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Lanosterol metabolism and sterol regulatory element binding protein (SREBP) expression in male germ cell maturation☆

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Abstract

Expression of genes involved in cholesterol biosynthesis in male germ cells is insensitive to the negative cholesterol feedback regulation, in contrast to cholesterol level-sensitive/sterol regulatory element binding protein (SREBP)-dependent gene regulation in somatic cells. The role of sterol regulatory element binding proteins in spermatogenic cells was an enigma until recently, when a soluble, 55 kDa cholesterol-insensitive form of SREBP2 (SREBP2gc) was discovered [Mol. Cell. Endocrinol. 22 (2002) 8478], being translated from a germ cell-specific SREBP2 mRNA. Our RT-PCR results also show that SREBP2 as well as SREBP1c mRNAs are detectable in prepubertal and postpubertal male germ cells while SREBP1a is not detected. Surprisingly, three SREBP2 immunoreactive proteins (72, 63 and 55 kDa), that are not present in mouse liver nuclei, reside in testis nuclei of prepubertal and adult mice. The 55 kDa protein is likely SREBP2gc, the other two isoforms are novel. HPLC measurements in liver and testes of fasted prepubertal and postpubertal mice showed no significant difference in cholesterol level. However, FF-MAS and lanosterol/testis-meiosis activating sterol (T-MAS) intermediates that are detectable mainly in testes, increase in fasted postpubertal mice which coincides well with the elevated level of 68 kDa SREBP2. Similar to SREBP2gc, the two novel SREBP2 immunoreactive proteins seem to be insensitive to the level of cholesterol.

Keywords: Cholesterol; SREBP; Testis; Male germ cell; MAS

1. Introduction

Spermatogenesis is a complex highly organized physiological process of male germ cell maturation [1]. The chief hormonal regulators of spermatogenesis are testosterone and follicle stimulating hormone (FSH) [2]. In addition to well-established endocrine control of spermatogenesis, there are also considerable data to indicate the paracrine/autocrine regulation of the process [3]. Although many factors have been implicated in the paracrine control of spermatogenesis, functional proof is only available for a few of them. Recently, the post-squalene cholesterol intermediate testis-meiosis activating sterol (T-MAS) was found to accumulate in testis, having the capacity to trigger resumption of oocyte meiosis in vitro [4].

In contrast to the very detailed knowledge of pre-squalene portion of the cholesterol biosynthetic pathway, the post-squalene portion is still poorly understood and it is believed to be committed to cholesterol. The discovery of two late cholesterol intermediates accumulating in gonads, being capable to initiate meiosis of mouse oocyte in vitro [4,5], pinpointed the possibility of additional physiological roles of late cholesterol intermediates. FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) was extracted from human preovulatory follicular fluid and T-MAS (4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol) from bull testicular tissue [4,5]. FF-MAS is derived from lanosterol by removal of the methyl group at the position 14α by lanosterol 14α-demethylase (CYP51), the only cytochrome P450 enzyme involved in the cholesterol biosynthetic pathway (Fig. 1). CYP51 is expressed in all tissues with the highest level in testis due to appearance of testis-specific transcripts [6]. T-MAS is produced via Δ14-reduction of FF-MAS (Fig. 1) [7,8] by sterol Δ14-reductase. Under normal metabolic conditions, rapid conversion of sterol intermediates to cholesterol results in their low concentration in liver, blood and other tissues. Exceptions include elevated level of MAS in testicular tissue and follicular fluid [9] and desmosterol in spermatozoa of the testis [10,11],

