented are mean±standard error of three independent experiments. Different letters indicate statistically significant differences (P<0.05), while the same letters indicate no statistically significant differences.

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Conclusions

To conclude, our current work provided a new evidence suggesting an additional role for LXR in positively regulating the SREBP-2 expression. Particularly, we have demonstrated that LXR agonists could stimulate PI3K/AKT/mTOR pathway, this suggested that PI3K/AKT/mTOR pathway seems to contribute to TO901317-induced cholesterol homeostasis. These findings indicate that LXR plays an important role in the regulation of cholesterol biosynthesis, meanwhile, this nuclear receptor also contacts with PI3K/AKT/mTOR pathway in cholesterol biosynthesis.
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