Regulation of the Rate-Determining Step in the Steroidogenic Cascade in Rat Leydig Cells

Regulatie van de
Snelheids-Bepalende Stap
in de Steroïdogene Cascade
in Leydig Cellen van de Rat

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam op gezag van
de Rector Magnificus Prof. Dr P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 30 oktober 1996 om 11.45 uur

door

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geboren te Vught

omslag:

'Mitochondrial Man'

door Katinka van Haren

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ENDOCRINOLOGY
ROTTERDAM

Dit proefschrift werd bewerkt binnen de Vakgroep Endocrinologie & Voortplanting van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam.

Contents

Abbreviations and Trivial Names

ACTH	adrenocorticotropic hormone
AMH	anti-müllerian hormone
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
CSCC	cholesterol side-chain cleavage
DAG	diacylglycerol
dbcAMP	dibutyryl adenosine cyclic-3',5'-monophosphate
DBI	diazepam-binding inhibitor
EGF	epidermal growth factor
FSH	follicle-stimulating hormone, or follitropin
GH	growth hormone
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
3ßHSD	3ß-hydroxysteroid dehydrogenase
17ßHSD	17ß-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IP_3	inositol triphosphate
kb	kilo base
kDa	kilo Dalton
LH	luteinizing hormone, or lutropin
LHRH	luteinizing hormone-releasing hormone
mRNA	messenger ribonucleic acid
nsL-TP	non-specific lipid transfer protein
P450c17	17α-hydroxylase cytochrome P450
P450arom	aromatase cytochrome P450
P450scc	cholesterol side-chain cleavage cytochrome P450
PBR	peripheral benzodiazepine receptor
SAP	steroidogenesis activator polypeptide
TGFß	transforming growth factor ß
TNFα	tumour necrosis factor α
SCCM	Sertoli cell-conditioned medium
SCP_2	sterol carrier protein 2
StAR	steroidogenic acute regulatory protein
VDAC	voltage-dependent anion channel

Chapter 1

Introduction

1.1. Testicular steroid production

The testis consists of two compartments: the seminiferous tubules and the interstitium. The primary function of the seminiferous tubules is the production of spermatozoa. The developing germ cells are embedded in Sertoli cells, which form the blood-testis barrier by specialized junctions in-between Sertoli cells (De Kretser and Kerr, 1988). The seminiferous tubules are surrounded by a basal lamina and peritubular myoid cells. The interstitium contains Leydig cells (Leydig, 1850), blood vessels, lymphatics, and macrophages. The main function of the interstitial tissue is production of androgens by the Leydig cells.

1.1.1. Leydig cells

Androgens, produced by the Leydig cells, are involved in initiation and maintenance of spermatogenesis (Clermont and Harvey, 1965), development of the internal and external genitalia, the secondary sexual characteristics, and the musculoskeletal system, and feedback inhibition in the gonadal-hypothalamo-pituitary axis.

Androgen action is mediated by the androgen receptor, an intracellular protein that is activated upon hormone binding. The hormone-receptor complex can subsequently regulate the transcription of androgen responsive genes, which results in changes in cell function (Beato, 1989). Androgen action in androgen target cells is determined mainly by the presence of the androgen receptor, but also by the enzyme 5α-reductase. This enzyme, responsible for the conversion of testosterone into 5α-dihydrotestosterone, is present in most androgen target tissues. Both testosterone and 5α-dihydrotestosterone can bind to the androgen receptor. 5α-Dihydrotestosterone is a more potent androgen (Grino *et al.*, 1990), and it binds with a higher affinity to the androgen receptor than testosterone (Veldscholte, 1993).

Luteinizing hormone (LH), produced by the pituitary gland, is the main regulator of Leydig cell steroidogenesis (Bartke *et al.*, 1978; Hall, 1988). The production and release of LH are under the control of luteinizing hormone-releasing hormone (LHRH, also known as gonadotropin-releasing hormone: GnRH), a decapeptide produced by the hypothalamus (Hodgson *et al.*, 1983; Fink, 1988). In response to LH, the plasma testosterone level increases and acts as a negative feedback signal to inhibit the release of both LHRH and LH (Cunningham and Huckins, 1979). Although LH is the main regulator of Leydig cell steroidogenesis, there is abundant evidence, both from *in vivo* and *in vitro* experiments, that the effect of LH can be modulated by other hormones and by local factors produced in the testis (see 1.2.3.).

1.1.2. The steroidogenic cascade

For Leydig cell steroidogenesis, cholesterol is the obligate precursor. Cholesterol may be obtained from three different sources:

1) **Lipoproteins** - Leydig cells are able to take up cholesterol from plasma lipoproteins. Both MA-10 Leydig tumour cells (Freeman and Ascoli, 1983) and pig Leydig cells (Benahmed *et al.*, 1983) use low density lipoproteins, while rat Leydig cells preferentially use high density lipoproteins (Chen *et al.*, 1980).
2) **Intracellular stores** - In Leydig cells of various species, stores of esterified cholesterol are found in lipid droplets in the cytoplasm (Almahbobi *et al.*, 1993). However, compared to the steroidogenic adrenal cells or luteinized granulosa cells, lipid droplets in Leydig cells are far less prominent. Even though Leydig cells do not store large quantities of cholesteryl esters, they can utilize these esters. Upon hormone stimulation the stores are depleted (Christensen, 1975) and the cellular amount of free cholesterol is increased, through cAMP-dependent protein kinase activation of cytoplasmic cholesterol ester hydrolase (Trzeciak and Boyd, 1973; Pedersen *et al.*, 1980) and suppression of acyl-CoA:cholesterol acyltransferase (Mikami *et al.*, 1984). Cholesterol can also be stored as unesterified cholesterol in the plasma membrane. MA-10 Leydig tumour cells preferentially use this nonesterified cholesterol as a substrate for steroid production (Freeman, 1987).
3) ***De novo* synthesis** - Rat Leydig cells contain all the enzymes necessary for *de novo* synthesis of cholesterol. Newly synthesized cholesterol is either used immediately for steroid production or stored in membrane systems. The morphological characteristics of Leydig cells are consistent with a large synthetic capacity to produce cholesterol, in that the endoplasmic reticulum is particularly prominent (Iehihara, 1970; Russo, 1971). Although different cholesterol sources can be important for Leydig cell steroidogenesis (Hall, 1994), rat Leydig cells preferentially use intracellularly produced cholesterol as substrate for androgen production (Charreau *et al.*, 1981; Van der Molen and Rommerts, 1981).

Steroid production does not take place until cholesterol is transported to a pool where it is available for metabolism by the cholesterol side-chain cleavage (CSCC) enzyme. This steroidogenic pool is located in the inner mitochondrial membrane (Seybert *et al.*, 1979; Privalle *et al.*, 1987). Prior to the accumulation of cholesterol into this steroidogenic pool, cholesterol is transported to a pre-steroidogenic pool (Stevens *et al.*, 1993), which is located in the outer mitochondrial membrane (Privalle *et al.*, 1983, 1987). The

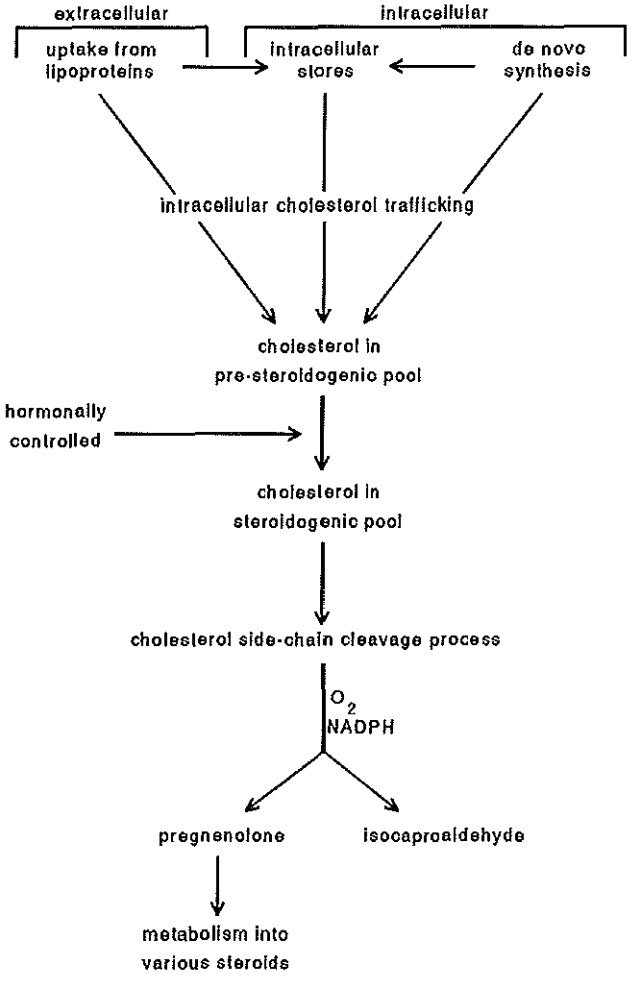

Fig. 1. Supply of cholesterol for steroidogenesis. Cholesterol from extracellular sources (lipoproteins) or intracellular sources (lipid droplets, plasma membrane, de novo synthesis) is transported to the pre-steroidogenic pool located in the outer mitochondrial membrane. From this pool, cholesterol is translocated to the steroidogenic pool in the inner mitochondrial membrane, where subsequent cleavage of cholesterol into pregnenolone and isocaproaldehyde takes place by the cholesterol side-chain cleavage enzyme.

cholesterol that enters this pool in the outer mitochondrial membrane is derived either from intracellular or extracellular sources (Fig. 1). Cholesterol transport to this pre-steroidogenic pool is independent of protein synthesis but involves the cytoskeleton. This has been concluded from experiments using various compounds which disrupt the cytoskeleton (Crivello and Jefcoate, 1980; Hall, 1984; Nagy and Freeman, 1990a,b). In addition, in adrenal cells there is a close association of lipid droplets and mitochondria with intermediate

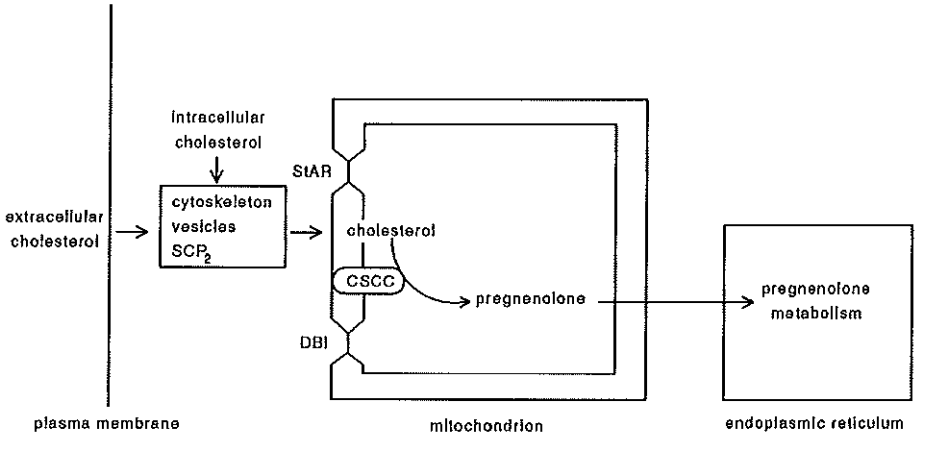

Fig. 2. Control of intracellular and intramitochondrial cholesterol trafficking. Intracellular transport of cholesterol towards the mitochondria may involve the cytoskeleton, vesicles, and/or SCP_2. Intramitochondrial transport of cholesterol, from the outer to the inner mitochondrial membranes, may require steroidogenic acute regulatory protein (StAR), and/or diazepam-binding inhibitor (DBI). These proteins facilitate intramitochondrial cholesterol translocation probably via the induction of contact sites.

filaments, suggesting that these filaments could be involved in cellular cholesterol transport (Almahbobi *et al.*, 1993). Transport of cholesterol from the outer to the inner mitochondrial membranes (translocation to the steroidogenic pool) is difficult; the aqueous space in-between

the membranes prevents rapid cholesterol movements. Since cholesterol is a hydrophobic compound, diffusion of cholesterol through an aqueous phase is extremely slow (Phillips *et al.*, 1987; Schroeder *et al.*, 1991; Rennert *et al.*, 1993). Such a low rate of diffusion cannot provide sufficient substrate to account for the rapid and large increase in steroid production observed in steroidogenic cells after hormone stimulation. To explain hormonal stimulation of steroidogenesis, an alternative mechanism for rapid transport of cholesterol across the

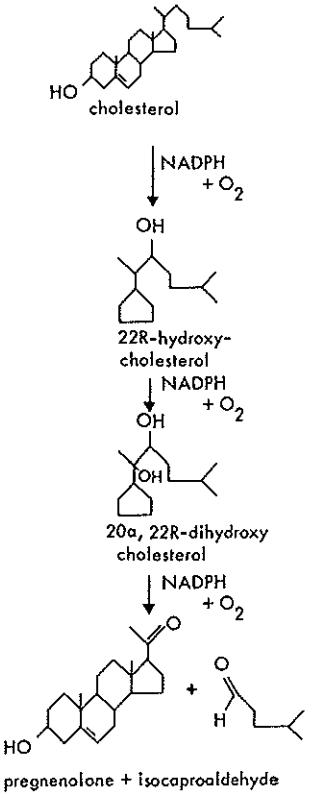

Fig. 3. Steps in the cholesterol side chain cleavage reaction. In this reaction, two hydroxylated sterol intermediates, 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol, are produced by the CSCC enzyme. 22R-Hydroxycholesterol has been used as a relatively soluble exogenous substrate to saturate cholesterol side-chain cleavage enzyme.

aqueous space between the outer and inner mitochondrial membranes must be present. Different specific proteins may be involved in this process (Fig. 2; see also 1.2.1.).

The conversion of cholesterol into pregnenolone and isocaproaldehyde is catalyzed by the cholesterol side-chain cleavage (CSCC) enzyme, a complex which consists of three different proteins: the cytochrome P450scc enzyme, which is a heme protein, and two associated electron transport proteins, viz. the flavoprotein NADPH-adrenodoxin reductase and the iron sulfur protein adrenodoxin (Omura *et al.*, 1966). The overall reaction proceeds via three sequential oxidations: hydroxylations at the 22R and 20α positions, and a cleavage of the 20-22 carbon-carbon bond (Burstein and Gut, 1976; Hume and Boyd, 1978). Each step utilizes

molecular oxygen and two electrons, originating from NADPH by the action of NADPH-adrenodoxin reductase and adrenodoxin (Fig. 3). In studies utilizing purified cytochrome P450scc reconstituted into phospholipid vesicles, it was found that the two hydroxylated steroid intermediates, 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol, produced in the side-chain cleavage reaction, are bound to the enzyme several orders of magnitude more tightly than cholesterol (Lambeth *et al.*, 1982). Thus, significant accumulation of

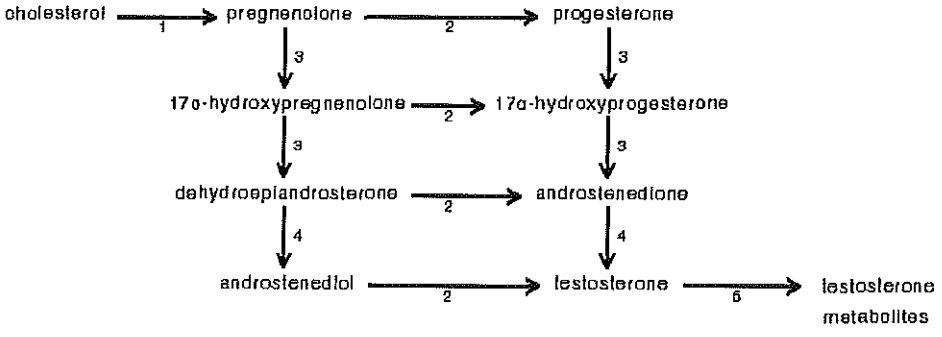

Fig. 4. Steroidogenic pathways operating in the testis. Enzymes: 1) cholesterol side-chain cleavage enzyme (P450scc); 2) 3ß-hydroxysteroid dehydrogenase (3ßHSD); 3) 17α-hydroxylase (P450c17); 4) 17ß-hydroxysteroid dehydrogenase (17ßHSD); 5) 5α-reductase and aromatase cytochrome P450 (P450arom).

intermediate sterols does not occur, and once the first hydroxylation has taken place, the steroid is committed for complete conversion to pregnenolone. 22R-hydroxycholesterol is frequently used as an exogenous substrate for cultured steroidogenic cells, to saturate the CSCC enzyme. This compound is more water soluble than cholesterol and can cross the aqueous space between the outer and inner mitochondrial membranes easily, in contrast to the more hydrophobic cholesterol (Sinensky, 1981; Toaff *et al.*, 1982). Therefore, the rate of conversion of exogenous 22R-hydroxycholesterol into pregnenolone is independent of any transport mechanisms between the mitochondrial membranes, but is only determined by the CSCC enzyme activity.

Subsequent conversion of pregnenolone into testosterone is dependent on the action of various metabolizing enzymes in the endoplasmic reticulum. The 17α-hydroxylase cytochrome P450 enzyme (P450c17)(Nakajin and Hall, 1981; Nakajin *et al.*, 1981; Hall, 1991) catalyzes two reactions: hydroxylation of the C_{21}-steroid progesterone (17α-hydroxylase activity) followed by a cleavage reaction ($C_{17\text{-}20}$ lyase activity) to yield C_{19}-steroids. The conversion of pregnenolone into progesterone is catalyzed by the enzyme 3ß-hydroxysteroid dehydrogenase (3ßHSD). As a result, there are two pathways for the formation of C_{19}-steroids: the delta 4

or progesterone pathway, and the delta 5 or pregnenolone pathway. The relative contribution of the pathways is species-specific; *e.g.*, in rat Leydig cells the delta 4 pathway is prominent (Samuels, 1960), whereas in human (Weusten, 1989), dog (Eik-Nes and Hall, 1962), rabbit (Hall *et al.*, 1964), and pig (Onoda *et al.*, 1987) the delta 5 pathway is more pronounced. The conversion of androstenedione into testosterone is mediated by the enzyme 17ß-hydroxysteroid dehydrogenase (17ßHSD). The enzymes 5α-reductase and aromatase cytochrome P450 (P450arom) can further metabolize testosterone, to generate 5α-dihydrotestosterone or estradiol (Fig. 4).

The relative amount of testosterone produced by Leydig cells changes during testicular development. In Leydig cells of immature rats (20-40 days old) 5α-androstane-3α,17ß-diol and 3α-hydroxy-5α-androstan-17-one (androsterone) are present in much larger amounts than testosterone, whereas the relative amount of testosterone is higher in Leydig cells from older rats (Lacroix *et al.*, 1975; Purvis *et al.*, 1978; Shan *et al.*, 1993). These 5α-reduced androgens are mainly formed via a 5α-pregnane pathway, which does not require testosterone as an intermediate. In this pathway, progesterone is metabolized to various pregnane compounds, which can subsequently be converted to androsterone and androstanediols (Moger and Armstrong, 1974; Van der Molen and Rommerts, 1981).

Testosterone production by Leydig cells can be regulated at different levels of the steroidogenic cascade:

1) cholesterol transport to the CSCC enzyme;
2) CSCC enzyme activity;
3) conversion of pregnenolone into testosterone;
4) metabolism of testosterone.

The net production of testosterone by Leydig cells is obviously favoured by a high activity of enzymes that synthesize testosterone and a low activity of the enzyme that metabolizes testosterone.

1.1.3. Rate-determining step in the steroidogenic cascade

Testosterone is an intermediate in a cascade of steroidogenic reactions, but not an end product (Fig. 5, I). If metabolic conversions of testosterone are absent, the rate of testosterone production depends on a certain rate-limiting step in the cascade. In the literature, the CSCC enzyme activity is often identified as the rate-limiting step, but this is only valid if the subsequent enzymes have a higher conversion capacity than the CSCC enzyme (Fig. 5, II). However, this is not always the case, since several C_{21} and C_{19}

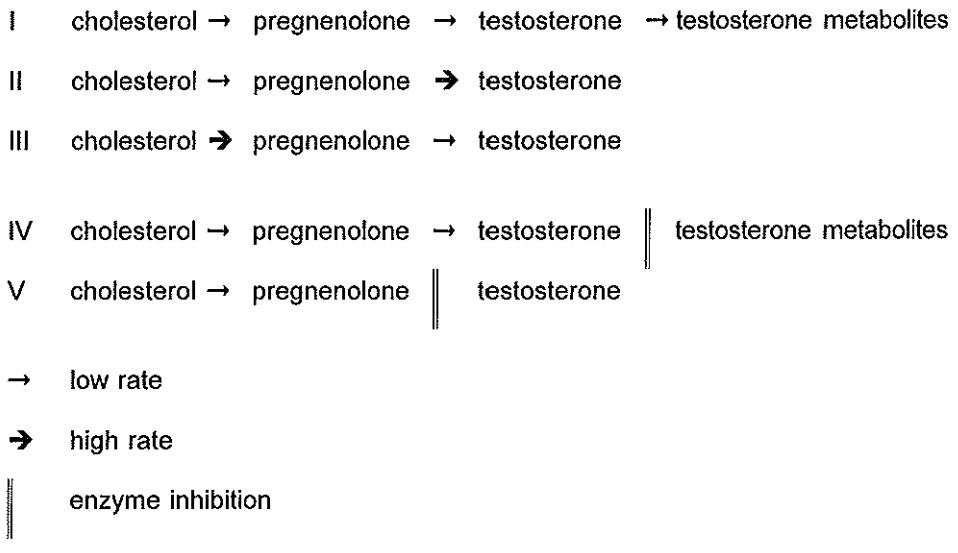

Fig. 5. ***Rate-limiting steps of the steroidogenic cascade.*** *Depending on the rate of metabolism, the rate-limiting step can be at the level of CSCC enzyme or somewhere else in the cascade. Several situations are possible.*

I testosterone is not an end product but an intermediate in a cascade of steroidogenic conversions. This is unique for the testis of immature rats, which metabolizes testosterone into various testosterone metabolites.

II the CSCC enzyme activity is rate-limiting in the conversion of cholesterol into testosterone, since the other steroidogenic enzymatic reactions have a higher capacity. In this situation, the steroid production rate is low (basal steroid production) and the rate-determining step (CSCC enzyme activity) is the rate-limiting step.

III the microsomal enzymes which metabolize pregnenolone into testosterone are rate-limiting in the steroidogenic cascade. This occurs when the steroid production rate is high (for example in the presence of 22R-hydroxycholesterol).

