Insulin sensitivity of hepatic glucose and lipid metabolism in animal models of hepatic steatosis
Grefhorst, Aldo
Chapter 7

Induction of hepatic lipogenic gene expression upon pharmacological inhibition of glucose-6-phosphate translocase is independent of liver X receptor alpha

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In preparation
Abstract

In mammals, hepatic expression of lipogenic genes is regulated by the coordinate action of the transcription factors sterol-regulatory element-binding protein-1c (SREBP-1c), carbohydrate responsive element binding protein (ChREBP) and liver X receptor (LXR). The “master-switch” LXR controls not only transcription of Srebp-1c and Chrebp but also ChREBP activity. Treatment of mice with S4048, a pharmacological inhibitor of glucose-6-phosphate translocase, results in increased hepatic triglyceride concentrations associated with markedly enhanced transcription of lipogenic genes. We used C57BL/6J OlaHsd mice as well as LXRα-/- mice and their wild-type littermates to investigate which lipogenic transcription factor is critical for S4048-induced de novo lipogenesis and hepatic steatosis. S4048-treatment resulted in similar induction of hepatic steatosis and lipogenic gene expression in all strains of mice, i.e., does not require the presence of LXRα. Expression of the ChREBP target gene pyruvate kinase was significantly increased, but expression of Srebp-1c and its target gene glucokinase was not affected or even decreased by S4048. Nuclear translocation of ChREBP was not affected, probably related to concomitantly increased AMPK activity. These data strongly indicate involvement of ChREBP but not of SREBP-1c and LXRα in S4048-induced de novo lipogenesis and hepatic steatosis.
Introduction

In fed conditions, liver and muscle store excess glucose as glycogen, but this storage capacity is limited (1). A certain amount of glucose is therefore broken down in the glycolytic pathway to yield pyruvate and, after passing the tricarboxylic acid cycle, acetyl-CoA moieties that can be used for production of fatty acids and triglycerides (TGs) in a process called de novo lipogenesis (DNL). Hepatic transcription of genes encoding for enzymes involved in DNL is tightly controlled by three transcription factors, i.e., sterol-regulatory element-binding protein-1c (SREBP-1c) (2), carbohydrate responsive element binding protein (ChREBP) (3,4), and liver X receptor (LXR) (5,6).

SREBP-1c, a basic-helix-loop-helix-leucine-zipper transcription factor bound in inactive form to the ER membrane, is known to facilitate transcription of many genes involved in DNL (2), including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and acyl-CoA synthase (ACS) upon its activation. Both transcription and activation of SREBP-1c itself is tightly regulated by insulin (7).

ChREBP binds as a heterodimer with Mlx (8) to the carbohydrate response element (ChoRE) composed of two E-box (5’-CACGTG-3’) or E-box-like sequences present in promoters of target genes. Upon increasing intracellular levels of the glucose metabolite xylulose-5-phosphate (Xu5P), protein phosphatase-2A (PP2A) becomes active and dephosphorylates ChREBP. Translocation of ChREBP into the nucleus is enhanced upon dephosphorylation of Ser196. Dephosphorylation of Ser196 and Thr666 enhances the binding activity of ChREBP to DNA (9). AMP-activated protein kinase (AMPK) phosphorylates ChREBP on Ser196 and protein kinase A (PKA) phosphorylates ChREBP on both Thr666 and Ser196 and can thus inhibit ChREBP-mediated gene transcription (10). Activity of AMPK is increased upon elevated energy-usage (e.g., exercise) (11). Remarkably, most genes regulated by SREBP-1c also are regulated by ChREBP (12), i.e., Fas, Acc and Acs. Expression of the gene encoding pyruvate kinase (PK) is regulated by ChREBP, but not by SREBP-1c (10). In contrast, expression of the gene encoding glucokinase (GK) is regulated by SREBP-1c, but not by ChREBP (13).

