Lanosterol 14 α -demethylase (CYP51), NADPH–cytochrome P450 reductase and squalene synthase in spermatogenesis: late spermatids of the rat express proteins needed to synthesize follicular fluid meiosis activating sterol

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Abstract

Lanosterol 14 α -demethylase (CYP51) is a cytochrome P450 enzyme involved primarily in cholesterol biosynthesis. CYP51 in the presence of NADPH–cytochrome P450 reductase converts lanosterol to follicular fluid meiosis activating sterol (FF-MAS), an intermediate of cholesterol biosynthesis which accumulates in gonads and has an additional function as oocyte meiosis-activating substance. This work shows for the first time that cholesterologenic enzymes are highly expressed only in distinct stages of spermatogenesis. CYP51, NADPH–P450 reductase (the electron transferring enzyme needed for CYP51 activity) and squalene synthase (an enzyme preceding CYP51 in the pathway) proteins have been studied. CYP51 was

detected in step 3–19 spermatids, with large amounts in the cytoplasm/residual bodies of step 19 spermatids, where P450 reductase was also observed. Squalene synthase was immunodetected in step 2–15 spermatids of the rat, indicating that squalene synthase and CYP51 proteins are not equally expressed in same stages of spermatogenesis. Discordant expression of cholesterologenic genes may be a more general mechanism leading to transient accumulation of pathway intermediates in spermatogenesis. This study provides the first evidence that step 19 spermatids and residual bodies of the rat testis have the capacity to produce MAS sterols *in situ*.

Journal of Endocrinology (2000) **166**, 463–474

Introduction

Lanosterol 14 α -demethylase (CYP51) is a member of the diverse cytochrome P450 superfamily and is involved in cholesterol biosynthesis in mammals (Nelson 1999). CYP51 resides in the endoplasmic reticulum (Trzaskos *et al.* 1986) and oxidatively demethylates substrates in the presence of NADPH, molecular oxygen and the microsomal electron transferring enzyme, NADPH–cytochrome P450 reductase (Fischer *et al.* 1991, Shyadehi *et al.* 1996). Lanosterol, the major endogenous substrate for mammalian CYP51, is synthesized from squalene through sequential epoxidation and cyclization reactions that are catalyzed by squalene epoxidase and oxidosqualene cyclase (Fig. 1). Squalene, which is the first pathway intermediate committed to the formation of cholesterol, is produced directly from the branch-point substrate, farnesyl pyrophosphate,

through the action of squalene synthase (Fig. 1A). CYP51 removes the 14 α -methyl group from lanosterol, forming 4,4,-dimethyl 5 α -cholesta, 8,14,24-diene-3 β -ol (Fig. 1B), also known as follicular fluid meiosis activating sterol (FF-MAS). At least six enzymatic steps after CYP51 are needed for the production of cholesterol from its precursors (Fig. 1C).

Cholesterol biosynthesis and its coordinate regulation by the sterol regulating element binding protein (SREBP)-dependent pathway is well characterized in the liver (Goldstein & Brown 1990, Brown & Goldstein 1998), but very little is known about this process in the male gonad, especially in germ cells. Human sperm lysates were shown to have the capacity to synthesize cholesterol from [¹⁴C]acetate *in vitro*, yet the activity seems to be too low to contribute significantly to the free cholesterol content of the sperm (Gunasegaram *et al.* 1995, Cross 1998). In

A **B** **C**

Figure 1 Enzymatic conversion of farnesyl pyrophosphate to cholesterol. (A) Conversion of farnesyl pyrophosphate to lanosterol. Farnesyl pyrophosphate is formed from acetate through a multistep pathway that includes the key intermediates mevalonate and isopentenyl pyrophosphate (not shown). (B) Lanosterol 14 α -demethylase (CYP51) reaction. The methyl group at position 14 is subjected to three consecutive cytochrome P450 oxidation cycles, the intermediates of the reaction being the alcohol and aldehyde. The carbon at position 14 is removed as formic acid during the third oxidation. In each cytochrome P450 cycle, a pair of electrons is transferred from NADPH to NADPH–cytochrome P450 reductase and on the cytochrome P450 enzyme, CYP51. There is no catalytic activity in the absence of NADPH–cytochrome P450 reductase. (C) Conversion of FF-MAS to cholesterol. At least six enzymes after CYP51 participate in cholesterol biosynthesis. The precise order of the last three reactions might be tissue-specific and remains a matter of discussion. T-MAS is another sterol having *in vitro* meiosis activating activity.