The amount of different steroids can be modulated by inhibition of steroidogenic enzymes. This facilitates the measurement of steroidogenic activity in Leydig cells. The use of these inhibitors results in the following situations:

IV testosterone is the end product in the steroidogenic cascade. This occurs normally in mature rats, or in immature rats in the presence of 5α-reductase inhibitors.

V pregnenolone is the end product in the steroidogenic cascade. This situation occurs after blocking conversion of pregnenolone into pregnenolone metabolites by inhibition of 3βHSD and P450c17 enzymes.

precursors of testosterone can be measured in testis tissue of rats and humans (De la Torre *et al.*, 1982; Punjabi *et al.*, 1983; Grizard *et al.*, 1987). The presence of these precursors of testosterone indicates that there may be a rate-limiting step in-between the formation of pregnenolone and testosterone (Fig. 5, III). Hence, the rate-limiting step can be either at the level of the CSCC enzyme (when the steroid production rate is low) or somewhere else in the cascade (when the steroid production rate is high, for instance in the presence of a high substrate concentration 22R-hydroxycholesterol). We prefer to define the CSCC enzyme activity as the rate-determining step of the steroidogenic cascade, since the cleavage rate of cholesterol determines the rate of steroid hormone production.
For studying regulation of Leydig cell steroidogenesis it is important to measure all steroids. Since measurement of testosterone does not always reflect the total steroidogenic activity, we have tried to measure the rate-determining step of the steroidogenic cascade, the CSCC enzyme activity, directly. Effects of hormones on the supply of substrate (cholesterol) and the amount of the CSCC enzyme have been investigated (Chapters 3, 4, and 5).

1.1.4. Measurement of Leydig cell steroid production

The total steroidogenic activity of Leydig cells can be estimated by measurement of all steroids produced. This includes measurement of testosterone, as well as the precursors and metabolites of testosterone (including the 5α-reduced androgens). Due to the variety of steroids which can be produced by Leydig cells, measurement of all steroids may require much time and effort.
Net testosterone production has been used by many investigators, as an index of the steroidogenic activity of Leydig cells. Although testosterone is one of the most important biologically active androgens and frequently also the most abundant steroid secreted by Leydig cells, measurement of this steroid alone may underestimate the total steroidogenic Leydig cell activity since the production of precursors and/or metabolites of testosterone is not included. Verhoeven and Cailleau (1983) have used Na-4-methyl-4-aza-3-oxo-5α-pregnane-20(S)-carboxylate to inhibit the enzyme 5α-reductase, to get a better impression of total androgen production (Fig. 5, IV). However, using this method, the precursors of testosterone must still be measured in separate assays. It seems better to completely inhibit metabolism of pregnenolone, and to measure the accumulated pregnenolone. In this thesis, we have established conditions for blocking the conversion of pregnenolone into pregnenolone metabolites using enzyme inhibitors without any adverse effect on the CSCC enzyme activity (Fig. 5, V). This assay system was applied for measuring endogenous Leydig cell steroidogenesis as well as 22R-hydroxycholesterol-supported Leydig cell steroid production (Chapter 3).

1.2. Regulation of the steroidogenic cascade by LH

LH is the most important regulator of Leydig cell steroidogenesis, which stimulates androgen production at different levels of the steroidogenic cascade via two different mechanisms: a rapid effect (within seconds or minutes) and a slower action (after several hours). Short-term stimulation of steroid production is exerted via an increased supply of cholesterol to the CSCC enzyme (Hall *et al.*, 1979; Simpson, 1979). There is growing evidence that this process involves production and/or modification (phosphorylation) of proteins, which facilitate the transport of cholesterol to the CSCC enzyme in the inner mitochondrial membrane (Boujrad *et al.*, 1993; Clark *et al.*, 1994). The long-term stimulating effect of LH depends on transcriptional activation of specific genes encoding steroidogenic enzymes and accessory proteins (Hall, 1994)(Fig. 6).

The short-term and long-term effects of LH are both initiated by binding of the circulating hormone to the LH receptor, located in the plasma membrane (Segaloff and Ascoli, 1993). Upon binding of LH to the receptor, several second messenger systems are activated. The cyclic AMP level is increased (Janszen *et al.*, 1976; Themmen *et al.*, 1985, 1986a; Sullivan and Cooke, 1986), and stimulation of the phospholipase C pathway (as assessed by measuring inositol phosphates (IP_3), diacylglycerol (DAG), and/or intracellular Ca^{2+}) also may play a role (Gundermann *et al.*, 1992a,b). The activation of these different signal transduction systems in Leydig cells causes phosphorylation (Epstein and Orme-Johnson, 1991a; Chaudhary and Stocco, 1991) and/or synthesis of specific regulatory proteins and steroidogenic enzymes (Cooke *et al.*, 1976; Bakker *et al.*, 1983a,b; Themmen *et al.*, 1986b; Miller, 1989).

1.2.1. Short-term effects of LH

Short-term activation of steroid production by LH is mediated via an enhanced cholesterol transport from the outer to the inner mitochondrial membranes (Hall *et al.*, 1979; Simpson, 1979). The following observations indicate that a rapidly-turning-over protein is required for this process. First, rapid hormone stimulation of steroidogenesis in cultured cells can be inhibited by inhibitors of protein synthesis such as cycloheximide (Hall and Eik-Nes, 1962; Ferguson, 1963) which do not affect the CSCC enzyme activity itself (Arthur and Boyd, 1976). Furthermore, treatment of adrenal cells with adrenocorticotropic hormone (ACTH) increases the concentration of cholesterol in the inner mitochondrial membrane (Nakamura *et al.*, 1980), whereas treatment with ACTH and inhibitors of protein synthesis increases the concentration of cholesterol in the outer but not in the inner mitochondrial membranes (Privalle *et al.*, 1983; Ohno *et al.*, 1983). Pregnenolone production does not take place until cycloheximide has been removed and the cells are stimulated with hormone (Stevens *et al.*,

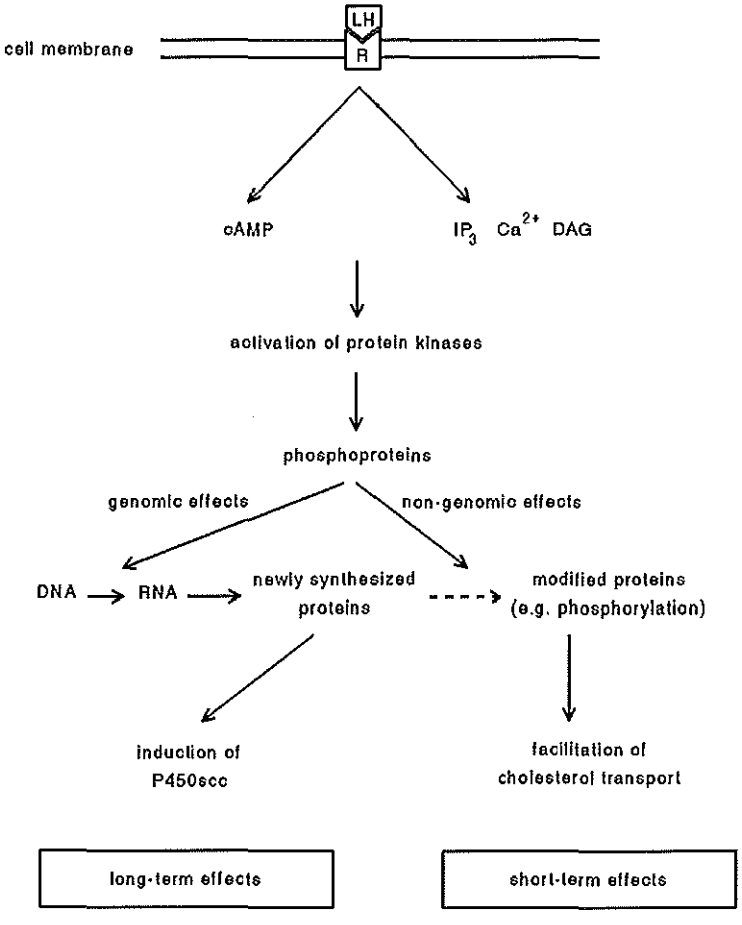

Fig. 6. Short-term and long-term regulation of steroidogenic activity in Leydig cells by LH. The gonadotropic hormone LH can stimulate Leydig cell steroidogenesis via two different mechanisms: rapid non-genomic action and slower genomic action. The rapid response to LH, mediated by cAMP (and/or other second messengers), protein phosphorylation, and protein modifications, but without direct involvement of RNA synthesis, occurs within minutes. This sequence of events leads to enhanced trafficking of cholesterol to cholesterol side-chain cleavage enzyme. Long-term effects of LH are mediated by cAMP, protein phosphorylation, gene expression, and protein synthesis, and require several hours. One of these long-term actions is induction of P450scc enzyme.

1993). Various proteins have been suggested to be important for intracellular transport of cholesterol in steroidogenic cells. These include steroidogenic acute regulatory protein (StAR) and diazepam-binding inhibitor (DBI), which appear to be unique to steroidogenic cells (see below). Moreover, COS cells engineered to make steroids by transfection with constructs expressing cytochrome P450scc and adrenodoxin but lacking StAR or DBI, do not respond to dibutyryl cyclic AMP with enhanced endogenous steroidogenesis. However, in the presence of a soluble hydroxycholesterol substrate, steroid production in these transfected cells can be measured (Zuber *et al.*, 1988).

A family of mitochondrial proteins and phosphoproteins with a molecular weight of approximately 30 kDa has been described. These proteins appear rapidly after hormone stimulation in rat adrenal cells (Nakamura *et al.*, 1978; Krueger and Orme-Johnson, 1983; Alberta *et al.*, 1989; Epstein *et al.*, 1989), the corpus luteum (Pon and Orme-Johnson, 1986), and in mouse Leydig cells (Pon *et al.*, 1986; Epstein and Orme-Johnson, 1991b). The appearance of these proteins is inhibited by cycloheximide (Epstein and Orme-Johnson, 1991b). Clark and Stocco (1995) have recently identified StAR, which appears to be an important candidate 30 kDa protein in intramitochondrial cholesterol transport. This protein has been purified from MA-10 cells and its cDNA has been cloned (Clark *et al.*, 1994). The cDNA encodes a protein with a N-terminal mitochondrial targeting sequence. Transient expression of StAR cDNA in MA-10 cells and COS-1 cells results in enhanced steroidogenesis (Clark *et al.*, 1994; Sugawara *et al.*, 1995). The mRNA that encodes StAR is abundant in adrenal glands and gonads, but not in placenta (Sugawara *et al.*, 1995). Several possible explanations for the ability of the placenta to produce progesterone in the absence of StAR have been proposed (Strauss *et al.*, 1996). For example, the mass of the placenta could compensate for the absence of facilitators to move cholesterol to the inner mitochondrial membrane since some cholesterol is able to move from the outer to inner membranes in COS cells transfected with P450scc and adrenodoxin but not with StAR (Lin *et al.*, 1995). Another possibility is that other mechanisms as described in this section facilitate sterol delivery in the placenta. Direct evidence for an indispensable role of StAR in adrenal and gonadal steroidogenesis has been provided by Lin *et al.* (1995). These authors found that a defect in steroidogenesis in congenital lipoid adrenal hyperplasia patients, which could not be explained by a mutation in one of the genes encoding steroidogenic enzymes, resides in a defect in the protein StAR. Stocco and Clark (1996a,b) have proposed that StAR induces contact sites between the inner and outer mitochondrial membranes, thereby facilitating intramitochondrial transport of cholesterol.

A great deal of attention has also been given to a possible role of the peripheral benzodiazepine receptor (PBR) in short-term regulation of steroidogenesis, as reviewed by Papadopoulos and Brown (1995). Although PBR is present in mitochondria of most cell

types, a high number of receptors was found especially in steroidogenic tissues (Papadopoulos *et al.*, 1990). Stimulation of steroid production in response to PBR ligands was observed in mouse MA-10 Leydig tumour cells and Y-1 adrenal tumour cells (Mukhin *et al.*, 1989; Papadopoulos, 1993), bovine primary adrenal cell cultures (Yanagibashi *et al.*, 1989), human placental tissue (Barnea *et al.*, 1989), and rat ovarian granulosa cell lines (Amsterdam and Suh, 1991). The endogenous PBR ligand DBI, also stimulates mitochondrial steroidogenesis (Yanagibashi *et al.*, 1989; Papadopoulos *et al.*, 1992). Depletion of DBI from MA-10 Leydig tumour cells, using an antisense oligonucleotide method, inhibited short-term stimulation of progesterone production by hCG, whereas the cAMP level and CSCC enzyme activity were not affected (Boujrad *et al.*, 1993). More evidence for a role of DBI/PBR in steroidogenic regulation was the finding that an 8.2 kDa peptide purified from bovine adrenal cells, which stimulates mitochondrial pregnenolone production (Yanagibashi *et al.*, 1988), is virtually identical to DBI (Besman *et al.*, 1989). PBR is associated with a voltage-dependent anion channel protein (VDAC) in the mitochondrial membranes (McEnery *et al.*, 1992). VDAC is preferentially located at contact sites of the inner and outer mitochondrial membranes (Garnier *et al.*, 1994), which have been suggested to be involved in intramitochondrial cholesterol transfer (Jefcoate *et al.*, 1992). PBR/VDAC may form a pore in the outer mitochondrial membrane through which cholesterol and other molecules can be translocated to the inner mitochondrial membrane (Papadopoulos *et al.*, 1994). However, there are several inconsistencies in connection with an important role of DBI/PBR in the short-term regulation of steroidogenesis. Since DBI has a half-life of 3-6 h and is not induced by hormones or cAMP analogues in steroidogenic cells, it seems not likely that DBI is the long-sought cycloheximide sensitive factor (Brown *et al.*, 1992). Furthermore, rat adrenal DBI and PBR levels are reduced dramatically nine days after hypophysectomy; subsequent administration of ACTH to these hypophysectomized rats resulted in an increase in steroidogenesis which peaked within one hour, while both PBR and DBI mRNA and protein levels showed no increase for approximately 12 hours (Cavallaro *et al.*, 1993). This indicates that steroidogenesis and PBR/DBI levels are not temporally related to acute steroidogenesis induced by ACTH. To explain this inconsistency, a model has been proposed wherein tropic hormone action induces conformational changes either in PBR or its mitochondrial microenvironment, thus modulating the affinity of PBR for DBI, which then triggers intramitochondrial cholesterol transport (Boujrad *et al.*, 1993).

Another peptide, steroidogenesis activator polypeptide (SAP) with a low molecular weight (3.2 kDa), identified both in rat adrenal cells and in the H-540 rat Leydig cell tumour, increased steroid production when added to isolated mitochondria (Pedersen and Brownie, 1983, 1987; Xu *et al.*, 1991). This peptide was found to be present only in steroidogenic cells, and its level could be increased by tropic hormone stimulation through a cycloheximide

sensitive mechanism (Pedersen, 1987; Mertz and Pedersen, 1989; Frustaci *et al.*, 1989). However, in the recent literature no new information on SAP has been given.

Sterol carrier protein 2 (SCP_2), also called non-specific lipid transfer protein (nsL-TP), is a low molecular weight protein (13 kDa) which has been found in liver and in various steroidogenic tissues. SCP_2 enhances the transfer of phospholipids, cholesterol and glycolipids between membranes *in vitro* (Bloj and Zilversmit, 1977, 1981; Poorthuis *et al.*, 1981; Trzaskos and Gaylor, 1983), and stimulates enzymatic reactions involved in cholesterol biosynthesis (Johnson and Shah, 1973) and mitochondrial production of steroids (Chanderbhan *et al.*, 1982; Van Noort *et al.*, 1988a; McNamara and Jefcoate, 1989; Yamamoto *et al.*, 1991). It has been suggested that the stimulatory effect of LH on rat Leydig cells is associated with an intracellular redistribution of SCP_2, which contributes to an increase in cholesterol trafficking to the mitochondria (Van Noort *et al.*, 1988a). Initially, the cellular location of SCP_2 in rat testis has been studied by measuring the amount of protein in cytosolic fractions from different testicular cell types, as an estimate of loosely bound SCP_2. The level of SCP_2 was found to be high in Leydig cell cytosol, but SCP_2 was virtually absent from Sertoli- and germ cell cytosol, in accordance with the suggestion that SCP_2 is important for steroidogenesis (Van Noort *et al.*, 1986). However, analysis of the subcellular location of SCP_2 in different rat tissues, including rat liver, adrenal gland, and testis, revealed that SCP_2 was predominantly membrane-associated in all tissues except for the liver (Van Amerongen *et al.*, 1989; Van Heusden *et al.*, 1990a). In addition, immunogold labelling using SCP_2 antibody revealed dense labelling of peroxisomes in rat liver and adrenal gland (Van der Krift *et al.*, 1985; Keller *et al.*, 1989; Van Amerongen *et al.*, 1989). Measuring SCP_2 exclusively in the cytosol of testicular cells, as performed by Van Noort *et al.* (1986), may have given insufficient information on the cellular and subcellular location of SCP_2 in rat testis. In this thesis, we have therefore reinvestigated the cellular and subcellular location of SCP_2 in Leydig cells and other testicular cells, before and after hormonal stimulation, using different biochemical techniques (Chapter 2).

1.2.2. Long-term effects of LH

Similar to short-term effects, long-term effects of LH are also for a great part mediated by an increased cAMP level, which results in enhanced synthesis of the P450 enzymes involved in testosterone production (Simpson *et al.*, 1987, 1990; Miller, 1989). These long-term effects are exerted at the level of gene expression. Increased expression of different mRNAs in both adrenocortical cells and in tumour Leydig cells can occur within several hours after the start of tropic hormone stimulation, which is slow, relative to the fast (< 1 h) activation of many non-steroidogenic genes by dbcAMP in other tissues (John *et al.*, 1986; Mellon and Vaisse, 1989; Hales *et al.*, 1990). An increase of the P450scc level 24 hours after treatment

with hCG has been demonstrated in mature rat and immature pig Leydig cells (Mason *et al.*, 1984; Anderson and Mendelson, 1985). Increased transcription of the P450scc gene after administration of dbcAMP has been shown in MA-10 cells (Mellon and Vaisse, 1989) and in mouse Leydig cells (Payne, 1990). Moreover, expression of P450c17 mRNA and *de novo* synthesis of this enzyme also are cAMP-dependent in mouse and porcine Leydig cells (Anakwe and Payne, 1987; Orava *et al.*, 1989; Payne and Sha, 1991). Another steroidogenic enzyme that is transcriptionally regulated by cAMP, is 3ßHSD (Keeney and Mason, 1992a,b).

1.2.3. Modulation of LH action by local factors

Steroidogenic enzyme induction does not only depend on tropic action of LH, but paracrine factors can also take part in regulation of gene expression. Factors produced by seminiferous tubules or Sertoli cells are important for regulation of Leydig cell testosterone production (Skinner, 1991; Saez, 1994). Indications for functional interaction between Sertoli cells and Leydig cells have been obtained from studies in which immature rats were hypophysectomized. Hypophysectomy caused a rapid and almost complete loss of Leydig cell responsiveness to LH. Subsequent administration of follicle-stimulating hormone (FSH) resulted in an increase in number and steroidogenic activity of Leydig cells (Odell and Swerdloff, 1976; Van Beurden *et al.*, 1976; Teerds *et al.*, 1989; Vihko *et al.*, 1991). Moreover, morphological studies indicated mild hyperplasia of Leydig cells after FSH treatment (Kerr and Sharpe, 1985). These actions of FSH are mediated by Sertoli cells, which exclusively express the FSH receptor (Dorrington *et al.*, 1975; Heckert and Griswold, 1991).

In vitro studies have provided evidence that Sertoli cells or seminiferous tubules produce both stimulatory and inhibitory activities, which modulate Leydig cell testosterone production. A paracrine action of Sertoli cells on Leydig cells has first been reported by Johnson and Ewing (1973), who demonstrated that FSH enhanced testosterone production by perfused rabbit testes. A Sertoli cell-mediated stimulatory effect of FSH on fetal rat testis steroid production has been reported using recombinant FSH (Lecerf *et al.*, 1993). Experiments on cocultures of Leydig cells with Sertoli cells or seminiferous tubules revealed that Sertoli cells generally increase the basal and hormone stimulated Leydig cell steroid production (Reventos *et al.*, 1983; Saez *et al.*, 1989). The stimulatory action of Sertoli cells on Leydig cell steroidogenesis could be enhanced in the presence of FSH (Behnamed *et al.*, 1985). On the other hand, studies utilizing conditioned culture medium derived from Sertoli cells or seminiferous tubules showed not only stimulatory (Verhoeven and Cailleau, 1985, 1990; Onoda *et al.*, 1991; Papadopoulos, 1991) but also inhibitory activities (Syed *et al.*, 1988; Vihko and Huhtaniemi, 1989; Zwain *et al.*, 1991) on Leydig cell steroid production. One of

the reasons for these discrepant findings might be that in most studies testosterone production was used as a parameter to investigate the modulating activities of Sertoli cell products. Since testosterone production depends on a steroidogenic cascade with multiple regulation points, it is difficult to perform reproducible experiments on the effect of a mixture of regulatory compounds on testosterone production (see 1.1.3.). In this thesis, we have therefore focused on regulation of the CSCC enzyme activity (the rate-determining step of steroidogenesis) by one specific compound, IGF-I, and by a mixture of factors, that is secreted by cultured Sertoli cells.

1.3. Aim and scope of this thesis

As discussed in the previous sections, LH and local factors may regulate Leydig cell steroidogenesis at different points of the steroidogenic cascade. In this thesis, the hormonal regulation of Leydig cell steroidogenesis was investigated at the level of the CSCC enzyme, since this is the rate-determining step for steroid production. The conversion rate of cholesterol into pregnenolone by the CSCC enzyme depends on the amount of substrate (cholesterol) that gains access to the enzyme, and the amount of CSCC enzyme in the intramitochondrial membrane. These aspects are addressed in two parts of this thesis:

1) the role of SCP_2 in short-term hormonal action: transport of cholesterol to the CSCC enzyme, and
2) long-term effects of LH and paracrine factors on CSCC enzyme induction.

This is described in the following chapters:

Chapter 2	Cellular and subcellular localization of SCP_2 in the rat testis, particularly in Leydig cells.
Chapters 3 and 4	Conditions for measurement of endogenous CSCC enzyme activity and CSCC enzyme induction.
Chapter 4	Role of IGF-I in LH induction of CSCC enzyme.
Chapter 5	Role of Sertoli cell factors in LH induction of CSCC enzyme.