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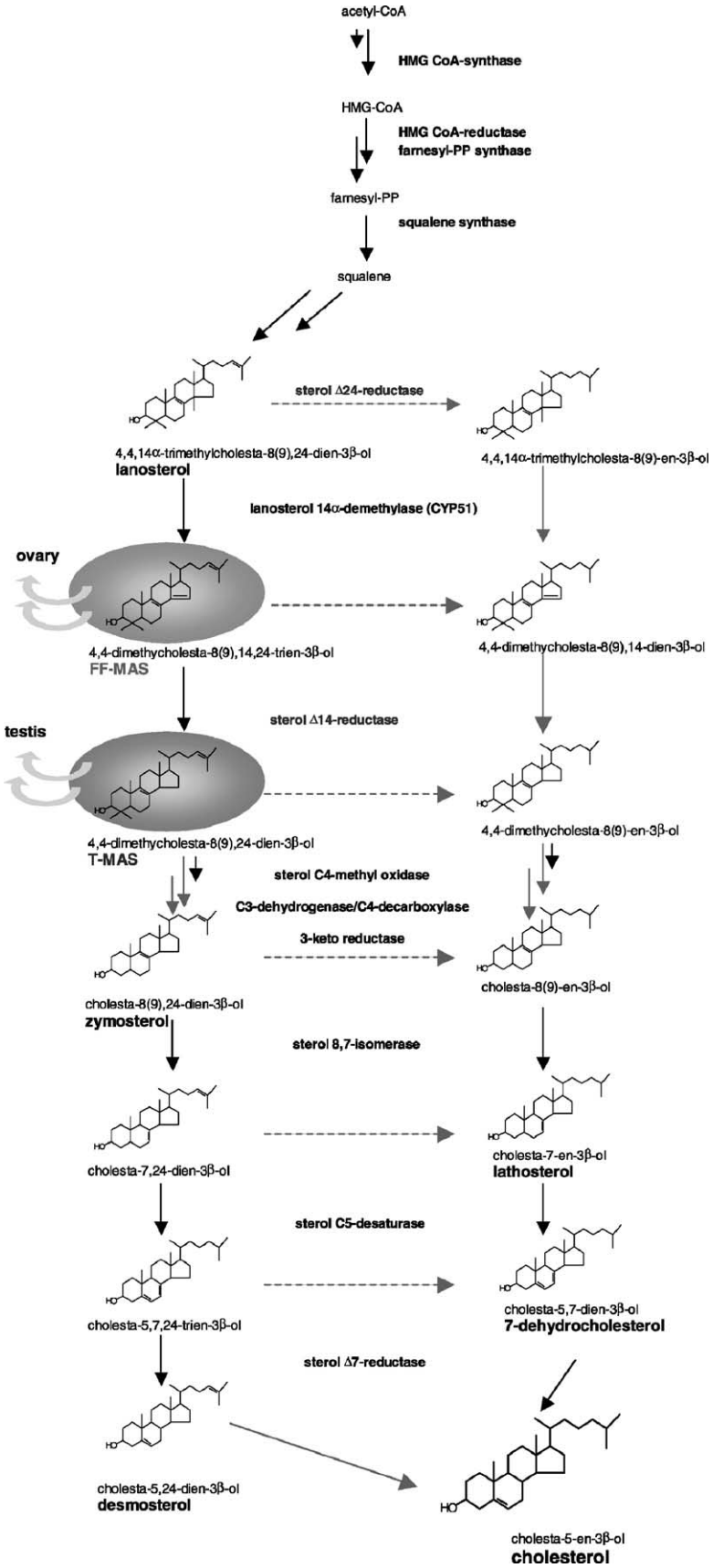

Fig. 1. The cholesterol biosynthetic pathway. All carbon atoms of cholesterol originate from acetate. After cyclization of squalene to lanosterol, the exact sequence of reactions is not clear and it seems to be tissue-specific.

developing brain [12] and human milk [13]. However, the mechanism by which the level of MAS in testis is regulated remains an unanswered question. Lindenthal et al. propose inhibition of two enzymatic steps in cholesterol biosynthesis, the 4,4-demethylation and Δ24-reduction, by a combined action of progestins [14]. However, results from the mouse oocyte assays showed that neither progesterone or 17α-OH-pregnenolone, nor the combination of both, influenced resumption of meiosis [15]. By studying the cholesterogenic gene expression in rat testis throughout sexual maturation, we have discovered that the transcriptional regulation in testis is, in contrast to the liver, not coordinated (discordant) [16]. Expression of pre-MAS genes (Fig. 1) is upregulated, whereas post-MAS genes are expressed at low level or even decrease during sexual maturation of the animal [16]. We have proposed that the discordant transcriptional regulation of cholesterogenic genes represents the first regulatory level that contributes to T-MAS accumulation in the testis [16].

Cholesterol biosynthesis is generally regulated on the transcriptional level by membrane bound transcription factors of the sterol responsive element binding protein (SREBP) family. SREBPs coordinate the homeostasis of two major building blocks of membranes; fatty acids and cholesterol [17]. SREBPs are involved also in glucose metabolism, which provides a link between carbohydrate and lipid metabolism [18]. The existence of three members of the SREBP family is well established. SREBP1a and -1c are produced from a single gene by alternative splicing, and differ only in their NH2-terminal transactivation domain. SREBP2 is encoded by a separate gene. All three proteins share a common tripartite structure consisting of a N-terminal transactivation domain, a hydrophobic region containing two transmembrane regions, and a C-terminal regulatory domain [17]. SREBP1a and -1c mRNAs are differently expressed in various mouse tissues and cultured cells. Obvious predominance of SREBP1c over SREBP1a was established for the majority of mouse tissues analyzed, while SREBP1a predominated in cultured cells. Interestingly, predominance of SREBP1a transcripts was likely not due to the rapid cell growth as a similar SREBP1c/SREBP1a ratio was observed also in fully differentiated cells [19]. SREBP1c was found to be especially highly expressed in the mouse liver, which is not surprising, keeping in mind the role of the liver in fatty acid synthesis. Transgenic mice technology demonstrated that SREBP1 isoforms are preferentially responsible for energy metabolism, including fatty acid synthesis and glucose/insulin metabolism, whereas the role of SREBP2 is in activation of genes involved in cholesterol biosynthesis [20]. Overexpression of SREBP1a profoundly activates both genes of cholesterol biosynthesis as well as fatty acid biosynthesis [21].