LXR has been identified as an oxysterol-activated nuclear receptor (14-16) that, after ligand-binding, forms a heterodimer with the retinoid X receptor (RXR). The LXR/RXR heterodimer binds to a LXR response element (LXRE), resulting in adaptation of gene transcription (17). LXR controls expression of various genes involved in DNL, in part via stimulation of Srebp-1c transcription (5,18,19), but also by direct effects on transcription of Fas (20). In experiments with Srebp-1c-/- mice, it was shown that pharmacological LXR activation increased Acc expression independent of SREBP-1c (21). The promoter region of the ACC-gene lacks an LXRE, suggesting indirect, SREBP-1c-independent LXR-effects. Recently, it was shown that LXR also regulates mRNA levels of ChREBP (22). Moreover, it was speculated that ChREBP activity was mediated by LXR via effects on AMPK (22).
S4048 is a pharmacological inhibitor of glucos-6-phosphate (G6P) translocase (G6PT) and, when given to animals, provides a model of glycogen storage disease type I (GSDI). GSDI patients have a defect in the glucose-6-phosphatase (G6Pase) enzyme complex, consisting of G6PT and G6P hydrolase (G6PH). As a result of this defect, the patients do not only show increased hepatic glycogen levels, but also massively increased DNL (23). Treatment of rats with S4048 resulted in elevated hepatic G6P levels (24) and increased lipogenesis (25), resulting in hepatic steatosis. Because Xu5P is an intermediate in the pentose phosphate pathway that is markedly enhanced upon increasing G6P levels (26), the data gathered so far strongly suggest a role of ChREBP in the S4048-induced lipogenesis, but the exact roles of the other lipogenic transcription factors have remained unclear. In the present study, we therefore questioned which lipogenic transcription factor(s) is critically involved in S4048-induced DNL and hepatic steatosis.

**Material and methods**

**In vivo experiments**

Male C57BL/6J OlaHsd mice (Harlan, Horst, The Netherlands) and LXRa -/- mice and their wild-type littermates on a mixed C57BL/6J Sv129/OlaHsd background were housed in a light- and temperature controlled facility and were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) and had free access to water. The mice received humane care and experimental procedures were in accordance with local guidelines for use of experimental animals. S4048 was infused to the mice via a permanent catheter in the right atrium of the heart (27) from which the entrance was attached to the skull. The mice were allowed a recovery period of at least five days after surgery. Mice were kept in metabolic cages during the experiment and the preliminary fasting period, allowing frequent collection small tail blood samples under conscious and unrestrained conditions (28). After nine hours of fasting, the mice were infused for six hours with S4048 (a generous gift of Aventis Pharma, Frankfurts, Germany) (5.5 mg S4048/ml PBS with 6% DMSO, 0.135 ml/h). Blood glucose levels were measured in a small tail blood sample that was taken every hour during the experiment. After the six hours of infusion, the mice were killed by cardiac puncture and the liver was immediately removed, weighed and freeze clamped. The liver was powdered and stored in separate portions until later analyses.

**Hepatic analyses**

Hepatic concentrations of TG, free cholesterol and total cholesterol were measured using commercial kits (Roche Diagnostics, Mannheim, Germany, and Wako Chemicals, Neuss, Germany) after lipid extraction according to Bligh and Dyer (29). Phospholipid content of the liver was determined according to Böttcher et al. (30) after lipid extraction. Protein concentrations in livers were determined according to Lowry et al. (31) using bovine serum albumin (Pierce, Rockford, IL) as standard. Hepatic glycogen and G6P levels were determined as described by Bergmeyer (32).

**Pharmacological LXR activation**

Male C57BL/6J mice received 10 mg/kg of the LXR agonist T0901317 (kindly donated by Organon Laboratories, Oss, The Netherlands) or its solvent by gavage daily for 4 days. T0901317 was dissolved in DMSO and Chremophor (both Sigma, St. Louis, MO, USA) in 5% mannitol/water, to a final concentration of 2.5 mg/ml. On the morning of the fifth day, the liver was quickly removed, weighed and frozen in separate portions for RNA isolation.
RNA isolation and measurement of mRNA levels by Real Time-PCR (Tagman)

mRNA expression levels in liver was measured by realtime-PCR as described before (6). PCR results were normalised to β-actin mRNA levels. The sequences of the primers and probes used are listed in table 7.1.