Chapter 2

Sterol Carrier Protein (Non-Specific Lipid Transfer Protein) is Localized in Membranous Fractions of Leydig Cells and Sertoli Cells but not in Germ Cells

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Biochimica et Biophysica Acta
(1992) 1124:288-296

Abstract

The cellular and subcellular distribution of sterol carrier protein 2 (SCP_2; nsL-TP) was reinvestigated in rat testicular cells by Western blotting and immunocytochemistry, using the affinity purified antibody against rat liver SCP_2. Western blot analysis revealed high levels of the protein in the somatic cells of the testis *e.g.* Leydig and Sertoli cells whereas it could not be detected in germ cells. This cellular localization of SCP_2 was confirmed by Northern blotting. Immunocytochemical techniques revealed that in Leydig cells, immunoreactive proteins were concentrated in peroxisomes. Although SCP_2 was also detected in Sertoli cells, a specific subcellular localization could not be shown. SCP_2 was absent from germ cells. Analysis of subcellular fractions of Leydig cells showed that SCP_2 is membrane bound without detectable amounts in the cytosolic fraction. These results are at variance with data published previously which suggested that in Leydig cells a substantial amount of SCP_2 was present in the cytosol and that the distribution between membranes and cytosol was regulated by luteinizing hormone. The present data raise the question in what way SCP_2 is involved in cholesterol transport between membranes in steroidogenic cells but also in non-steroidogenic cells.

Introduction

Sterol carrier protein 2 (SCP_2), also called non-specific lipid transfer protein (nsL-TP), is a low molecular weight (Mr 14 kDa) protein that enhances the transfer *in vitro* of phospholipids, cholesterol and glycolipids between membranes (Bloj and Zilversmit, 1977,1981; Noland *et al.*, 1980; Poorthuis *et al.*, 1981; Trzaskos and Gaylor, 1983). By transferring sterols, it can stimulate enzymatic reactions involved in cholesterol biosynthesis (Johnson and Shah, 1973) and the mitochondrial production of steroids from cholesterol (Chanderbhan *et al.*, 1982; Van Noort *et al.*, 1988a; McNamara and Jefcoate, 1989; Yamamoto *et al.*, 1991). In a previous study, in which SCP_2 levels were measured in cytosolic fractions derived from various testicular cells, it was found that the protein is present at high levels in Leydig cells, but is virtually absent from Sertoli and germ cells (Van Noort *et al.*, 1986). Moreover, it was observed that, after incubation of Leydig cells with luteinizing hormone (LH), the amount of SCP_2 increased in the cytosolic fraction and decreased to low levels in the membranous fractions. It has been suggested that SCP_2 as a soluble, or as a loosely membrane bound protein, facilitates the supply of cholesterol to the cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme in mitochondria of Leydig or adrenal cells (Chanderbhan *et al.*, 1982; Vahouny *et al.*, 1983; Van Noort *et al.*, 1988a).

In a recent study of the subcellular distribution of SCP_2 in various rat tissues, it was found that SCP_2 was predominantly present in the membranous fractions (Van Heusden *et al.*, 1990a). Also in steroidogenic tissues such as the testis, adrenals and in Chinese hamster ovary cells, SCP_2 was mainly found in the membranous fractions (Chanderbhan *et al.*, 1986; Van Amerongen *et al.*, 1989; Van Heusden *et al.*, 1990a,b). In liver tissue and Leydig cells a substantial amount of the protein was located in the peroxisomes (Tsuneoka *et al.*, 1988; Keller *et al.*, 1989; Mendis-Handagama *et al.*, 1990a,b).

Measuring SCP_2 in cytosolic fractions may therefore only give partial information about the amount of protein in a particular cell or tissue. In the present study, we have reinvestigated the cellular and subcellular localization of SCP_2 in testicular cells with particular reference to the Leydig cells. The distribution of SCP_2 was compared with that of the P450scc enzyme, the rate-determining enzyme in steroidogenesis which is localized in mitochondria of steroidogenic cells (Van der Vusse *et al.*, 1973; Moyle *et al.*, 1973; Farkash *et al.*, 1986). Western blotting, Northern blotting and immunocytochemistry were used for detection of SCP_2 (Van der Krift *et al.*, 1985; Ossendorp *et al.*, 1991).

Materials and Methods

Materials

SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl-1-(2H)-naphtalenone), an inhibitor of P450c17 activity, was a gift of Ciba-Geigy, Basle, Switzerland. Epostane or WIN-32729 (4α,5α-epoxy-17ß-hydroxy-4ß, 17α-dimethyl-3-oxo-androstane-2α-carbonitrile), an inhibitor of 3ßHSD activity, was a gift from Sterling-Wintrop, New York, USA. 22R-hydroxycholesterol and bovine serum albumin (BSA, fraction V) were purchased from Sigma, St. Louis, MO, USA. Nitrocellulose was a product from Schleicher and Schuell (Dassel, FRG). Goat anti-rabbit IgG conjugated to horseradish peroxidase was from Nordic Immunological Laboratories (Tilburg, The Netherlands). Goat anti-rabbit IgG conjugated to alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (BCIP), p-nitro blue tetrazolium chloride (NBT) and molecular weight standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis were obtained from BioRad Laboratories (Richmond, USA). Percoll and CNBr-activated Sepharose 4B were derived from Pharmacia (Uppsala, Sweden). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, UK). Biotrans nylon membrane was a product from ICN, Pall Biosupport, New York, USA.

Cell isolation and characterization of cell fractions

Leydig cells were obtained from Wistar mature rats 11-15 weeks of age, immature rats 21 days old, or the Leydig cell tumour H-540 (Cooke *et al.*, 1979). Leydig cells were isolated through collagenase treatment of decapsulated testes or tumour fragments for 18 min at 37 °C in a shaking water bath (80 cycles/min) as described previously (Rommerts *et al.*, 1985). The immature rat Leydig cells and the tumour Leydig cells were respectively 20-30% and 100% pure. In the procedure for isolation of Leydig cells from mature rats a Ficoll purification step was routinely employed (Rommerts *et al.*, 1985). This preparation contained 80-90% Leydig cells. Seminiferous tubules and Sertoli cells were isolated from testes of 21 days old Wistar rats as described by Oonk *et al.* (1985). Briefly, seminiferous tubules were isolated through collagenase treatment of decapsulated testes for 30 min at 32 °C in a shaking water bath (120 cycles/min). Sertoli cells were isolated using a double collagenase digestion method after which Sertoli cell clusters were obtained. These clusters were washed and fragmentated by mechanical agitation using a Dounce homogenizer (10-15 strokes). The preparation contained at least 95% Sertoli cells. Pachytene spermatocytes and round spermatids from 28 and 40 days old Wistar rats respectively, were isolated by velocity sedimentation at unit gravity (STA-PUT) and further purified by Percoll gradient centrifugation (Jutte *et al.*, 1985). The purity of both cell fractions was approximately 90%.

Enriched Sertoli cell fractions from mature rats were obtained from Leydig and germ cell-depleted testes after a single intraperitoneal injection of the Leydig cell toxicant ethane dimethylsulphonate (EDS) (75 mg/kg body weight) (Molenaar *et al.*, 1985) that was administered to rats irradiated prenatally (Beaumont, 1960). The steroidogenic activities of the different cell fractions were estimated by incubating the freshly isolated cells in a shaking water bath at 37 °C in RPMI medium (GIBCO, USA), 0.1% (w/v) BSA supplemented with 22R-hydroxycholesterol (19 μM), as a soluble substrate for the P450scc enzyme and a mixture of epostane (5 μM) and SU-10603 (5 μM), to inhibit the conversion of pregnenolone (Van Haren *et al.*, 1989). After 1 h, the media were collected for determination of pregnenolone by radioimmunoassay (Verjans *et al.*, 1973).

Preparation of homogenates and subcellular fractions

Homogenates (20%, w/v) of various testicular cell types were prepared in 0.25 M Sucrose containing 5 mM EGTA and 1 mM Tris-HCl, pH 7.5 (SET) by sonication at 0 °C (5 x 10 sec, amplitude 16 microns, frequency 60 Hz). Leydig tumour cells were homogenized by applying 10 strokes with a Dounce-Wheaton glass homogenizer (clearance 0.025 mm). Subcellular fractions were prepared by differential centrifugation as described previously for liver and adrenal glands (Van Amerongen *et al.*, 1989). The nuclear pellet (N-fraction) was obtained after centrifugation at 1000 x g for 10 min, the mitochondrial pellet (M-fraction), lysosomal pellet (L-fraction), microsomal pellet (P-fraction) and the cytosol (S-fraction) were obtained after centrifugation at 8,500 x g for 10 min, 20,000 x g for 20 min and 100,000 x g for 60 min respectively. All pellets were resuspended in SET. Protein was determined according to a modification (Van Amerongen *et al.*, 1989) of the method of Lowry *et al.* (1951).

Density gradient centrifugation

Homogenates from Leydig cell tumour tissue (20%, w/v) were prepared in 0.25 M Sucrose containing 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM Tris-HCl (pH 7.4) using a potter-Elvehjem homogenizer (three strokes at 1000 rpm). The homogenates were centrifuged for 10 min at 2,500 x g to remove nuclei and unbroken cells. An aliquot (0.8 ml) of the supernatant was layered on 7 ml of a Percoll solution (35%, w/w, in SET). After centrifugation for 75 min at 20,000 rpm in a rotor 50 (Beckman), 0.5 ml fractions of the gradient were collected. In these fractions, catalase activity was measured as described by Holmes and Masters (1970).

RNA extraction and hybridization

Total RNA was isolated from rat liver, total testis tissue and from various testicular cell types according to Auffray and Rougeon (1980). Briefly, RNA was extracted with phenol and chloroform, precipitated in ethanol containing 0.16 M sodium acetate, and stored in 70% ethanol at -20 °C. For hybridization experiments, RNA was separated on a denaturing 0.7% agarose gel containing 6.9% formaldehyde and ethidium bromide (0.1 mg/150 ml gel) and transferred to a Biotrans nylon membrane by capillary blotting. After fixing the RNA blot by baking at 80 °C for 2 h, the blot was prehybridized for about 2 h in 5 x SSPE (1 x SSPE contains 0.15 M NaCl, 0.01 M $NaH_2PO_4.H_2O$, 1 mM EDTA, pH 7.5), 5 x Denhardt's solution (1 x Denhardt's contains 0.02% w/v Ficoll, 0.02% w/v polyvinyl pyrrolidone, 0.02% w/v BSA), 0.2% sodium dodecyl sulfate (SDS), 100 μg herring sperm DNA/ml and 50% (v/v) formamide at 42 °C. Hybridization was performed in the same solution to which a [α-^{32}P]dCTP labelled 724 bp cDNA probe was added (random prime labelling, S.A. > $1x10^9$ cpm/μg), which encodes SCP_2 (Ossendorp *et al.*, 1990), at 42 °C overnight. Following hybridization, the blot was washed twice with 2 x SSC (1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 20 min, once with 0.1 x SSC/0.1% SDS at room temperature for 20 min, and finally with 0.1 x SSC/0.1% SDS at 55 °C for 12 min. Autoradiography was performed for 120 h at -50 °C.

Production and purification of antibodies

The immunization procedure using purified rat SCP_2 as an antigen and the isolation of total rabbit IgG were performed as described previously (Teerlink *et al.*, 1984). A specific IgG fraction was obtained by affinity chromatography using rat SCP_2 covalently coupled to Sepharose 4B. The rabbit anti-rat P450scc antiserum was prepared as described by Farkash *et al.* (1986). The polyclonal antibody against catalase was a generous gift of Dr. G. Posthuma (Dept. of Cell Biology, Medical School, Utrecht University, The Netherlands).

Enzyme immunoassay

Levels of SCP_2 were determined by an enzyme immunoassay according to Teerlink *et al.* (1984). Samples were heated for 5 min at 100 °C and centrifuged at 10,000 x g for 5 min to remove cross-reactive proteins (high molecular weight cross-reactive material). Prior to heating, the mixtures were diluted in phosphate-buffered saline (PBS) containing BSA to an end concentration of 1 mg BSA/ml. The supernatant fractions were transferred to glass tubes and further diluted with PBS containing BSA (1 mg/ml). The amount of SCP_2 adsorbed to a plastic surface in the presence of a large excess of BSA was measured using a polyclonal antibody against rat liver SCP_2, and a second anti-rabbit antibody linked to peroxidase.

Immunoblotting

Proteins were separated by SDS polyacrylamide gel electrophoresis on 7.5-25% (w/v) gradient gels (Laemmli, 1970) followed by blotting onto nitrocellulose. Immunological detection was performed with the polyclonal antibody against rat liver SCP_2 (dilution 1:600) and goat anti-rabbit IgG conjugated to alkaline phosphatase as described by Van Heusden *et al.* (1990a).

Immunocytochemistry

Testis tissue derived from adult rats was fixed by perfusion, using 0.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in Na-cacodylate buffer (0.1 M, pH 7.4) during 1 h at room temperature. After dehydration, the material was either embedded in Lowicryl HM 20 (Chemishe Werke Lowi, Walt Kraiburg, BRD) for the detection of SCP_2 immunoreactivity, or in LR White (The London Resin Co. Ltd., Woking, Surrey, England) for the detection of P450scc immunoreactivity. Ultrathin sections were cut and either incubated with the polyclonal antibody against rat liver SCP_2 (dilution 1:20) or with a polyclonal antibody against rat ovarium P450scc (dilution 1:20). The dilution of 1:20 for both antibodies was most appropriate, as established after series of dilution experiments. Binding of the antibodies was visualized using a pig anti-rabbit second antibody and 10 nm protein A gold (SCP_2) (Geuze *et al.*, 1983) or a goat anti-rabbit antibody conjugated to 10 nm gold particles (P450scc; Janssen Pharmaceutica, Beerse, Belgium).

Leydig cells were isolated from testes of adult rats using procedures described by Rommerts *et al.* (1985). Fixation was performed using 0.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde. The fixed cells were washed, harvested using a disposable cell scraper, and embedded in 10% (w/v) gelatin. Gelatin blocks and sections were cut according to Posthuma *et al.* (1987). These ultrathin cryosections were incubated with the antibody against rat liver SCP_2 and with a polyclonal antibody against catalase, a marker enzyme for peroxisomes. Both antibodies were visualized using 10 or 5 nm protein A gold respectively (Geuze *et al.*, 1983). The specificity of the antibodies was checked by either omitting the first antibody or by replacing it by pre-immune serum.

Results

Localization of SCP_2 by immunocytochemistry

The cellular and subcellular distribution of SCP_2 in testis tissue was investigated by immunocytochemistry. Plastic embedded ultrathin sections or cryosections of rat testicular tissue were incubated with the antibody against SCP_2. In Leydig cells, small organelles, presumably peroxisomes, were intensely labelled with gold particles. Small clusters of grains were found in the cytoplasm, nuclei and mitochondria (Fig. 1A). In the Sertoli cells, heavily labelled organelles such as found in Leydig cells were absent. On the other hand, the distribution and intensity of the labelling in these cells was rather similar as in the Leydig cells and no particular subcellular localization could be discerned (Fig. 1B). The dispersion of gold particles in spermatogonia was not different from background levels (Fig. 1C). In spermatids no specific labelling could be detected; only non-specific labelling around the acrosome was found (results not shown). The co-localization of the SCP_2 antibody and the catalase antibody, visualized by gold particles with a diameter of 10 nm and 5 nm respectively, confirmed that the organelles in mature rat Leydig cells

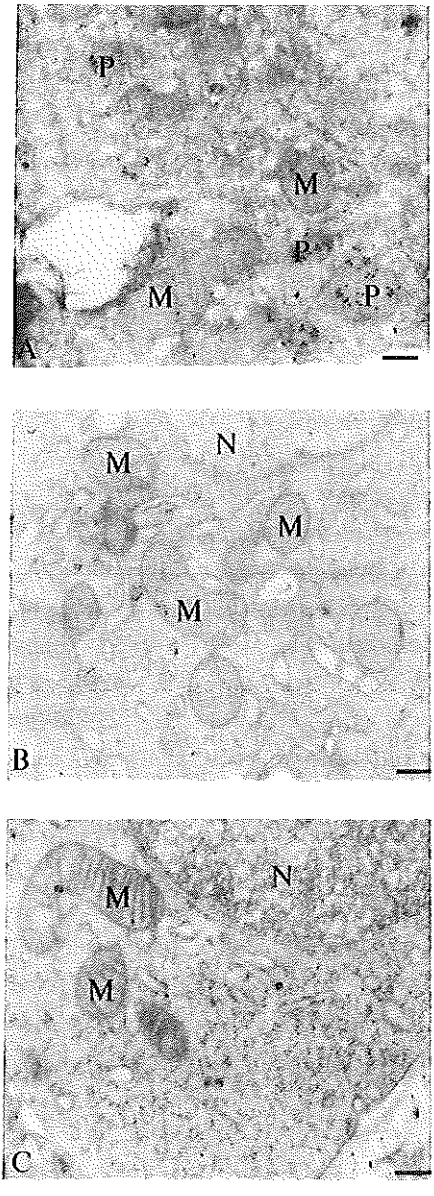

Fig. 1. Distribution of gold particles in testicular cells after incubation with SCP_2 antibody. A, Leydig cell; B, Sertoli cell; C, spermatogonium. Abbreviations used: P=peroxisome; M=mitochondrion; N=nucleus. Bars: A=210 nm; B=360 nm, C=250 nm.

which contained most gold particles were indeed peroxisomes (Fig. 2).

A similar distribution pattern for SCP_2 and catalase was found in tumour Leydig cells. In Sertoli cells and in spermatogonia specific binding of the catalase antibody could not be detected, indicating that these cells most likely do not contain catalase bound to peroxisomes (data not shown).

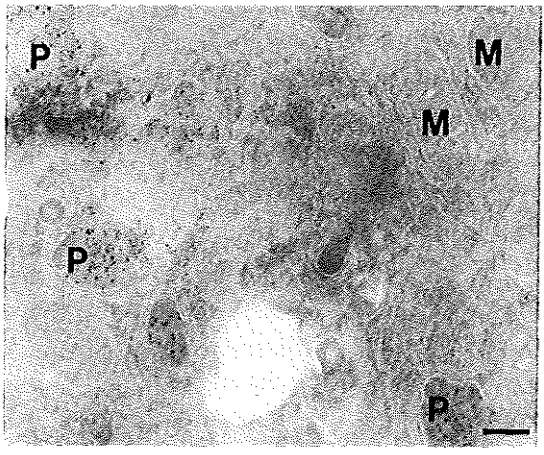

Fig. 2. Distribution of gold particles in Leydig cells with a large diameter (10 nm), visualizing the localization of the SCP_2 antibody and a small diameter (5 nm), visualizing the catalase antibody. Abbreviations used: P=peroxisome; M=mitochondrion. Bar=170 nm.

The immunocytochemical procedure was also performed with an antibody against the P450scc enzyme, known to be localized specifically in Leydig cell mitochondria (Van der Vusse *et al.*, 1973; Moyle *et al.*, 1973). Immunoreactivity of the P450scc enzyme was predominantly found within the mitochondria of Leydig cells (Fig. 3) while immunoreactivity was absent from Sertoli cells and spermatogonia (data not shown).

Cellular localization of SCP_2 by Western blotting

The cellular distribution of SCP_2 was also investigated by Western blotting. Various fractions enriched with specific testicular cell types were used: Leydig cells, seminiferous tubules, spermatocytes, spermatids and Sertoli cells.

In order to establish whether Leydig cells were concentrated in the Leydig cell fraction, the P450scc enzyme activity in the various cell preparations was measured by the conversion of added substrate (22R-hydroxycholesterol) into pregnenolone. The steroidogenic activity was

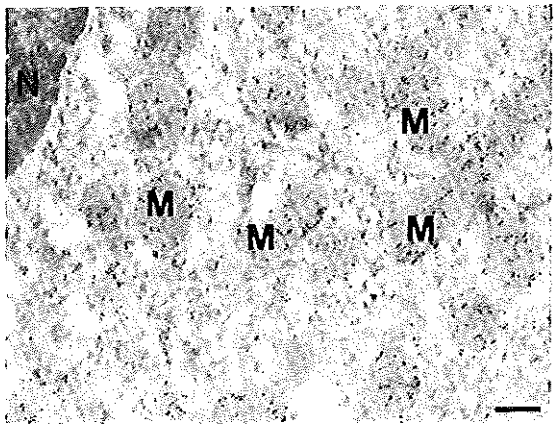

Fig. 3. Distribution of gold particles in Leydig cells after incubation with the P450scc enzyme antibody. Abbreviations used: P=peroxisome; M=mitochondrion; N=nucleus. Bar=293 nm.

almost exclusively present in the Leydig cell preparation. The low P450scc enzyme activities in germ and Sertoli cells indicate that Leydig cells are virtually absent from these fractions (Table 1).

The same cell preparations were used for Western blotting, employing the polyclonal antibody against SCP_2 which was also used for the immunocytochemistry. The predominant immunolabelled protein in Leydig cells from immature and mature rats possessed a molecular mass of 14 kDa, which corresponds with the molecular weight of SCP_2 (Fig. 4, lanes A-C). Immunoreactive proteins of higher molecular mass could also be detected. However, the cross-reacting protein with a molecular mass of 58 kDa, which is frequently found together with the 14 kDa protein, was barely detectable in Leydig cells.

A similar pattern of immunoreactive proteins was present in the fraction of seminiferous tubules (lane G). After separation of this tissue in a germ cell fraction, containing spermatocytes and spermatids and an enriched Sertoli cell fraction, it became evident that the 14 kDa protein in the seminiferous tubules was localized in Sertoli cells (lane F) and not in the germ cells (lane D, E). Enriched Sertoli cell preparations obtained from mature animals also contained a 14

cell fraction	nmol pregnenolone/mg protein/h
Leydig cells from mature rats	16.8 ± 7.7
Leydig cells from immature rats	17.6 ± 2.3
Leydig cells from tumour tissue	55.1 ± 8.7
seminiferous tubules	<0.1
spermatocytes	<0.1
spermatids	0.3 ± 0.3
immature Sertoli cells	<0.1
mature Sertoli cells	<0.1

Table 1. Cholesterol side-chain cleavage activity in different cell fractions. Cell fractions were incubated for 1 h at 37 °C with 22R-hydroxycholesterol (19 μM) as substrate and SU-10603 (5 μM) and epostane (5 μM) as inhibitors of pregnenolone metabolism. The amount of pregnenolone formed was measured by radioimmunoassay. Mean values ± SD (six determinations of two experiments) are shown.

