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Selective Proteolytic Processing of Rat Hepatic Sterol Regulatory Element Binding Protein-1 (SREBP-1) and SREBP–2 During Postnatal Development*

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Sterol regulatory element-binding protein-1c (SREBP-1c) plays a major role in hepatic lipogenic gene expression. In adult animals, insulin and oxysterols induce SREBP-1c gene transcription, whereas polyunsaturated fatty acids suppress the nuclear content of SREBP-1c through pre-translational regulatory mechanisms. A decline in nuclear SREBP-1 is associated with suppression of hepatic lipogenesis. In contrast to adult rats, hepatic lipogenesis in preweaned neonatal rats is low. Ingestion of milk fat by the neonate may contribute to low hepatic lipogenesis. In this report, we tested the hypothesis that low lipogenic gene expression prior to weaning correlates with low $mRNA_{SREBP\text{-}1c}$, as well as low precursor and nuclear forms of SREBP-1. In contrast to expectations, levels of $mRNA_{SREBP\text{-}1c}$ and the 125-kDa SREBP-1 precursor in livers of preweaned rats was comparable with adult levels. Despite high levels of SREBP-1 precursor, mature (65 kDa) SREBP-1 was not detected in rat liver nuclei prior to 18 days postpartum. Weaning rats at 21 days postpartum was accompanied by a rise in nuclear SREBP-1 levels as well as increased lipogenic gene expression. In contrast, SREBP-2 was present in rat liver nuclei, and its target gene, *HMG-CoA reductase*, was expressed above adult levels prior to weaning. These studies indicate that, prior to weaning, SREBP-2 but not SREBP-1 is proteolytically processed to the mature form. As such, SREBP-2-regulated genes are active. Failure of SREBP-1 to be processed to the mature form <18 days postpartum correlates with low hepatic lipogenic gene expression. This mechanism differs from the hormonal and fatty acid-mediated pre-translational control of SREBP-1c in adult liver.

Hepatic *de novo* lipogenesis is under complex hormonal and dietary regulation in adult animals (1). Lipogenic gene expression is induced by insulin, thyroid hormone, and dexamethasone and is suppressed by hormones elevating hepatocellular cAMP levels (1, 2). Dietary carbohydrate induces lipogenic genes, whereas dietary polyunsaturated fatty acids (PUFAs) suppress transcription of hepatic lipogenic genes. One of the key transcription factors controlling hepatic *de novo* lipogenesis is sterol regulatory element-binding protein-1c (SREBP-1c) (3, 4). SREBP-1c is a member of a family of basic helix-loop-helix leucine zipper transcription factors involved in fatty acid, triglyceride, and cholesterol synthesis.

SREBPs are translated as ~125-kDa precursors (pSREBP) attached to the endoplasmic reticulum (ER) (4, 5). After proteolytic processing in the Golgi, the nuclear form, nSREBP (~65 kDa), accumulates in nuclei where it binds sterol regulatory elements in promoters of many genes involved in fatty acid, triglyceride, and cholesterol synthesis. The proteolytic processing of SREBP is mediated by at least three proteins, *i.e.* SREBP-cleavage activating protein (SCAP), site-1 protease (S1P), and site-2 protease (S2P) (4). The escort of SREBP from the ER to the Golgi by SCAP is inhibited by the accumulation of sterols in cells, thus preventing maturation of SREBP to a form regulating gene transcription. Germline modification of mice has shown that SCAP and S1P are important for the processing of both SREBP-1 and SREBP-2 (4, 6, 7).

Whereas SREBP-2 is involved in cholesterol synthesis, SREBP-1 plays a central role in lipogenesis (3, 4). SREBP-1c is the predominant SREBP-1 subtype expressed in adult rodent liver (3, 4). Transcription of the *SREBP-1c* gene is induced by oxysterols through LXR (8, 9,11) as well as insulin (12–15). Insulin induction of LXRα gene transcription might also contribute to this regulatory scheme (15). Much of insulin action on lipogenic gene transcription has been ascribed to the insulin-mediated induction of SREBP-1c (12–14). Studies with primary rat hepatocytes suggest that SREBP-1 may be constitutively processed to the nuclear form (12).