Nuclear translocation of ChREBP

Hepatic nuclear extracts of livers were made according to Itoh et al. (33). In short, powdered livers were homogenized with pre-chilled glass douncers in a cytosolic lysis buffer containing 10 mM Hepes, pH 7.5; 1.5 mM MgCl₂; 10 mM KCl; and protease inhibitors (Complete, Roche Diagnostics). The homogenate was centrifuged 5 min 5,000 rpm at 4°C. The centrifugation procedure was repeated with the received supernatant. The supernatant thus received contained the cytosolic fraction. The pellet from the first centrifugation step was dissolved in a nuclear extract buffer containing 50 mM Tris HCl, pH 7.2; 140 mM NaCl; 2 mM EDTA; 1% N-P450; and protease inhibitors (Complete, Roche Diagnostics), and incubated for 30 minutes on ice followed by centrifugation at 15,000 rpm at 4°C for 5 min. The supernatant retrieved is the nuclear fraction. Protein content was determined using BCA-kit (Pierce) and equal amounts of protein were used to determine ChREBP by Western blotting, using an antibody raised in rabbit (Novus Biologicals, Littleton, CO).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5’ to 3’)</th>
<th>GenBank™ accession no.</th>
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<tr>
<td>β-Actin</td>
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</tr>
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<td></td>
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<td></td>
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<td>NM 133360</td>
</tr>
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<td></td>
<td>Forward: CACACAGACCTGTGTTGGACT  Reverse: CACCATGCCCCACCTCGTTCAC  Probe: CAGGAAGGCGGTTCACTCCACCAG</td>
<td>NM 133904</td>
</tr>
<tr>
<td>Fas</td>
<td>Forward: GGCAATAGTGGGCACACTCTT  Reverse: CCGTCAAGCAGAGAGCCTCT  Probe: CCATCTGATAGGCAAGCTCTC</td>
<td>NM 007988</td>
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<td>G6pt</td>
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<td>Forward: GATGCTTGGCAGAGACACTCTT  Reverse: GCTGCTGCATAGGCTTCGGG  Probe: CAGGCTTTGCCATCTCCACACC</td>
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<td>Srebp-1c</td>
<td>Forward: GGAGGCTTGGATTGACACTC  Reverse: CCGTGCTGCTCCGGGACACCC  Probe: CATCTGCACAGACAGCAGATGACTCC</td>
<td>AF286470</td>
</tr>
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<td>Gk</td>
<td>Forward: CCTGCGCTTCACCCTCTCTT  Reverse: GAGGCTTGAGAGCCATTGGT  Probe: CACGAAGACATAGACAAGGGCATCCTGCTC</td>
<td>NM 01292</td>
</tr>
</tbody>
</table>

Table 7.1. Primers and probes used for realtime-PCR analysis.
**Hepatic insulin signaling experiment**

After 5.45 hours of S4048-infusion, two C57BL/6J mice in each group (vehicle and S4048) received an i.p. injection of 200 µl water containing 50 mU insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), 2.7 µg somatostatin (UCB, Breda, The Netherlands), 10% glucose and 1% bovine serum albumine (Sigma-Aldrich, St. Louis, MO). Fifteen minutes after the injection, the mice were killed by cardiac puncture and the liver was immediately removed, weighed and freeze clamped until later analysis.

Hepatic lysates were made in buffer (30 mM Tris.Cl pH 7.4; 2.5 mM EDTA pH 8.0; 150 mM NaCl; 1.3 M glycerol; 0.5 mM Na3VO4; 5 mM NaF; 5 mM MgCl2; and protease inhibitors (Complete, Roche Diagnostics)) and cleared by centrifugation. Protein content was determined using BCA-kit (Pierce) and equal amounts of protein were used to determine PKB-Ser473 and αAMPK-Thr172 by Western blotting, using antibodies against protein kinase B-Ser473P (PKB-Ser473P) and αAMPK-Thr172P raised in rabbit (Cell signaling, Beverly, MA).