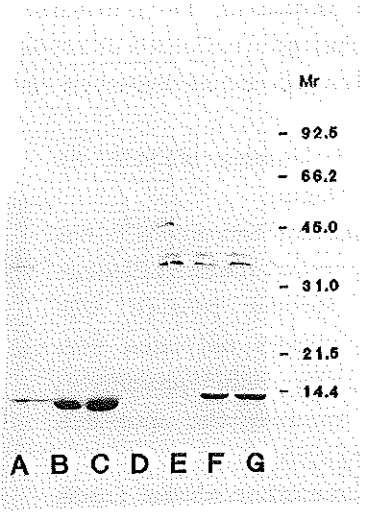

Fig. 4. SCP_2 protein in various testicular cells. Western blot developed with SCP_2 antibody after SDS-polyacrylamide gel electrophoresis of homogenates (100 μg protein) of Leydig cells from immature rats (lane A), Leydig cells from mature rats (lane B and C), spermatocytes (lane D), spermatids (lane E), and Sertoli cells (lane F) and seminiferous tubules of 21 days old rats (lane G).

kDa immunoreactive protein (data not shown). Other immunoreactive proteins of approximately 40 kDa were present in both the Sertoli cell and spermatid enriched cell preparations, but nearly absent from the spermatocyte enriched fractions.

Levels of SCP_2 mRNA in various testicular cell types

To obtain complementary data concerning the cellular distribution of SCP_2 in the testis, levels of SCP_2 mRNA were measured in total RNA isolated from rat liver (which served as a control), rat testis, and various testicular cell types. The SCP_2-encoding 724 bp cDNA which was used as a probe, recognized in liver mRNA species of 1.1, 1.7, 2.4 and 3.0 kb (Fig. 5, lane A). This agrees with recent studies in which it was reported that SCP_2 cDNA recognizes 4 mRNAs of which the two smaller mRNAs encode the 14 kDa SCP_2 whereas the larger ones encode the 58 kDa protein which was found to cross-react with the SCP_2 antibody (Ossendorp *et al.*, 1991; Seedorf and Assmann, 1991). In total testis, hardly any SCP_2 mRNAs could be detected. However, in preparations from various isolated cells specific mRNAs could be detected. The 1.1 and 1.7 kb mRNAs were clearly detectable in tumour Leydig cells (lane B) whereas in Sertoli cells these mRNAs were only found after 3 days culture (lane C, D). In spermatids, no SCP_2 mRNA was present (lane E). The 28 S rRNA band is visible in all lanes, probably due to non-specific binding of the probe to the rRNA. Ethidium bromide staining of the RNA revealed that equal amounts of mRNA were present in the different lanes of the blots (results not shown). These data support the observation that SCP_2 is present in the somatic cells, but not in the germ cells of the testis.

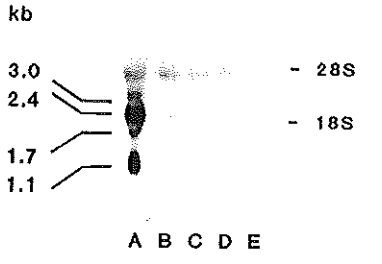

Fig. 5. SCP_2 mRNA in various cell types. Northern blot analysis of total RNA extracted from liver tissue (lane A), tumour Leydig cells (lane B), freshly isolated Sertoli cells from 21 days old rats (lane C), 3 days culture of Sertoli cells isolated from 21 days old rats (lane D) and spermatids (lane E). For analysis, 35 μg of total RNA was applied per lane and analyzed using a 724 bp cDNA fragment corresponding to SCP_2 mRNA.

Subcellular localization of SCP_2 by Western blotting

The subcellular distribution of SCP_2 in Leydig cells, as evaluated by immunocytochemistry, was also investigated by Western blotting and enzyme immunoassay. For this purpose,

tumour Leydig cells were used since large quantities of pure Leydig cells can be obtained and because these cells have also been used in previous studies (Van Noort *et al.*, 1988a,b). Estimation of SCP_2 levels in the cytosolic fraction of tumour Leydig cells by Western blotting yielded reproducible results and very low levels of SCP_2 (less than 25 ng/mg protein) were detected. The low cytosolic levels of this protein were not influenced by incubating the cells with LH. These observations are in sharp contrast with results obtained previously, when SCP_2 could be measured reproducibly with this enzyme immunoassay in cytosolic fractions: values of 200 ng SCP_2/mg protein in control cells and 400 ng SCP_2/mg protein in cells stimulated with LH were found (Van Noort *et al.*, 1988b). After additional application of the enzyme immunoassay to the same cytosolic samples as used for Western blotting, apparent levels of SCP_2 ranging from 100-800 ng SCP_2/mg protein were obtained. We have not been able to find an explanation for these discrepancies. It may be possible that depending on the sample preparation or coating to the plastic wells immunoreactive components interfere in the enzyme immunoassay. The possibility that some residual endogenous peroxidase activity interferes in the enzyme immunoassay has been excluded since no signal was detectable when both the first and the second antibody were omitted. Since the enzyme immunoassay appears not to be reliable for measuring levels of SCP_2 in testicular cells, we have not employed this technique further in the present study.

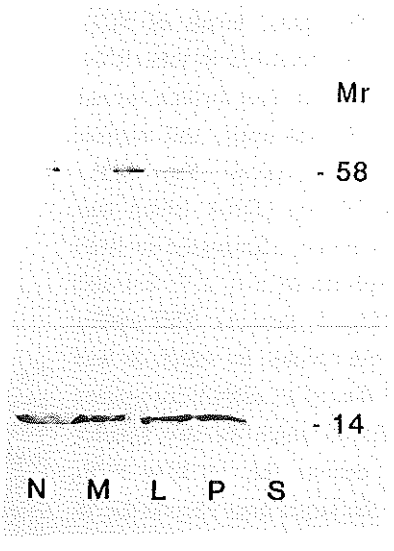

Fig. 6. Subcellular localization of SCP_2 in tumour Leydig cells. Western blot developed with SCP_2 antibody after SDS-polyacrylamide gel electrophoresis of 200 µg protein of different subcellular fractions of tumour Leydig cells. N, nuclear; M, mitochondrial; L, lysosomal; P, microsomal and S, cytosolic fraction.

Western blot analysis of all the subcellular fractions of tumour Leydig cells showed that an immunoreactive protein with a molecular mass of 14 kDa was present in the membranous fractions including the nuclear (N), mitochondrial (M), lysosomal (L) and microsomal (P) fractions, but not in the cytosolic (S) fraction (Fig. 6). Similarly, the immunoreactive protein of 58 kDa was present in all the membranous fractions, particularly in the mitochondrial fraction and absent from the cytosolic fraction.

Since the membranous fractions are not pure and a possible peroxisomal localization of the 14 and 58 kDa immunoreactive proteins is of particular interest, a post-nuclear fraction from tumour Leydig cells was further fractionated by centrifugation on a Percoll gradient. Peroxisomes were concentrated in fractions 7-11 as became clear from the catalase distribution (Fig. 7). Free catalase activity was concentrated in fractions 14-16 (the top of the gradient). Western blotting of all these Percoll fractions showed that the bulk of the 14 kDa immunoreactive protein and the 58 kDa immunoreactive protein were present in the fractions which also contained catalase activity (fractions 7-11, Fig. 7). A more careful inspection of the data shows that the 58 kDa protein is shifted to fractions of higher density when compared with the distribution pattern of the catalase activity and the 14 kDa immunoreactive protein.

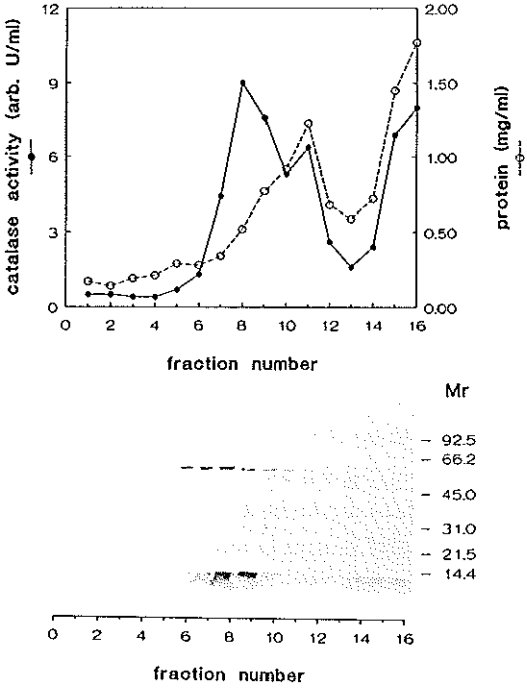

Fig. 7. Subcellular distribution of the 58 kDa immunoreactive protein, the 14 kDa SCP_2 and catalase activity. Subcellular fractions of tumour Leydig cells separated after Percoll density centrifugation were analyzed. Upper panel: catalase activity and protein concentration. Lower panel: Western blot analysis of 0.07 ml aliquots of each fraction. Fractions are numbered starting from the bottom of the gradient.

Discussion

In this report, the cellular and subcellular localization of SCP_2 in testicular cells has been reinvestigated, since it had become apparent that our previous observations as obtained by enzyme immunoassay (Van Noort *et al.*, 1988a) did not correlate with recent studies of other steroidogenic tissues (Chanderbhan *et al.*, 1986; Van Amerongen *et al.*, 1989; Van Heusden *et al.*, 1990a). Because SCP_2 in testicular samples could not be measured quantitatively by enzyme immunoassay, two other techniques, Western blotting and immunocytochemistry, were used to study the distribution of SCP_2.

All antibodies against purified SCP_2 obtained thus far, cross-react with the 58 kDa protein, while this protein contains the complete SCP_2 sequence at its carboxy-terminus (Ossendorp *et al.*, 1991). Therefore immunocytochemical studies of the (sub)cellular localization of SCP_2 are often difficult to interpret (Van der Krift *et al.*, 1985; Tsuneoka *et al.*, 1988; Van Amerongen *et al.*, 1989; Keller *et al.*, 1989; Mendis-Handagama *et al.*, 1990b). This problem is circumvented by the use of Western blotting, since with this technique SCP_2 and cross-reacting proteins are separated before the antibody is applied. On the other hand, Western blotting requires disruption of the cells, whereas immunocytochemical techniques are applied to cells with intact (though fixed) subcellular structures. A disadvantage of immunocytochemistry is that interactions between antibodies and SCP_2 in embedded in tissue sections may be restricted to matrix components (Van Amerongen *et al.*, 1989). Hence, the interpretation of the data obtained by immunocytochemistry as well by Western blotting depends on the specificity of the SCP_2 antibody used (Teerlink *et al.*, 1984).

According to the Western blots, the 14 kDa protein SCP_2 was present in Leydig and Sertoli cells but absent from germ cells. Cross-reacting proteins with molecular weights of approximately 38-40 kDa could be detected in spermatids but also in other testicular cell types. The 58 kDa cross-reacting protein was almost undetectable in all testicular cell types whereas low amounts of the 58 kDa protein were found in tumour Leydig cells.

mRNA encoding SCP_2 was clearly present in tumour Leydig cells, which is consistent with the presence of SCP_2 in Leydig cells (Van Noort *et al.*, 1986; Mendis-Handagama *et al.*, 1990a,b). Surprisingly, in Sertoli cells the expression of SCP_2 mRNA could, for unknown reasons, only be discerned after a 3 day culture period. The fact that expression of SCP_2 mRNA as well as the expression of the 58 kDa protein mRNA were clearly absent from spermatids, suggests that the cross-reacting proteins with a molecular weight of 38-40 kDa, as detected with Western blotting, probably represent some non-specific signal.

With the immunocytochemical studies both the cellular and subcellular localization of SCP_2 can be determined. The usefulness of this technique was evaluated by assessing the cellular

and subcellular distribution of the P450scc enzyme, which acts as an acceptor of (SCP_2 transported?) cholesterol. This enzyme was, as expected, exclusively located in the mitochondria of the Leydig cells. Immunocytochemical analysis of SCP_2 showed that the distribution and intensity of gold particles in the Leydig cells and Sertoli cells were rather similar, except for the peroxisomal concentration in Leydig cells. The cytoplasmic pattern of SCP_2 labelling is not specific for Leydig cells or Sertoli cells. Keller *et al.* (1989) have reported that SCP_2 immunoreactivity in hepatocytes is not only present within the peroxisomes but also in the mitochondria and other parts of the cytoplasm. Due to the absence of labelled peroxisomes the total amount of immunoreactivity in Sertoli cells appears lower when compared to Leydig cells. This is in contrast with results obtained from Western blotting which showed similar amounts of SCP_2 immunoreactivity in both cell types. These apparent differences must be interpreted with caution. As has already been mentioned, parts of SCP_2 in embedded tissue sections may be masked. Moreover, immunocytochemistry can be evaluated only quantitatively after fulfilling several criteria, to exclude variations in labelling efficiency in different intracellular compartments (Posthuma *et al.*, 1988). In light of these observations it may be possible that changes in the density of gold particles over peroxisomes, under the influence of LH, as observed by Mendis-Handagama *et al.* (1990a), may not reflect changes in the local amount of SCP_2. All together, the immunocytochemical and blotting data show that SCP_2 is not only present in the Leydig cells but also in Sertoli cells whereas the germ cells do not contain SCP_2. The presence of SCP_2 was not been observed previously in Sertoli cells by us (Van Noort *et al.*, 1986), neither by others.

The subcellular localization of SCP_2 as determined by cell fractionation of tumour Leydig cells and Western blotting revealed that SCP_2 and the 58 kDa cross-reacting protein were present in the membranous fractions whereas both proteins were absent from the cytosolic fraction. In previous studies employing the enzyme immunoassay, significant amounts of SCP_2 have been measured in the cytosolic fractions of Leydig cells and this level was increased after incubation of the cells with LH (Van Noort *et al.*, 1988a,b). The present data obtained with a (presumably more reliable) blotting technique showed that SCP_2 could not be detected in the cytosolic fraction, neither before nor after stimulation with LH. A concentration of SCP_2 in membranous fractions has been described for other rat tissues (Chanderbhan *et al.*, 1986; Van Amerongen *et al.*, 1989; Van Heusden *et al.*, 1990a). From studies on liver tissue and Leydig cells it was inferred that substantial amounts of the protein were located in the peroxisomes (Tsuneoka *et al.*, 1988; Keller *et al.*, 1989; Mendis-Handagama *et al.*, 1990a,b), whereas in the adrenal gland SCP_2 was mainly found in the mitochondrial or peroxisomal/microsomal fractions (Chanderbhan *et al.*, 1986; Van Amerongen *et al.*, 1989). Our immunocytochemical data indicate that most of the immunoreactivity in Leydig cells is concentrated in the peroxisomes. Similarly, after density

centrifugation of a Leydig cell homogenate it was established that both SCP_2 and the 58 kDa protein sedimented together with the peroxisomes. A small amount of the 58 kDa protein was shifted to fractions of higher density in which the amount of SCP_2 was very low, implicating that both proteins may be slightly different distributed. A primary localization of SCP_2 to peroxisomes seems concordant with the finding that peroxisome-deficient cells lack SCP_2, probably due to a disturbed processing of the precursor protein (Van Amerongen *et al.*, 1987; Van Heusden *et al.*, 1990b; Suzuki *et al.*, 1990). Moreover, a tripeptide sequence related to the peroxisomal targeting sequence has been detected within the SCP_2 molecule (Gould *et al.*, 1989; Ossendorp *et al.*, 1990). Nevertheless, other studies employing liver and testis tissue have indicated that the bulk of SCP_2 is located extraperoxisomal (Van der Krift *et al.*, 1985; Van Heusden *et al.*, 1990a). Similarly, in the present study high amounts of SCP_2 were found in Sertoli cells, a cell type which most likely does not contain peroxisomes and is not active in cholesterol metabolism (Byskov *et al.*, 1986). It appears from these data that the amount and subcellular distribution of SCP_2 could depend more on the cell type than on the presence of peroxisomes.

The predominant localization of SCP_2 in membranous fractions does not favour SCP_2 to act as a soluble intracellular carrier protein for the supply of cholesterol to the P450scc enzyme in the mitochondria. It appears more likely that the protein, attached to membranes, facilitates the transfer of cholesterol between different, closely appositioned, organelles inside the cell. This model was already proposed earlier by Van Noort (1989). In this model, the transfer activity of SCP_2 at critical junctions (near mitochondria in steroidogenic cells) could be more important for the transport of cholesterol than the total amount of SCP_2 present in the cell or in a particular organelle. SCP_2 may therefore be considered as a permissive protein allowing adequate cholesterol metabolism in steroidogenic cells. If this model is correct, the minimal amount of SCP_2 required for normal cholesterol transport must be established. At this moment the existence of several immunoreactive SCP_2 species in different subcellular pools complicates the studies on the functional role of SCP_2 in steroidogenic cells. In view of the complicated nature of this protein in biochemical assays and biological function we propose to read the abbreviation SCP as seriously complicated protein instead of sterol carrier protein.

Acknowledgements

We thank J.R.C.M. van Beckhoven for preparing the affinity purified antibody against SCP_2 and for help during the course of this work. The assistance of L.J. Blok, B.P. Neve, G. Posthuma, L. Van Haaster and E. Spek is gratefully acknowledged. This research was supported by a grant to G.P.H. Van Heusden from the Dutch Heart Society (Nederlandse Hart Stichting; grant number 88.040). F.F.G. Rommerts and D.M. Stocco were in part supported by NATO grant nr. CRG 880703.

Chapter 3

Measurement of Steroidogenesis in Rodent Leydig Cells: a Comparison between Pregnenolone and Testosterone Production

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Molecular and Cellular Endocrinology
(1989) 65:157-164

This publication was included in the present thesis
with permission of the "College voor Promoties".

Summary

The efficiency and specificity of inhibition of pregnenolone metabolism in mature, immature rat Leydig cells, mouse and tumour Leydig cells by SU-10603, a P450c17 inhibitor and epostane (WIN 32729), a 3βHSD inhibitor were studied.
Metabolism of ^{14}C pregnenolone by mature rat Leydig cells was inhibited for more than 95% in the presence of 20 μM SU-10603 and 5 μM epostane. The sum of the different steroids produced by Leydig cells from immature rats incubated in the presence of a 5α-reductase inhibitor was only 80% of the pregnenolone production in the presence of SU-10603 and epostane. Pregnenolone metabolism could also be inhibited in tumour Leydig cells but not in mouse Leydig cells. Pregnenolone and testosterone production by Leydig cells from mature rats were similar when steroidogenesis is maximally stimulated by LH. However, in the presence of LH and BSA or 22R-hydroxycholesterol and BSA pregnenolone production was resp. 1.7 and 6 fold higher than the testosterone production.
The data show that for measuring the steroidogenic activity of Leydig cells estimation of pregnenolone production is more reliable than measuring testosterone production. At high activities of CSCC the conversion of pregnenolone into testosterone may become the rate-limiting step for testosterone production. Under all conditions the conversion of cholesterol into pregnenolone is the (hormonal regulated) rate-determining step for steroidogenesis.

Introduction

The oxidative cleavage of the side-chain of cholesterol in the mitochondria of Leydig cells and the ensuring production of pregnenolone is the first step of the steroidogenic process. Pregnenolone is further converted into androgens by different enzymes in the endoplasmic reticulum.

For accurate measurement of the steroidogenic activity of Leydig cells, all steroids produced must be estimated. This includes measurement of testosterone but also precursors (such as progesterone, 17α-hydroxypregnenolone) and metabolites of testosterone (*e.g.* dihydrotestosterone, androstanediol) (Chubb and Ewing, 1981; Sheffield and O'Shaughnessy, 1988). In many studies on regulation of Leydig cell function, the testosterone production is used as the parameter of the steroidogenic activity since testosterone is one of the main biologically active androgens and also frequently the most abundant steroid secreted by the cells. However, Leydig cells may secrete many precursors and/or metabolites of testosterone and in such cases testosterone may be a minor secretion product. In Leydig cells of immature rats (30-40 days old) 5α-reduced androgens are formed via a 5α-pregnane pathway (Moger and Armstrong, 1974; Van der Molen and Rommerts, 1981). At this age the 3α-androstanediol production accounts for 80% of the total steroid secretion (Purvis *et al.*, 1978).

Furthermore, estimations of endogenous steroids in testis tissue from normal mature rats have shown that in addition to metabolites of testosterone precursor steroids may be as abundant as testosterone (Punjabi *et al.*, 1983; Tapanainen *et al.*, 1984). Similarly in men the intratesticular levels of precursor steroids, such as pregnenolone, dehydroepiandrosterone and 17-hydroxyprogesterone, are higher than testosterone (De la Torre *et al.*, 1982).

Thus the steroidogenic activity of Leydig cells may be underestimated when only testosterone production is measured. This deficiency can be overcome by measuring all the main steroids produced (Verhoeven and Cailleau, 1983).

An alternative approach is to prevent formation of the different steroids and measuring pregnenolone production under conditions of inhibition of pregnenolone metabolism. For practical purposes this method seems preferable over measuring all the possible steroids which can be produced and we have used this method in many investigations on regulation of steroidogenesis in isolated Leydig cells. In these studies we have used a combination of SU-10603, an inhibitor of P450c17 and WIN 19578 (cyanoketone), an inhibitor of 3βHSD activity (Van der Vusse *et al.*, 1974; Brinkmann *et al.*, 1984). Since the supply of cyanoketone via donations is extremely limited, we have searched for an alternative inhibitor which can block the pregnenolone metabolism in combination with SU-10603 without interfering with the cholesterol side-chain cleavage enzyme or other cellular activities which are important for regulation of steroid production. Epostane could be a good candidate. It is

a potent inhibitor of 3βHSD in isolated Leydig cells (Lambert *et al.*, 1987) and it has also been applied in women to reduce progesterone synthesis and terminate pregnancy in the first eight weeks (Crooij *et al.*, 1988).

We have studied at which concentrations of inhibitors pregnenolone conversion can be inhibited without adverse effects on the cholesterol side-chain cleavage (CSCC). In addition, the production rates of pregnenolone and testosterone have been compared at various activities of CSCC.