contrast to the majority of tissues in which cholesterol is the main product, male and female gonads over-produce two late intermediates of the cholesterol biosynthetic pathway, follicular fluid meiosis activating sterol (FF-MAS; 4,4-demethyl-5 α -cholesta-8,14,24-triene-3 β -ol) and testis meiosis activating sterol (T-MAS; 4,4-dimethyl-5 α -cholesta-8,24-diene-3 β -ol) (Byskov *et al.* 1995, 1999). Both tissue-isolated FF-MAS (Byskov *et al.* 1995), the product of lanosterol 14 α -demethylase (CYP51) and chemically synthesized FF-MAS (Grondahl *et al.* 1998), and T-MAS, the product of the sterol $\Delta 14$ -reductase, stimulate reinitiation of meiosis in mouse

oocytes *in vitro* and are believed to have important, but not yet fully understood, roles in fertilization (Byskov *et al.* 1995, 1999, Grondahl *et al.* 1998).

The first mammalian CYP51 gene was characterized in 1994 (Aoyama *et al.* 1994) and extensively studied since then (Aoyama *et al.* 1996, Rozman *et al.* 1996a,b, Strömstedt *et al.* 1996, Noshiro *et al.* 1997). The gene shows remarkable conservation between species: for example, the amino acid homology between the human and the rat genes is 93% (Strömstedt *et al.* 1996). CYP51 mRNA is expressed in all mammalian tissues, the expression being an order of magnitude greater in male germ

cells of the testis compared with any other tissue/cell type (Strömstedt *et al.* 1998). Testis is also the organ in which the greatest concentrations of accumulating MAS sterols have been detected (Byskov *et al.* 1999). Thus, it seems likely that overexpression of CYP51 mRNA influences accumulation of MAS sterols in the testis. *In situ* hybridization studies show a stage-specific expression of CYP51 mRNA in rat (Strömstedt *et al.* 1998) and mouse (Rozman *et al.* 1999) testis, with the greatest amounts in postmeiotic germ cells, the spermatids. The earliest detection of CYP51 mRNA is in round spermatids of steps 3–5. Thereafter, expression increases, reaching the highest level in elongating spermatids of steps 7–14. CYP51 mRNA expressed in germ cells is translated into the protein, as increased CYP51 activity is observed in germ cells of sexually mature rats (Strömstedt *et al.* 1998).

This study investigated which cell types of the testis have the greatest potential to synthesize MAS sterols *in situ*, by describing immunolocalization of lanosterol 14 α -demethylase and NADPH-cytochrome P450 reductase – two enzymes needed to produce FF-MAS in the rat testis. By also monitoring squalene synthase, we have been able to describe the stage-specific expression of post-farnesyl pyrophosphate enzymes of cholesterol biosynthesis in testicular germ cells, giving insights into the molecular basis by which accumulation of intermediates in cholesterol biosynthesis can occur.

Materials and Methods

Animals and tissue recovery

Rat testicular tissue was obtained from sexually mature (90-day-old) male Wistar rats after perfusion fixation using Bouin's solution as described before (Millar *et al.* 1993). Rat liver was obtained from sexually mature 90-day-old animals and was immersion-fixed in Bouin's solution for 6 h. After fixation, all tissues were either stored in 70% ethanol until further processing or immediately processed into paraffin wax using a Shandon tissue processor (Millar *et al.* 1993).

Purification of the human CYP51 protein and preparation of antibodies

Human CYP51 was expressed in *Escherichia coli* Topp3 strain as described previously (Strömstedt *et al.* 1996). Four histidine codons were introduced into the 3'-end of the cDNA 3 by standard cloning procedures (Imai *et al.* 1993), to facilitate protein purification. The *E. coli* cells expressing human CYP51 were collected by centrifugation at 2000 g for 10 min at 4 °C. All purification steps were performed at 4 °C. The cell pellet was resuspended at 10 ml/g of cell weight in 100 mM Tris-HCl buffer pH 7.4 containing 500 mM sucrose and 1 mM EDTA.

