Differential Regulation of Sterol Regulatory Element-binding Protein 1c Transcriptional Activity by Insulin and Liver X Receptor during Liver Development*

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Sterol regulatory element-binding proteins (SREBPs) are transcription factors involved in the synthesis of cholesterol and fatty acids. In adults, the isoform SREBP-1c is the predominant transcript in the liver of fed animals, and it activates triglyceride production from glucose when diet is enriched in carbohydrates. Studies have shown that SREBP-1c expression is dependent on insulin but also on the availability of oxysterols, ligands of the nuclear liver X receptor (LXR). The aim of this study was to investigate the regulation of the hepatic SREBP-1c expression in vivo in situations where drastic nutritional and hormonal changes occur, from the gestation to the weaning period. In this paper, we report the discovery of LXR-independent SREBP-1c transcriptional activity during late gestation. In utero insulin injection prior to the natural rise in insulin in late gestation triggers SREBP-1c mRNA elevation, nuclear SREBP-1c binding activity, and expression of its target genes independently of LXR transactivation. On the other hand, during suckling, we observed strong SREBP-1c mRNA expression despite very low plasma insulin, an expression that may be due to LXR transactivation. In contrast to insulin, LXR is not sufficient to trigger nuclear SREBP-1c binding activity and target gene induction. This could be due to the concomitant induction of INSIG-2a by LXR and subsequent retention of SREBP-1c in the endoplasmic reticulum.

During development, the organism must continuously adapt its metabolism to the nutritional environment because the availability and quality of the nutrients varies widely throughout the different developmental stages. In rodents, as for most mammals, the fetus receives through the placenta a diet enriched in carbohydrates but very poor in fat (1). Immediately after birth, the maternal supply of substrates ceases abruptly, and the newborn is fed at intervals with milk, a high fat, low carbohydrate diet (2, 3). Toward the end of the suckling period, the milk is progressively replaced by the solid food diet of the adult, enriched in carbohydrates. The adaptation to these changes in nutrition requires important modifications of glucose and fatty acid metabolism in the liver orchestrated mainly through hormonal variations.

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In the adult, the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) has been shown to play a key role for the control of glucose and fatty acid metabolism in the liver (4, 5). The SREBP-1c transcription factor belongs to the basic helix-loop-helix leucine zipper family. It is synthesized as a precursor form anchored in the endoplasmic reticulum (ER). In the ER, SREBPs form a complex with the SREBP cleavage-activating protein. SREBP cleavage-activating protein escorts the SREBPs to the Golgi apparatus where they are processed by two proteases. After proteolytic cleavage, the mature transcriptionally active form of SREBP migrates into the nucleus where it can bind to the sterol regulatory element (SRE) or E boxes of target genes (6–8). INSIG-1 and INSIG-2 proteins have been identified as proteins that promote SREBP retention in the ER through their interaction with SREBP cleavage-activating protein, thus preventing SREBP translocation to the Golgi apparatus for proteolytic processing (9). It has recently been shown that a liver-specific isoform derived from the INSIG-2 gene called INSIG-2a is selectively down-regulated by insulin (10).

SREBP-1c expression is itself under the control of the nutritional and hormonal status of the animal. Indeed, previous studies have shown that SREBP-1c expression is transcriptionally stimulated by insulin (11, 12). In vivo, a reduction of liver SREBP-1c mRNA is observed during fasting, whereas in mice refed a high carbohydrate diet, a strong increase of the SREBP-1c transcript occurs (11). SREBP-1c has been shown to be a major mediator of insulin action on the expression of glucokinase and lipogenesis-related genes in cultured rat hepatocytes (13) and in vivo (14). In the adult liver, insulin induces SREBP-1c transcription, but it has also been suggested that insulin enhances nuclear abundance of SREBP-1c (15, 16).

