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Localization of hormone-sensitive lipase to rat Sertoli cells and its expression in developing and degenerating testes

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Abstract Using in situ hybridization, hormone-sensitive lipase was found to be expressed in a stage-dependent manner in Sertoli cells of rat testis. No expression was found in Leydig cells but expression in spermatids could not be excluded. These results suggest a role for hormone-sensitive lipase in the metabolism of lipid droplets in Sertoli cells, in contrast to its previously proposed function in steroid biosynthesis. The expression of testicular hormone-sensitive lipase mRNA and protein, both larger in size compared to other tissues, coincided with the onset of spermatogenesis and was dependent on scrotal localization of the testis, suggesting a temperature-dependent, pretranslational regulation of expression.

Key words: Hormone sensitive lipase; Cholesterol ester hydrolase; Testis; Sertoli cell; In situ hybridization

1. Introduction

Like other steroidogenic tissues, testes accumulate cholesterol esters together with phospholipids and triacylglycerols in intracellular lipid droplets. These stores are thought to be important for steroid biosynthesis and spermatogenesis [1–5]. Spermatogenesis is severely impaired by temperature elevation, and we have previously shown that experimental cryptorchidism in the rat leads to a decrease in testicular cholesterol esterase activity, concomitant with increased lipid accumulation in Sertoli cells [6,7]. There are many reports on the presence of neutral cholesterol esterase activity in steroidogenic tissues, including testis. An early report by Durham and Grogan [3] describes the presence of two distinct cholesterol ester hydrolases in testis; one temperature-stable form, found in both Sertoli cells and Leydig cells, and one temperature-labile form, found only in Sertoli cells. They also suggested that a rapid inactivation of the temperature-labile enzyme was a primary event in the impairment of spermatogenesis in cryptorchid animals.

The cholesterol ester, hydrolase, previously described by us, exhibited the biochemical properties typical of hormone-sensitive lipase (HSL; EC 3.1.1.3), including the key feature of HSL of being phosphorylated and activated by cAMP-dependent protein kinase [6,8]. The presence of HSL in testis, as well as in adrenal glands and ovaries, has been confirmed using anti-HSL antibodies [9] and HSL cDNA probes [10]. Interestingly, both the mRNA and protein in testis are considerably larger than in other HSL-expressing tissues; the testicular 130 kDa protein is encoded by a 3.9 kb mRNA, as compared to 84 kDa and 3.3 kb, respectively, in adipose tissue.

Although it has been definitively established that HSL is expressed in the steroidogenic tissues, its function in these tissues is still unclear. It has been proposed that HSL primarily exhibits its cholesterol ester hydrolase activity, releasing free cholesterol from stored cholesterol esters to be used as a substrate in the biosynthesis of steroids. For two reasons this is an appealing hypothesis. First, it justifies the high cholesterol esterase activity of HSL, which in fact is as high as its activity against triacylglycerols [11], a unique feature among the mammalian lipases. Second, it raises the interesting possibility that cholesterol mobilization in steroidogenic tissues, in analogy with lipolysis in adipocytes, is a hormonally controlled event through cAMP-mediated phosphorylation and activation of HSL [11]. However, there is no experimental support for a hormone-dependent cholesterol mobilizing role of HSL in steroidogenic tissues. The larger size of testicular HSL mRNA and protein also raises the possibility that testis HSL has different, or additional, roles, as well as a different regulation compared to HSL in other steroid-producing tissues.

To start exploring the role and regulation of HSL in the testis the present study was undertaken. We demonstrate here that testis HSL is expressed in Sertoli cells, and not in Leydig cells, which would be expected from its proposed role in steroid biosynthesis. Furthermore, we show that HSL, both in postnatal development and in experimental cryptorchidism, is regulated at the pretranslational level through a temperature-dependent mechanism.