Materials and Methods

Materials

Bovine serum albumin (BSA, fraction V) was obtained from Sigma. Ovine LH (oLH, NIH-oLH S23) was a gift from the Endocrinological Study Section of the NIH, U.S.A. SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridy)-1-(2H)-naphtalenone), an inhibitor of P450c17 activity, was a gift from Ciba-Geigy, Basle, Switzerland. Epostane or WIN-32729 (4α,5α-epoxy-17β-hydroxy-4β,17α-dimethyl-3-oxo-androstane-2α-carbonitrile), an inhibitor of the 3βHSD was a gift from Sterling-Wintrop, New York, U.S.A. The 5α-reductase inhibitor Na-4-methyl-4-aza-3-oxo-5α-pregnane-20(S)-carboxylate was kindly provided by Dr. E.H. Cordes (Merck Sharp & Dohme Research Laboratories, Rahway, NJ). 22R-hydroxycholesterol was purchased from Ikapharm, Ramat-Gan, Israel. Fetal calf serum (FCS) was obtained from Gibco Limited, Paisly, U.K. [4-^{14}C] pregnenolone, S.A. 56 mCi/mmol was purchased from Amersham International.

Methods

Leydig cells were isolated from Wistar mature rats 11-15 weeks, immature rats 3-6 weeks, the Leydig cell tumour H-540 or mice aged 10-20 weeks by procedures described in detail previously (Rommerts *et al.*, 1985). In brief, isolated cells were allowed to attach to Costar 48-well plates ($2.5 \cdot 10^5$ Leydig cells/well) in modified Eagle's medium with Earle's salts and non-essential amino acids containing streptomycin (100 μg/ml), penicillin (100 IU/ml), fungizone (0.6 μg/ml) and 1% (v/v) fetal calf serum (MEM/FCS). After 1 h of incubation non-attached cells were removed by washing and all subsequent incubations were carried out in 400 μl MEM/FCS at 32 °C or 37 °C in air containing 5% CO_2. The rate of attachment of mouse Leydig cells was better than reported previously (Rommerts *et al.*, 1985) and approximately 50% of cells could be recovered after 1 h.

At the end of the incubations the media were collected for determination of pregnenolone or testosterone by radioimmunoassay (Verjans *et al.*, 1973; Van der Vusse *et al.*, 1975). The antiserum against pregnenolone cross reacted for less than 5% with dehydroepiandrosterone, testosterone, 5α-dihydrotestosterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone, 20-dihydro-pregnenolone; 12% with progesterone and 59% with 5α-pregnanolone.

The efficiency of the inhibitors was tested after incubating rat Leydig cells (approx. $1.5 \cdot 10^6$ cells) in 2 ml MEM/FCS at 32 °C for 90 min with ^{14}C pregnenolone (150 pmol) in the absence of the inhibitors or in the presence of SU-10603 (20 μM) and epostane (5 μM). Then the medium was removed and the dishes were washed twice with n-hexane to remove the ^{14}C-labelled metabolites present in the cells. Subsequently 9 tritiated marker steroids were added to the collected media and hexane-rinses to monitor procedural losses. The mixtures were

extracted twice with diethylether. Radioactively labelled steroids were analyzed by HPLC using a diol column (Hibar LiChrosorb diol, Merck, Rahway, NJ, 5 μm) and samples were eluted with a n-hexane-isopropanol gradient as described by Weusten *et al.* (1987).
For a comparison between the total amount of steroids produced in the presence of the 5α-reductase inhibitor or in the presence of SU-10603 and epostane, Leydig cells of 19 day old Wistar rats were isolated and purified by Percoll gradient centrifugation (Verhoeven and Cailleau, 1985) and subsequently cultured for 6 days (Verhoeven and Cailleau, 1988). On day 7 the cells were washed 3 times in the presence of 0.5 μM 5α-reductase inhibitor or with a mixture of 20 μM SU-10603 and 5 μM epostane. Subsequently the cells were incubated for 24 h at 32 °C in the presence of a phosphodiesterase inhibitor (IBMX 0.1 mM) and either 0.5 μM 5α-reductase inhibitor or 5 μM epostane and 20 μM SU-10603. The different combinations of steroids were measured in the incubation medium by specific radioimmunoassays as described by Verhoeven and Cailleau (1985). Pregnenolone was directly measured by radioimmunoassay as described above.

Results

The efficiency of epostane, as an inhibitor of the 3βHSD and SU-10603, as an inhibitor of P450c17 were evaluated by studying the conversion of ^{14}C pregnenolone by normal mature rat Leydig cells.

After incubation of cells in the absence of the inhibitors, approximately 70% of the added

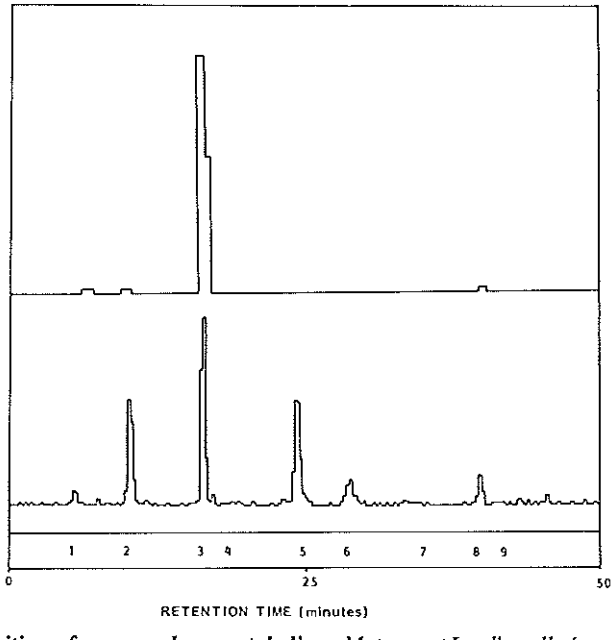

Fig. 1. Inhibition of pregnenolone metabolism. Mature rat Leydig cells (approx. 1.5·10^6 cells) were incubated for 90 min with ^{14}C labeled pregnenolone (150 pmol) in the presence of SU-10603 (20 μM) and epostane (5 μM) (top) or without the inhibitors (middle). The chromatograms show the position of ^{14}C-labeled steroids. The positions of tritiated marker steroids indicated by numbers at the bottom: 1, progesterone; 2, androstenedione; 3, pregnenolone; 4, dehydroepiandrosterone; 5, testosterone; 6, 17α-hydroxyprogesterone; 7, androstenediol; 8, 17α-hydroxy-pregnenolone; 9, estradiol.

^{14}C pregnenolone was metabolized. The main products were testosterone (30%), androstenedione (20%) and 17α-hydroxyprogesterone (10%) (Fig. 1). After addition of the inhibitors less than 5% of the ^{14}C pregnenolone was metabolized.

The specificity of the inhibitors was evaluated by studying the time course of the pregnenolone production of mature rat Leydig cells stimulated by LH. The steroid production was linear for at least 4 hours of incubation indicating that non specific inhibitory effects did

not develop in this period (Fig. 2). Possible inhibitory side effects of SU-10603 and epostane on the endogenous LH stimulated pregnenolone production were also measured by studying the effects of increased concentrations of inhibitors. Pregnenolone production by Leydig cells isolated from mature rats in the presence of 20 μM SU-10603 was constant between 5-20 μM epostane. In the presence of 5 μM epostane pregnenolone production was constant between 1 and 40 μM SU-10603 (Fig. 3A). The pregnenolone production by Leydig cells from immature rats was maximal in the presence of 5 μM epostane and 5 μM SU-10603. At higher concentrations of SU-10603 than 5 μM the pregnenolone production was inhibited

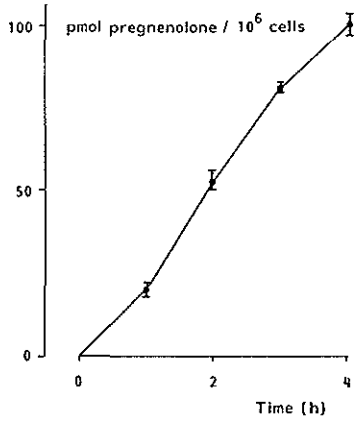

Fig. 2. Time course study of the pregnenolone production. Mature rat Leydig cells were incubated with LH (100 ng/ml) in the presence of SU-10603 (20 μM) and epostane (5 μM). Mean values ± SD (n = 3). In three other cell preparations linear relation between time and steroids levels was also observed but the rates of steroid production were different.

epostane (μM)	SU-10603 (μM)	tumour Leydig cells (pmol/10^6cells/h)		mouse Leydig cells (pmol/10^6cells/h)	
		pregnenolone	testosterone	pregnenolone	testosterone
0	0	<4	<4	<4	48.9±13.6
0	20	2.7±0.4		5.8±1.0	6.6± 2.1
1	20	16.8±2.4		22.2±1.0	<4
5	20	16.7±2.3		26.9±0.8	<4
20	20	13.8±1.6		22.7±4.5	<4
5	0	<4		3.2±0.2	<4
5	5	17.7±0.5		23.5±2.3	<4
5	20	16.7±2.3		26.9±0.8	<4
5	40	14.0±1.9		22.3±4.8	<4

Table 1. Steroid production by Leydig cells isolated from Leydig cell tumour or mice. Cells were incubated for 60 min with LH, 1000 ng/ml (tumour) or 100 ng/ml (mice) in the presence of increasing concentrations of epostane with 20 μM SU-10603 or increasing concentrations of SU-10603 with 5 μM epostane. Mean values ± SD (n=3).

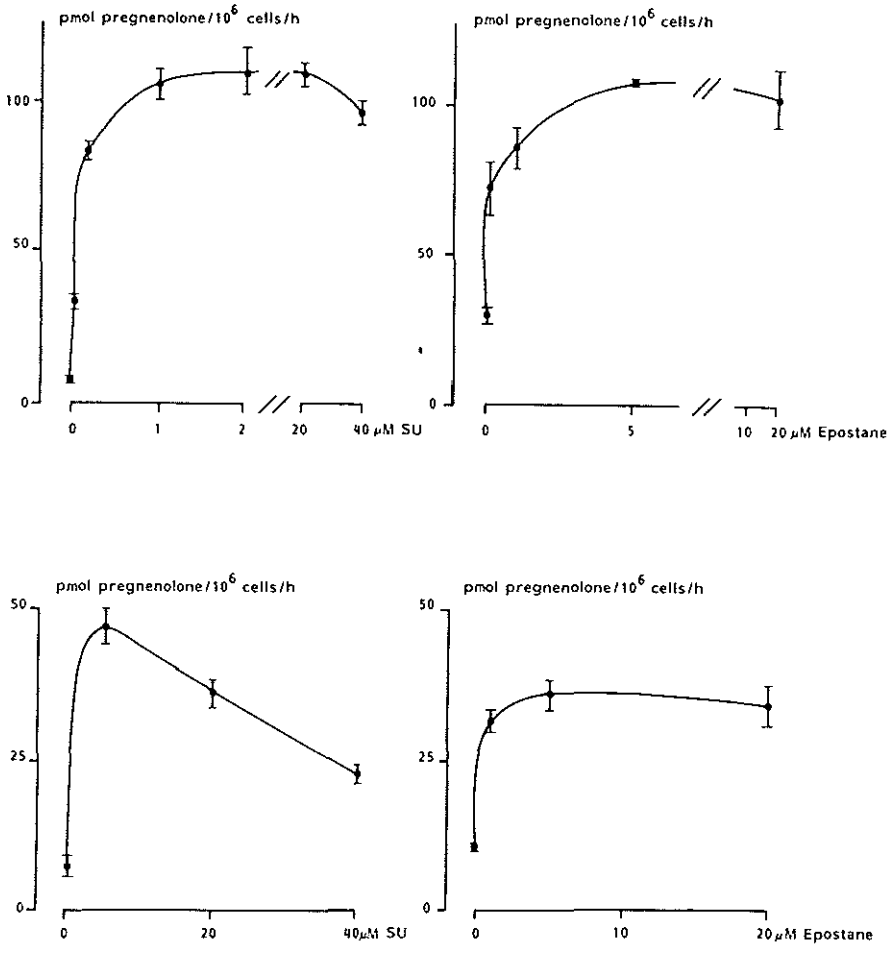

Fig. 3. Pregnenolone production by Leydig cells isolated from mature rats (top) or immature rats (bottom). The cells were incubated for 60-90 min with LH (100 ng/ml) in the presence of increasing concentrations of SU-10603 with epostane 5 µM (left panel) or with increasing concentrations epostane with SU-10603 20 µM (right panel). A: Mean values ± SD (n = 3). B: Mean values ± SD (n = 6) of two different cell preparations.

(Fig. 3B). In tumour Leydig cells the LH stimulated pregnenolone production increased more than 6 fold after addition of 1-20 μM epostane and 5-20 μM SU-10603 (Table 1). Pregnenolone production in mouse Leydig cells is always less than testosterone production (Table 1) indicating that the inhibitors either do not completely inhibit pregnenolone conversion or that they inhibit the cholesterol side-chain cleavage activity.

Pregnenolone production by isolated mitochondria from Leydig cells of mature rats or from tumour Leydig cells was not increased after addition of inhibitors at concentrations up to 20 μM SU-10603 and 5 μM epostane (data not shown).

The pregnenolone production by Leydig cells of immature rats in the presence of SU-10603 and epostane was also compared with the production of steroids in the presence of a 5α-reductase inhibitor. Under all conditions the sum of the measured steroids was approximately 80% of the pregnenolone production (Table 2).

	Steroid production in the presence of a 5α-reductase inhibitor (pmol steroid/mg protein)		Steroid production in the presence of SU and epostane (pmol steroid/mg protein)	
	Control	LH	Control	LH
Pe	85±13	970±13	2341±6	18000±389
P+17-OHP+20-OHP	325±50	3754±159	3±1	29±1
T+A^c	1451±73	10804±433	25±4	178±8
C19+C21	1776±147	14558±395	28±4	207±8
Pe+C19+C21	1861±160	15528±350	2369±89	18207±383

Pe=pregnenolone; P=progesterone; 17-OHP=17α-hydroxyprogesterone; 20-OHP=20α-hydroxyprogesterone; T=testosterone; A^c=androstenedione; C21=P+17-OHP+20-OHP; C19=T+A^c.

Table 2. Comparison of total steroid production by immature rat Leydig cells after 24 h incubation without or with LH (100 ng/ml) in the presence of a 5α-reductase inhibitor (0.5 μM) or a combination of SU-10603 (20 μM) and epostane (5 μM). Mean values ± SD (n=6).

Finally the pregnenolone and testosterone production by Leydig cells from mature rats have been compared at various rates of steroid production. For this purpose endogenous steroidogenic activities were stimulated with LH and with or without BSA (Melsert *et al.*, 1988) or the CSCC enzyme activity was supported with 22R-hydroxycholesterol, an intermediate in the conversion of cholesterol into pregnenolone. Similar amounts of pregnenolone and testosterone were formed when steroid production was stimulated by LH. However, when steroidogenesis was further stimulated when BSA was added to LH only pregnenolone production was increased while the testosterone production remained unchanged (Fig. 4). When cells were incubated in the presence of 22R-hydroxycholesterol pregnenolone

production was 10 fold more active than in the presence of LH. This steroidogenic activity was underestimated when testosterone was estimated. When the steroidogenic activity was further stimulated by incubating the cells with BSA and 22R-hydroxycholesterol this disparity between testosterone and pregnenolone production was further magnified and the testosterone production was only 20 % of the pregnenolone production (Fig. 4).

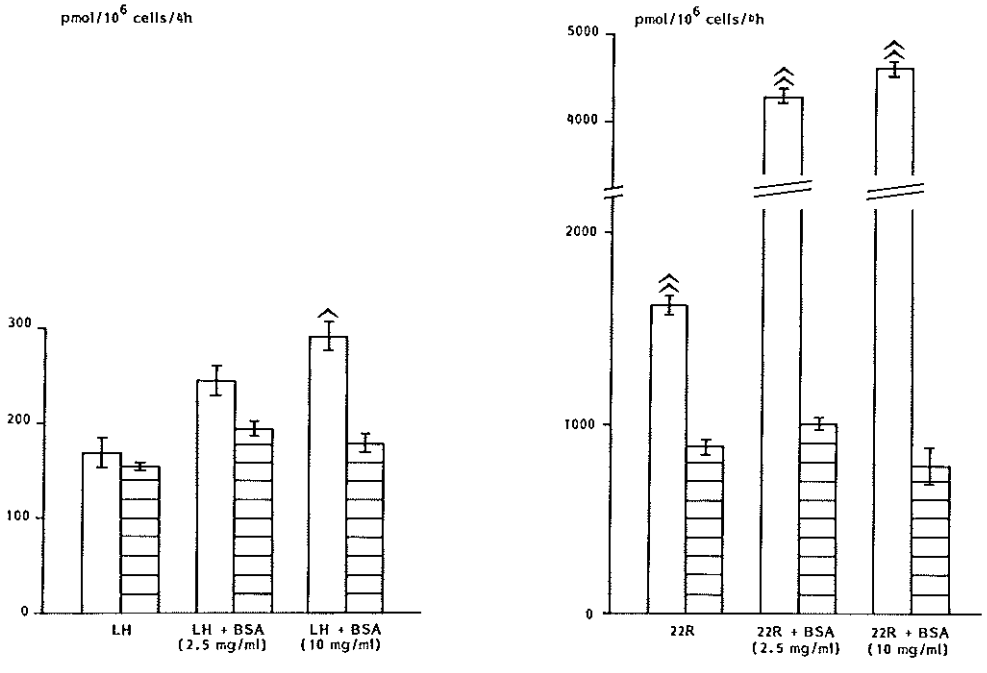

Fig. 4. Comparison of the pregnenolone production in the presence of SU-10603 (20 μM) and epostane (5 μM) (open bars) and testosterone production (hatched bars) by mature rat Leydig cells at different cholesterol side chain activities. Cells were incubated for 4 h at 32 °C in the presence of BSA (2.5 mg/ml or 10.0 mg/ml) and LH (100 ng/ml) (left panel) or 22R-hydroxycholesterol (right panel). The control values were 8.1 ± 0.8 pmol pregnenolone and 18.0 ± 2.4 pmol testosterone/10^6 cells/4 h. Mean values ± SD (n = 3), this figure shows a representative experiment of 2 different celpreparations. ∧ P < 0.05 when compared with the testosterone production in the presence of LH and BSA (10 mg/ml). ⩓ P < 0.001 when compared with the testosterone production in the presence of 22R-hydroxycholesterol. Significance of differences was determined by Bonferroni t-test.

Discussion

Studies on measuring the CSCC enzyme activity in rat Leydig cells can be facilitated by measuring the pregnenolone production instead of measuring the sum of all steroids formed. Specific inhibition of pregnenolone metabolism can be accomplished with SU-10603 and epostane. For mature rat and tumour Leydig cells the optimal concentrations are 20 μM SU-10603 and 5 μM epostane whereas in immature rat Leydig cells optimal concentrations are 5 μM SU-10603 and 5 μM epostane. Although the levels of pregnenolone were higher than the sum of other steroids including testosterone, it can not be concluded that pregnenolone conversion is inhibited completely. The formation of some 5α-reduced compounds can not be excluded. Higher doses SU-10603 apparently inhibited the CSCC enzyme activity in immature rat Leydig cells. For mice Leydig cells it was not possible to establish conditions for specific inhibition of pregnenolone metabolism.

In a recent study on the characterization of inhibitors of pregnenolone metabolism in pig Leydig cells WIN-24540 (trilostane), an inhibitor of the 3βHSD and spironolactone, an inhibitor of P450c17 were used. It was found that the pregnenolone production in the presence of these inhibitors was less than the testosterone production and the authors concluded that the inhibitors were non-specific in their actions (Penhoat *et al.*, 1988). We have shown that inhibitors of pregnenolone conversion which do not inhibit CSCC enzyme activity in rat Leydig cells may inhibit the CSCC enzyme activity in cells from mice. It may therefore be possible that specificity and effectiveness of the inhibitors are greatly depending on the species used.

In vitro studies with rat Leydig cells showed no differences between pregnenolone and testosterone production rates under basal conditions and in the presence of LH indicating that under these conditions the capacity of the enzymes in the endoplasmic reticulum is sufficient to convert all pregnenolone into testosterone. However, when steroidogenesis was further stimulated with BSA and/or 22R-hydroxycholesterol, the capacity for conversion of pregnenolone was much lower than the rate of pregnenolone production, although it can not be excluded that 22R-hydroxycholesterol might act as a competitive inhibitor for the 3βHSD. High levels of C_{21} precursor steroids have also been detected in testis tissue of rats after administration of high doses of hCG. These effects by "lesions" in the steroid converting enzymes have been explained by desensitization (Quinn and Payne, 1985). However, appreciable amounts of C_{21} precursors have also been measured in testis tissue from normal rats and from humans without extra stimulation by gonadotropins.

Thus 17α-hydroxylation or desmolase activity may be the rate-limiting step in the testosterone biosynthesis *in vivo* and *in vitro* (Belanger *et al.*, 1980; Punjabi *et al.*, 1983), especially when the rate of formation of pregnenolone is high. The conversion of cholesterol

into pregnenolone has often been termed as the rate-limiting step of steroidogenesis since the conversion of cholesterol into pregnenolone is the most important step for hormonal control of the steroidogenesis. However, in light of the data discussed above it seems better to identify this step as the rate-determining step of steroidogenesis.

For studies on the mechanism of regulation of side-chain cleavage process, measurement of pregnenolone production is most preferable. For physiological studies on steroidogenesis by Leydig cells, measurement of the biological active steroids such as testosterone and 5α-dihydrotestosterone is better. In these studies it must not be forgotten that the rate of androgen production depends on the activity of the rate determining step at the mitochondrial level as well as on the possible rate-limiting steps at the level of the enzymes in the endoplasmic reticulum.

Chapter 4

Luteinizing Hormone Induction of the Cholesterol Side-Chain Cleavage Enzyme in Cultured Immature Rat Leydig Cells: no Role of Insulin-like Growth Factor-I?

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Molecular and Cellular Endocrinology
(1992) 87:57-67

Summary

Long-term inductive effects of LH on cholesterol side-chain cleavage (CSCC) enzyme activity were studied, using cultured Leydig cells isolated from 21 days old rats. Particular reference was given to the role of IGF-I as an autocrine or paracrine modulator or as an essential extracellular mediator of LH action.