In addition to insulin, hepatic SREBP-1c is also under control of the oxysterols, which are ligands of the nuclear liver X receptor (LXR) (17). LXR directly activates SREBP-1c transcription through two LXRE-binding sites present in the SREBP-1c promoter. α and β isoforms of LXR form heterodimers with the ubiquitous dimerizing partner, RXR (18). Apart from activation of lipogenesis through SREBP-1c, the LXR transcription factors are crucial because they regulate multiple genes involved in cholesterol metabolism and transport, including cholesterol 7α hydroxylase, the rate-limiting enzyme in bile acids production (19), and genes such as the 

1 The abbreviations used are: SREBP-1c, sterol regulatory element-binding protein 1c; ABC-A1, ATP-binding cassette transporter A1; EMSA, electromobility shift assay; ER, endoplasmic reticulum; FAS, fatty acid synthase; GK, glucokinase; INSIG, insulin-induced gene; LPL, lipoprotein lipase; LXR, liver X receptor; LXRE, liver X receptor-binding element; pc, post-coitum; SRE, sterol regulatory element.
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EXPERIMENTAL PROCEDURES

Animals—C3H mice (Charles River) were used in this study. The animals were housed in a controlled environment (constant temperature and humidity, light from 7 a.m. to 7 p.m.) and fed ad libitum with a standard chow diet: composition (calories) 65% carbohydrates, 11% lipids, and 24% proteins (UAR A 03).

Adult Mice—Adult mice were fasted for 16 h and then refed with standard chow and water containing 20% sucrose for 6 h. After cervical dislocation, the livers were collected for RNA isolation.

Fetuses—Following mating, noon of the day of the appearance of a vaginal plug was taken to be 0.5 day of gestation. Fetuses at stages 16.5, 17.5, and 19.5 days post coitum (pc) were removed from uteri, transferred to dishes containing phosphate-buffered saline, sacrificed, and dissected. The liver was collected for RNA and nuclear protein extraction. For insulin injection (Actrapid®, NovoNordisk), the abdominal wall of pregnant mice was incised under anesthesia, and subcutaneous injection on P6 polyacrylamide beads columns (Bio-Rad). For binding assays of SREBP-1, nuclear extracts (4 μg) were incubated in a buffer containing 1% Ficoll, 10 mM Tris, pH 7.4, 50 mM NaCl, 6.5 ng/μl poly(dI-dC), 0.3 mM MgCl2, 20 μM β-mercaptoethanol for 10 min at room temperature. The labeled DNA (0.1 ng) was then added with or without LXR (Santa Cruz Biotechnology) or SREBP-1 antibody (IgG 2A4; Interchim) for 20 min at room temperature. LXR electromobility shift assays (EMSA) were performed according to Yoshikawa et al. (23).

Insulin Immunoassay—The blood samples were centrifuged at 1500 × g for 15 min at 4 °C, and supernatants were used for insulin immunoassay (Insulin CT, Cis Bio International).

RESULTS

Liver SREBP-1c Binding Activity Increases with Plasma Insulin during Fetal Development—To investigate the mechanisms involved in the regulation of SREBP-1c expression during development, we first measured hepatic SREBP-1c mRNA in fetal livers from 16.5 days pc to day 1 after birth. Fig. 1A shows that SREBP-1c is expressed in the fetal liver at 16.5 days pc. Earlier stages showed no detectable SREBP-1c mRNA (results not shown). SREBP-1c mRNA levels increase from 16.5 days pc to 19.5 days pc and then fall dramatically at birth. The level of SREBP-1c mRNA expression at day 1 after birth is the lowest detected throughout our experiments including in the fasted mice (data not shown). These variations in SREBP-1c mRNA expression closely paralleled insulin plasma levels (Fig. 1A), before and after birth. Two SREBP-1 isoforms are derived from a single gene by the use of two distinct promoters and yield two differently regulated isoforms, SREBP-1a and SREBP-1c. In our experiments, SREBP-1a mRNA levels were low and did not change significantly between 16.5 days pc and 19.5 days pc (Fig. 1A).