2. Material and methods

2.1. Animals

Male Sprague–Dawley rats of various ages were used in the different experiments. Newborn rats were subjected to unilateral gubernaculotomy in order to prevent testicular descent [12] and their testes were examined at adult age. In adult rats, unilateral cryptorchidism was induced by relocating one testis into the abdomen [13] for up to 4 days. In order to deplete the Leydig cells, ethane dimethane sulphonate (EDS, 75 mg/kg in dimethyl sulfoxide/H_2O, 1:3) was administered by intraperitoneal injection [14] and the animals were examined after 3 days. In order to deplete germ cells, rats of day 13 of pregnancy were given Busulphan (10 mg/kg in Tween 80 [15]). When 52 days old, the male

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Abbreviations: EDS, ethane dimethane sulphonate; DFP, diisopropyl fluorophosphate; HSL, hormone-sensitive lipase; SDS-PAGE, polyacrylamide gel-electrophoresis in sodium dodecyl sulphate.

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offspring were treated with EDS and examined 3 days later. The EDS and Busulphan treatments were verified by light microscopy of Bouin-fixed, metacrylate resin-embedded, hematoxylin/eosin-stained sections.

2.2. In situ hybridization

Testes from intact, adult rats were used. The specimens were fixed overnight in buffered 4% paraformaldehyde (pH 7.2), dehydrated and embedded in paraffin. Sections (4 μm) were cut in a microtome and mounted on chrome/alum-coated slides. Two synthetic 30-mer oligodeoxyribonucleotide probes were designed, complementary to the nucleotide sequences 762–791 and 2283–2312, respectively, of the rat HSL cDNA [16]. These nucleotide sequences showed no homology with any other mammalian cDNA sequences, as established by a computerized search (GenEMBL, June 1994). The probes were end-tailed with [^{35}S]dATP using a terminal transferase (both supplied by NEN-duPont), yielding a specific activity of approximately 2×10^9 cpm/μg, and purified through Chroma spin-10 columns (Clontech). The hybridization protocol used has previously been decribed in detail [17]. Briefly, the sections were deparaffinized and hydrated. Prior to hybridization the sections were digested by proteinase K (10 μg/ml; Sigma) and acetylated by 0.25% acetic anhydride in 0.1 M triethanolamine. Hybridization was carried out overnight at 37°C in sealed moisturizing chambers, using a probe concentration of approximately 1.0 pmol/ml. The sections were washed 4 times in 0.15 M NaCl, 0.015 M sodium citrate, 55°C (15 min each wash), followed by a 30 min wash in the same buffer at room temperature. The slides were dipped in Ilford's K-5 film emulsion, exposed for 3 weeks and developed in Kodak D-19. For control purposes, hybridization was also performed after incubation in RNase A (45 μg/ml; Sigma) for 30 min at 37°C or in the presence of a 100-fold excess of unlabeled probe.

2.3. RNA isolation, Northern blot and slot-blot analyses

Testis tissue was removed from rats that had been sacrificed by a blow on the neck. Total RNA was prepared either by the guanidinium thiocyanate/CsCl method [18] or the method of Chomczynski and Sacchi [19]. For Northern blot analysis, 10 μg of total RNA was subjected to agarose electrophoresis under denaturing conditions using 2.2 M formaldehyde. The RNA was transferred to nylon membranes and crosslinked by UV-light. Probes for hybridization were the following: full-length or a 1895 nt (595–2489) fragment of rat HSL cDNA, labelled with [^{32}P]dCTP to about 10^9 cpm/μg; the oligonucleotide representing nt 2283–2312 of rat HSL cDNA, end-labelled with [^{32}P]dATP to a specific activity of approximately 10^8 cpm/μg. Hybridization was carried out at 60°C and membranes were washed in 30 mM NaCl, 3 mM sodium citrate, 0.1% SDS at 60°C before subjected to autoradiography using Kodak XAR 5 film or digital imaging (Fujix BAS 2000). For analysis of slot blots, membranes were incubated with ^{32}P-labeled 1895 nt HSL DNA probe as above and autoradiographed. RNA was quantified by scanning densitometry.