The CSCC enzyme activity was measured using an excess of 22R-hydroxycholesterol as substrate to saturate the enzyme, and inhibitors of pregnenolone metabolism to concentrate all the products of the enzyme reaction in pregnenolone. The rate of sterol conversion into pregnenolone (CSCC enzyme activity) reflected the amount of cytochrome P450scc (P450scc), as was shown by Western blotting. In cells cultured without LH, the CSCC enzyme activity decreased to 10% on day 7 of the culture period. In the presence of various doses of LH ranging from 0.01-100 ng/ml, the CSCC enzyme activity also diminished during the first 3 days of culture, but during the following days, the amount of CSCC enzyme was stimulated by LH. In contrast to the absence of any LH effect on the activity of the CSCC enzyme during the first days of the culture, the endogenous steroid production (no added 22R-hydroxycholesterol) could be stimulated at least 10 fold by high doses of LH. When LH (1 ng/ml) was added to cells which had been cultured for 7 days without hormones, CSCC enzyme activity was elevated 8 fold after 4 days of exposure of LH. These effects of LH could be mimicked by dbcAMP (0.5 mM). No evidence could be provided that IGF-I plays any role in the LH induction of the CSCC enzyme; neither the addition of exogenous IGF-I or analogs that do not bind to IGF-I binding proteins (IGFBPs) nor the inactivation of endogenous IGF-I action (through binding to IGFBP and antibodies to IGF-I or via masking of IGF-I receptor by antibodies) could influence the LH induced CSCC enzyme activity.

The present data raise the question under which conditions IGF-I is capable of modulating Leydig cell steroidogenesis.

Introduction

Luteinizing hormone (LH) is probably the most important regulator of steroidogenesis in Leydig cells. The regulatory effects are mediated by two different modes of hormone action, a rapid non-genomic effect and a slower genomic action. The acute response to LH, mediated by cAMP, protein phosphorylation and protein synthesis but without the involvement of RNA synthesis occurs within minutes (Cooke *et al.*, 1975; Rommerts *et al.*, 1983; Themmen *et al.*, 1986a). This sequence of events leads to enhanced trafficking of cholesterol to the rate-determining enzyme of the steroidogenic cascade, the cholesterol side-chain cleavage (CSCC) enzyme, localized inside the mitochondria (Van der Vusse *et al.*, 1973; Moyle *et al.*, 1973). The pregnenolone formed is subsequently metabolized into androgens, by various enzymes in the endoplasmic reticulum. These conversions may become rate-limiting for the production of androgens, when the rate of formation of pregnenolone is higher than the conversions in the endoplasmic reticulum (Van Haren *et al.*, 1989). Long-term effects of LH are mediated by cAMP, protein phosphorylation, gene expression and protein synthesis and require several hours (Mellon and Vaisse, 1989; Orava *et al.*, 1989). One of the biological responses of this hormonal effect is an increase in the synthesis of various steroidogenic enzymes (Simpson *et al.*, 1990).

The continuous requirement of LH for maintenance of steroidogenic enzyme activities has been demonstrated in hypophysectomized rats. Following hypophysectomy, the activity of the CSCC enzyme diminishes to less than 1% in a period of 33 days (Stocco *et al.*, 1990). The steroidogenic enzyme activities of testicular Leydig cells can be restored by administration of LH/hCG to rats (Purvis *et al.*, 1973; Wing *et al.*, 1984; Stocco *et al.*, 1990). Similar findings on the regulation of CSCC enzyme activity have been made *in vitro* with isolated Leydig cells (Mason *et al.*, 1984; Anderson and Mendelson, 1985; Orava *et al.*, 1989). Surprisingly, the activity of the CSCC enzyme in isolated mouse Leydig cells seems not to be dependent on LH (Anakwe and Payne, 1987).

Locally produced factors can amplify or inhibit the stimulating effects of LH on steroidogenic enzymes. In this respect, the role of insulin-like growth factor-I (IGF-I) seems of particular interest, since:

1) a significant amount of this growth factor is present in the testis (D'Ercole *et al.*, 1984) and is produced by Leydig, Sertoli and peritubular cells (Cailleau *et al.*, 1990; Naville *et al.*, 1990; Lin *et al.*, 1990);
2) Leydig cells possess IGF-I receptors (Lin *et al.*, 1986a,b) and

3) an enhanced LH stimulated androgen production under the influence of IGF-I has been reported (Adashi *et al.*, 1982; Kasson and Hsueh, 1987; Benahmed *et al.*, 1987; Verhoeven and Cailleau, 1990).

Since IGF-I is produced by Leydig cells and also acts on Leydig cells it can be considered as an autocrine modulator of LH action. It may theoretically even be possible that the action of LH on steroidogenesis not only depends on a cascade of intracellular events but that LH action also requires an extracellular loop, via secretion of IGF-I which then acts through IGF-I receptors at the cell surface. We have therefore investigated the long-term inductive effects of LH on the CSCC enzyme activity as the rate-determining enzyme of the steroidogenic cascade, with particular reference to the role of IGF-I.

It is difficult to obtain specific information about the CSCC enzyme activity by measuring androgens since these steroids are not the direct products of the enzyme reaction. The amount of androgens produced may be more a reflection of the rate-limiting steps of enzymes in the endoplasmic reticulum rather than the enzymatic rate-determining step in the mitochondria (Van Haren *et al.*, 1989). This holds especially for Leydig cells from immature rats in which testosterone production constitutes approximately 20% of the total steroid production (Purvis *et al.*, 1978). This problem can be solved by measuring the main steroids produced, as carried out by Verhoeven *et al.* (1982, 1990), but this is a rather complicated procedure. Measurement of pregnenolone in the presence of inhibitors of pregnenolone metabolism seems a better alternative. Furthermore, in the presence of an excess of 22R-hydroxycholesterol pregnenolone production is limited by the amount of CSCC enzyme and is independent of the available amounts of the endogenous substrate cholesterol (Van Haren *et al.*, 1989). Immunotechniques such as Western blotting have often been used to detect amounts of specific proteins including P450scc (Anderson and Mendelson, 1985; Anakwe and Payne, 1987), but this technique may have limitations for sensitive and quantitative estimations of the amount of the CSCC enzyme.

In order to study the regulation of the mitochondrial CSCC enzyme in Leydig cells by endocrine, paracrine or autocrine factors, we have employed and evaluated an *in situ* enzyme assay for the CSCC enzyme. Leydig cells from immature rats have been used for these studies since these cells remain hormone responsive and active in steroid production during culture for at least 10 days (Verhoeven *et al.*, 1982).

Materials and Methods

Materials

Ovine LH (oLH, NIH-oLH S23) was a gift from the National Hormone and Pituitary Program (Baltimore, MD). SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone), an inhibitor of P450c17 activity, was a gift from Ciba-Geigy, Basle, Switzerland. Epostane or WIN-32729 (4α,5α-epoxy-17ß-hydroxy-4ß,17α-dimethyl-3-oxo-androstane-2α-carbonitrile), an inhibitor of the 3ßHSD, was a gift from Sterling-Wintrop, New York, NY, USA. Bovine serum albumin (BSA, fraction V), 22R-hydroxycholesterol and 1-methyl-3-isobutylxanthine (MIX) were purchased from Sigma, St. Louis, MO, USA. IGF-I, desamino-(1-3)-IGF-I (des-IGF-I) and long R^3 IGF-I (long-IGF-I) were from GroPep, Adelaide, Australia. The IGF-I receptor antibody was obtained from Oncogene Science, Manhasset, NY, USA. The antibody to IGF-I was a generous gift of Dr. J. Closset (Clinical and Experimental Endocrinology, Centre Hospitalier Universitaire B23, Liège, Belgium). IGFBP-1 was kindly donated by Dr. S.L.S. Drop (Dept. of Paediatric Endocrinology, Erasmus University Rotterdam, The Netherlands). The purification procedure of this binding protein has been described by Liu *et al.* (1991).

Cell isolation and culture

Leydig cells were isolated from Wistar rats, either immature (21-23 days) or mature (11-15 weeks), by procedures described in detail previously (Rommerts *et al.*, 1985). In brief, isolated cells were allowed to attach to Costar 48-well plates (10^5 cells/well) in RPMI medium containing streptomycin (100 μg/ml), penicillin (100 IU/ml) and 0.1% (w/v) BSA. After 1 h of incubation, non-attached cells were removed by washing and the cells attached to the dishes were either used immediately or, where indicated, after several days of culture. Cultures were performed at 32 °C in air containing 5% CO_2 and the control medium or medium supplemented with LH (0.01-100 ng/ml) were refreshed at days 3 and 5.

Characterization of the Leydig cell cultures

The percentage of Leydig cells, as established by staining for 3ßHSD activity (Molenaar *et al.*, 1983), ranged from 20-30% in the Leydig cell preparation derived from immature rats. In Leydig cell preparations obtained from mature rats, 80-90% of the cells stained for 3ßHSD and/or esterase activity (Rommerts *et al.*, 1973). Most of 3ßHSD negative cells had morphological characteristics of mesenchymal cells (Kerr and Sharpe, 1985), whereas macrophages and Sertoli cells were absent.

The percentage of damaged cells was estimated, using a histochemical NADH-dependent test for intracellular diaphorase activity (Rommerts *et al.*, 1985). Both in the freshly isolated cell

preparation and in the cells cultured for 7 days, the percentage of diaphorase positive cells was $<1\%$. In addition, morphological signs of degeneration were absent during the culture period and no changes in the cellular protein and DNA levels were found after culturing cells for 7 days with or without LH (1 ng/ml). The LH stimulated cAMP response also remained constant during the culture period (Table 1).

CSCC enzyme activity assay

The CSCC enzyme activity was estimated by measurement of the conversion rate of a saturating level of 22R-hydroxycholesterol into pregnenolone. The cells were incubated in the presence of 22R-hydroxycholesterol (19 μM) as substrate and with SU-10603 (20 μM or 5 μM for mature and immature rat Leydig cells, respectively) and epostane (5 μM) as inhibitors of pregnenolone metabolism (Van Haren *et al.*, 1989). After 3 h, the media were collected and stored at -20 °C until analysis. The amount of pregnenolone was measured by a radioimmunoassay (Van der Vusse *et al.*, 1975). In other experiments, the testosterone production was also measured by a radioimmunoassay (Verjans *et al.*, 1973).

Immunoblotting

Proteins were separated by SDS polyacrylamide gel electrophoresis (7.5% acrylamide) followed by horizontal semi-dry electroblotting onto nitrocellulose membrane filter (BA 85, 0.45 μm, Schleicher and Schuell, GmbH, Dassel, Germany) according to Szewczyk and Kozloff (1985). After blotting for 45 min at 5 mÅ/cm^2 using E & K Scientific Products (Saratoga, Ca) apparatus, the filter was quenched (1 h) by 5% skimmed milk solution in PBS and incubated with rabbit antiserum against rat P450scc (1:10,000 dilution, Farkash *et al.*, 1986) for 2 h. Thereafter, excess antibody was washed (4 x 10 min) with PBS containing 0.1% Tween-20, and the filter was further incubated for 2 h with horseradish peroxidase conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) at 1:5,000 dilution. After washing, the immunocomplex with P450scc was detected by enhanced chemiluminescence (ECL) detection kit (Amersham, UK). The filter was exposed to Kodak x-ray film (Eastman Kodak, Rochester, NY, USA) for 15 sec.

Estimation of cAMP

The cAMP level was estimated in the culture medium, after incubating the cells for 3 h in medium containing MIX (200 μM) with or without LH (100 ng/ml). For the determination of cAMP, a cAMP assay kit was used (Amersham International plc), with the lower limit of detection at 0.5 pmol and an intra-assay variation of 11%.

Estimation of cellular protein and DNA

The Leydig cell cultures were washed 3 times with RPMI medium and dissolved subsequently in 1 M NaOH. The amount of cellular protein was determined according to Bradford (1976), using BSA (fraction V) as standard. The DNA content was measured by a fluorometric assay using 4',6-diamidino-2-phenylindole (DAPI) as a fluorescent dye (Toebosch *et al.*, 1987).

Statistical methods

Data are expressed as mean ± SD. Statistical differences were determined by analysis of variance with the Tukey's Honestly Significant Difference test to discern individually significant effects.

Results

Validation of the CSCC enzyme activity assay

Measurement of the CSCC enzyme activity in intact cells was performed by estimating the pregnenolone production in the presence of an excess of substrate 22R-hydroxycholesterol, which is more water soluble than cholesterol. Under this condition, the conversion of 22R-

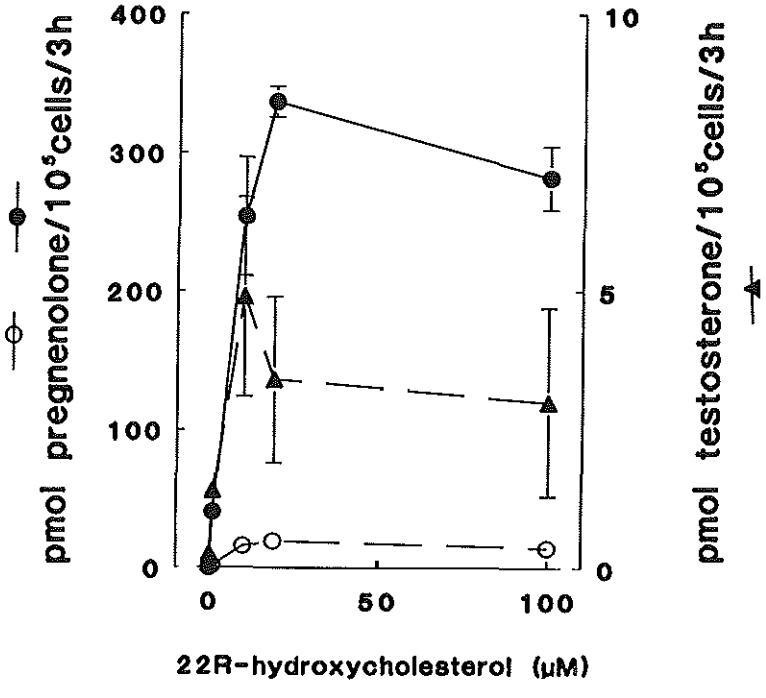

Fig. 1. 22R-hydroxycholesterol-supported steroid production. Immature rat Leydig cells were incubated for 3 h with increasing concentrations of 22R-hydroxycholesterol (0-100 μM). Steroids were estimated in the absence (— —) or presence of SU-10603 (5 μM) and epostane (5 μM) (——). Mean values ± SD of incubations performed in triplicate are shown.

hydroxycholesterol reflects the CSCC enzyme activity (amount) and is independent of the available amount of endogenous cholesterol. Metabolism of 22R-hydroxycholesterol to pregnenolone was dose dependent, and saturation of the CSCC enzyme was reached at 10 μM 22R-hydroxycholesterol. An excess of 19 μM 22R-hydroxycholesterol was used in all

the subsequent experiments. In the presence of 22R-hydroxycholesterol as substrate, and SU-10603 and epostane as inhibitors of pregnenolone metabolism, approximately 300 pmol pregnenolone was produced in 3 h by 10^5 Leydig cells. Cells incubated with 22R-hydroxycholesterol but without inhibitors of pregnenolone metabolism produced only 15 pmol pregnenolone and 3 pmol testosterone. Thus, testosterone production reflected only 1% of the rate of 22R-hydroxycholesterol-supported steroidogenic activity (Fig. 1).

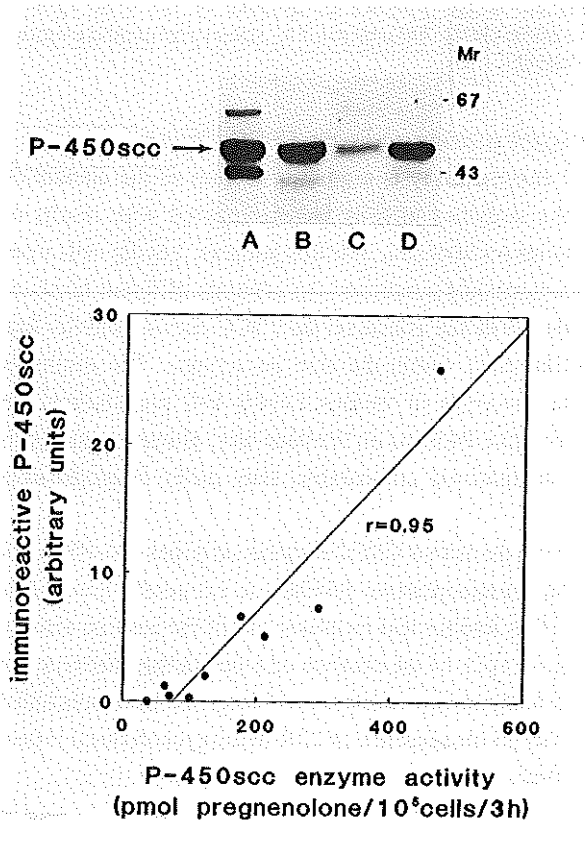

*Fig. 2. **Relationship between 22R-hydroxycholesterol supported pregnenolone production and cellular P450scc content.** Upper panel. Western blot developed with P450scc antibody after SDS-polyacrylamide gel electrophoresis of 10 μg protein of rat ovarian tissue or 100 μg protein of immature rat Leydig cells. Lane A, ovary; lane B, freshly isolated Leydig cells; lane C, cells cultured for 9 days without LH; lane D, cells cultured for 7 days without LH followed by 48 h treatment with LH (1 ng/ml). Lower panel. Freshly isolated Leydig cells, cells cultured for 7 days in the absence or presence of LH (1 ng/ml) or cells cultured for 7 days without LH followed by 48 h treatment with LH (1 ng/ml) were incubated with 22R-hydroxycholesterol (19 μM), SU-10603 (5 μM) and epostane (5 μM) for 3 h and the production of pregnenolone was estimated. In the same cell preparation immunoreactive P450scc was quantified by scanning laser densitometry after immunoblotting. Arbitrary units were assigned.*

Conversion of 22R-hydroxycholesterol into pregnenolone was linear with time for at least 4 h. After the addition of aminoglutethimide (500 μM), a specific inhibitor of P450scc, the enzyme activity was reduced to 10% of the control value (data not shown). This demonstrates

Cell preparation	Protein (μg)	DNA (μg)	cAMP (pmol/3h)	
			-LH	+LH
Freshly isolated	29.8±3.2	1.8±0.6	0.9±1.3	54.0±16.0
Cultured without LH	27.1±4.4	1.5±0.3	<0.1	102.0±24.0
Cultured with LH (1 ng/ml)	32.4±3.2	1.6±0.3	1.2±1.8	94.0±28.0

Table 1. Protein and DNA contents and cAMP production by interstitial cells isolated from immature rats, immediately after isolation and after a culture period of 7 days. For measurement of cAMP production cells were incubated in the presence or absence of LH (100 ng/ml) supplemented with 1-methyl-3-isobutylxanthine (MIX; 200 μM) for 3 h. Values represent the mean ±SD (n=6) of 2-3 different cell preparations.

that metabolism of 22R-hydroxycholesterol is catalyzed by the CSCC enzyme.

In order to verify whether this CSCC enzyme assay is a good reflection of the amount of P450scc protein, estimations of enzyme activities in cells were compared with results of immunoblotting after separation of proteins on SDS gels. For this purpose, Leydig cells were cultured for several days under various hormonal conditions, to generate different levels of CSCC enzyme activity. Details about the hormonal effects on the CSCC enzyme activity that occur during these incubations are being dealt within the next paragraphs. The results in Fig. 2 show that the different enzyme activities in various Leydig cell preparations are correlated with the amount of immunoreactive P450scc on Western blot. However, although the results of the two techniques are qualitatively comparable, they differ in quantitative terms.

Especially in Leydig cell preparations with decreased CSCC enzyme activities (50-100 pmol pregnenolone/10^5 Leydig cells/3 h) the immunoreactive P450scc is extremely low and is clearly underestimated or not detected. This implies that measurement of the enzyme activity is more sensitive than Western blotting. In spite of the absence of a parallelism between the results of immunoblot and the enzyme assay at low levels of P450scc, we have concluded that changes in the CSCC enzyme activity in the presence of 22R-hydroxycholesterol reflect changes in the cellular content of P450scc.

P450scc content during culture

Leydig cells cultured without or with LH remained viable for 7 days, in terms of a constant protein and DNA content as well as a more than 50 fold cAMP response after addition of LH (Table 1). During primary culture of Leydig cells in the absence of LH, the CSCC enzyme content declined progressively, as indicated by the decrease in the conversion of exogenous 22R-hydroxycholesterol into pregnenolone (Fig. 3A). In the presence of the standard dose of LH (1 ng/ml), the drop in CSCC enzyme activity during the first 3 days was

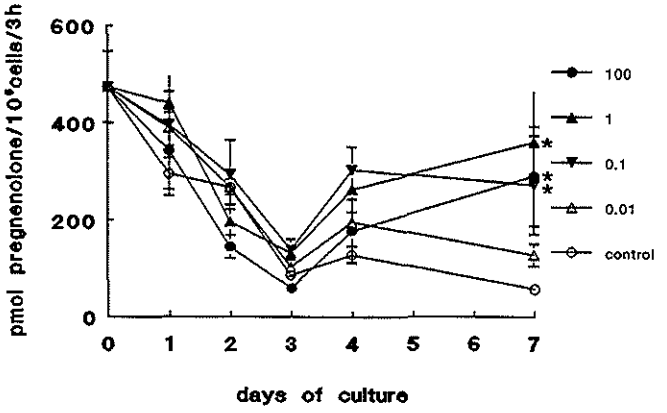

Fig. 3A.

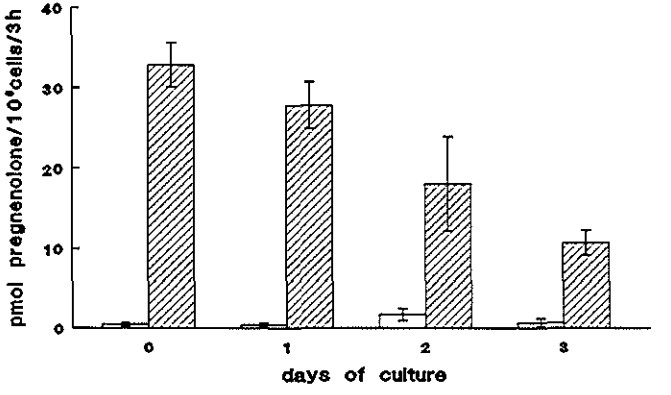

Fig. 3B.

Fig. 3. Changes in the amount of CSCC enzyme activity during culture.

*Fig. 3A. Immature rat Leydig cells were cultured for 7 days in the absence or presence of increasing concentrations of LH (0.01-100 ng/ml). The CSCC enzyme activity was estimated with excess of 22R-hydroxycholesterol (19 μM) and SU-10603 (5 μM) and epostane (5 μM). Mean values ± SD (n=3-6) of two different cell preparations are shown. * $p < 0.01$ versus zero dose.*

Fig. 3B. Endogenous steroid production by Leydig cells after several culture periods (0-3 days) without LH. The cells were incubated for 3 h in the absence (open bars) or presence of LH (100 ng/ml; hatched bars) and SU-10603 (5 μM) and epostane (5 μM) but without 22R-hydroxycholesterol. Mean values ± SD (n=4) of two different cell preparations are shown.

similar as that in cells incubated without LH. However, during the next 4 days (day 4-7), cells incubated continuously with LH showed an increase in enzyme content. When experiments were repeated with higher and lower doses of LH a similar drop in enzyme content was observed during the first days although the amount of P450scc at day 7 may depend on the dose of LH.