We then assessed the potential SREBP-1c binding activity in the liver during development by EMSA using the SRE-binding site of the fatty acid synthase (FAS) gene (Table II). As shown in Fig. 1B, SREBP-1c binding activity was detected from 17.5 days pc. For SREBP-1c mRNA levels, there is a gradual increase in nuclear binding of SREBP-1 to the SRE probe from 16.5 to 19.5 days pc followed by a dramatic fall at birth. The specificity of the binding activity is confirmed by SREBP-1 antibody competition of the 19.5 days pc binding activity (Fig. 1B).


table

| Table I | Sequences of the forward and reverse primers used in real time RT-PCR |
|---------|---------------------------------------------------------------|
| ARNm   | Sequences of forward and reverse primers (5' to 3') |
| 18 S   | GGAGACCTAGAAAGGCG |
| ABC-A1 | GGAGACCTAGTTCCAGTCG |
| Fatty acid synthase | TTCGTTTTTCAGTTGCTCG |
| Glucokinase | GCCCGTATGCTGCTGGA |
| INSIG-1 | TCAGACCTAGGGCGATG |
| INSIG-2a | TCGACGTAGTACTGATG |
| Lipoprotein lipase | CTAACCTCTTAAACGAGAGC |
| LXRα | GCCACCTGCCTCCTCA |
| LXRβ | GCACGCTTTGACTCTTTCG |
| SREBP-1a | TGTGAGATGTGACACAC |
| SREBP-1c | AGCGACGATGAGAGGCG |

Nuclear Extracts Preparation—The livers were removed and quickly rinsed in ice-cold phosphate-buffered saline with the protease inhibitors pepstatin (5 μg/ml), leupeptin (8 μg/ml), and aprotinin (2 μg/ml). The livers were then transferred into a beaker, on ice, containing the homogenization buffer (2 μm sucrose, 10 μm Hepes, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol) and minced with scissors. After processing in a Dounce apparatus in fresh buffer on ice, the homogenates were layered on 2 μm sucrose cushions and centrifuged at 80,000 × g for 35 min at 0 °C. The nuclear pellets were then resuspended in a buffer containing 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl2, resuspended in a 20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol buffer, and incubated 15 min on ice. After spinning 5 min at 10,000 rpm, aliquots of the supernatants were stored at −80 °C. The protein content was determined as described by Bradford, using bovine serum albumin as a standard.

Electromobility Shift Assay—The DNA probes were prepared by annealing both strands of the SRE and LXR binding sequences (Table II) (23) and were labeled by fill-in with [α-32P]dCTP followed by purification on P6 polyacrylamide beads columns (Bio-Rad). For binding assays of SREBP-1, nuclear extracts (4 μg) were incubated in a buffer containing 1% Ficoll, 10 μM Tris, pH 7.4, 50 mM NaCl, 6.5 ng/μl poly(dI-dC), 0.3 mM MgCl2, 20 μM β-mercaptoethanol for 10 min at room temperature. The labeled DNA (0.1 ng) was then added with or without LXR (Santa Cruz Biotechnology) or SREBP-1 antibody (IgG 2A4; Interchim) for 20 min at room temperature. LXR electromobility shift assays (EMSA) were performed according to Yoshikawa et al. (23).

Statistical Analyses—The results are expressed as the means ± S.E. Statistical significance was assessed using a Mann-Whitney nonparametric test.
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The lowest band (Fig. 1B, band NS) is not displaced by the specific antibody and thus corresponds to a nonspecific binding activity. These experiments provide evidence that SREBP-1c mRNA and nuclear binding to the SRE increase progressively from mid-gestation to late gestation and drop just after birth in parallel with plasma insulin.

In the adult liver, SREBP-1c is a major transcriptional mediator of insulin action on glycolytic and lipogenic enzyme genes such as the glucokinase (GK) and FAS genes (12, 13, 16). A similar pattern as described for SREBP-1c mRNA and binding activity was observed for GK and FAS mRNA expression, characterized by an increase between 16.5 days pc and 19.5 days pc and a drastic fall at birth (Fig. 1C). These results suggest that GK and FAS are SREBP-1c target genes during late gestation and that SREBP-1c expression is itself related to plasma insulin concentrations.