Regulation of cholesterol homeostasis and the mechanism by which cells sense the level of cholesterol are now well understood. SREBPs are synthesized as 125 kDa precursor proteins residing in ER membranes. The precursor is transported to the Golgi by a chaperone protein, SREBP-activating protein (SCAP) and is cleaved by two proteinases to release the mature 68 kDa N-terminal transcriptionally active part [17,22]. Once the cleavage has occurred, SREBP activates its target genes. SREBPs belong to bHLH-Zip family of transcription factors, which bind to sterol responsive element (SRE) motifs in the promoters of SREBP-responsive genes [17]. Processing of SREBP is regulated on the basis of the cholesterol negative feedback loop. SCAP, an eight transmembrane protein, containing a sterol-sensing domain, is crucial for the cholesterol level-based regulation. Alteration in cholesterol content in the membrane promotes changes in physical properties of the membrane and in this way prevents the SCAP-SREBP complex from reaching the Golgi apparatus under cholesterol-rich conditions [23]. INSIG proteins are responsible for ER-retention of the SCAP-SREBP complex [24,25].

The role of SREBPs in cholesterogenesis of male germ cell and testis was not studied in depth until recently, when a novel isoform of male germ cell-enriched transcription factor SREBP2 was characterized [26]. This protein, SREBP2gc, is expressed in a stage-dependent matter and is, in contrary to the somatic isoform, insensitive to the level of cholesterol. SREBP2gc is translated as a soluble, mature form of SREBP2, lacking a membrane spanning and SCAP-binding domain [26].

In the current study, we describe that only SREBP2 and SREBP1c mRNAs (but not SREBP1a mRNA) are expressed in mouse liver and testis of prepubertal and sexually mature mice. By studying the expression of SREBP2 protein, that is responsible for regulation of cholesterol biosynthesis, we observed four SREBP2 immunoreactive proteins. Three of the SREBP2 immunoreactive proteins reside only in testis of prepubertal and adult mice but not in the liver and all seem to be insensitive to the level of cholesterol. We also describe a correlation between the increase of the cholesterol level-dependent form of SREBP2 and intermediates of cholesterol biosynthesis during starvation.

2. Materials and methods

2.1. RNA isolation and RT-PCR

Tissues (liver, testis) were collected from normally fed adult and prepubertal male mice. Germ cells and interstitial cells were isolated from whole testis, by previously described procedures [27]. Total RNA was prepared from mouse liver, testis, germ cells and interstitial cells, using TriReagent kit (Sigma, St. Louis, MO). In order to remove possible DNA contamination, RNA samples were purified by DNase treatment (RQ1 RNase-Free DNase, Promega). Amplification of SREBP1a, -1c and SREBP2 fragments was done by one-step RT-PCR using rTth DNA polymerase (Gene Amp, EZ rTth RNA PCR Kit, Perkin-Elmer) and specific primers. The RT-PCR amplification conditions were:

60 °C for 60 min; 94 °C for 1 min; 32 cycles of 94 °C, 15 s; 56 °C, 30 s; 72 °C for 7 min. Sequences of primers were chosen according to the previously described procedure (Shimomura et al., 1997): (1) 5′-TAG TCC GAA GCC GGG TGG GCG CCG GCG CCA T-3′ (sense), 5′-GAT GTC GTT CAA AAC CGC TGT GTG TCC AGT TC-3′ (antisense) for amplification of SREBP1a fragment; (2) 5′-ATC GGC GCG GAA GCT GTC GGG GTA GCG TC-3′ (sense), 5′-ACT GTC TTG GTT GTT GAT GAG CTG GAG CAT-3′ (antisense) for amplification of SREBP1c fragment; (3) 5′-CAC AAT ATC ATT GAA AAG CGC TAC CGG TCC-3′ (sense), 5′-TTT TTC TGA TTG GCC AGC TTC AGC ACC ATG-3′ (antisense) for amplification of SREBP2 fragment. Mouse β-actin was used as an internal control and its fragment was amplified with primers: 5′-GGT CGT ACC ACA GGC ATT GTG ATG-3′ (sense), 5′-GGA GAG CAT AGC CCT CGT AGA TGG-3′ (antisense). RT-PCR products were analyzed by capillary electrophoresis (AbiPrism 310 Genetic Analyzer, Perkin-Elmer). Semi-quantitative analysis of specific SREBP transcript (SREBP1a, -1c, SREBP2) expression in mouse tissues was finally calculated by normalization to internal control, i.e. by comparing quantified peak areas of individual specific RT-PCR products to peak areas of internal control (mouse β-actin) RT-PCR products. Quantitative data were thus expressed in arbitrary units as mean values and standard deviations, based on data from three to six RT-PCR experiments performed for each SREBP transcript in each analyzed tissue/cell type sample.