Lysozyme was added to a final concentration of 0.5 mg/ml. An equal volume of ice-cold 0.1 mM EDTA, pH 8, was slowly added and the mixture stirred for 30 min. The spheroplasts were collected by centrifugation at 2000–5000 g for 15 min and resuspended at 5 ml/g in potassium phosphate buffer pH 7.4 containing 30% glycerol, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg/ml DNase and 10 mM lanosterol. Spheroplasts were homogenized using a Teflon homogenizer. Triton X-114 (Boehringer Mannheim) was added to a final concentration of 0.7% and the mixture was stirred for 20 min (Halkier *et al.* 1996). The P450-rich phase was separated by centrifugation at 100 000 g for 30 min and diluted 1:10 in potassium phosphate buffer pH 7.4 containing 20% glycerol, 0.1 mM PMSF, 0.4% Triton X-100 and 10 mM lanosterol. The diluted P450 suspension was applied to a Ni²⁺ nickel nitrilotriacetic acid (NTA) affinity column (Qiagen). The column was washed with 50 mM potassium phosphate buffer pH 7.4 containing 500 mM NaCl, 20% glycerol, 50 mM glycine, 0.4% Triton X-100 and 10 mM lanosterol. The human CYP51 was eluted with the same buffer containing 40 mM histidine instead of glycine. The CYP51 protein was >90% pure by SDS-PAGE and silver staining. Antibodies against the human CYP51 were raised by injecting New Zealand rabbits with 0.5 mg Ni²⁺NTA-eluted protein mixed with one volume of Freund's complete adjuvant (Sigma). The same amount of protein was used for a booster 4 weeks later, as a mixture with Freund's incomplete adjuvant (Sigma). The antisera collected from a bleed 4 weeks after the second injection were assayed by immunoblot analysis and seen to recognize both the purified and the tissue-derived CYP51 (Fig. 2).

Preparation of the human squalene synthase protein and antibodies

The human squalene synthase protein used in preabsorption studies is a doubly truncated form of the membrane-bound protein that has been rendered soluble by removing 30 amino acids from the N-terminus and 46 amino acids from the C terminus (Thompson *et al.* 1998). The protein, which was overexpressed in *E. coli* and purified to homogeneity as described (Thompson *et al.* 1998), was homogenous on SDS-PAGE and mass spectroscopy with the measured mass of 39 196 Da, and was maintained at –20 °C at a protein concentration of 4 mg/ml in 20 mM Tris pH 7.4, 5 mM DTT, 2 mM MgCl₂, 0.5 M NaCl and 50% glycerol. Squalene synthase antibody was raised in New Zealand white rabbits against peptide-keyhole limpet hemocyanin (KLM) conjugates corresponding to the C terminus (CH₃CO-CDSDPS SSKTRQIISTIRTQN-COOH) of the truncated protein. The antiserum specifically recognizes the human squalene synthase protein,

Figure 2 Detection of CYP51 (A), NADPH-P450 reductase (B) and squalene synthase (C) proteins by immunoblot analysis. Lane 1, rat germ cell proteins (10 μ g); lane 2, the purified human CYP51 protein (85 ng); lane 3, rat liver microsomal (100 000 g) proteins (10 μ g); lane 4, rat germ cell proteins (10 μ g); lane 5, purified truncated $\sim$ 39 kDa human squalene synthase protein (100 ng); lane 6, mouse liver microsomal proteins (10 μ g). Rainbow protein molecular mass markers (Amersham) are shown at the left (kDa). Human and rat CYP51 proteins have a molecular mass of $\sim$ 55 kDa, rat NADPH-P450 reductase protein a molecular mass of 72–74 kDa (Guengerich *et al.* 1981), and squalene synthase is detected as a protein of $\sim$ 60 kDa in rat and mouse.

both in purified fractions and in cell lysates, at dilutions between 1:200 and 1:2500, and also crossreacts with the rat enzyme.

Preparation of the rat NADPH-cytochrome P450 reductase protein and antibodies

The antibodies kindly provided by Dr F P Guengerich (Vanderbilt University) were raised against the rat liver NADPH-cytochrome P450 reductase purified from liver microsomes as described elsewhere (Guengerich *et al.* 1981). The antibody recognizes a single protein by immunoblot analysis (Guengerich *et al.* 1987). Recombinant rat NADPH-cytochrome P450 reductase was expressed in *E. coli* and purified as described elsewhere (Jenkins & Waterman 1998). For the preabsorption studies, 45.1 mM protein was used.