SREBP-1c Expression Occurs in the Absence of LXRs Binding to LXRE during the Fetal Development in the Liver—In addition to insulin, the nuclear receptor LXR is also able to control SREBP-1c gene expression in the adult liver (24–26). Two isoforms of LXR are known. LXRα is primarily expressed in the liver, whereas LXRβ expression is ubiquitous with low levels in the adult liver (27). Expression of LXRα remains low from day 16.5 to day 19.5 pc when compared with the values observed after birth (Fig. 2A). LXRβ expression increases between day 16.5 and 19.5 pc and shows minor variations thereafter.

The potential binding activity of the LXR nuclear receptors was then assessed in the fetal liver by EMSA, using one functional LXRE-binding site of the SREBP-1c promoter (23) (Table II). Despite LXRα and β mRNA expression, there was no detectable LXRα or β binding activity between 16.5 and 19.5 days pc, whereas it was clearly present in the 21-day-old weaned mouse. Thus, SREBP-1c gene expression during fetal life is independent of both LXRα and β binding on its promoter at these stages.

In Utero Insulin Injections Can Trigger a Premature Increase of the SREBP-1c mRNAs and Expression of SREBP-1c Target Genes in the Fetus—To determine the involvement of insulin in the transcriptional regulation of SREBP-1c during fetal life, insulin was injected in utero at 16.5 days pc, a developmental stage characterized by low plasma insulin, low SREBP-1c mRNA level, and the absence of LXRs binding on the LXRE of the SREBP-1c gene. Within 4 h, insulin administration to the 16.5-day-old fetuses induces an increase of SREBP-1c mRNA (Fig. 3A). As shown by EMSA (Fig. 3B), a precocious strong binding activity of SREBP-1 is also triggered by the insulin injection. In contrast, no LXR binding activity was detected upon insulin injection (Fig. 3C). Moreover, no effect on LXR mRNA levels was observed (data not shown). Therefore, insulin is able to trigger a premature increase of SREBP-1c mRNA in the 16.5 day pc fetal liver in the absence of any LXR binding activity (Fig. 3C). This transcriptional activation of the SREBP-1c gene by insulin is followed by an increase in expression of SREBP-1c target genes, FAS and GK (Fig. 3D).

LXR Binding to SREBP-1c LXRE Could Explain High SREBP-1c mRNA Levels during the Suckling Period—Directly after birth, during the suckling period, pups ingest milk, which is a high fat, low carbohydrate diet, inducing low plasma insu-
lin and high plasma glucagon levels. At weaning, the transition from a milk diet to a high carbohydrate, low fat diet (animal chow) is responsible for a rise in plasma insulin and a decrease in plasma glucagon (28, 29).

Following our observation of the insulin-mediated regulation of SREBP-1c in the fetal liver, we then went on to examine the hepatic expression of SREBP-1c mRNA at days 1, 8, and 13 after birth (suckling period) and after weaning. Surprisingly, hepatic SREBP-1c mRNA levels were high during the suckling period, reaching a level of expression even higher than levels observed in weaned mice (Fig. 4A) or high carbohydrate-refed adult mice (data not shown). The low plasma insulin concentrations during suckling (under 20 microunits/ml) cannot account for this peak of SREBP-1c expression; thus another factor must be responsible for this induction.

Given that LXR is known to increase SREBP-1c transcription in the adult, we then hypothesized that this transcription factor could be responsible for the elevated hepatic SREBP-1c mRNA level during suckling. LXRα mRNA expression during suckling is increased 2-fold between days 1 and 8 after birth and peaks at day 13. LXRα expression decreases somewhat after weaning (Fig. 5A). At the same time, no statistically significant variation of LXRβ mRNA expression is observed (Fig. 5A). To assess LXR binding activity during suckling, we performed EMSA with nuclear extracts from days 1 to 15 after birth and from weaned mice (Fig. 5B). Contrary to what we observed during gestation, a strong LXR binding activity is detectable throughout the suckling period. Moreover, LXR target genes such as lipoprotein lipase (LPL) (30) and ABC-A1 (20, 31) have significantly elevated mRNA levels during suckling in comparison with weaned values and show the same expression pattern as SREBP-1c (Fig. 4B). The stronger decrease in the expression of SREBP-1c, LPL, and ABC-A1 mRNA in comparison with weaned values and show the same expression pattern as SREBP-1c (Fig. 4B). Thus, during the suckling period and in contrast with the late gestation, LXR appears the more likely activator of SREBP-1c expression.