Unsaturated fatty acids have been reported to suppress nuclear SREBP levels in HepG2 cell, rat primary hepatocytes, and rat liver (16–21). However, in the liver, nuclear levels of SREBP-1c but not of SREBP-2 are suppressed by PUFAs (18–21). Feeding rodents diets supplemented with polyunsaturated fatty acids or treating primary hepatocytes with PUFA will suppress $mRNA_{SREBP\text{-}1c}$ and lead to a decline in both the precursor and nuclear forms of SREBP-1c (18–20). Clarke and co-workers (20) have reported that the principal mechanism for the pre-translational control involves PUFA-enhanced $mRNA_{SREBP\text{-}1c}$ turnover. Overexpression of nSREBP-1c in pri-

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[1] The abbreviations used are: PUFA, polyunsaturated fatty acids; SREBP, sterol regulatory binding protein; pSREBP, precursor SREBP; nSREBP, nuclear form of SREBP; ER, endoplasmic reticulum; SCAP, SREBP-cleavage activating protein; LXR, liver X receptor; S1P, site-1 protease; RT, reverse transcription; GPAT, glycerophosphate acyl transferase; PPAR, peroxisome proliferator activated receptor; FAS, fatty acid synthase; ABCG5, ATP-binding cassette-G5, CYP7A, cytochrome P450 7A (7α-hydroxylase); CYP4A, cytochrome P450 4A; HMG-CoA, hydroxymethylglutaryl coenzyme A; mtHMG-CoA, mitochondrial HMG-CoA; HNF, hepatocyte nuclear factor.

mary hepatocytes or *in vivo* eliminates the PUFA effects on several lipogenic genes, indicating that SREBP-1c is a key target for PUFA suppression of *de novo* lipogenesis (19, 21).

During postnatal development of rats and mice, hepatic lipogenesis is low prior to weaning at 21 days postpartum (22–29). The activities of key enzymes, as well as their mRNAs, are very low during the suckling phase. Where examined, low lipogenic gene expression is due to low transcription rates (24). Based on studies with adult animals (2), low lipogenic gene transcription in newborns has been attributed to the ingestion of a high fat milk diet (26).

Because PUFA controls nuclear SREBP-1c levels through a pre-translational regulatory mechanism (18–20), we were interested in determining whether low hepatic lipogenic gene expression in preweaned animals correlates with low $mRNA_{SREBP\text{-}1c}$. Contrary to expectations, our studies indicated that both $mRNA_{SREBP\text{-}1}$ and pSREBP-1c are well expressed in rat liver prior to weaning. Only nSREBP-1c levels are low in neonatal liver, reflecting abrogated maturation of pSREBP-1 to nSREBP-1.

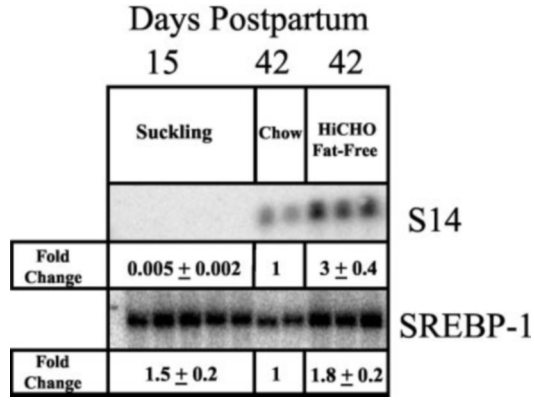

FIG. 1. **Expression of S14 and SREBP-1 mRNA in rat liver.** RNA was extracted from five suckling rats (15 days postpartum), two chow-fed rats, and three rats fed a high carbohydrate (*HiCHO*) fat-free diet for 7 days. The chow and high carbohydrate fat-free fed animals were 42 days postpartum. RNAs were separated electrophoretically, transferred to nitrocellulose, and probed with [^{32}P]cDNAs for S14 and SREBP-1c. Levels of expression were quantified by PhosphorImager analysis. The results are representative of at least two separate studies.