Immunocytochemistry

Sections 5 μ m thick were mounted on slides coated with 3-aminopropyl triethoxysilane (TESPA; Sigma) and dried overnight at 37 $^{\circ}$ C. Before incubation with primary antibody, sections were dewaxed, rehydrated in graded ethanols, washed in water and 0.05 M Tris-HCl (pH 7.4)–0.85% NaCl (TBS) and endogenous peroxidases were

blocked by incubating the sections for 30 min in 1% H₂O₂ in TBS. Sections were subjected to antigen retrieval (Shi *et al.* 1993) by microwaving in 0.01 M citrate buffer (pH 6.0) on full power for 20 min, and were thereafter left standing for 20 min without disturbance. Sections were then washed for 5 min in TBS and blocked in TBS containing native goat serum (Dako, Copenhagen, Denmark) diluted 1:5 in TBS. The rabbit polyclonal antibodies against human CYP51 protein were used at a dilution of 1:20, the rabbit polyclonal antibodies against human squalene synthase were used at a dilution of 1:100, and the rabbit polyclonal antibodies against human NADPH-cytochrome P450 reductase were used at a dilution of either 1:100 or 1:200. The immunostaining procedure was similar with all antibodies, which were diluted in TBS containing normal goat serum (5:1, v/v) before incubation on sections under plastic coverslips overnight at 4 $^{\circ}$ C. The following day, coverslips were removed and sections were washed twice in TBS (5 min each wash), incubated for 30 min with a 1:60 dilution of goat anti-rabbit IgG (Dako) in TBS and then, washed again in TBS (twice for 5 min). For detection of bound antibodies, sections were incubated with a 1:100 dilution of rabbit peroxidase-antiperoxidase complex (Dako) in TBS for 30 min and then washed twice in TBS (5 min each). Color was developed by incubating sections in a mixture of 0.05% (w/v)

3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 5–15 min, sections were washed in distilled water, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Specificity of the antibodies was evaluated by using rabbit serum instead of primary antibodies and by pre-absorbing the primary antibodies overnight with a 20-fold excess of the corresponding protein or peptide before staining. Photomicrographs were taken using a Nikon microphot FXA.

Preparation of rat germ cell protein extracts

Germ cells from two sexually mature Sprague-Dawley rats (Harlan) were prepared in each experiment as described elsewhere (Strömstedt *et al.* 1998). Briefly, decapsulated testes were thoroughly minced with an array of sealed razor blades and treated with DNase and trypsin. This procedure destroys most of the Sertoli and Leydig cells, but germ cells remain intact (Meistrich *et al.* 1973). Our preparations of germ cells contained premeiotic spermatocytes in addition to postmeiotic round and elongating spermatids (Strömstedt *et al.* 1998). No Sertoli cells were observed; Leydig cells were present at less than 1% of the total cell population (Strömstedt *et al.* 1998). Germ cells were pelleted, washed and suspended in 5 ml of assay buffer and hand-homogenized on ice by at least 70 strokes. The 1500 g supernatant was prepared and the protein content measured (Bio-Rad, Hemel Hempstead, UK). Contamination with testis somatic cells was determined by morphological examination of air-dried smears stained with periodic acid Schiff-hematoxylin (Meistrich *et al.* 1973).

Preparation of microsomes, protein extracts and CYP51 activity

Liver protein extracts were prepared from the same sexually mature, 52–56-day-old rats as described previously for the testis protein extracts (Strömstedt *et al.* 1998). Two 52–56-day-old sexually mature Sprague-Dawley rats (Harlan) were killed in each of three independent experiments. The livers were removed, washed three times with 4 °C 0.25 M sucrose, minced with razor blades, diluted 1:20 with 4 °C assay buffer (100 mM potassium phosphate buffer pH 7.4, 0.1 M DTT, 0.1 mM EDTA, 20% glycerol) and homogenized by 10 strokes of a Dounce tissue homogenizer, on ice. Cell debris was removed by centrifugation for 15 min at 1500 g and 4 °C and protein content of the supernatant measured (Bio-Rad). Microsomal proteins for immunoblot analysis were prepared by recentrifuging the supernatant at 100 000 g and 4 °C for 1 h and suspending the S-100 pellet in one half of the initial amount of assay buffer. Adult mouse liver