Despite High SREBP-1c mRNA Expression, Glucokinase and Fatty Acid Synthase Gene Expression Remains Very Low throughout the Suckling Period—To determine whether the high level of SREBP-1c mRNA during the suckling period is followed by the transcriptional activation of its potential target genes, we measured GK and FAS expression at days 1, 8, and 13 after birth and at weaning by real time quantitative PCR (Fig. 6). As previously described (32–34), GK and FAS mRNA
levels remain very low during the suckling period and increase only at weaning when the animal switches to a high carbohydrate diet.

Recently, it has been shown by Botolin and Jump (35) that the SREBP-1c precursor protein was present in the ER of suckling rats, indicating that SREBP-1c mRNA is well translated at that time but absent from the nuclei. We have also detected high levels of expression of SREBP-1 precursor protein in the suckling mice (data not shown). In EMSA analysis of SREBP-1c binding activity of nuclear extracts from mouse livers, no SREBP-1 binding is detected during the suckling period, whereas a shift is evident using extracts from weaned mice (Fig. 7). This indicates an absence of nuclear binding of SREBP-1c on SRE probes during suckling. These results reveal that the absence of hepatic SREBP-1c target gene induction during suckling, despite elevated SREBP-1c mRNA and ER precursor protein, is due to the absence of nuclear SREBP-1 binding activity. Thus, these studies indirectly suggest that a specialized mechanism should promote SREBP-1c retention in the ER preventing nuclear translocation.

INSIG-2a but Not INSIG-1 Might Be Implicated in the Lack of SREBP-1c Binding Activity during the Suckling Period—INSIGs are recently discovered ER-specific proteins that function as SREBPs precursor retention factors, preventing their cleavage in the Golgi apparatus and further nuclear translocation. INSIG-1 and INSIG-2a are the two isoforms predominantly expressed in the liver. Because insulin is able to down-regulate INSIG-2a mRNA expression and because insulin plasma concentration during the suckling period is very low,
we hypothesized that INSIG-2a expression could be high during suckling and may interfere with SREBP-1c cleavage. We first measured hepatic INSIG-2a mRNA levels from 16.5 days pc to weaned mice (Fig. 8A). INSIG-2a expression is barely detectable during gestation in the liver. INSIG-2a expression increases gradually throughout suckling until day 15, when expression is maximal, and falls at the weaning/suckling transition. Thus, elevated INSIG-2a levels during suckling could be at least partly responsible for the retention of SREBP-1c into the ER compartment. It is interesting to note that INSIG-1 mRNA displays a totally opposite expression profile (Fig. 8A); high during gestation, INSIG-1 mRNA levels drop at birth and remain low throughout suckling until weaning when expression increases again.

**LXR Synthetic Agonist T0-901317 Induces INSIG-2a mRNA—** Because LXR binding activity and INSIG-2a mRNA are both elevated during the suckling period, we tested the hypothesis that LXR might regulate the INSIG-2a gene expression. We force-fed adult mice a LXR agonist, T0-901317. The results show a 3-fold induction of hepatic INSIG-2a mRNA after 12 h of LXR agonist treatment (Fig. 8B).

**DISCUSSION**

In this study, we have shown that insulin stimulates SREBP-1c transcriptional activity in the absence of LXR binding activity, and this results in the increased expression of SREBP-1c target genes during the late gestation period. In contrast, the suckling period is a unique situation in which a high LXRA transcriptional activity but a low plasma insulin concentration, and insulin administration is sufficient to stimulate SREBP-1c expression and transcriptional activity. It is interesting that in contrast with what has been observed in the adult liver (17, 24, 26), SREBP-1c mRNA primary expression occurs in the complete absence of LXRa binding activity. It has been recently reported (36, 37) that insulin activates SREBP-1c expression through LXR binding on the SREBP-1 promoter. In our experiments, it is clear that during late gestation LXR binding activity is absent, and thus the contribution of LXR on insulin-induced SREBP-1c transcription must be negligible at this stage of development.