MATERIALS AND METHODS

Animals—Female Sprague-Dawley rats with litters were obtained from Charles River Laboratories (Kalamazoo, MI) and maintained on a Tek-Lad chow diet, *ad libitum*. Male rats at 15, 18, and 21 days postpartum were used for this analysis and compared with adult male rats (≥30 days postpartum).

Cell Extracts and Western Blotting—Extracts of rat liver were prepared by homogenizing tissue in Buffer A (0.25 M sucrose, 10 mM Tris-Cl, pH 7.5, and 3 mM $MgCl_2$ plus the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), pefabloc (0.1 mM), pepstatin (5 μg/ml), leupeptin (5 μg/ml), and aprotinin (2 μg/ml)). The homogenate was centrifuged (300 × *g* for 5 min at 2 °C.). The supernatant was then centrifuged (100,000 × *g* for 1 h at 4 °C) to obtain microsomes. The pellet from the first centrifugation was resuspended in Buffer A, adjusted to 1% Nonidet P-40, and homogenized. The homogenate was centrifuged (300 × g for 5 min at 2 °C.). The supernatant was retained for analysis. The nuclear pellet was resuspended in Buffer B (50 mM Hepes, pH 7.4, 0.1 M KCl, 3 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol plus the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), pefabloc (0.1 mM), pepstatin (5 μg/ml), leupeptin (5 μg/ml), and aprotinin (2 μg/ml)), adjusted to 0.4 M ammonium sulfate, and centrifuged at 100,000 × *g* for 60 min. The supernatant was used for analysis of nuclear proteins.

Proteins (50–100 μg) were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPAGE 4–10% polyacrylamide Bis-Tris, Invitrogen) and transferred to nitrocellulose membranes. Membranes were incubated with antibodies for SREBP-1 (IgG-2A4) or SREBP-2 (IgG-7D4) obtained from the supernatants of the hybridoma cell lines CRL 2121 and CRL2198, respectively (American Type Culture Collection, Manassas, VA), and CYP4A (Affinity Bioreagents, Inc., Warrendale, PA) or HNF-4α (Santa Cruz Biotechnology). The anti-mouse secondary antibody was obtained from Bio-Rad). The detection system employed the SuperSignal West Pico chemiluminescence kit (Pierce).

Plasmids—cDNAs for SREBP-1c, fatty acid synthase, S14, and cytochrome P450 4A (CYP4A) were described previously (19). cDNAs for HMG-CoA reductase and mtHMG-CoA reductase were prepared by RT-PCR and subcloned into a pSp72 vector. Sequence analysis was verified at the Michigan State University Genomic Core facility. The primers used to generate HMG-CoA reductase are as follows: sense, 5′-GTGGCCTCCATTGAGATCCGGAGGATCCAA-3′; and antisense, 5′-GGATCGCCATCCCACGCGCTATATTCTCCC-3′. The primers used to generate mtHMG-CoA synthase are as follows: sense, 5′-GATGTGGGCATCCTTGCCCTGGAGGTCTAC-3′; and antisense, 5′-AGTTGGCAGCGTTGAAGAGGGAGG CAGTGC-3′.