microsomal (100 000 g) proteins were prepared by the same procedure. The CYP51 activity assay and HPLC separation of sterols were performed exactly as described previously (Strömstedt *et al.* 1996). Each reaction contained 3–8 mg of the liver total protein, 1.6×10^5 c.p.m. pure [3 H]24,25-dihydrolanosterol (a gift of Dr Trzaskos, DuPont-Merck), 25 nmol each of a mixture of unlabeled lanosterol and 24,25-dihydrolanosterol in Triton WR 1339 (16 mg/ml in acetone), and inhibitors NaCN (0.5 mM) and AY9944 (0.1 mM; a gift of Dr Trzaskos, DuPont-Merck) to block further metabolism of reaction products.

Immunoblot analysis

Proteins were separated on 8–10% SDS polyacrylamide gels and transferred by the Sammy Dry electroblotting apparatus (Schleicher & Schuell, Dassel, Germany) to Optitran BA-S 83 nitrocellulose membranes using the continuous transfer buffer (50 mM Tris, 39 mM glycine, 20% methanol) at 3.5 mA/cm². The air-dried membrane was incubated for 1 h in 10 ml 5% enhanced chemiluminescence (ECL) blocking agent (Amersham) in TBS-T (20 mM Tris pH 7.4, 300 mM NaCl, 40 mM KCl, 0.1% Tween 20) buffer. The blocking agent solution was decanted and membranes incubated in 10 ml of the primary antibody solutions in TBS-T buffer for 1 h: (a) rabbit polyclonal antibodies against human CYP51 diluted 1:300; (b) rabbit polyclonal antibodies against human squalene synthase diluted 1:200; (c) rabbit polyclonal antibodies against rat NADPH-P450 reductase diluted 1:1500. Three washing steps in 10 ml fresh TBS-T buffer (15 min–1 h and twice for 5 min) preceded the 1-h incubation in 10 ml of the secondary antibody solution (rabbit IgG horseradish peroxidase-linked whole antibody, diluted 1:3000 in TBS-T buffer). Membranes were then washed three times in TBS-T buffer as before. Detection of proteins was performed by the ECL western blotting kit (Amersham) using autoradiography as described by the manufacturer. When more than one protein was analysed on the same membrane, the first antibody was removed completely by incubating the membrane in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 at 50 °C for 30 min. The membrane was then washed twice for 10 min in TBS-T buffer at room temperature. The subsequent steps from blocking to ECL detection were same as described above.

Results

Immunodetection of CYP51 enzyme in testis and liver

Rat germ cell proteins were subjected to immunoblot analysis using rabbit anti-human CYP51 antibody. The human histidine-conjugated, bacterially expressed CYP51 protein had a molecular mass of ~55 kDa (Fig. 2A, lane 2), which is in accordance with the predicted value

A

Figure 3 (A)

deduced from the cDNA. A protein of identical size was detected in rat germ cell preparations (Fig. 2A, lane 1) and in rat liver (not shown). Using this antibody, the highest level of immunodetectable CYP51 protein was found in Leydig cells of sexually mature rat testis (Fig. 3A, a,b

arrowheads) and also in haploid germ cells. Interestingly, in germ cells the CYP51 protein was not localized only to the cytoplasm where the endoplasmic reticulum resides (double arrows), but also to the acrosomal region of round and elongating spermatids (arrows, Fig. 3A, a,b; Fig. 3B).

Figure 3 (B)

Figure 3 (A) Immunodetection of CYP51 in rat testis (a,b,d) and liver (c,e). (a,b,c) Stained with the anti-human CYP51 antibody; (d,e) stained with the same antibody preadsorbed with the purified CYP51 protein. Arrows, acrosomes of round and elongated spermatids; open arrowheads, staining in step 19 spermatids and residual bodies; arrowheads, staining in Leydig cells; doubleheaded arrows, staining of cytoplasm. Note an intense 'ring' of staining in the cytoplasm of elongated spermatids in (a). The cytoplasm of round spermatids shows low levels of the CYP51 protein, seen as a weak 'ring' of staining, separating spermatocytes and spermatids. Bar represents 50 μ m. (B) Stage-specific immunodetection of CYP51 in spermatogenesis of the rat. Spermatogenesis stages are indicated by roman numerals. Bar represents 50 μ m. Staining of the basal cell types is considered to be non-specific, as it was not completely removed by the preadsorption experiment.