2.2. Protein extraction and Western blotting

Nuclear proteins were prepared from liver and testis of normal fed and starved (12 h) prepubertal (25–30 days old) and adult (65–70 days old) CBA male mice (pool of 5–10 animals) as previously described [28] with slight modification. During feeding–fasting experiments, animals were housed in an ambient temperature and maintained on daylight schedule in ordinary cages containing sawdust. Tissues were homogenized in buffer containing 10 mM HEPES-KOH at pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM sodium EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1× cocktail of protease inhibitors (Sigma). The homogenate was centrifuged at 3300 × *g*, 4 °C for 15 min. The pellets were resuspended and incubated 1 h on ice in an equal volume of buffer containing 20 mM HEPES-KOH at pH 7.6, 25% (v/v) glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 1× cocktail of protease inhibitors (Sigma). The nuclei were collected by centrifuging the suspension for 30 min at 15,000 × *g*. The abundance of mature SREBP2 was determined by Western blotting.

Nuclear extracts (100 μg per lane) were separated on 8% SDS-polyacrylamide gels and transferred onto PVDF membranes (Amersham). Blocking step and dilution of antibody was done in 1× PBS buffer with 0.1% Tween and 5% dry milk. Immunoreactive SREBP2 was identified by incubating the blot overnight with monoclonal antibody against hamster SREBP2 (AA 32–250), IgG-7D4 prepared from mouse hybridoma cell line (ATCC No. CRL-2198). To estimate the quality of nuclear proteins, membrane was stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl (6.7) and incubated with anti-CREM-1 antibody (Santa Cruz, SC-440). To estimate cross-reaction between SREBP1a protein and anti-SREBP2 antibody, recombinant SREBP1a (AA 1–460) and SREBP2 (AA 1–481) proteins were prepared in *Escherichia coli* as 6× His-tagged fusion proteins (expression plasmids were a gift of T.F. Osborne, University of California, Irvine, CA, USA) and purified by Ni^{2+} affinity chromatography by QIAexpressionist system (Qiagen). Ten micrograms of each recombinant protein was loaded per lane and detected after incubation with the anti-SREBP2 antibody. Protein visualization was performed using a chemiluminiscence Western blotting detection system (Amersham).

2.3. Sterol extraction and HPLC analysis

Testis and liver tissue of 100–500 mg from normal fed and (12 or 24 h) starved prepubertal and adult CBA male mice was homogenized in 1 ml of cold PBS. Sterols were extracted as previously published [16,29]. Homogenate of 1 ml was extracted in 2.5 ml of 25% isopropanol:75% *n*-heptane, 100 ml of 0.3 M NaH_2PO4 (pH 1.0) was added and the solution was subjected to vigorous shaking (1800 rpm, several hours). The organic phase was separated by centrifugation (2000 × *g*, 10 min) and transferred to a fresh vial. Secondary extraction was preformed on the remaining water phase. Organic phases were pooled, dried in speed-vac and reconstituted in mobile phase for HPLC straight phase (SP) assay. Samples were loaded onto a ChromSpherSi, 5 mM, 150 mm × 3.0 mm column (Chrompack) running in 0.5 M isopropanol in *n*-heptane (v/v) at 1 ml/min. The windows containing 4,4-dimethylsterols and cholesterol (window 2–6 ft) were collected automatically. Individual SP elution windows were dried and subjected to reverse phase (RP) separation by reconstitution in acetonitrile and loading onto a LiChrospher RP-8, 5 μm, 150 mm × 3.0 mm HPLC column running in (v/v) 93% acetonitrile (Fischer Scientific, UK):7% water at 100 ml/min, room temperature. Quantification was done by comparing the eluted peaks with runs of commercial standards lanosterol (Sigma L5768, USA) and cholesterol (Steraloids C6760, USA) or laboratory standards FF-MAS and T-MAS [29]).

3. Results

3.1. RT-PCR analysis of SREBP mRNA expression in testis and spermatogenic cells in comparison to liver

To study the expression pattern of SREBP mRNA in different cell types of testis, we performed RT-PCR

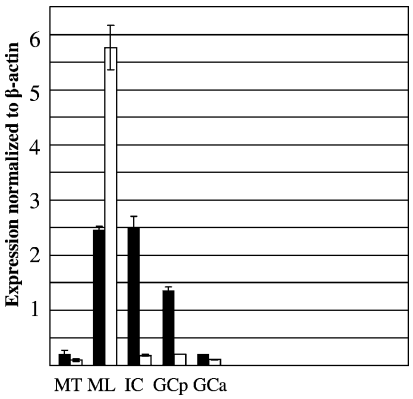

Fig. 2. SREBP mRNA expression in testis and spermatogenic cells in comparison to liver. Semi-quantitative analysis of SREBP2 (black bars) and SREBP1c (open bars) expression in mouse testis (MT), mouse liver (ML), interstitial cells (IC), prepubertal germ cells (GCp) and sexually mature germ cells (GCa) of male mice. All values were normalized to β-actin levels. The columns represent the mean values ± S.D., calculated from 3–6 independent RT-PCR experiments.