2.4. Immunoprecipitation and Western blot analysis

Immunoprecipitation was performed essentially as in [20]: 1 mg of protein was incubated overnight with approximately 300 μg anti-HSL antibody [21], followed by incubation with protein A. Precipitates were washed, fractionated by SDS-PAGE [22], and electroblotted onto nitrocellulose membranes. HSL protein was detected with anti-HSL antibody [21], followed by incubation with [^{125}I]protein G [23] and autoradiography. Protein measurements were performed according to Bradford [24].

2.5. Enzyme preparation and HSL activity

1(3)-Mono-[^{3}H]oleoyl-2-*O*-oleylglycerol and [^{3}H]trioleoylglycerol were synthesized as in [25,26]. The HSL preparation used for determination of enzyme activity was a 100,000 × *g* supernatant fraction (10%) in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml antipain, 1 μg/ml pepstatin and 10 μg/ml leupeptin. The HSL used for activation was the pH 5.2 precipitate of a 100,000 × *g* supernatant, resuspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithioerythritol, 10 μg/ml antipain, 1 μg/ml pepstatin and 10 μg/ml leupeptin, cf. [11]. Enzyme was assayed against cholesteryl oleate [6], and against monooleoylalkylglycerol and trioleoylglycerol [11,25,26], at 25°C for 30 min. Enzyme activity is expressed in units, 1 unit being equivalent to the release of 1 μmol free fatty acid/min at 25°C. For enzyme activation studies, a pH 5.2 precipitate was phosphorylated by incubation with ATP and the catalytic subunit of cAMP-dependent protein kinase, prepared from rat adipose tissue as in [27]. Lipase activity was measured against trioleoylglycerol [8]. In inhibition experiments, supernatants from normal and cryptorchid testes were preincubated with 0.1 mM DFP or increasing concentrations of NaF for 30 min. Activity was measured against cholesteryl oleate.

3. Results

3.1. In situ hybridization

Both HSL oligonucleotide probes extensively labelled cells in the seminiferous tubules but not cells in the interstitial tissue. The grains were numerously distributed over the luminal parts of the epithelium, while labelling of the basal parts was sparse. This suggests that Sertoli cells express HSL (Fig. 1). The intensity of the labelling was stage dependent; the lowest intensity of labelling was observed in stages III–VII, i.e. prior to spermiation, whereas the highest intensity was observed in stages X–XIV, i.e. after spermiation (Fig. 2). Due to the limited resolution of autoradiography it is, however, not possible to exclude the possibility that the cytoplasm of the elongated spermatids could also express HSL.

Hybridization following RNase treatment or with an excess of unlabelled probe in the hybridization buffer resulted in loss of autoradiographical labelling of the sections (not illustrated). The same probes were also used for in situ hybridization in sections from rat supraepididymal fat, yielding perinuclear labelling of the adipocytes (not illustrated). In Northern blot analysis, using RNA from rat testis and adipose tissue, the oligonucleotide probe tested recognized a 3.9 kb mRNA in the testis and a 3.3 kb mRNA in adipose tissue, as expected (not illustrated).

3.2. EDS treatment

Depletion of Leydig cells (verified by light microscopy) through treatment with EDS affected neither the amount of HSL mRNA, its activity (not illustrated) nor protein content (Fig. 3). In Busulphan- and EDS-treated animals almost no germ cells and no Leydig cells were seen but these testes still expressed HSL mRNA, although at lower levels than in intact testes (not illustrated).

3.3. Developmental expression of HSL in the testes

The presence of HSL in rat testes at different developmental stages was analyzed with regard to mRNA expression and HSL enzyme activity (Fig. 4). The testis-specific mRNA of 3.9 kb could not be detected until 40 days of age, using a full-length HSL cDNA probe. A high and stable expression was maintained thereafter, at least until the age of 10 months. Enzyme activity measurements showed that before the age of 35 days, the activity towards cholesteryl oleate was less than 10% of the adult value. Thereafter a rapid increase in activity occurred. This activity was due to HSL, as confirmed by a simultaneous increase in diacylglycerol activity using as substrate a diacylglycerol analogue with high specificity for HSL [11]. The identity of testicular HSL was further confirmed by biochemical characteristics. The enzyme could be activated 102% (mean from two experiments) by incubation with ATP and the catalytic subunit of cAMP-dependent protein kinase, cf. [8]. Furthermore, the enzyme activity was inhibited 76% by 0.1 mM DFP, 72% by 100 mM NaF (cf. [11]) and 71% by the antibody towards rat adipose tissue HSL, cf. [9]. The residual activity