The regulation of SREBP-1c after birth displays drastic and unexpected changes throughout the suckling period. Our studies support the recent findings of Botolin and Jump (35), which show that despite high SREBP-1c mRNA content and SREBP-1c precursor protein, the SREBP-1c mature form and SREBP-1c target genes are very low. However, in the previous study, the authors gave no explanation as to why this may occur. During the suckling period, plasma insulin concentration is very low, and thus another factor must be responsible for the induction of SREBP-1c transcription. Here, we propose that LXR, a known activator of SREBP-1c transcription, is responsible for the high SREBP-1c gene expression during the suckling period. This is supported by the fact that we observed high LXRA mRNA, high LXR binding activity, and high expression of other known LXR target genes such as ABC-A1 and LPL during that period. Furthermore, milk is rich in cholesterol, which could induce an increased level of oxysterols, the natural LXR ligands. Oxysterols, through binding to the LXR, trigger mainly the expression of enzymes involved in bile acids production. Because the diet of suckling animals is extremely rich in triglycerides, the hepatic production of bile acids is an absolute requirement for complete fat digestion.
We also propose that the absence of SREBP-1c nuclear form during the suckling period is linked to the induction of INSIG-2a through LXR stimulation (Fig. 8B). During suckling and at weaning, the pattern of INSIG-2a expression is similar to the expression of known LXR target genes, including SREBP-1c. The presence of INSIG-2a during the suckling period would then explain the absence of the nuclear mature form of SREBP-1c and thus the absence of SREBP-1c target gene expression. At weaning, the decrease in the diet cholesterol content would reduce the synthesis of LXR ligands, and the increase in plasma insulin would lead to a rapid decrease in INSIG-2a (Fig. 8A) and thus the appearance of a SREBP-1c binding activity in the nucleus (Fig. 7). Moreover, our observations have shown an opposite expression profile for INSIG-1 and INSIG-2a mRNA. This suggests that in contrast with INSIG-2a, INSIG-1 does not interact with SREBP-1c to induce its retention in the ER. In fact, SREBP-1c is present in liver nuclei during late gestation and at weaning despite elevated
INSIG-1 mRNA level. Conversely, because mature SREBP-2 is present in the liver nuclei during suckling (35), this tends to indicate that INSIG-2a does not interact with SREBP-2.

What could be the physiological significance of this unique regulation of SREBP-1c during suckling? SREBP-1c can be considered as a transcription factor reflecting carbohydrate availability. Its transcription is induced by insulin in the liver (13), and its target genes include GK, the first enzyme of glucose utilization necessary for both glycogen and lipid synthesis. SREBP-1c together with the glucose-responsive carbohydrate-responsive element-binding protein transcription factor induces also lipogenic genes such as FAS, acetyl CoA carboxylase, and stearoyl CoA desaturase 1 (38–40). Finally, SREBP-1c is also able to repress genes involved in hepatic glucose production such as the phosphoenolpyruvate carboxykinase (41, 42). SREBP-1c expression is also inducible in the presence of a high cholesterol diet through LXR activation. This link between cholesterol and lipid metabolism could allow achievement of a proper cellular cholesterol/fatty acid ratio, an important membrane parameter. However, when a high cholesterol/triglyceride, low carbohydrate diet is consumed. At weaning, when the mechanism is switched on each time a high cholesterol, triglyceride/low carbohydrate diet is absorbed (suckling period), fatty acid synthesis is not anymore necessary, and low glucose availability precludes lipid synthesis from this substrate. Induction of SREBP-1c transcriptional activity in these conditions would be deleterious because it would favor glucose utilization and repress glucose production by the liver in a period of glucose shortage, leading to hypoglycemia. The mechanism that we are describing with a potential role for INSIG-2a would then be a safety mechanism allowing escape from such an adverse situation. One would predict that this mechanism is switched on each time a high cholesterol, triglyceride/low carbohydrate diet is consumed. At weaning, when the plasma insulin concentration increases, this leads to a decrease of INSIG-2a, then allowing the maturation and translocation of SREBP-1c to the nucleus, where it activates its target genes.

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