CYP51 protein was immunodetected also in residual bodies (Fig. 3A, b, open arrowheads; Fig. 3B, stage VII–VIII), but only background levels were found in early germ cells, the spermatogonia and spermatocytes. The specificity of staining was checked in control testis sections

in which the primary antibody was replaced with non-immune serum (not shown) or with CYP51 protein preadsorbed antibodies (Fig. 3A, d). By careful examination of testis slides containing germ cells in different stages of their development (Fig. 3B), the pattern of CYP51

Figure 4 Pattern of immunolocalization of squalene synthase (dark gray), CYP51 (medium gray) and NADPH–cytochrome P450-reductase (light gray) proteins during spermatogenesis of the rat. A map of spermatogenesis shows the stages of the cycle of the seminiferous epithelium indicated by roman numerals (Russell *et al.* 1990). The arrow indicates a schematic tracing of transilluminated living seminiferous tubule at the same stages of the cycle (Parvinen & Vanha-Perttula 1972). Arabic numerals present steps of spermiogenesis. Step 19 spermatids release their cytoplasm as residual bodies and move to the lumen of the seminiferous epithelium. Note the coexpression of CYP51 and NADPH–cytochrome P450 reductase in step 19 spermatids.

protein immunodetection in spermatogenesis in rat was established (Fig. 4, medium gray colour). The greatest concentrations of CYP51 protein were detected in round spermatids of stages III–VII (steps 3–7), in elongated spermatids of stages VIII–XIV (steps 8–14) and in stage VII (step 19). Low levels of CYP51 protein were observed also in elongated spermatids of stages I–VI (steps 15–18).

Immunodetection of CYP51 protein was monitored also in liver of chow-fed rats. A low level of expression was observed in the majority of hepatocytes, but some contained significantly greater concentrations of CYP51 protein (Fig. 3A, c, arrow). No positive signal was detected in control liver sections when primary antibody was replaced with preadsorbed antibody (Fig. 3A, e).

Immunodetection of NADPH–cytochrome P450 reductase in the testis

Using the anti-rat NADPH–P450 reductase antibody, NADPH–P450 reductase was detected as a protein of ~72 kDa in rat liver (Fig. 2B, lane 3) and rat male germ cells (Fig. 2B, lane 4). A high concentration of NADPH–cytochrome P450 reductase protein was detected in Leydig cells (Fig. 5a, arrowhead) and in residual bodies (Fig. 5a, arrow) inside the seminiferous tubules. Staining was also found in elongated spermatids of stages VII–VIII (step 19). Despite the fact that all germ cells probably express low levels of the ubiquitous NADPH–P450 reductase, high levels of the enzyme were detectable exclusively in mature, step 19 elongated spermatids, which are ready to be released to the lumen (Fig. 4, light gray colour). No NADPH–cytochrome P450 reductase protein

was detected in sections incubated with non-immune serum (not shown) or with preadsorbed antibodies (Fig. 5b, rat).

Immunodetection of squalene synthase

Squalene synthase is the third housekeeping cholesterologenic enzyme of which expression in spermatogenesis has been studied. The purified human truncated squalene synthase protein has a molecular mass of ~39 kDa (Fig. 2C) and is easily detected by the anti-human squalene synthase antibody raised against the C-terminal peptide of the protein. Squalene synthase is detected as a protein of ~60 kDa in mouse and rat liver (Fig. 2C, lane 6) and in germ cells (not shown). The greatest concentrations of squalene synthase protein were immunodetected in Leydig cells (Fig. 6a, arrowheads) and in the acrosomal region (arrow) of round and elongated spermatids (Fig. 6a). No immunostaining was detected using non-immune rabbit serum (not shown) or preadsorbed antibodies (Fig. 6b). The pattern of immunolocalization of squalene synthase during spermatogenesis in rat is shown in Fig. 4 (dark gray color). The protein was detected continuously from stage II–XIV (steps 2–14) round and elongated spermatids and in stage I (step 15) elongated spermatids, but was not detected in later steps of spermatogenesis.