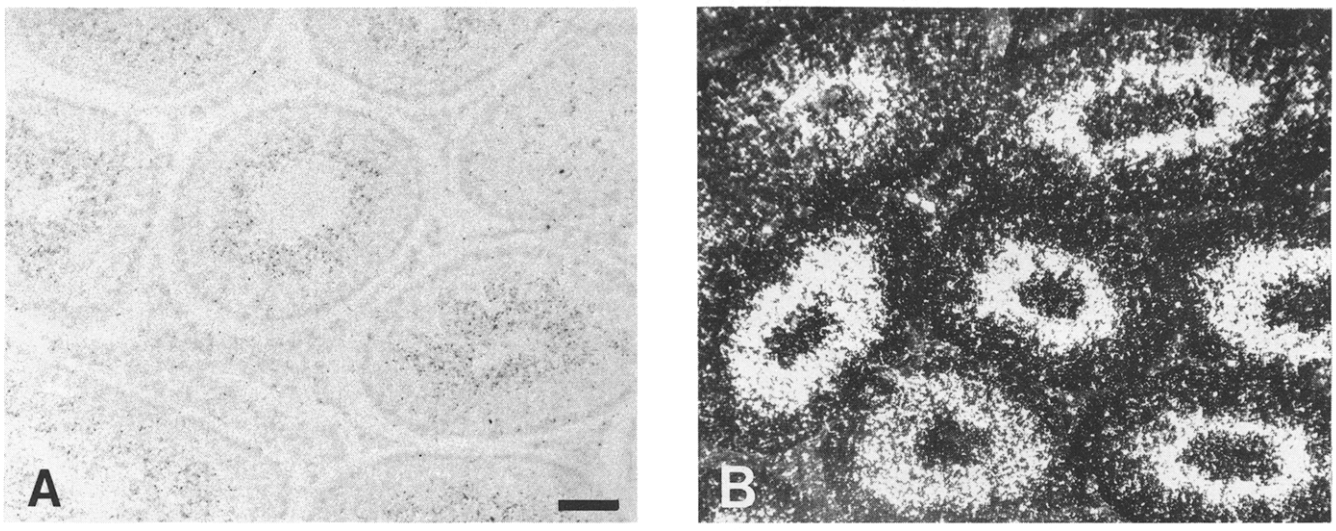

Fig. 1. Rat testis. In situ hybridization with a ^{35}S-labeled HSL oligonucleotide probe as viewed in light field (A) and dark field (B). Note the variable distribution of autoradiographic grains in the seminiferous tubules and the lack of grains in the interstitium. Bar = 100 μm.

observed indicates the presence of an additional lipase/esterase since HSL is known to be more than 90% inhibited by the above agents.

3.4. HSL in normal and cryptorchid testes

The regulation of HSL was further investigated in rats made unilaterally cryptorchid either at birth (primary) or when adult (secondary). Using Northern blot analysis, comparisons between the scrotal and abdominal testes RNA from primary cryptorchid rats revealed a high expression of the 3.9 kb HSL mRNA in the scrotal location, whereas in the abdominal testis no expression of HSL mRNA was found (Fig. 5). The same