RNA Analysis—Livers were extracted with Triazol as described and used for Northern analysis (19). RNA was separated electrophoretically in denaturating agarose gels, transferred to nitrocellulose, and probed with [^{32}P]cDNAs. Levels of hybridization were quantified using a PhosphorImager 820 (Amersham Biosciences). RT-PCR analysis (Superscript One-Step RT-PCR with platinum *Taq*, Invitrogen) of the SREBP-1 subtype used the SREBP-1a specific primer (5′-ATGGACGAGCTGGCCTTCGGTGAGGCG-3′), and the SREBP-1c-specific primer (5′-GATTGCACATTTGAAGACATGCTT-3′) was used for sense strand synthesis (30). The internal primer (5′-GGGTCCTCCCAGGAAGGCTTCCAGAGA-3′) was used for antisense strand synthesis and is common to both the SREBP-1a and SREBP-1c transcripts. Total liver RNA from 15-, 18-, 21-, and 30-day-old rats was used as template. Amplified DNAs were separated electrophoretically and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Post-translational Processing of SREBP-1c Regulates Nuclear SREBP-1c Levels during Postnatal Development—In this report, we tested the hypothesis that low levels of hepatic lipogenesis prior to weaning correlate with low $mRNA_{SREBP\text{-}1c}$, which leads to a decline in pSREBP-1 and nSREBP-1. Levels of mRNA encoding SREBP-1 and the lipogenesis-associated protein S14 were measured in 15- and 42-day-old rats and 42-day old rats, respectively, fed a high carbohydrate-fat free diet for 5 days (Fig. 1). S14 was used because SREBP-1c induces S14 gene transcription (19, 31). The high carbohydrate fat-free diet was used to illustrate the effect of a fat-free diet on hepatic SREBP-1 and S14 mRNA levels. Hepatic $mRNA_{S14}$ is essentially absent at 15 days postpartum (Fig. 1). At this age, S14 gene transcription is not detectable (24). In contrast to $mRNA_{S14}$, hepatic $mRNA_{SREBP\text{-}1}$ in 15 day old rats is ~1.5-fold higher than that seen in 42-day-old chow-fed rats and is comparable with the level seen in rats fed a high carbohydrate fat-free diet for 5 days. RT-PCR analysis using SREBP-1a- and SREBP-1c-specific primers indicated that SREBP-1c represents >90% of the SREBP-1 expressed in rat liver at 15 days postpartum (not shown). The ratio of SREBP-1c to SREBP-1a was not different at 15 days postpartum and in adults. Thus, SREBP-1c is the predominant SREBP-1 transcript expressed in neonatal and adult liver.

Insulin-mediated induction of S14 and SREBP-1 gene transcription accounts for the elevated levels of S14 and SREBP-1c mRNAs in livers of adult animals fed high carbohydrate fat-free diets (12–14, 31, 32). However, high SREBP-1c mRNA levels prior to weaning cannot be ascribed to elevated insulin, because blood insulin levels are typically low, and the liver displays elevated ketogenesis (26, 28, 29). Thus, factors controlling hepatic SREBP-1c mRNA levels in the suckling animal differ from that seen in the adult rat.

Because low lipogenic gene expression cannot be explained by pre-translational suppression of $mRNA_{SREBP\text{-}1c}$, we examined hepatic precursor and nuclear SREBP-1 and SREBP-2