**Statistics**

All values represent mean ± standard deviation for the number of animals indicated. Statistical analysis was assessed by Mann-Whitney U test. Level of significance was set at p<0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, USA).

**Results**

*ChREBP but not SREBP-1c is involved in S4048-induced DNL and hepatic steatosis*

Previous experiments from our laboratory (24,25) showed that pharmacological inhibition of G6PT with S4048 resulted in severe hepatic steatosis in rats. In the present study, conscious, unrestrained mice were infused during 6 hours with S4048. In S4048-treated C57BL/6J OlaHsd mice, blood glucose levels were significantly reduced within 1 hour (figure 7.1). At the end of the 6-hour infusion, blood glucose levels were 64% lower in the treated mice compared to untreated mice. In livers of S4048-treated mice, concentrations of glycogen, G6P and TGs were increased compared to control mice (table 7.2). Hepatic cholesterol and phospholipid levels were not affected.

In the livers of S4048-treated mice, expression of genes encoding enzymes involved in DNL, i.e., *Fas, Acc1* and *Acc2*, was markedly increased compared to control mice (figure 7.2), suggesting that enhanced DNL contributes to S4048-induced hepatic steatosis in mice. Expression of the genes encoding the G6Pase enzyme complex, G6PT and G6PH, was also increased upon G6PT inhibition. Compared to control mice, hepatic mRNA levels of *Chrebp* and *Pk* in S4048-treated mice were increased by 90% and 400%, respectively. Taken together, this strongly suggests that S4048-induced hepatic steatosis is related to ChREBP-induced DNL, because *Pk* gene expression is regulated by ChREBP but not by SREBP-1c (10). Moreover, upon S4048-infusion, expression of *Srebp-1c* was not affected and mRNA level of its target gene *Gk* was even reduced by 70% (figure 7.2). This suggests that SREBP-1c is not involved in S4048-induced hepatic steatosis, because *Gk* expression is regulated by SREBP-1c and not by ChREBP (13).

To investigate whether ChREBP protein nuclear translocation was affected upon S4048-infusion, we determined the cytosolic and nuclear ChREBP protein levels by Western blotting. Figure 7.3 shows that most of the ChREBP protein is located in the cytosol and that G6PT inhibition, surprisingly, does not affect the subcellular translocation of ChREBP.
Lipogenic effects of glucose-6-phosphatase inhibition independent of LXRα

Table 7.2. Hepatic parameters of C57BL/6J OlaHsd mice treated with 30 mg·kg\(^{-1}\)·h\(^{-1}\) glucose-6-phosphate translocator inhibitor S4048 or vehicle.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>S4048</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liverweight (% of body weight)</td>
<td>5.2 ± 2.2</td>
<td>5.1 ± 0.5</td>
</tr>
<tr>
<td>Proteins (mg/g liver)</td>
<td>194 ± 20</td>
<td>222 ± 33</td>
</tr>
<tr>
<td>Triglycerides (nmol/mg liver)</td>
<td>25.7 ± 6.9</td>
<td>60.3 ± 22.6 *</td>
</tr>
<tr>
<td>Free cholesterol (nmol/mg liver)</td>
<td>8.1 ± 1.5</td>
<td>8.2 ± 1.6</td>
</tr>
<tr>
<td>Cholesterylester (nmol/mg liver)</td>
<td>1.9 ± 0.7</td>
<td>2.5 ± 1.7</td>
</tr>
<tr>
<td>Phospholipids (nmol/mg liver)</td>
<td>37.7 ± 11.8</td>
<td>40.3 ± 5.8</td>
</tr>
<tr>
<td>Liver glycogen (nmol/mg liver)</td>
<td>16.1 ± 3.4</td>
<td>254.8 ± 85.4 *</td>
</tr>
<tr>
<td>Liver G6P (nmol/g liver)</td>
<td>21.4 ± 15.0</td>
<td>574.9 ± 133.7 *</td>
</tr>
</tbody>
</table>

Figure 7.1. Relative blood glucose levels in C57BL/6J OlaHsd mice during of 30 mg·kg\(^{-1}\)·h\(^{-1}\) glucose-6-phosphate translocase inhibitor S4048 or vehicle. Data are mean ± S.D.; n=7; *, p<0.05 S4048 vs. vehicle; #, P<0.05 vs. t=0.