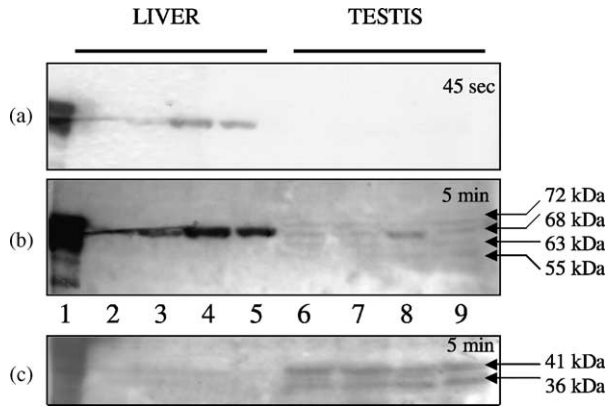

Fig. 3. SREBP2 mature protein in nuclear extracts of mouse liver and testis. 100 μg liver and testis nuclear extracts of normally fed, sexually mature (65–70 days, lanes 2 and 6) and prepubertal (20–25 days, lanes 3 and 7) male mice as well as of 12 h starved adult (lanes 4 and 8) and young (lanes 5 and 9) animals was examined. SREBP2 immunoreactive proteins were detected using IgG-7D4 antibody: (a) exposition 45 s, (b) exposition 5 min. To evaluate the quality of nuclear proteins, the same membrane was exposed to CREM-1 antibody (c). 1, SREBP2 recombinant protein.

experiments. Fig. 2 shows semi-quantitative data on expression of specific SREBP isoforms in mouse liver and testis. SREBP1c and SREBP2 were found to be expressed in all analyzed tissues, i.e. in mouse liver, testis, prepubertal and adult germ cells, and interstitial cells. SREBP1a was below the detection limit of our method. Higher expression of SREBP2 in comparison with SREBP1c was observed in all testis samples (interstitial cells > prepubertal germ cells > adult germ cells > whole testis). On the contrary, the expression of SREBP1c was found to predominate over SREBP2 in mouse liver samples.

3.2. Western blot analysis of SREBP2 protein expression in liver and whole testis

Liver and testis nuclear extracts of normal fed and starved (12 h) prepubertal (25–30 days old) and sexually mature (65–70 days) CBA male mice were prepared and immunoblots were performed to determine the SREBP2 protein. Starved mice were housed in cages, having the access to water and sawdust. SREBPs are synthesized as membrane attached precursor forms. Precursor protein is cleaved to release approximately 470 N-terminal amino acids which represents the mature transcription factor, migrating on SDS gels as 68 kDa [30]. In liver nuclear extracts we detected SREBP2 protein as a single lane migrating as 66–68 kDa. The expression level of mature SREBP2 protein in the liver of prepubertal animals shows a slight (1.4-fold) increase after 12 h starvation (Fig. 3a and b; lanes 3 and 5) compared to three-fold increase in liver of sexually mature animals (Fig. 3a and b; lanes 2 and 4). SREBP2 protein was detected also in whole testis nuclear extracts. The level of mature SREBP2 in testis nuclear extracts (Fig. 3b; lanes 6–9) was found to be approximately 10 times lower compared to the liver (Fig. 3b; lanes 2–5). In contrast to the liver, where 68 kDa protein was the only SREBP2 immunoreactive form, additional SREBP2-immunoreactive bands were noticed in testis nuclear extracts. According to our Western analysis, at least four SREBP2 isoforms exist in the testis, migrating on SDS gels as 72, 68, 63 and 55 kDa proteins. The 55 kDa protein (Fig. 3b), corresponds to the recently discovered male germ cell specific SREBP2 isoform, SREBP2gc [26]. SREBP2gc of 55 kDa was detected in nuclear extracts of sexually mature animals as well as in nuclei of 25–30 days old prepubertal animals. This finding is consistent with the developmental expression of SREBP2gc in male germ cells since this protein is first detected on postnatal day 17 in mouse testis, when pachytene spermatocytes become predominant [26]. The somatic 68 kDa SREBP2 also exists in testicular nuclei and likely belongs to somatic cell types of the testis (Sertoli cells, Leydig cells, peritubular cells, etc.). Elevated level of the 68 kDa SREBP2 was detected in sexually mature starved animals (Fig. 3b; lanes 6 and 8). This is in accordance with the cholesterol level-dependent activation of SREBP cleavage. Interestingly, the 68 kDa SREBP2 isoform of sexually immature male mice does not show a cholesterol level-dependent regulation. Our experiments show two novel SREBP2 isoforms, 72 and 63 kDa SREBP2 proteins. The starving–feeding experiment in prepubertal and sexually mature animals indicate that the two novel SREBP2 isoforms are also cholesterol level-independent. With the intention to estimate the quality of nuclear extracts from liver and testis, the membrane has been stripped and another immunodetection with the anti-CREM antibody has been performed. Two CREM-immunoreactive proteins have been detected in testis (Fig. 3c; lanes 6–9), one of them (36 kDa) likely corresponding to CREMτ, a transcription activator expressed mainly in haploid male germ cells. As expected, CREM proteins are below level of detection in