To examine whether the refractoriness of the cells to the enzyme inductive effects of LH

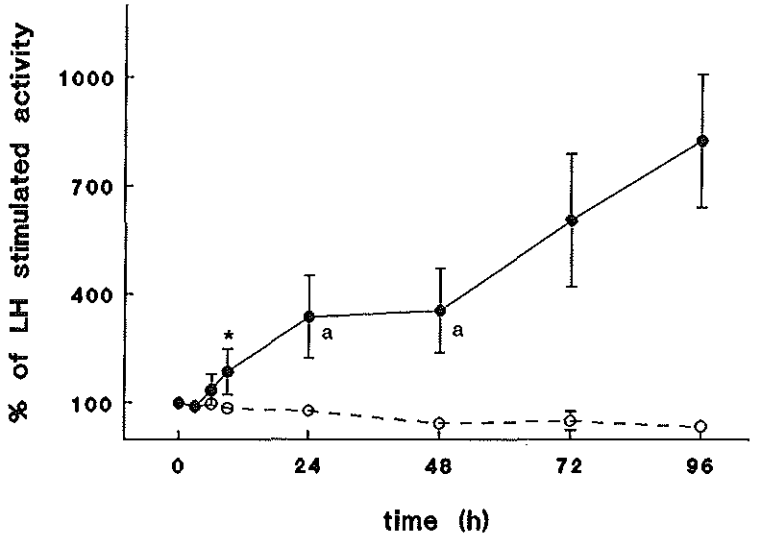

*Fig. 4. Restoration of the amount of CSCC enzyme by LH. Immature rat Leydig cells were cultured for 7 days without LH. On day 7 the cells were incubated with (●-●) or without (○-○) LH (1 ng/ml) and at the various time points the 22R-hydroxycholesterol supported CSCC enzyme activity was estimated. The 22R-hydroxycholesterol supported steroid productions at day 0 and day 7 were 512 ± 84 and 31 ± 10 pmol pregnenolone/10^5 cells/3 h, respectively. Mean values ± SD (n=3-9) of three different cell preparations are shown. * $p < 0.01$ versus time 0. [a] The two marked points are not significantly different.*

during the first culture days is caused by receptor defects, the short-term stimulatory effect of LH on the endogenous steroid production (no substrate added), was also studied. Cells cultured without LH for 0, 1, 2 or 3 days were incubated for 3 h with a maximally stimulating dose of LH (100 ng/ml), and the amount of endogenously produced pregnenolone was estimated. Irrespective of the duration of the culture period, the acute stimulation of

steroid production by LH was always more than 10 fold although the absolute amounts of endogenously produced pregnenolone decreased from 33 pmol at day 0 to 11 pmol pregnenolone at day 3 of culture (Fig. 3B). This decrease in pregnenolone production parallels the decrease in the CSCC enzyme content, as described above, indicating that the amount of the CSCC enzyme limits both the LH stimulated endogenous pregnenolone production and the 22R-hydroxycholesterol-supported steroid production.

Restoration of P450scc content

To investigate whether LH can stimulate synthesis of P450scc in quiescent cells, Leydig cells were preincubated for 7 days without LH. After addition of LH (1 ng/ml) to these cells a time-dependent increase in CSCC enzyme content (3 fold stimulation after 2 days and a 8 fold stimulation after 4 days) occurred. The kinetics of this enzyme induction process are peculiar. The enzyme content increases in two phases, with a plateau between 24 and 48 hours. Stimulatory effects on enzyme induction were also obtained with 0.5 mM dbcAMP (data not shown). In the control cells, the basal P450scc content further declined to 35% of the amount at day 7 (Fig. 4).

P450scc content in cultured mature rat Leydig cells

The long-term effects of LH on CSCC enzyme content were also studied in Leydig cells from mature rats since these cells can be prepared with higher purities. Both in control cells and in LH (1 ng/ml) treated cells, the CSCC enzyme content declined rapidly and already at day 2 of the culture the enzyme activity was reduced to only 10%. LH could not restore the CSCC enzyme, as found in the immature rat Leydig cell preparation (Fig. 5).

Role of IGF-I

To investigate whether IGF-I can modulate the LH effects or plays an essential role in LH induction of the CSCC enzyme, two strategies were followed. The level of IGF-I was increased by addition of exogenous IGF-I or IGF-I analogs, or IGF-I action was inhibited by adding IGF-I binding protein (IGFBP) or antibodies (to IGF-I or to IGF-I receptor). Addition of exogenous IGF-I (1 to 100 ng/ml) in the absence or presence of increasing concentrations of LH (0.01-1 ng/ml) to Leydig cells precultured for 7 days did not further stimulate the LH induction of the CSCC enzyme (data not shown). The effects of two IGF-I analogs which are more potent than intact IGF-I due to a reduced affinity for IGFBPs were also investigated.

The analogs used were desamino-(1-3)-IGF-I (des-IGF-I), lacking the first three amino acid residues of IGF-I, and long R^3 IGF-I (long-IGF-I), with an arginine substitution of the glutamate at position 3. At concentrations of 1-100 ng/ml, neither of these analogs stimulated CSCC enzyme activity (data not shown). To examine whether IGF-I could increase the basal and/or LH stimulated endogenous steroid production, immature rat Leydig cells were incubated either at day 0 or at day 2 with IGF-I (100 ng/ml) or IGF-I analogs (100 ng/ml)

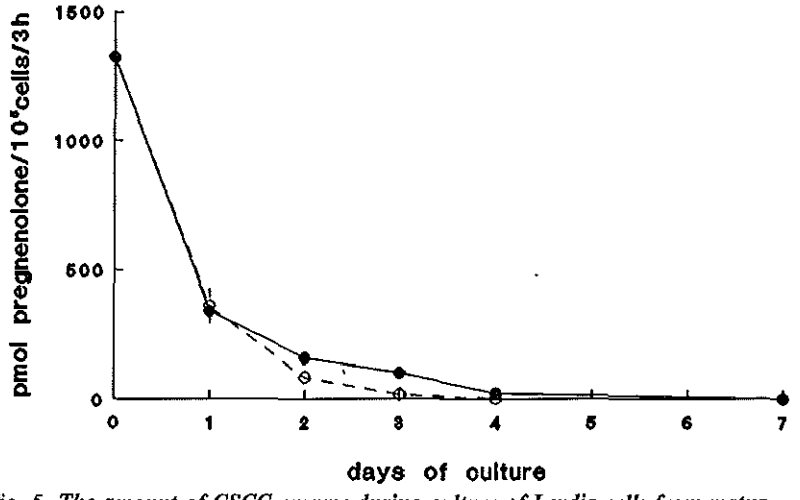

Fig. 5. The amount of CSCC enzyme during culture of Leydig cells from mature rats. Cells were cultured for 7 days in the absence (○-○) or presence (●-●) of LH (1 ng/ml). Mean values ± SD of incubations performed in triplicate are shown.

for 48 h, followed by 3 h treatment without or with LH (100 ng/ml) in the presence of pregnenolone metabolism inhibitors. No stimulatory effects of these different IGF-I preparations on the endogenous pregnenolone production could be demonstrated (data not shown). Although we could not demonstrate any effect of IGF-I on steroidogenic activities, effects of IGF-I have been shown on ^{3}H-thymidine incorporation in similar preparations of immature rat Leydig cells (Khan *et al.*, 1992).

To examine the effect of blockers of IGF-I action, experiments were carried out with IGFBP. Immature rat Leydig cells were cultured in the presence of increasing concentrations of LH (0.0001-1 ng/ml), with or without IGFBP (800 ng/ml), a dose known to inhibit IGF-I action in chicken embryo fibroblasts (Liu *et al.*, 1991). Similarly, the IGFBP preparation isolated by Ui *et al.* (1989) blocks endogenous IGF-I action in rat granulosa cells maximally at a dose

of 400 ng/ml. In this experiment, IGFBP did not diminish the induction of the CSCC enzyme activity by LH (Fig. 6). In other experiments, neither the addition of excess IGF-I antiserum nor the addition of excess antiserum to IGF-I receptors diminished the induction of the CSCC enzyme activity by LH (data not shown).
All these findings taken together, indicate that IGF-I apparently plays no role in the induction of the CSCC enzyme in Leydig cells from immature rats cultured for 7 days. A few attempts

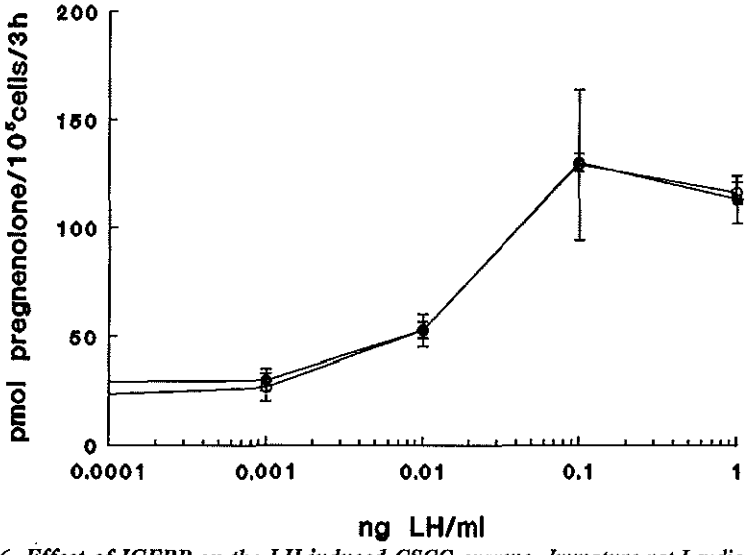

Fig. 6. Effect of IGFBP on the LH induced CSCC enzyme. Immature rat Leydig cells were cultured for 7 days in the absence of LH. On day 7 the cells were incubated with increasing concentrations of LH (0.0001-1 ng/ml) in the presence (●-●) or absence (○-○) of IGFBP (800 ng/ml) for 48 h. Mean values ± SD of incubations performed in triplicate are shown.

were made to investigate whether IGF-I could influence P450scc at earlier time points (culture days 2-4), but this could not be demonstrated.

Discussion

Long-term effects of LH on CSCC enzyme induction, with particular reference to the role of IGF-I, as an autocrine modulator or as extracellular mediator of LH action, have been studied. CSCC enzyme activity was measured using an excess of 22R-hydroxycholesterol as substrate, and inhibitors of pregnenolone metabolism, to accumulate all the products of the enzyme reaction in pregnenolone. Changes in the CSCC enzyme activity correlated with the cellular immunoreactive P450scc content, indicating that the rate of sterol conversion into pregnenolone reflects the amount of CSCC enzyme.

Correlations between 25-hydroxycholesterol-supported CSCC enzyme activity and P450scc content have also been described earlier, for rat luteal mitochondria (Toaff *et al.*, 1982), swine granulosa cells (Veldhuis *et al.*, 1986) and rat Leydig cells (Anderson and Mendelson, 1985; Georgiou *et al.*, 1987).

When immature rat Leydig cells were cultured in the absence of tropic hormone, the CSCC enzyme content was reduced to 10% at day 7 of culture. After subsequent treatment of the cells with LH for 4 days, the amount of CSCC enzyme was restored, to approximately 50% of the level present in freshly isolated cells. Similar effects could be obtained by the addition of dbcAMP, indicating that the inductive effect of LH on the CSCC enzyme was mediated by cAMP as second messenger. In bovine cells, the cAMP dependent regulation of genes encoding steroidogenic enzymes (*e.g.* P450scc) has been studied in detail (Simpson *et al.*, 1990). On the other hand, a constitutive synthesis of the P450scc enzyme has been reported for isolated mouse Leydig cells and in corpora lutea of pregnant rats (Anakwe and Payne, 1987; Hickey *et al.*, 1989). This suggests that, in addition to LH, other mechanisms, largely independent of cAMP, could be involved in regulation of the P450scc enzyme. Evidence for such mechanisms has also been provided by studies utilizing ovarian cells. For instance, IGF-I and estradiol can also induce synthesis of the P450scc enzyme (Veldhuis *et al.*, 1982, 1986; Toaff *et al.*, 1983; Magoffin *et al.*, 1990; Urban *et al.*, 1990).

It was surprising to observe that during the first part of the culture period the Leydig cells were refractory to stimulatory effects of LH on the CSCC enzyme induction. Only after a culture period of 3 days was the enzyme content increased under the influence of LH. These data are comparable with an earlier report by Hsueh (1980), showing that testosterone production by rat testis cells in response to hCG was also temporally diminished, although this period lasted 6 days. A factor which can contribute to this type of refractoriness could be the steroid dependent inactivation of the P450scc enzyme, as described by Georgiou *et al.* (1987). However, it appears difficult to explain why after 3 days of culture this steroid dependent inactivation of P450scc does not occur any more. The resistance to the stimulatory

effects of LH could neither be explained in terms of desensitization events in the plasma membrane such as loss of LH receptors, since a short-term stimulatory effect of LH on the endogenous pregnenolone production could still be observed. It may be possible that the long-term effect of LH on the CSCC enzyme induction requires other intracellular or extracellular factors, which may be absent during the first days of culture, but this hypothesis could not be substantiated.

Since CSCC enzyme induction may involve IGF-I in addition to LH, we have investigated whether IGF-I plays an essential role. IGF-I is produced by Leydig cells and can act on Leydig cells and has therefore been considered as an important autocrine modulator by many investigators (Adashi *et al.*, 1982; Lin *et al.*, 1986a,b; Kasson and Hsueh, 1987; Benahmed *et al.*, 1987; Verhoeven and Cailleau, 1990). Similar suggestions have been made for IGF-I action in granulosa cells (Veldhuis *et al.*, 1986; Adashi *et al.*, 1988, 1991). In contrast to these opinions which favour a role of IGF-I as an intragonadal modulator we could not obtain any evidence for a role of IGF-I in the LH induction of the CSCC enzyme; neither exogenous IGF-I nor the addition of potent IGF-I analogs increased the basal or LH induced CSCC enzyme activity. Moreover, the absence of any inhibitory effect of IGFBP on the LH induced CSCC enzyme activity also argued against an essential role of IGF-I. However, the latter finding must be carefully interpreted. Although the IGFBP preparation used in the present paper strongly inhibits IGF-I action on chicken embryo fibroblasts (Liu *et al.*, 1991), in accordance with other reports which describe inhibitory activities of distinct IGFBPs (Ritvos *et al.*, 1988; Frauman *et al.*, 1989; Koistinen *et al.*, 1990), stimulatory activities of IGFBPs have also been reported (Elgin *et al.*, 1987; Busby *et al.*, 1988). Therefore, attempts were made to block IGF-I action by the addition of IGF-I antiserum or antiserum to the IGF-I receptor. The negative outcome of these experiments further supports our conclusion that IGF-I plays no role in the LH induction of the CSCC enzyme in Leydig cells from immature rats. This conclusion may be in contradiction with the previously cited references which favour a stimulatory action of IGF-I on steroid production. Especially Verhoeven and Cailleau (1990), which have used a similar interstitial cell preparation of immature rats, were able to show that IGF-I stimulated the endogenous steroid. However, the following should not be overlooked:

1) the endogenous production of steroids does not only depend on CSCC enzyme activity but the available amount of cholesterol as substrate is often a major limiting factor and
2) the enzymatic activities in the endoplasmic reticulum which convert pregnenolone into androgens are often rate-limiting whereas the CSCC enzyme activity is the rate-determining factor (Van Haren *et al.*, 1989).

Although we found no evidence for any stimulatory effect of IGF-I on substrate availability, this may constitute an important regulatory site for other growth factors. The inhibitory

activity of TNFα on the LH stimulated testosterone production in porcine Leydig cells caused by a decreased cholesterol availability is a good example in this respect (Mauduit *et al.*, 1991). A technical limitation of this study is that experiments have been carried out in a time frame of 48 h, after incubating Leydig cells for several days without LH. However, these conditions were necessary for accurate measurement of enzyme induction.

A fundamental aspect required for a proper evaluation of effects of growth factors is the influence of context (composition of other signalling molecules) for the ultimate response of the cell. Sporn and Roberts (1988) have shown that the specific biological effects of growth factors depend very much on the previous exposure to and presence of other signalling molecules. This implies that the potential regulatory effects of IGF-I could be masked by other growth factors. The absence of any regulatory role of IGF-I in the LH induction of P450scc may therefore be caused by products secreted by the many interstitial cells other than Leydig cells that are present in the cell preparation. In this connection it must be stressed that the reported studies on the modulatory effects of IGF-I in Leydig cells have often been carried out in an artificial context; absence of surrounding cells and naturally occurring proteins. Such studies give therefore only information on the potential role of IGF-I in chemically defined culture media, but this function may not be expressed *in vivo*. Since in most studies using optimal *in vitro* conditions effects of IGF-I on steroid production hardly exceed more than a 2 fold stimulation, it may be questioned whether IGF-I is a major regulator of steroid production in a physiological context.

Acknowledgement

We thank J. Anton Grootegoed for the constructive comments.

Chapter 5

Inhibition of the Luteinizing Hormone-Dependent Induction of Cholesterol Side-Chain Cleavage Enzyme in Immature Rat Leydig Cells by Sertoli Cell Products

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European Journal of Endocrinology
(1995) 132:627-634

Abstract

The modulation of the luteinizing hormone (LH) induction of cholesterol side-chain cleavage (CSCC) enzyme in immature rat Leydig cells was studied using rat Sertoli cell-conditioned medium (SCCM), which stimulates short-term endogenous steroid production.
LH increased the CSCC enzyme activity 10 fold in cells cultured for 7 days in the absence of hormones. This enzyme induction was almost completely abolished in the presence of SCCM. The inhibition was dose-dependent (half-maximal effect at 5 μg protein/ml) and parallelled by a decrease in the amount of cytochrome P450scc (P450scc) enzyme. There were no indications for loss of cell viability. The inhibitory action of SCCM could be localized at the level of the adenylate cyclase activation and at steps beyond the cAMP production. The inhibition was not specific for Sertoli cell products since conditioned media from different cell lines and media from isolated rat hepatocytes displayed similar effects. Trypsin treatment of SCCM destroyed the activity whereas the bioactivity could resist heating for 5 min at 100 °C. Generally occurring (growth) factors, such as EGF or TNFα, may have contributed to the observed inhibitory effects of SCCM.
These inhibitory effects of Sertoli cell products *in vitro* are in contrast with stimulatory effects of Sertoli cells on Leydig cell steroidogenesis *in vivo* after FSH administration.

Introduction

Luteinizing hormone (LH) is the main regulator of Leydig cell steroidogenesis. However, there is abundant evidence, both from *in vivo* and *in vitro* experiments, that the effects of LH can be modulated by factors produced by seminiferous tubules, reviewed by Skinner (1991). Especially Sertoli cell factors are important. Following administration of follicle-stimulating hormone (FSH) to immature hypophysectomized rats, the number and steroidogenic activity of Leydig cells increase, indicating a stimulatory influence of Sertoli cells on Leydig cell function (Odell and Swerdloff, 1976; Teerds *et al.*, 1989; Vihko *et al.*, 1991).

Factors present in medium obtained from Sertoli cell cultures or seminiferous tubules have also been shown to modulate steroid production *in vitro* but many different observations have been made: Leydig cell testosterone production could be either stimulated (Verhoeven and Cailleau, 1990; Onoda *et al.*, 1991; Papadopoulos, 1991) or inhibited (Syed *et al.*, 1988; Vihko and Huhtaniemi, 1989; Zwain *et al.*, 1991; Fillion *et al.*, 1994) by these factors. The reason for these opposed effects on Leydig cell steroidogenesis is not clear but differences in the composition of Sertoli cell products, culture conditions or differences in the methodology for steroid measurements may have contributed to these discrepancies.

In studies on the paracrine regulation of Leydig cell steroidogenesis the testosterone production has often been measured to obtain information on the activity of Leydig cells, since testosterone is one of the most important androgens. The rate of testosterone production depends on both the cholesterol side-chain cleavage (CSCC) enzyme activity, as the rate-determining step of the steroidogenic cascade, and on the activity of certain rate-limiting steroid metabolizing enzymes in the endoplasmic reticulum (Van Haren *et al.*, 1989). Thus, the rate of testosterone production may be controlled at different levels and this could be one of the reasons why so many conflicting data on paracrine regulation of testosterone production have been reported. Measurement of the individual enzymatic steps in the steroidogenic cascade could provide more precise information on the effects of paracrine factors on Leydig cell steroidogenesis. In this connection the measurement of the CSCC enzyme activity, the conversion rate of cholesterol into pregnenolone, is the first choice since this is the rate-determining enzyme of steroidogenesis.

Previously, we have shown that insulin-like growth factor-I (IGF-I) did not modulate the LH induction of the CSCC enzyme in cultured immature rat Leydig cells (Van Haren *et al.*, 1992). In the present study we have investigated whether the LH induction of CSCC enzyme can be modulated by medium from Sertoli cells, which stimulates the endogenous pregnenolone production in immature Leydig cells during short-term incubations (Grootenhuis *et al.*, 1990a).

Materials and Methods

Materials

Fetal calf serum (FCS) was obtained from Gibco, Paisly, UK. Ovine LH (oLH, NIH-oLH S20) was a gift from the National Hormone and Pituitary Program (Baltimore, MD). SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphtalenone), an inhibitor of P450c17 activity, was a gift from Ciba-Geigy, Basle, Switzerland. Epostane or WIN-32729 (4α,5α-epoxy-17ß-hydroxy-4ß,17α-dimethyl-3-oxo-androstane-2α-carbo-nitrile), an inhibitor of the 3ßHSD, was a gift from Sterling-Wintrop, New York, NY, USA. Epidermal cell growth factor (EGF), bovine serum albumin (BSA, fraction V), 22R-hydroxycholesterol and 1-methyl-3-isobutylxanthine (MIX) were purchased from Sigma, St. Louis, MO, USA. Tumour necrosis factor-α (TNFα) was a generous gift of Dr. G. J. Van Steenbrugge (Dept. of Urology, Erasmus University Rotterdam, The Netherlands). Transforming growth factor-ß (TGFß) was obtained from Biomedical Technologies Inc., Stoughton, USA. Activin was kindly donated by H.M.J. VanderStichele (Innogenetics, Belgium).