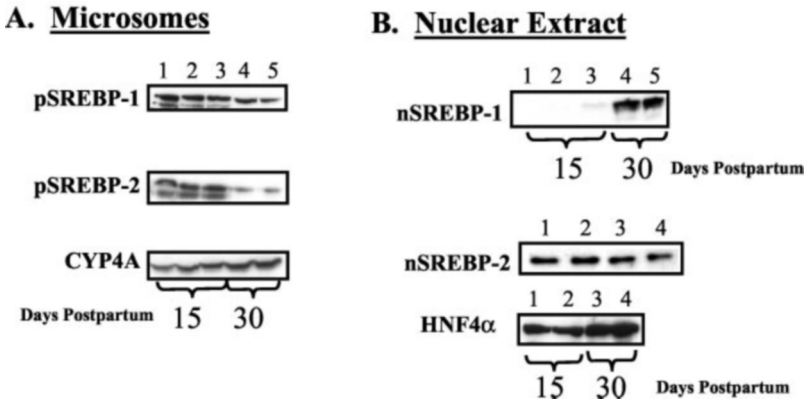

FIG. 2. **Expression of SREBP-1 and SREBP-2 in hepatic microsomes and nuclear extracts from 15- and 30-day-old rats.** Microsomal and nuclear extracts were prepared from two adult (30 days postpartum) and three preweaned (15 days postpartum) male rats as described under "Materials and Methods." Proteins from microsomal (*panel A*) and nuclear (*panel B*) extracts were separated electrophoretically, transferred to nitrocellulose, and reacted with antibodies for SREBP-1, SREBP-2, HNF-4, and CYP4A. The results are representative of two separate studies.

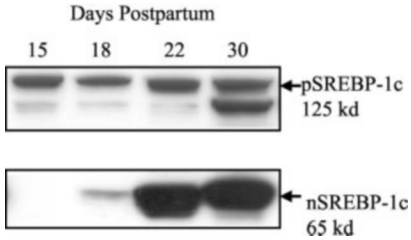

FIG. 3.**Time course of hepatic SREBP-1 appearance in microsomal and nuclear fractions during postnatal development.** Microsomal and nuclear extracts (one animal per time point) were prepared from animals 15, 18, 22, and 30 days postpartum. Proteins from microsomal (pSREBP-1c, 125 kDa) and nuclear (nSREBP-1c, 65 kDa) extracts were separated electrophoretically, transferred to nitrocellulose, and reacted with an SREBP-1 antibody. These results are representative of four separate sets of animals at each time point.

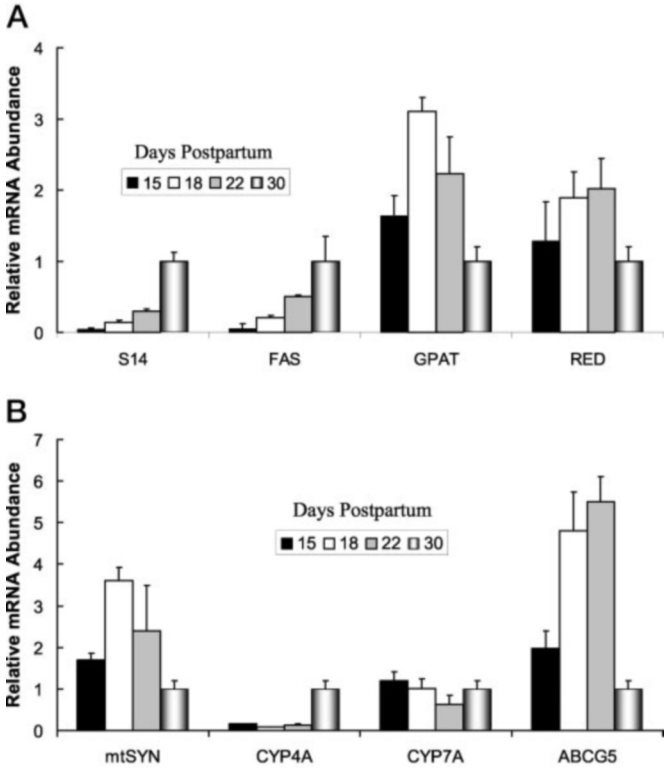

FIG. 4. **Time course of hepatic lipogenic gene expression.** RNAs were extracted from livers of male rats 15, 18, 22, and 30 days postpartum and separated electrophoretically. After transfer to nitrocellulose, blots were probed with ^{32}P-cDNAs. *A*, S14; fatty acid synthase (*FAS*); glycerophosphate acyl transferase (*GPAT*); and HMG-CoA reductase (*RED*). *B*, mitochondrial HMG-CoA synthase (*mtSYN*); cytochrome P450 4A (*CYP4A*); cytochrome P450 7A (*CYP7A*); and ATP-binding cassette-G5 (*ABCG5*); 15 (*black bar*), 18 (*white bar*), 22 (*gray bar*), and 30 days postpartum (*shaded bar*). Results are expressed as relative mRNA abundance, *i.e.* relative to adult levels at 30 days postpartum animal. Three animals are used at each time point. Mean ± standard deviation.