Figure 7.2. Changes in hepatic gene expression patterns upon treatment with 30 mg·kg\(^{-1}\)·h\(^{-1}\) glucose-6-phosphate translocase inhibitor S4048. Results were normalized to β-actin mRNA levels, data from untreated mice defined as ‘1’. Pk, pyruvate kinase; Acc, acetyl-CoA carboxylase; Fas, fatty acid synthase; G6pt, glucose-phosphate translocase; G6ph, glucose-6-phosphate hydrolase; Chreb, carbohydrate responsive element binding protein; Srebp-1c, sterol-regulatory element-binding protein-1c; Gk, glucokinase. Data are mean ± S.D.; n=5; *, p<0.05.
Increased AMPK-phosphorylation upon S4048-treatment

To test whether AMPK activity was changed and whether insulin could suppress this activation, as reported by Witters and Kemp (34), hepatic AMPK phosphorylation was determined by Western blotting in livers from control and S4048-treated mice with or without insulin injection (figure 7.4). Remarkably, in livers of mice not treated with insulin, the phosphorylation at Thr172 of the \( \alpha \)-subunit of AMPK was clearly increased by S4048-treatment mice. Phosphorylation subsequently decreased to the same extent in control and S4048-treated mice. Moreover, insulin-induced phosphorylation and activation of PKB, the main transducer of insulin-effects on lipid and glucose metabolism (35), was not affected in the S4048-induced fatty livers.

Pharmacological LXR activation regulates expression of the ChREBP target gene \( Pk \)

Treatment of C57BL/6J mice with the pharmacological LXR ligand T0901317 for 4 days resulted in increased hepatic mRNA levels of \( Pk \) (figure 7.5). Pharmacological LXR activation did not affect \( Chrebp \) mRNA levels. Taken together, these results show that LXR-induced DNL might, in part, be mediated via ChREBP.

S4048-induced de novo lipogenesis and hepatic steatosis is independent of LXR\( \alpha \)

To test whether S4048-induced hepatic steatosis is dependent on LXR\( \alpha \), the isoform most likely involved in regulation of lipogenic gene expression, LXR\( \alpha \ -/- \) mice and their wild-type littermates were treated with S4048. Infusion of S4048 resulted in hypoglycemia in all mice with no differences between the knockouts and the wild-type littermates (figure 7.6). After 6 hours of infusion, blood glucose levels were decreased by 45% and 43% in LXR\( \alpha \) +/- and LXR\( \alpha \) -/- mice, respectively. Hepatic glycogen, G6P and TG levels increased upon S4048-
infusion in both knockouts and wild-types (table 7.3), although hepatic TG levels upon S4048-treatment remained somewhat lower in the LXRα +/+ mice. The hepatic cholesterylester content was not affected by S4048 in the wild-types, but rose 3.4-fold in the LXRα -/- mice.

To test whether LXRα deficiency affected S4048-induced transcription of lipogenic genes, we measured hepatic gene expression by realtime RT-PCR (figure 7.7). Expression of Pk, Acc1, Acc2 and Fas was induced by S4048 with no differences between LXRα +/- and LXRα +/- mice. Thus, S4048-induced expression of lipogenic genes is independent of LXRα. In both type of mice, S4048-infusion induced transcription of G6pt, G6ph and Chrebp. Srebp-1c mRNA levels were lower in LXRα -/- mice compared to the wild-types, and its expression decreased even more upon S4048-infusion. mRNA levels of Gk were not affected upon S4048-infusion in either type of mice.

Comparable with the C57BL/6J OlaHsd mice, translocation of ChREBP protein was not affected by S4048 (figure 7.8): there was also no difference between LXRα +/- and LXRα -/- mice in this respect.