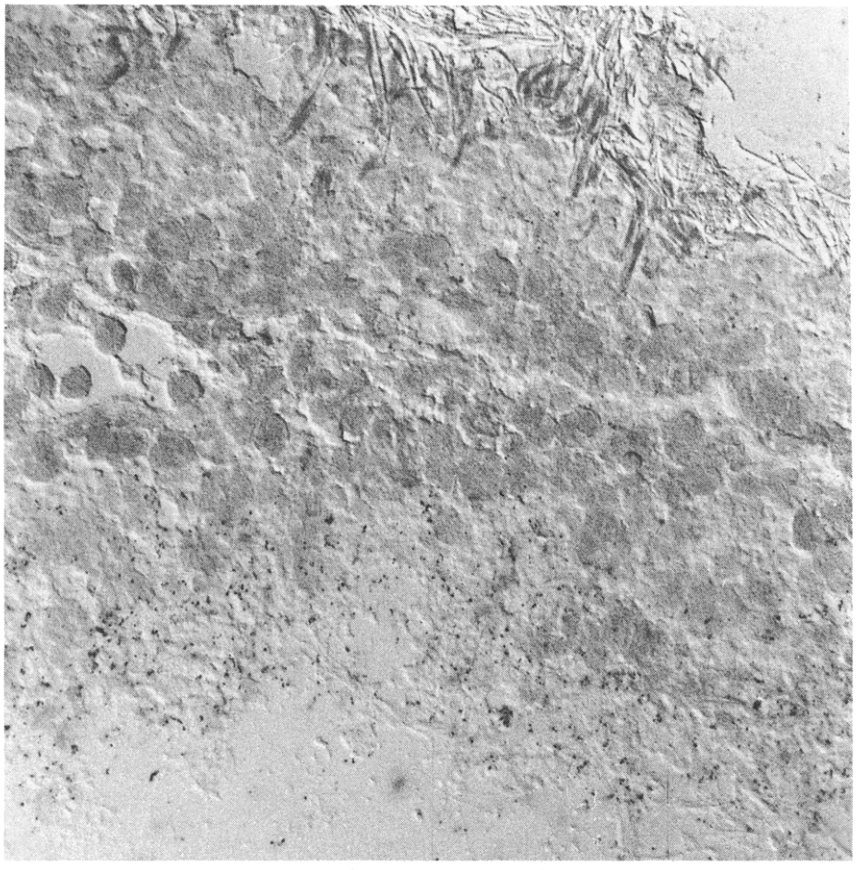

Fig. 2. Detail of rat testis from Fig. 1 (400× magnification, viewed in differential interference contrast). In the upper part is a tubule in stage VII and in the lower part a tubule in stage X. Note the low expression of HSL (autoradiographic grains) prior to spermiation.

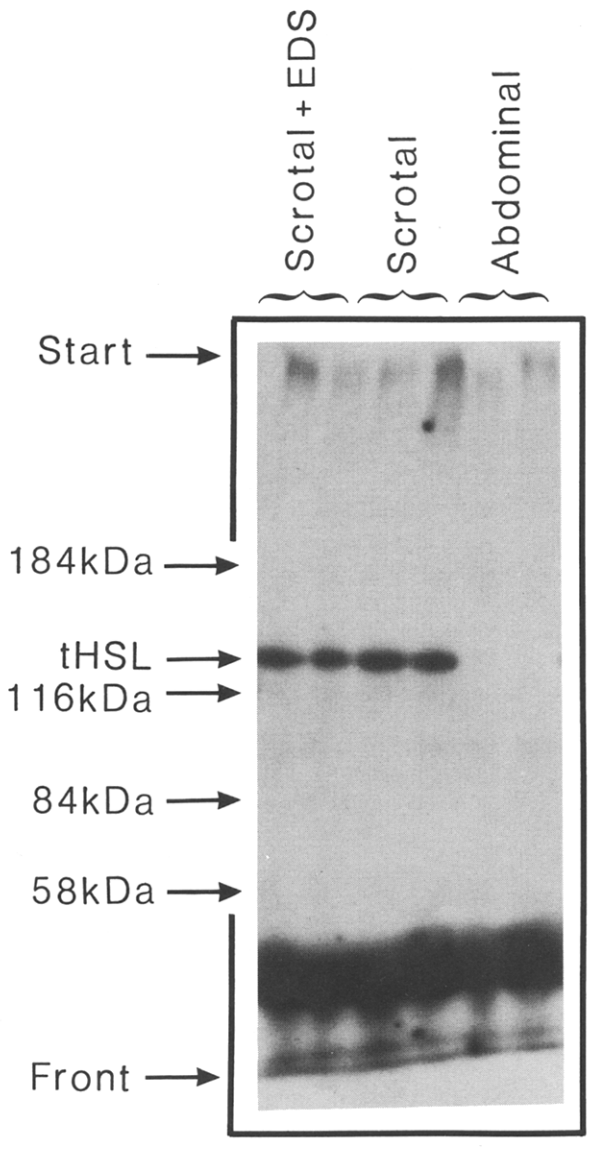

Fig. 3. Western blot analysis of scrotal and abdominal testis protein from primary cryptorchid and EDS treated rats.