levels in 15- and 30-day-old rats (Fig. 2). Precursor levels of SREBP-1 and SREBP-2 were ~1.5- and 2-fold higher in livers of 15-day old rats when compared with adults. As a control, the microsomal monooxygenase CYP4A was ~2-fold higher in livers derived from 30-day-old animals than in those from 15-day-old animals. Although nSREBP-2 was present in nuclear extracts obtained from both 15- and 30-day-old animals, nSREBP-1 was not detected in hepatic nuclei isolated from 15-day-old rats. However, nSREBP-1 was present in nuclear extracts from 30-day-old animals. HNF-4α levels were ~2-fold higher in 30- *versus* 15-day-old rats.

These studies indicate that SREBP-2 matures to the nuclear form, but SREBP-1 maturation is abrogated, leading to little or no nSREBP-1 accumulation in hepatic nuclei of 15-day-old rats. The absence of nSREBP-1c in nuclei correlates with low hepatic *de novo* lipogenesis in the neonate. Thus, the principal mechanism accounting for low lipogenic gene expression in hepatic nuclei prior to weaning is due, at least in part, to low nSREBP-1 but not low $mRNA_{SREBP\text{-}1}$ or pSREBP-1. The mechanism controlling hepatic nSREBP-1 levels in neonatal and adult liver is clearly different.

Developmental Regulation of SREBP-1c Maturation Correlates with the Induction of Hepatic Lipogenic Gene Expression—Lipogenic gene expression, *i.e.* S14 and FAS, increases dramatically when rats are weaned (22–25). To determine whether SREBP-1 maturation followed this same time line, we examined SREBP-1 protein levels in hepatic microsomes and nuclei of animals at 15, 18, 22, and 30 days postpartum. Although these animals are normally weaned at 21 days of age, they begin to ingest solid food between 18 and 21 days postpartum. Microsomal pSREBP-1 remained unchanged over the 15-, 18-, 22-, and 30-day-old period (Fig. 3), a finding that correlates with the modest changes in $mRNA_{SREBP\text{-}1c}$ in 15-day-old and adult animals (Fig. 1). Hepatic nuclear nSREBP-1 was not detected at 15 days postpartum (Figs. 2 and 3) but was detected at 18 days postpartum. By 22 days of age, nSREBP-1 levels are comparable with adult levels. The maturation of SREBP-1c parallels the dietary switch from a high fat milk diet to a control chow diet.

To determine whether the change in nSREBP-1 correlated with the onset of lipogenic gene expression, we measured the expression of three SREBP-1 target genes (*S14*, *FAS*, and *GPAT*) as well as the SREBP-2 regulated gene *HMG-CoA reductase* (Fig. 4*A*). The mRNAs encoding S14 and FAS increased progressively from very low levels at 15 days of age to high levels at 30 days of age. These changes correlated with increased nSREBP-1 levels (Fig. 3). In contrast, glycerophos-

phate acyl transferase (GPAT) mRNA levels were above adult levels at 15–22 days postpartum. Unlike *de novo* lipogenesis, GPAT is required for the synthesis of phospholipids as well as triglycerides. The growing liver likely requires GPAT expression throughout all phases of development. Expression of the SREBP-2-regulated transcript, HMG-CoA reductase, was also above adult values during the 15–22 day old period, a finding consistent with abundant nSREBP-2 (Fig. 2) and elevated cholesterol synthesis in neonatal liver (28).