CYP51 enzymatic activity in rat liver and in testis

CYP51 enzymatic activity was measured in total liver protein extracts and in liver microsomes of sexually mature

Figure 5 Immunodetection of NADPH-cytochrome P450 reductase in rat testis. (a) Stained with the antibody raised against rat NADPH-cytochrome P450-reductase. (b) Stained with the same antibody preadsorbed with purified rat NADPH-cytochrome P450 reductase. Arrows, staining in elongated spermatids and residual bodies; arrowheads, staining in Leydig cells. Bar represents 50 μ m.

Figure 6 Immunodetection of squalene synthase in rat testis. (a) Stained with the antibody raised against the C-terminal peptide of a truncated squalene synthase protein (Thompson *et al.* 1998). (b) Stained with the same antibody preadsorbed with the purified truncated squalene synthase protein. Arrows, staining in acrosomes of round and elongated spermatids; arrowheads, staining in Leydig cells. Bar represents 50 μ m.

rats. The average values and standard errors of the means were 12.9 ± 4.8 pmol/mg per min for CYP51 activity in total liver proteins and 20.8 ± 8.6 pmol/mg per min for activity in liver microsomes. In the same group of sexually mature animals, the activities were 10.3 ± 2.8 pmol/mg per min in total testis proteins and 2.4 ± 0.9 pmol/mg per min in germ cell proteins (Strömstedt *et al.* 1998).

Discussion

It is widely accepted that the presqualene portion of cholesterol biosynthesis generates intermediates that have additional biological roles (precursors of heme and ubiquinone synthesis, farnesylation and geranylation of proteins, etc.). However, according to current dogma, the postsqualene portion of this pathway is committed to cholesterol biosynthesis. Recent isolation of two intermediates of the postsqualene portion of this pathway that have the capacity to reinitiate meiosis, from human follicular fluid (FF-MAS) and from bull testis (T-MAS) (Byskov *et al.* 1995), demonstrated that late intermediates of cholesterol biosynthesis may also accumulate and may serve roles not committed to cholesterol, at least in gonads.

Our goal was to establish which tissues or cell types allow production of sterols by the CYP51-catalyzed reaction, lanosterol 14 α -demethylation. Our previous studies had unexpectedly shown the level of CYP51 mRNA to be one order of magnitude greater in testis than in liver of normally fed, sexually mature rats (Strömstedt *et al.* 1998). In contrast to the expression of CYP51 mRNA, its enzymatic activity in liver exceeds that in testis by about 30%, the Leydig cells and postmeiotic germ cells contributing mainly to this activity (Strömstedt *et al.* 1998). This suggests that in testis only a small portion of CYP51 mRNA may be translated into protein, that translation itself may be stage-delayed, or that the CYP51 protein may have a rapid turnover. A comparison of the profile of expression of CYP51 mRNA (Strömstedt *et al.* 1998) with that of protein (this work) has shown both mRNA and protein to be detectable at the early round spermatid stage. Thus the CYP51 message is translated to protein with no time delay, and translation also continues in later stages that lack mRNA synthesis (stage VIII and on, step 8–19 of spermiogenesis). We believe that high levels of CYP51 mRNA are needed to assure sufficient template for protein synthesis after transcription is shut off. We are so far unable to explain why the CYP51 mRNA level (Strömstedt *et al.* 1998), the CYP51 protein level (this study) and the MAS sterol concentration (M Baltsen, M Parvinen and A-G Byskov, unpublished data) decrease in step 15–18 (stages I–VI) elongating spermatids. The background levels of CYP51 mRNA present at these stages seem to be sufficient to allow translation to protein even in step 19 spermatids. Whereas in the liver all hepatocytes appear to express CYP51 (this study) and

NADPH-cytochrome P450 reductase proteins (Taira *et al.* 1980, Wolf *et al.* 1984), this seems to be true only for certain cell types of the testis. Besides being present in Leydig cells, large amounts of both proteins reside in step 19 elongating spermatids and residual bodies. This suggests that the absence (or very low levels) of CYP51 enzyme at particular stages of spermatogenesis may not be the only reason for lower CYP51 enzyme activity in testis. The electron-transferring enzyme NADPH-P450 reductase, which is needed for CYP51 activity, may also be a limiting factor.