difference was detected also at the protein level using Western blot analysis, where the anti-HSL antibody detected a protein of approximately 130 kDa in the scrotal testis but no HSL protein in the abdominal testis (Fig. 3).

The enzyme activity in the abdominal testis was found to be 25% of that on the scrotal side, when measured against cholesterol ester, tri- and diacylglycerol. No impairment of the abdominal enzyme activity was observed in inhibition experiments using NaF and anti-HSL antibodies, whereas the scrotal enzyme activity was inhibited 75% and 71%, respectively, by the same reagents (data not illustrated). In the secondary cryptorchid testes the amount of HSL mRNA, protein and activity rapidly decreased (Fig. 6). The decline in HSL mRNA appeared earlier than the decline in activity, which is in accordance with the assumption of the presence of an additional esterase, different from HSL, in the testis. From the total activity measured, the NaF-resistant activity was subtracted thus giving a measure of HSL activity, which decreases with time similarly to the decrease in HSL mRNA (Fig. 6).

4. Discussion

In situ hybridization demonstrated the localization of testicular HSL mRNA principally to Sertoli cells, whereas HSL mRNA was not detected in Leydig cells. The expression of HSL is stage dependent and apparently closely linked to the amount

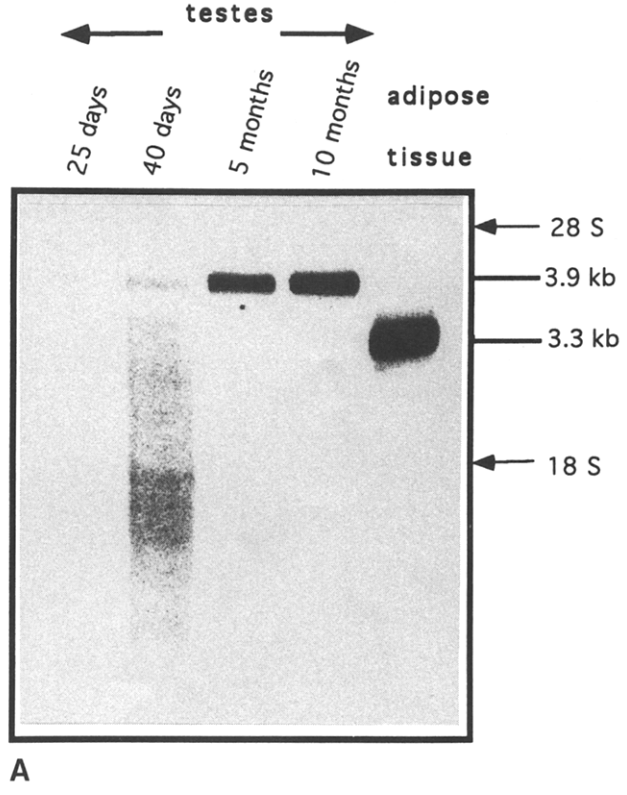

Fig. 4. Testicular HSL in the developing rat. (A) Northern blot analysis with total RNA from testes of rats at different ages and from adipose tissue of an adult rat. Hybridization was carried out using the full length rat HSL cDNA and visualized by digital imaging of ^{32}P. (B) Cholesterol ester hydrolyzing activity, expressed in μU/mg protein, measured in testes from rats of different ages. The results are means of 6 testes.