the liver nuclei (Fig. 3c; lanes 2–4). To check the specificity of anti-SREBP2 antibody, SREBP1a and SREBP2 recombinant proteins were transferred to membrane and incubated with the antibody. The anti-SREBP2 antibody does not cross-react with the SREBP1a protein, suggesting that all observed immunoreactive proteins are indeed isoforms of SREBP2 (not shown).

3.3. Identification of cholesterol and sterol intermediates in mouse liver and testis by HPLC analysis

The amount of cholesterol (Fig. 4a), FF-MAS (Fig. 4b) and lanosterol/T-MAS (Fig. 4c) was measured in liver and testis of normally fed sexually mature (65–70 days, lanes 2 and 6) and prepubertal (20–25 days, lanes 3 and 7) mice as well as in livers and testis of 12 h starved adult (lanes 4 and 8) and young (lanes 5 and 9) animals. Cholesterol is the predominant sterol in both tissues. At the level of non-esterified cholesterol, we were not able to notice a significant change despite the starvation. FF-MAS was detected only in testis and its amount is two orders of magnitude lower compared to cholesterol, which is in accordance with the proposed function of both sterols. Cholesterol is an abundant structural molecule as well as precursor of steroid hormone, etc. while FF-MAS is an intermediate and a possible signalling molecule. The amount of FF-MAS in testis of sexually mature animals during fasting increased (Fig. 4; lanes 6 and 8) which coincides with elevated level of 68 kDa SREBP2 protein in testis of starved adult animals (Fig. 3b; lanes 6 and 8). Lanosterol and T-MAS coeluted in our experiments. The peak of lanosterol/T-MAS shows a similar configuration as FF-MAS, its level being elevated in testis of starved adult animals.

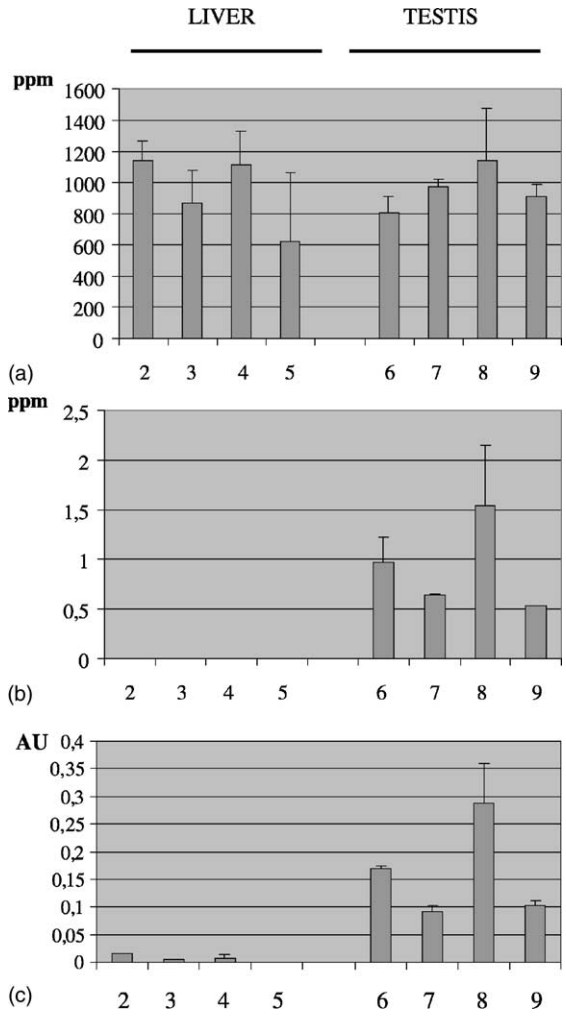

Fig. 4. Identification of cholesterol and sterol intermediates in mouse liver and testis by HPLC analysis. The amount of cholesterol (a), FF-MAS (b) and lanosterol/T-MAS (c) was measured in liver and testis of normally fed, sexually mature (65–70days, lanes 2 and 6) and prepubertal (20–25 days, lanes 3 and 7) male mice as well as of 12 h starved adult (lanes 4 and 8) and young (lanes 5 and 9) animals. Cholesterol and FF-MAS values are expressed in μg/g of wet weight tissue (parts per million—ppm). The values of the peak representing the sum of coeluting lanosterol and T-MAS are presented in arbitrary units (AU).