The mRNA encoding *mtHMG-CoA synthase*, a PPARα-regulated gene, is high prior to weaning (Fig. 4*B*). High levels of mtHMG-CoA synthase are consistent with elevated ketogenesis associated with suckling rats (26, 28). In contrast, another PPARα-regulated gene, *CYP4A*, is low prior to weaning. This apparent paradox is explained by the fact that low blood levels of insulin prior to weaning promote ketogenesis (26, 28, 29). Long-chain PUFAs are PPARα ligands and induce CYP4A gene transcription (33, 34). Milk fats are enriched in short to medium chain saturated fatty acids. Based on structural studies, these fatty acids are likely not good ligands for PPARα (23). The LXR-regulated transcripts (35), CYP7A (bile acid synthesis), and ABCG5 (cholesterol efflux) are well expressed throughout the 15–30-day postpartum period. Thus, the expression of genes encoding proteins involved in cholesterol synthesis (*HMG-CoA-reductase*), bile acid synthesis (*CYP7A*), and cholesterol efflux (*ABCG5*) are well expressed in liver prior to weaning.

Conclusion—Our studies provide the first *in vivo* evidence for differential regulation of hepatic SREBP-1 and SREBP-2 proteolytic processing. SREBP-2 is expressed as both pSREBP-2 and nSREBP-2 in livers derived from 15-day-old preweaned animals. Moreover, its target gene, *HMG-CoA reductase*, is well expressed in the liver prior to weaning. In contrast, hepatic lipogenic gene expression (FAS and S14) is very low prior to weaning. Low lipogenic gene expression correlates with low nSREBP-1 levels. The mechanism limiting nSREBP-1 in preweaned animals involves abrogated conversion of pSREBP-1 to nSREBP-1 (Figs. 2 and 3) and not pretranslational suppression of $mRNA_{SREBP\text{-}1}$ as seen in the adult (18–20). The physiological consequence of selective processing of SREBP-1 and SREBP-2 is a shift of hepatic metabolism toward cholesterol synthesis, *i.e.* HMG-CoA reductase, and away from *de novo* lipogenesis, *i.e.* S14 and FAS (Figs. 2–4).

Because SCAP and S1P are required for both SREBP-1 and SREBP-2 processing (4, 6, 7), it is unlikely that the selective blockade of SREBP-1 maturation is due to deficient SCAP or S1P. Alternatively, two other mechanisms might contribute to selective SREBP-1 and SREBP-2 proteolytic processing. Worgall *et al.* (17) reported that unsaturated fatty acid inhibition of SREBP processing was linked to sphingolipid metabolism and ceramide generation. This mechanism inhibited both SREBP-1 and SREBP-2 proteolytic processing without effects on SREBP-1 or SREBP-2 mRNAs.

An alternative mechanism involves the insulin regulated ER-associated proteins INSIG-1 and INSIG-2. INSIG-1 mRNA was originally isolated by Taub and co-workers (36) and found to be induced by insulin in Rueber H35 hepatoma cells. Yang *et al.* (37) and Yabe *et al.* (38) reported that INSIG-1 and INSIG-2 bind SCAP in the ER and block SREBP export to the Golgi for proteolytic processing. Janowski (10) recently reported that LXR agonists attenuate INSIG-1 gene expression (10). The LXR-regulated transcripts, ABCG5 and CYP7A1, are well expressed in neonatal liver implicating elevated hepatic oxysterols and active LXR (Fig. 4*B*). This observation, coupled with the fact that neonatal rats have low blood insulin levels and elevated hepatic ketogenesis (Refs. 28 and 29, and Fig. 4*B*), suggests that INSIG-1 or INSIG-2 might be low in the neonatal liver.

Clearly, more studies will be required to establish the roles sphingolipid metabolism, INSIG-1, and INSIG-2 play in the control of hepatic SREBP-1 and SREBP-2 proteolytic processing. We anticipate that definition of the molecular basis of differential processing of SREBP-1 and SREBP-2 in neonatal rat liver will provide important clues to selective control hepatic cholesterol and fatty acid synthesis.

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