Until this report, very little has been known about expression of cholesterologenic enzymes in testis. The major obstacle is the lack of immunoreactive antibodies with which to detect the transmembrane proteins involved in cholesterol biosynthesis. A fusion protein of bacterial anthranilate synthase and rat farnesyl pyrophosphate (FPP) synthase was used to prepare antibodies that made possible studies of sterol dietary regulation of the liver and testis FPP proteins, showing dietary response in liver but not in testis (Ashby & Edwards 1989). We have produced antibodies against the truncated human squalene synthase protein and the bacterially expressed human CYP51 protein, which enabled us to follow the expression of both cholesterologenic enzymes. Our results have shown that the two proteins are not co-expressed in all spermatogenesis stages, but both are expressed in liver. Previous studies of squalene synthase and CYP51 mRNA expression (Strömstedt *et al.* 1998) and the mechanism of CYP51 transcriptional regulation (Rozman *et al.* 1999) also indicated differences in regulation of the cholesterologenic pathway in liver compared with that in testis (germ cells). Male germ cell development proceeds for several weeks (Russell *et al.* 1990), and discordant expression of enzymes of cholesterol biosynthesis may lead to transient accumulation of intermediates in a particular cell type at a specific time during germ cell development. Whereas, in the liver, all cholesterologenic genes seem to be upregulated coordinately by the sterol/SREBP pathway with the aim of maintaining cholesterol synthesis, spermatids invoke different mechanisms to regulate expression of cholesterologenic genes, which may lead to accumulation of pathway intermediates (Rozman *et al.* 1999). The coexpression of CYP51 and of NADPH-cytochrome P450 reductase proteins in step 19 spermatids suggests that these haploid cells have the potential for *in situ* production of FF-MAS. MAS sterols are able to reinitiate meiosis in mouse oocytes *in vitro* (Byskov *et al.* 1995). As oocytes complete the secondary meiotic division only after fertilization, spermatid-produced MAS sterols might trigger the quiescent oocyte to complete the second meiotic division upon oocyte-sperm fusion. It is interesting to note that MAS sterol concentration is an order of magnitude greater in testis than in the ovaries (Byskov *et al.* 1999). In addition, the presence of functional CYP51 in residual bodies could be important for *in situ* MAS sterol

production and initiation of meiosis in the testis itself. After being separated from mature spermatids, residual bodies move towards the basement membrane, where they are degraded by Sertoli cells (Jegou 1993, Syed *et al.* 1993, 1995, Hakorvita *et al.* 1995). Accordingly, MAS sterols, delivered by residual bodies into the vicinity of premeiotic germ cells, could trigger meiosis.

The exact physiological significance of the expression of CYP51 and squalene synthase in male germ cells remains unknown. Studies with selective and potent squalene synthase inhibitors that have been developed as cholesterol-decreasing agents (Hayward *et al.* 1997) led to testicular germ cell lesions in animal toxicology studies (Camacho *et al.* 1998). This indicates that the postsqualene cholesterol biosynthetic pathway in germ cells is needed for normal spermatogenesis. Whether the endproduct, cholesterol, or one of the pathway intermediates is the key substance, remains to be determined.

In conclusion, this study has provided evidence that most mature, step 19 elongated spermatids and residual bodies of the rat testis have the capacity to produce MAS sterols *in situ*. It has also provided evidence that two key enzymes of cholesterol biosynthesis (squalene synthase and CYP51) are not expressed at same levels during all stages of spermatogenesis. Differential expression of late cholesterologenic genes may be a more general mechanism to explain the transient accumulation of cholesterologenic intermediates, with yet unrevealed roles, *in vivo*.

Acknowledgements

The authors would like to thank Dr Philippa Saunders (MRC – Reproductive Biology Unit, Edinburgh, Scotland) for providing rat tissue, Dr Fred P Guengerich (Vanderbilt University, School of Medicine, Nashville, TN, USA) for providing NADPH-P450 reductase antibody, Dr Jim Trzaskos (DuPont-Merck, Wilmington, DW, USA) for providing standards, substrates and inhibitors for lanosterol 14 α -demethylase assay, and Dr Jasna Šinkovec (Clinic for Gynaecology, Ljubljana, Slovenia) for use of the immunocytochemistry laboratory. We wish also to thank Dr William E Rainey (UT-Southwestern Medical Center, Dallas, TX, USA) for help in preparing the photomicrographs.

This work was supported by grants from the Ministry of Science of Slovenia Z1-7225-381 and SLO-US 0002, by grant 9650310N from the American Heart Association (Pfizer Award) and by grants from the Sigrid Jusélius Foundation.

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Received 28 January 2000

Accepted 6 April 2000