Figure 7.5. Changes in hepatic gene expression patterns upon treatment with the synthetic liver X receptor ligand T0901317. Results normalized to β-actin mRNA levels, data from untreated mice defined as ‘1’. Pk, pyruvate kinase; Chrebp, carbohydrate responsive element binding protein. Data are mean ± S.D.; n=3; *, p<0.05.

Figure 7.6. Relative blood glucose levels in LXRα +/- and LXRα -/- mice during infusion with 30 mg·kg⁻¹·h⁻¹ glucose-6-phosphate translocone inhibitor S4048. Data are mean ± S.D.; n=6; *, p<0.05 vs. t=0 in LXRα +/- mice; #, p<0.05 vs. t=0 in LXRα -/- mice.
In insulin sensitivity of hepatic glucose and lipid metabolism in animal models of hepatic steatosis

Table 7.3. Hepatic parameters of LXRα +/+ and LXRα -/- mice treated with or without 30 mg·kg⁻¹·h⁻¹ glucose-6-phosphate translocater inhibitor S4048.

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<tr>
<th></th>
<th>LXRα +/+</th>
<th>LXRα -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control S4048</td>
<td>Control S4048</td>
</tr>
<tr>
<td>Liverweight (% of body weight)</td>
<td>4.6 ± 0.4 4.6 ± 0.0</td>
<td>4.54 ± 0.2 4.3 ± 0.3</td>
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<tr>
<td>Proteins (mg/g liver)</td>
<td>180 ± 14 264 ± 15 *</td>
<td>186 ± 20 257 ± 27 *</td>
</tr>
<tr>
<td>Triglycerides (nmol/mg liver)</td>
<td>18.1 ± 11.0 38.1 ± 2.5 *</td>
<td>24.7 ± 5.6 71.2 ± 15.3 *</td>
</tr>
<tr>
<td>Free cholesterol (nmol/mg liver)</td>
<td>7.4 ± 0.6 8.6 ± 1.4</td>
<td>7.2 ± 0.4 7.6 ± 0.3</td>
</tr>
<tr>
<td>Cholesterylsteer (nmol/mg liver)</td>
<td>1.1 ± 0.1 1.9 ± 0.4</td>
<td>0.9 ± 0.4 3.1 ± 0.3 *</td>
</tr>
<tr>
<td>Phospholipids (nmol/mg liver)</td>
<td>44.0 ± 3.7 45.2 ± 3.7</td>
<td>39.5 ± 3.5 45.1 ± 5.0</td>
</tr>
<tr>
<td>Liver glycogen (nmol/mg liver)</td>
<td>78.8 ± 26.2 183.0 ± 6.1 *</td>
<td>101.6 ± 53.0 336.0 ± 40.1 *</td>
</tr>
<tr>
<td>Liver G6P (nmol/g liver)</td>
<td>262.5 ± 66.0 998.8 ± 288.9 *</td>
<td>340.7 ± 136.8 624.5 ± 50.6 *</td>
</tr>
</tbody>
</table>

Figure 7.7. Changes in hepatic gene expression patterns in LXRα +/+ and LXRα -/- mice upon treatment with 30 mg·kg⁻¹·h⁻¹ glucose-6-phosphate translocase inhibitor S4048. Results were normalized to β-actin mRNA levels, data from untreated mice defined as ‘1’. Pk, pyruvate kinase; Acc, acetyl-CoA carboxylase; Fas, fatty acid synthase; G6pt, glucose-phoshate translocase; G6ph, glucose-6-phosphate hydrolase; Chrebp, carbohydrate responsive element binding protein; Srebp-1c, sterol-regulatory element-binding protein-1c; Gk, glucokinase. Data are mean ± S.D.; n=7 (control mice); n=3 (S4048-treated mice); *, p<0.05 S4048 vs. control; #, P<0.05 vs. control LXRα +/+ mice.
Ligand effects of glucose-6-phosphatase inhibition independent of LXRα

![Image of Western blots](image)

**Figure 7.8.** ChREBP Western blots of cytosolic (C) and nuclear (N) liver extracts of LXRα +/+ and LXRα -/- mice treated with or without 30 mg·kg⁻¹·h⁻¹ glucose-6-phosphate translocase inhibitor S4048.