of lipid droplets in the Sertoli cell cytoplasm. The highest amount of lipid and HSL was observed in stages X–XIV and the lowest in stages III–VIII [13,28]. Against this background, and the observation that experimental cryptorchidism decreases HSL expression and increases the amount of lipid droplets in Sertoli cells [7,13,29], it appears that this enzyme is involved in the metabolism of lipids like triacylglycerols and cholesterol esters, present in these droplets. The HSL mRNA expression was, however, maximal in the luminal parts of the seminiferous tubules, in contrast to lipid droplets, that in some stages are located principally in the basal compartment of the tubules [28]. The possibility of the expressed protein being directed to the basal parts remains to be investigated by immunohistochemistry. In Sertoli cells, FSH is a main regulator of cAMP [30]. Since HSL is controlled by hormones in other tissues, it is likely that also testicular HSL activity is regulated by cAMP and thus controlled by FSH.

From the present study, it is not possible to exclude the interesting possibility that also some elongating spermatids could express HSL. Further studies using in situ-techniques

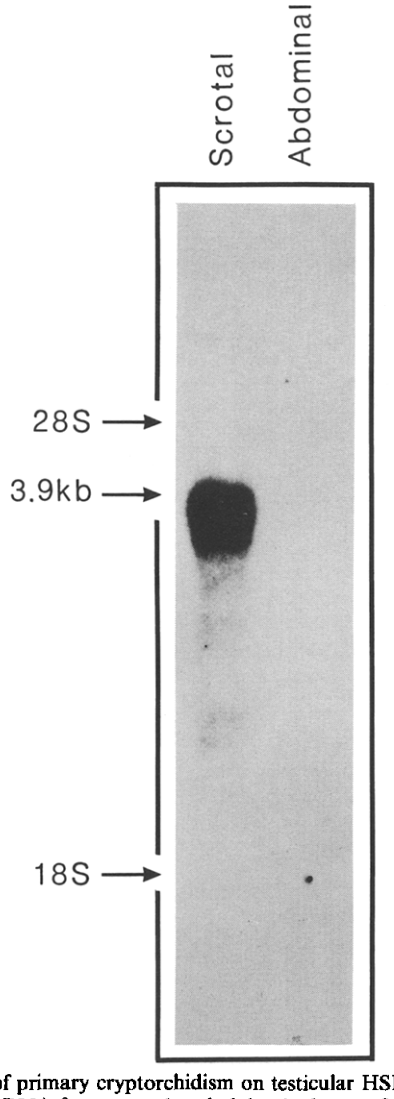

Fig. 5. Effect of primary cryptorchidism on testicular HSL. Northern blot with total RNA from scrotal and abdominal testes from primary cryptorchid rats. The blot was hybridized with the 1895 nt adipose tissue HSL cDNA and visualized by autoradiography.

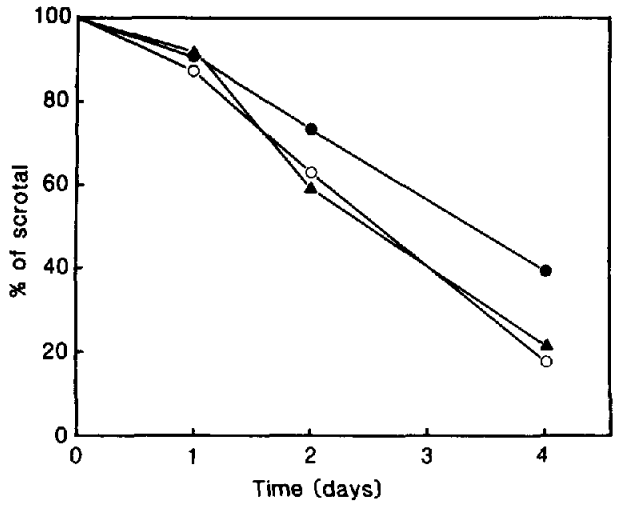

Fig. 6. Effect of secondary cryptorchidism on testicular HSL. HSL mRNA expression and cholesterol ester hydrolyzing activity was measured in scrotal and abdominal testes from unilaterally cryptorchid rats. The abdominal values are expressed as percentage of corresponding scrotal value. HSL mRNA (filled triangles) was quantified by scanning densitometry analyses of slot blots. Cholesterol ester hydrolase activity is shown both as total activity measured (filled circles) and as assumed HSL activity (open circles), i.e. the fraction of activity remaining after NaF inhibition (see text) subtracted from the total activity.

with higher resolution are needed to test this hypothesis. That mature Sertoli cells express HSL is, however, consistent with the observation that HSL expression is retained in animals treated with EDS (Leydig cell depleted) and Busulphan (germ cell depleted).