4. Discussion

SREBP transcription factors coordinately regulate the expression of genes involved in cholesterol homeostasis [31–33]. From the whole body perspective, cholesterol homeostasis maintains cholesterol level in physiological limits through coordination of all involved pathways, particularly the LDL receptor-mediated cholesterol uptake, biosynthesis de novo and export of cholesterol from cell and organism [34]. Regulation depends on the negative cholesterol feedback loop, being mediated by cholesterol level-sensitive transcription factors of the SREBP family.

Locally, on the level of a specific organ or even a specific cell, cholesterol homeostasis may be subordinate to other physiological/pathophysiological processes. In such cases, individual branches of the homeostatic process, such as cholesterol biosynthesis, might be regulated in a cholesterol level-independent manner. This means that genes involved in cholesterol biosynthesis might become transcriptionally activated also under conditions when cholesterol is not a limiting substance to the cell. Whenever cholesterol level-sensitive transcription factors of the SREBP family are responsible for transcriptional activation of cholesterogenic genes, a coordinate activation of all SREBP-responsive genes does exist [32,33,35]. It was believed until recently that SREBP-dependent activation arises solely in a cholesterol level-dependent manner. SREBP2 overexpressed in transgenic mice transactivates the entire pathway of cholesterol biosynthesis [33]. After permeability barrier perturbation in the epidermis, SREBP2 coordinately increases mRNA levels of HMG-CoA synthase, HMG-CoA reductase, FPP synthase and squalene synthase [36]. Using two independent prostate cancer cell lines, Heemers et al. demonstrated that coordinated stimulation

of lipogenic gene expression by androgens is a common phenomenon in androgen-responsive prostate tumor lines and involves activation of the sterol regulatory element binding protein pathway [37]. It is commonly accepted that the cholesterol-level/SREBP-dependent transactivation of cholesterogenic genes serves primarily to increase production of mRNAs encoding cholesterogenic enzymes, which consequently leads to an increased rate of cholesterol biosynthesis.

The second type of regulation described for cholesterogenic genes was the cholesterol level-independent/SREBP-independent type. Feeding cholesterol sufficient to increase the plasma level 30-fold did not prevent the increase in HMG-CoA reductase activity in the ovary of pregnant rabbits [38]. Steroidogenic factor 1 (SF-1), a nuclear receptor expressed in steroidogenic tissues with essential roles in steroid hormone biosynthesis, adrenal and gonadal development, conquers the cholesterol feedback inhibition and activates transcription of HMG-CoA synthase [39]. A decrease in the level of circulating cholesterol did not increase HMG-CoA reductase activity in normal rat testis [40]. Several physiologic and pathophysiologic conditions can result in cholesterol level/SREBP-independent transactivation of genes involved in cholesterol biosynthesis/metabolism. This results in a discordant regulation of the pathway, since under such conditions a unique cholesterol/SREBP-independent mechanism transactivates each pathway gene. The discordant regulation of HMG-CoA reductase and squalene synthase was observed during the host response to infection and inflammation [36,41,42]. Additionally, hepatomas (cancerous liver cells) lack the cholesterol feedback regulation since the cholesterol biosynthesis continues in spite of fasting or high cholesterol diet [43].

Cholesterogenic genes are furthermore discordantly expressed in testis during sexual development and no coordinate upregulation characteristic for the cholesterol-feedback mechanism is observed in germ cells [16]. Genes encoding pre-MAS enzymes (HMG-CoA synthase, HMG-CoA reductase, FPP synthase, squalene synthase, CYP51) are all upregulated, while two post-MAS genes (C-4 sterol methyl oxidase and sterol Δ7-reductase) are not upregulated in rat and human. Mice testis-specific transcripts of squalene synthase were found to be sterol unresponsive [44]. It was proposed initially that this transcriptional discrepancy in testis is cholesterol level/SREBP-independent. Nuclear extracts from mature male germ cells failed to show the presence SREBP1a in gel shift experiments [45].

The description of the soluble form of SREBP2 (SREBP2gc) in male germ cells [26] was a thrilling discovery, revealing the existence of cholesterol level-independent/SREBP2gc-dependent regulation of cholesterogenic genes. It seems possible that some of the above-described cholesterol level/SREBP-independent regulations indeed depend on cholesterol level-independent SREBPs. To date such SREBP isoforms were not reported in any other cell type except the mouse and rat male germ cells.

To understand the mechanism of the observed discordant male germ cell-specific transcriptional regulation of cholesterogenic genes [16], we tried to evaluate the role of SREBPs in the testis. As noted earlier, previous studies suggested that expression of cholesterogenic genes in male gonads is regulated through a cholesterol level/SREBP-independent mechanism. However, Shimomura et al. [19] using RNase protection assay showed that SREBP1a and SREBP1c are expressed in mouse testis. In our study, RT-PCR results show that both SREBP1c (but not SREBP1a) and SREBP2 transcripts are expressed in the whole testis and also in specific testis cell types (interstitial and germ cells). Semi-quantitative analysis of RT-PCR data showed higher SREBP2 expression when compared to SREBP1c expression in testis cells and tissue. The highest SREBP2/SREBP1c expression ratio was observed in interstitial cells, which are the site of testosterone synthesis. Although the role of SREBPs in cholesterol/fatty acid biosynthesis in somatic cells has been clearly established, the role of SREBPs in germ cells is not yet clear, despite the discovery of SREBP2gc [26].