**Discussion**

The regulation of hepatic lipid metabolism, and especially of DNL, is regulated by several transcription factors, including SREBP-1c, ChREBP and LXR. Numerous studies have addressed the roles of SREBP-1c and LXR in control of DNL and development of hepatic steatosis (2,36,37). Other studies have focussed on the interplay between these two factors (5,6,21). So far, few studies have addressed the role of ChREBP in control of DNL (9,12,13) and only very recently it was shown that LXR regulates ChREBP activation and transcription (22). The present study indicates that pharmacological inhibition of G6PT with S4048 in C57BL/6J OlaHsd mice, resulting in increased hepatic G6P and glycogen levels, induces DNL exclusively via ChREBP. Because Gk expression was not increased upon S4048-inhibition, SREBP-1c is probably not involved in S4048-induced hepatic steatosis. Moreover, LXRα does also not play a role: S4048-treatment had comparable hepatic effects in LXRα +/+ and LXRα -/- mice. Surprisingly, nuclear ChREBP translocation was not increased upon S4048-treatment. This is probably due to changes in phosphorylation state upon S4048-treatment. In livers of both control and S4048-treated mice, insulin decreased AMPK phosphorylation via its effects on PKB.

In previous experiments in rats, blood glucose levels of S4048-treated animals were 60% of that of control rats. (24). More drastic hypoglycemic effects were seen in the C57BL/6J mice (figure 7.1): 6 hours of S4048-infusion resulted in a 64% decrease of the blood glucose levels. The S4048-mediated increases of hepatic TG, G6P and glycogen concentrations were also more pronounced in C57BL/6J mice (table 7.2) than in rats (25). Compared to control rats, hepatic G6P, glycogen and TG levels increased 3-, 2.8-, and 4.6-fold respectively upon S4048-infusion in rats. In C57BL/6J mice, these increases were 27-, 16-, and 2.4-fold, respectively. The differences between S4048-mediated effects in rats and C57BL/6J mice could be due to the increased metabolic flux in mice compared to rats. Our laboratory used stable isotopes from intermediates in hepatic glucose metabolism to study in vivo hepatic glucose fluxes. The flux through G6Pase was 39.9 ± 3.8 µmol·kg⁻¹·min⁻¹ in Wistar rats (24) whereas this was 228 ± 16 µmol·kg⁻¹·min⁻¹ in C57BL/6J mice (38). When the G6Pase flux is higher, inhibition of this flux will lead to more drastic effects, i.e., more strongly increased hepatic G6P and glycogen levels after 6 hours of S4048-infusion. In the LXRα +/+ and LXRα -/- mice on a mixed C57BL/6J Sv129/OlaHsd background, however, blood glucose levels decreased only by 44% after 6 hours of S4048-infusion (figure 7.6). The elevations of hepatic G6P, glycogen and TG were also less severe in these mice (table 7.3), indicating lower hepatic glucose flux rates. Indeed, previous studies showed that hepatic G6Pase flux in Sv129 mice was ~70% of that of C57BL/6J mice (39).
Because SREBP-1c and ChREBP control transcription of largely the same genes, it is hard to distinguish effects of both transcription factors on lipogenic gene expression in vivo. However, Pk expression is regulated by ChREBP but not by SREBP-1c (10) and Gk expression is regulated by SREBP-1c but not by ChREBP (13). Focussing on changes in the expression of these two genes in C57BL/6J OlaHsd mice upon S4048 treatment (figure 7.2), it is evident that G6PT inhibition and hence increased G6P levels resulted in enhanced hepatic transcriptional activity of ChREBP but not of SREBP-1c. The fact that levels of the XulP precursor G6P were clearly increased upon S4048-infusion also indicates a role for ChREBP. Furthermore, the decreased Gk expression upon S4048 infusion could be the result of SREBP-1c mediated effects of insulin (40): it was shown in rats that S4048-infusion resulted in significantly reduced plasma insulin levels (24). Of course, studies with SREBP-1c -/-, ChREBP -/- and/or SREBP-1c -/- ChREBP -/- double knockout mice should be performed to investigate the roles of ChREBP and SREBP-1c in S4048-induced de novo lipogenesis. Using the LXRα +/- and LXRα -/- mice, it was already possible to exclude a role for LXRα in S4048-induced de novo lipogenesis. Effects of S4048 on hepatic gene expression and hepatic TG levels did not differ between LXRα +/- and LXRα -/- mice (figure 7.7 and table 7.3).