The identity of the cholesterol esterase with HSL was established both at the level of mRNA and protein. The previously observed larger sizes of both mRNA and protein [9,10] were confirmed (Figs. 3 and 4A); the enzyme showed biochemical properties similar to HSL in other steroid-producing tissues and adipose tissue. Among these were hydrolysis of di- and triacylglycerol as well as cholesterol ester, activation by cAMP-dependent phosphorylation, inhibition by NaF, DFP and antibodies against rat adipose tissue HSL.

In normal rats, the testicular descent into the scrotum is completed at about 35 days of age [31]. The initiation of HSL expression in the testes at this time-point (Fig. 4) thus coincides with the final establishment of the temperature difference between the body and the testis. Kraemer et al. [32] described a similar developmental regulation of a testicular mRNA claimed to be HSL, although with a size of 3.3 kb, i.e. the same size as the adipose tissue HSL mRNA. The reason for the discrepancy in the size of testicular HSL on Northern blots, in both cases probed with HSL cDNA, is obscure. In our laboratory, however, the 3.9 kb HSL mRNA is the only species seen on numerous blots from rat testes, irrespective of probe length.

The dependency on scrotal localization of testicular HSL expression was further investigated by experimental cryptorchidism, which was found to have a negative impact on HSL expression. The abdominal testes from rats operated at birth exhibited low amounts or absence of both hybridizing HSL mRNA and protein. When cryptorchidism was induced in adult rats, the levels were also rapidly reduced with time. A temperature dependent regulation, either on the transcriptional level

or on the level of mRNA stability, are indicated by these data. Sertoli cells have been reported to contain a unique temperature-labile, hormone (FSH) -induced cholesterol ester hydrolase, not present in Leydig cells [2,3,33–35]. However, the partial amino acid sequence reported [35] indicates that this cholesterol ester hydrolase is not HSL. The impairment of raised temperature on the temperature-labile enzyme was suggested to be a direct effect on protein stability, whereas our results rather indicate pretranslational regulatory mechanisms. Some enzyme activity remained in the abdominal testes of the cryptorchid rats, but it appeared to lack the biochemical properties typical of HSL. Durham and Grogan [3] also reported the presence of another esterase, shown to be hormonally induced by FSH as well as by LH, temperature-stable and not affected by cryptorchidism. This enzyme was present in both Sertoli cells and Leydig cells. Support for the presence of another cholesterol ester hydrolase, different from HSL, was provided also by Freeman and Ontko, who reported such an activity in a Leydig tumor cell line [36].

The previously proposed function of HSL in steroidogenic tissues is the mobilization of free cholesterol for steroid biosynthesis [4,32,34,37]. However, the lack of expression of HSL in Leydig cells demonstrated in this study, together with the apparent lack of steroid hormone synthesis from cholesterol in Sertoli cells [38], seriously question this proposal, at least with regard to testis. The results obtained instead point to a role of HSL in mobilization of lipids in the residual bodies of Sertoli cells. Whether the results presented here have any implications for the role of HSL in steroid-producing tissues other than testis, remains to be determined.

In conclusion, from data in the present report we propose a lipid mobilizing role for testicular HSL, instead of the previously suggested role in steroid biosynthesis. Also, the results concerning HSL expression in developing normal testes as well as in the degenerating testes of cryptorchid animals indicate a temperature dependent, pretranslational regulatory mechanism. The larger sizes of testicular HSL mRNA and protein, observed both in previous work and in the present, may have implications in this context and will be dealt with in future investigations on testicular HSL.

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