Our Western blot experiments revealed the mature (somatic) 68 kDa SREBP2 protein in nuclear extracts of mouse testis. This form of SREBP2 depends on cholesterol levels. During the experiment, fasted animals had free access to water and litter sawdust. In this way they likely received some carbohydrates through digestion of sawdust cellulose, which can explain the increase of 68 kDa SREBP2 protein in the liver. A decrease in SREBP2 protein has been reported in starved animals that were housed in metabolic cages, having the access only to water [32]. In our experiment, the quantity of the 68 kDa SREBP2 protein increased in fasting liver and testis of sexually mature male mice, but interestingly, did not increase in testis of prepubertal mice. One possible explanation is that testosterone production, that occurs only in sexually mature mice, requires increased cholesterol biosynthesis and uptake by Leydig cells [46–48] as well as by Sertoli cells [49]. The level of 68 kDa SREBP2 in testis nuclear extracts is about 10 times lower compared to the level in liver of normally fed animals, which is contrary to recently published results [26]. To verify the quality of our nuclear extracts, a control experiment with the anti-CREM antibody was performed. This antibody recognizes both inhibiting (CREMα, β, etc.) and activating (CREMτ) isoforms. Control experiment showed expected results—a neglectable level of CREM in the liver and high level in the testis (Fig. 3c). It is possible that the observed difference between SREBP2 levels in liver and testis between our work and the work of Wang et al. [26] result from different mouse strains and/or nutritional regime.

Cholesterol is the predominant sterol in liver as well as in the testis. The starve–feeding experiment did not reveal significant changes of the liver and testis cholesterol. Due to the high level of cholesterol and its esterified (storage) derivatives in tissues, differences in total cholesterol amount are likely difficult to be observed after 12–24 h

fasting. In contrast to cholesterol, intermediates of the cholesterol biosynthesis pathway are difficult to observe in livers of normally fed animals. Their quantity in testis is at least two orders of magnitude lower compared to cholesterol. The quantity of lanosterol/T-MAS intermediates in the testis is about 10-fold higher compared to the liver of normally fed mice. During the fasting period the level of intermediate sterols increases two to three-fold in testis of sexually mature male mice. Since FF-MAS is a direct product of a SREBP-responsive gene *CYP51*, elevated MAS levels coincide well with elevated level of the cholesterol level-dependent 68 kDa SREBP2 protein in testis of starved adult animals.

Besides the 68 kDa SREPB2, three other SREBP2-immunoreactive proteins were surprisingly detected in testis of CBA mice. One corresponds to the recently described germ cell-specific 55 kDa SREBP2gc form [26], while the other two forms (72 and 63 kDa) have not been detected before. The starve–feeding experiment shows no change in the level of 72 and 63 kDa SREBP2-immunoreactive proteins, similarly as no change is observed for the 55 kDa SREBP2gc (Fig. 3). SREBP2gc is generated by alternative splicing and is similar to mature, proteolitically processed SREBP2, lacking 20 amino acids at its C-terminus [26].

The observed differences in mRNA levels of cholesterogenic genes during development of rat testis [16] might result from different transcription rates for each gene. This can be achieved through unique DNA-multiprotein complexes that are formed at promoters of different cholesterogenic genes, combined with different mRNA stabilities. Unique DNA-multiprotein transactivation complexes that result in different mRNA levels for different pathway genes, might all include cholesterol level-independent SREBPs. However, it seems possible that cholesterol level-independent forms of SREBP2 (SREBP2gc in particular) contribute mainly to the basal expression level of SREBP-dependent genes in vivo. We propose that SREBP-coregulatory proteins, such as proteins of the CREB/CREM/ATF1, Sp1 or NF-Y families [50–52], are major transcription rate determinants in cholesterol level-independent conditions. SREBP-coregulatory proteins can be turned on by various physiological stimuli. For example the developmental switch from CREM inhibiting isoforms to the CREMτ activating isoform, enhances transcription of cholesterogenic CYP51 at least two orders of magnitude in haploid male germ cells [45,53]. Interestingly, other cholesterogenic genes are not activated by CREMτ and likely require other gene-specific mechanisms.

In conclusion, the previously described discordant activation of cholesterogenic genes in the testis [16] is cholesterol level-independent but may involve cholesterol level-independent SREBPs. It remains to be determined whether in addition to male germ cell development, other physiological/pathophysiological conditions do require cholesterol level-independent SREBPs. Early embryonal development, for example is one of the processes where the involvement of cholesterol level-independent SREBPs would not be surprising. It is important to underline that SREBP2gc might not be the only cholesterol level-independent SREBP isoform.

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