So far, the data suggested a role for ChREBP in S4048-induced DNL and we therefore measured ChREBP protein nuclear translocation in C57BL/6J OlaHsd mice as well as in LXRα +/- and LXRα -/- mice. (figures 7.3 and 7.8). However, no effects of S4048 were seen in either of the three types of mice. Figures 3 and 8 show clearly that ChREBP is predominantly present in the cytosol, as was already shown by Dentin et al. (41). The fact that the amount of ChREBP within the nucleus was not changed does not necessarily mean less active ChREBP because it is the dephosphorylation of ChREBP at Thr666 within in the nucleus that enhances its binding to the DNA (10). The phosphorylation state of Ser196 mediates its localization in the cytosol or nucleus (10). Until phospho-specific ChREBP antibodies are available, Western blotting techniques cannot be used to study the effects of S4048 on active ChREBP within the nucleus. In the future, studies with primary hepatocytes and stable cell-lines should be used to study the effects of S4048 on subcellular ChREBP protein localization and DNA-binding.

In the present study, we could not confirm the suggested role of ChREBP in LXR-induced hepatic DNL (22): pharmacological LXR activation nor LXRα deficiency affected hepatic Chrebp mRNA levels (figures 7.5 and 7.7). On the other hand, increased Chrebp mRNA levels upon S4048-infusion (figures 7.2 and 7.7) did not translate into increased ChREBP protein levels (figures 7.3 and 7.8). The effects of LXR activation on Pk mRNA levels (figure 7.5) are therefore presumably mediated by the ChREBP phosphorylation/dephosphorylation state. As mentioned, more advanced ChREBP antibodies and/or in vitro experiments should be used to study the effects of S4048 on ChREBP in more detail.

Using Western blotting techniques, we noticed increased phosphorylation of the α-subunit of AMPK upon S4048-treatment (figure 7.4), reflecting increased activity of this kinase. AMPK is known to regulate, at least, the phosphorylation of ChREBP on Thr666, thus reducing its binding to DNA (10). Activity of AMPK is enhanced upon increased AMP and/or decreased ATP levels, thus upon increased energy expenditure (11). In the present hepatic steatotic model, enhanced hepatic AMPK activity could be the result of the increased production of fatty acyl-CoA from fatty acids. This process, mediated by ACS, uses ATP and produces AMP (42). Expression of Acs is regulated by both SREBP-1c and ChREBP (7,12). Because insulin reduces AMPK-activity (34), we injected mice with insulin. In control and S4048-treated mice, insulin injection reduced AMPK phosphorylation. Moreover, the key-regulator of insulin signaling, PKB, was also showed increased phosphorylation upon insulin injection, with no difference between S4048-treated and control mice.
PKA is known to phosphorylate ChREBP at both Thr666 and Ser196 (10), suggesting that subcellular ChREBP localisation is presumably regulated by PKA and not by AMPK. Like AMPK, PKA activity is also increased upon increasing energy usage. Thus, enhanced AMPK and/or PKA activity might provide a mechanism to shut off ChREBP-induced DNL.

In conclusion, the present studies strongly suggest that ChREBP, but not SREBP-1c and LXRα, is involved in induction of DNL and development of hepatic steatosis upon pharmacological inhibition of G6PT. Hence, increased DNL in GSDI patients (23) might also be due to effects on ChREBP activity and/or transcription, suggesting that ChREBP might be an important target for future pharmacological interventions in these patients.

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