Leydig cell isolation and culture

Leydig cells were isolated from 21-23 days old Wistar rats by procedures described in detail previously (Rommerts *et al.*, 1985). In brief, isolated cells were allowed to attach to Costar 48-well plates (10^5 cells/well) in RPMI medium containing streptomycin (100 μg/ml), penicillin (100 IU/ml) and 0.1% (w/v) BSA. After 1 h of incubation, non-attached cells were removed by washing and the cells attached to the dishes were cultured at 32 °C in air containing 5% CO_2. The medium was replaced at day 3 and 5 with fresh medium and at day 7 by control medium or medium supplemented with LH (1 ng/ml) and/or various other compounds. After 48 h, the CSCC enzyme activity was estimated, unless specified otherwise.

Characterization of the interstitial cell culture

The percentage of Leydig cells, as established by staining for 3ßHSD activity (Molenaar *et al.*, 1983), ranged from 20-30%. The 3ßHSD negative cells had morphological characteristics of mesenchymal cells (Kerr and Sharpe, 1985). Macrophages and Sertoli cells could not be identified in the cell preparation.

The percentage of damaged cells was estimated with a histochemical NADH-dependent test for intracellular diaphorase activity (Rommerts *et al.*, 1985). Both in the freshly isolated cell preparation and in the cells cultured for 7-9 days without SCCM, the percentage of diaphorase positive cells was $<1\%$. There were no morphological signs of degeneration during the culture period in the presence of SCCM. In contrast, the morphology of the interstitial cells changed under the influence of SCCM from round cells towards a more

elongated 'fibroblastic' appearance. The cellular protein and DNA levels and the LH (100 ng/ml) stimulated cAMP production remained constant during culture (the stimulation was approximately 40 fold; see also Van Haren *et al.* (1992).

Cholesterol side-chain cleavage enzyme assay

The CSCC enzyme activity was estimated as described previously, by measuring the conversion rate of saturating levels of 22R-hydroxycholesterol into pregnenolone (Van Haren *et al.*, 1992). Briefly, the cells were incubated for 3 h in the presence of 22R-hydroxycholesterol (19 μM) as substrate and with SU-10603 (5 μM) and epostane (5 μM) as inhibitors of pregnenolone metabolism (Van Haren *et al.*, 1989). The amount of pregnenolone was measured by a radioimmunoassay (Van der Vusse *et al.*, 1975). Changes in this sterol-supported CSCC enzyme activity correlated over a broad range with the cellular immunoreactive cytochrome P450scc (P450scc) content, demonstrating that the rate of sterol conversion into pregnenolone is a good reflection of the amount of the CSCC enzyme (Van Haren *et al.*, 1992).

Western blotting

Western blot analysis, using rabbit antiserum against rat P450scc in an 1:10,000 dilution, has been described previously (Van Haren *et al.*, 1992).

Preparation of Sertoli cell-conditioned medium (SCCM)

Sertoli cells were isolated from testes of 21 days old Wistar rats as described by Grootenhuis *et al.* (1990b). Briefly, approximately $3 \cdot 10^6$ Sertoli cells were plated in plastic 150 cm^2 flasks (Costar, Cambridge, MA, USA) in Eagle's minimal essential medium (MEM) containing 1% (v/v) fetal calf serum (FCS). On the following day, the attached Sertoli cells were washed and cultured in 20 ml MEM without FCS for 4-5 weeks. In some experiments, human FSH (hFSH; 25 mU/ml, Metrodin, Serono, Geneva, Switzerland) was added to the culture medium. Media were renewed each 3 to 4 culture days and the spent media were stored at -20 °C. After thawing media were pooled and concentrated 50 fold by diafiltration. Finally, the concentrated media were exchanged with 5 ml MEM using disposable cartridges with YM10 membranes (molecular weight cut-off at 10 kDa). The protein concentration of the 50 fold concentrated SCCM was approximately 1 mg/ml. Four different SCCM preparations have been used in the present study. All these preparations inhibited the LH induction of CSCC enzyme to a similar extent.

Previously, SCCM has been shown to stimulate the endogenous pregnenolone production by freshly isolated Leydig cells within 4 h (Grootenhuis *et al.*, 1990a). These data could be confirmed in the present study.

After separation of the proteins in SCCM with SDS polyacrylamide gel electrophoresis followed by staining with PAGE Blue 83 (BDH, Poole, UK) at least 50 bands could be discriminated (data not shown). This indicates that more than 50 different proteins are present in the Sertoli cell culture medium.

Preparation of conditioned media from other cell types

The LNCaP (Lymph Node Carcinoma of the Prostate), NHIK (Norse Hydro's Institute for Cancer Research), COS (African Green Monkey Kidney cell line), and TM4 (Testis Mouse) cell lines were cultured in RPMI or MEM medium supplemented with 5 or 10% FCS until a confluent monolayer was obtained. The cells were washed twice with phosphate-buffered saline to remove serum and cultured subsequently for 4 days in MEM or RPMI without FCS but supplemented with 0.1% BSA. Primary cultures of immature rat Leydig cells and rat hepatocytes were cultured for 4 days in RPMI medium with 0.1% BSA. At the end of the culture period, media were collected and stored at -20 °C. After thawing, the media were concentrated 50 fold, as described for Sertoli cell medium. Due to the presence of 0.1% BSA in the media, the biological activities could not be expressed in terms of protein concentrations but as μl concentrated conditioned medium added to the Leydig cell cultures.

Estimation of cAMP

The cAMP level was estimated in the culture medium, after incubating the cells for 3 h in medium containing MIX (200 μM) with or without LH (100 ng/ml). For the determination of cAMP, a cAMP assay kit was used (Amersham International plc), with the lower limit of detection at 0.2 pmol and an intra-assay variation of 11%.

Statistical methods

Data are expressed as mean $\pm$ SD. Statistical differences were determined by analysis of variance with the Tukey's honestly significant difference test to discern individually significant effects, unless specified otherwise.

Results

Inhibition of the LH induction of CSCC enzyme activity by SCCM

Long-term regulatory effects of SCCM on the CSCC enzyme induction by LH were studied. For this purpose, Leydig cells cultured for 7 days in the absence of hormones were used because the CSCC enzyme activity in these cells is low and the response to the tropic action

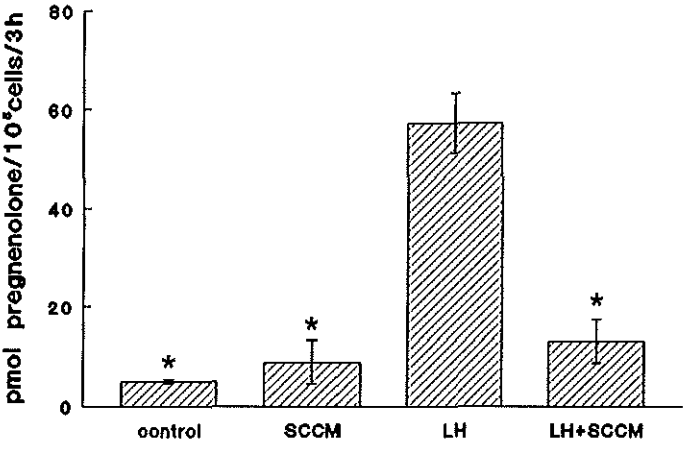

*Fig. 1. Modulation of P450scc enzyme activity by LH and SCCM. Immature rat Leydig cells were cultured for 7 days in the absence of LH. On day 7 the cells were further incubated without addition or with LH (1 ng/ml) and/or SCCM (122 μg protein/ml) for 48 h. After this incubation period of 48 h, the P450scc enzyme activity was estimated by measuring the 22R-hydroxycholesterol supported pregnenolone production, in the presence of inhibitors of pregnenolone metabolism. The 22R-hydroxycholesterol supported steroid productions at day 0 and day 7 were 367 ± 51 and 12.5 ± 2.4 pmol pregnenolone/10^5 cells/3 h, respectively. Mean values ± SD (n=4-6) of two different cell preparations are shown. *, significantly different from cells cultured in the presence of LH ($P < 0.01$).*

of LH high and also long-term effects of LH on CSCC enzyme induction can not be shown in freshly isolated cells (Van Haren *et al.*, 1992). Incubating these cells for 48 h in the presence of LH increased the CSCC enzyme activity 10 fold when compared to control cells. SCCM almost completely abolished the LH induction of the CSCC enzyme whereas it had no effect on the basal CSCC enzyme activity (Fig. 1). Comparison of the long-term

inhibitory effects of media from Sertoli cells cultured in the absence or presence of FSH (25 mU/ml) showed no differences in inhibitory activity (data not shown). Therefore, SCCM obtained from Sertoli cells cultured in the absence of FSH have been used in all the subsequent experiments. When the control and SCCM-inhibited cells were incubated with LH alone for an additional 48 h, the CSCC enzyme activity was increased 5 fold in both cells. However, the absolute level of pregnenolone formed was lower in the SCCM-inhibited cells

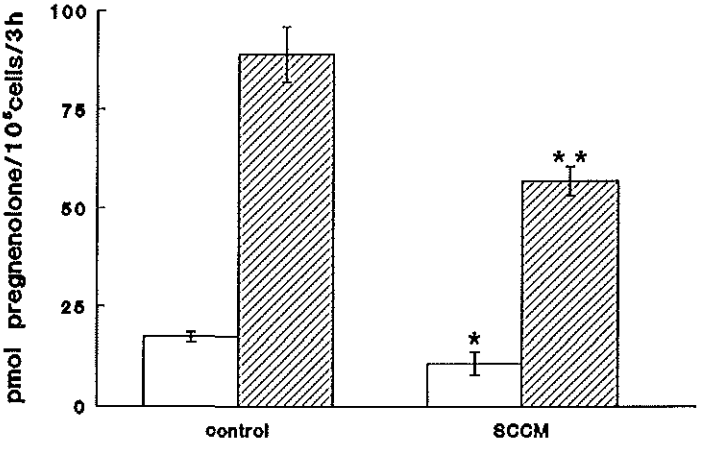

*Fig. 2. **Reversibility of SCCM action**. Immature rat Leydig cells were cultured for 7 days in the absence of LH. On day 7 the cells were incubated without or with SCCM (122 μg protein/ml) for 48 h. After this incubation period, cells were washed 3 times and further incubated without addition (open bars) or with LH (1 ng/ml; hatched bars) for an additional 48 h. The pregnenolone production was assayed as described in Fig. 1. Mean values ± SD of triplicate incubations are shown. * $P < 0.02$, ** $P < 0.002$ as compared with respective controls (Student's t-test).*

compared with control cells. This indicates that the inhibitory action of SCCM is at least partly reversible (Fig. 2). The action of SCCM was dose-dependent, and a half-maximal effect was observed at approximately 5 μg protein/ml (Fig. 3). To investigate whether the inhibition of the LH induction of CSCC enzyme activity by SCCM was due to a decrease in the amount of P450scc enzyme, the level of P450scc enzyme was also measured by Western blotting. It was shown that the increase of the amount of P450scc enzyme by LH could be

completely abolished by SCCM. Surprisingly, the amount of immunoreactivity in SCCM-inhibited cells was even lower than in control cells (Fig. 4).

Partial characterization of the inhibitory components in SCCM

Trypsin treatment of SCCM destroyed the activity whereas heating for 5 min at 100 °C had

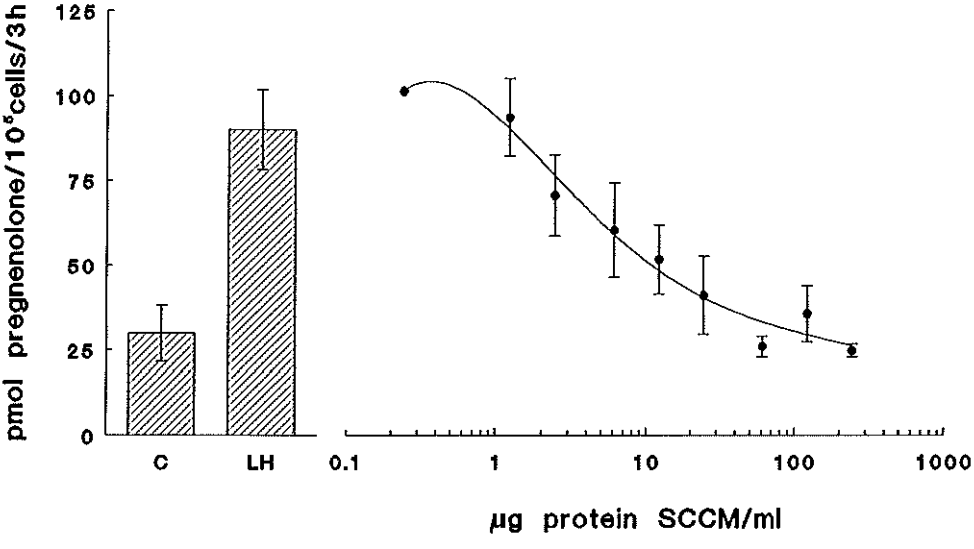

Fig. 3. Dose-dependent effects of SCCM on LH induction of P450scc enzyme. Immature rat Leydig cells were cultured and the pregnenolone production was assayed as described in Fig. 1. Cells were incubated with increasing concentrations of SCCM (0-244 µg protein/ml) in the presence of LH (1 ng/ml). Mean values ± SD (n=2-8) of two different cell preparations are shown.

no effect, indicating that part of the inhibition is caused by heat-resistant protein(s). Effects of conditioned media from four different cell lines (NHIK, COS, LNCaP and TM4), two primary cultures (rat hepatocytes and immature rat Leydig cells) and culture medium supplemented with 1% FCS were also studied. All the spent media from these incubations, except that from Leydig cells, inhibited the action of LH. Only FCS (1%) potentiated LH

action (Fig. 5). Since generally occurring (growth) factors might explain the effects of SCCM we have investigated the effects of a few candidate proteins. Tumour necrosis factor-α (TNFα; 1-100 ng/ml) and epidermal growth factor (EGF; 1-100 ng/ml) inhibited the induction of P450scc by LH whereas transforming growth factor-ß (TGFß; 0.1-10 ng/ml) and activin (1-100 ng/ml) had no effect (data not shown).

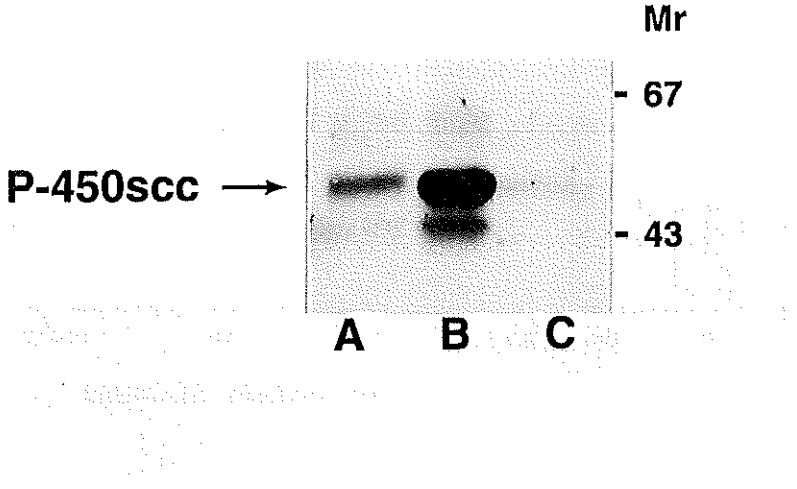

Fig. 4. Effect of SCCM on cellular P450scc content. Western blot developed with P450scc antibody after SDS-polyacrylamide gel electrophoresis of 100 µg protein of immature rat Leydig cells. Leydig cells were cultured as described in Fig. 1. Lane A, control; lane B, LH (1 ng/ml); lane C, LH (1 ng/ml) and SCCM (122 µg/ml).

Sites of action

To define whether the inhibitory activity is located at the level of LH receptor activation or at sites more distant in the signal transduction pathway, the effects of SCCM on the LH stimulated cAMP production and on the dbcAMP stimulated pregnenolone production were

investigated. LH stimulated the cAMP production approximately 100 fold, irrespective if cells had been cultured in the absence or in the presence of low levels of LH. The LH stimulated cAMP production was reduced to 50% of the maximal values when cells had been cultured for 48 h in the presence of SCCM. The LH stimulated cAMP production was reduced even more to 30% of the control values when cells had been cultured with LH and SCCM together (Fig. 6A). Evidence for more distant inhibitory effects of SCCM was

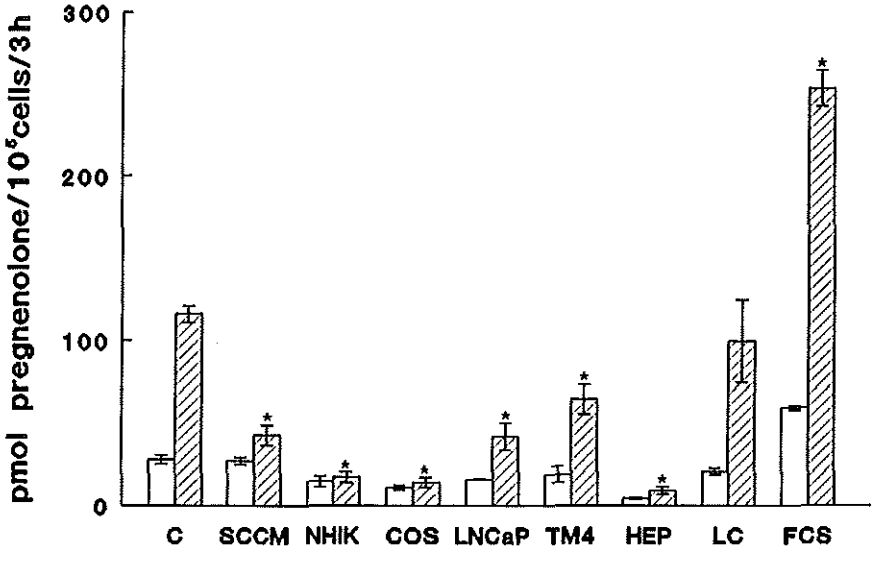

*Fig. 5. Modulation of LH dependent P450scc enzyme induction by conditioned media of various cell types. Immature rat Leydig cells were cultured and the pregnenolone production was assayed as described in Fig. 1. 50 µl of concentrated conditioned media derived from Sertoli cells, various cell lines (NHIK, COS, LNCaP and TM4), primary cultures of rat hepatocytes and immature rat Leydig cells or RPMI medium supplemented with 1% (v/v) FCS were added per 500 µl incubation medium supplemented without (open bars) or with LH (1 ng/ml; hatched bars). Mean values ± SD (n=3) of one cell preparation are shown. *, significantly different from cells cultured in the presence of LH without further additions (P < 0.01).*

obtained from studies employing dbcAMP as stimulatory agent for P450scc enzyme induction. This dbcAMP mediated enzyme induction, which bypasses LH receptor activation, was also inhibited to 35% by SCCM (Fig. 6B).

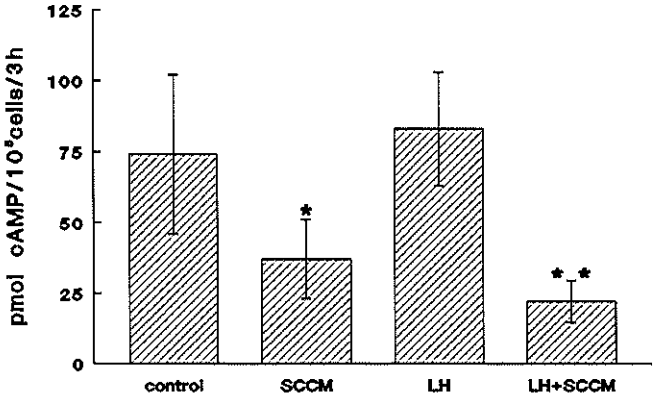

Fig. 6A.

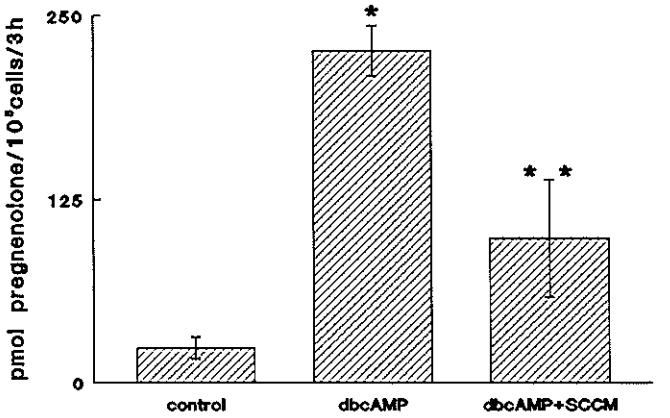

Fig. 6B.

Fig. 6. Inhibitory sites of SCCM action. *Immature rat Leydig cells were cultured for 7 days in the absence of LH.*
Fig. 6A. *On day 7 the cells were further incubated in fresh medium without additions or with LH (1 ng/ml) and/or SCCM (122 μg protein/ml) for 48 h. After this incubation period of 48 h, the media were removed and the cells were incubated for 3 h in fresh medium with LH (100 ng/ml), supplemented with 1-methyl-3-isobutylxanthine (MIX; 200 μM). The amount of extracellular cAMP formed was measured by radioimmunoassay. The control values were 0.3 (control), 0.3 (SCCM), 0.6 (LH) and 1.5 (LH+SCCM) pmol cAMP/10^5 cells/3 h. Mean values ± SD (n=6) of 3 different cell preparations are shown. *, significantly different from control ($P < 0.001$). **, significantly different from cells cultured in the presence of LH ($P < 0.001$).*
Fig. 6B. *On day 7 the cells were further incubated in fresh medium without additions or with dbcAMP (50 mM) and/or SCCM (122 μg protein/ml) for 48 h. Mean values ± SD (n=6) of 2 different cell preparations are shown. *, significantly different from control ($P < 0.01$). **, significantly different from control or cells cultured in the presence of dbcAMP ($P < 0.01$).*

Discussion

Culture medium obtained from Sertoli cells strongly inhibited the LH induction of CSCC enzyme in cultured immature rat Leydig cells via a decrease in the amount of P450scc enzyme. This inhibition was dose-dependent, without toxic effects on the interstitial cells and was parallelled by both an inhibition of cAMP production and a reduction of the dbcAMP stimulated pregnenolone production. This indicates that inhibition of LH dependent P450scc enzyme induction occurs both at a step before and beyond the adenylate cyclase complex. Evidence that seminiferous tubules factors can inhibit Leydig cell function through inhibition of adenylate cyclase has been reported previously (Syed *et al.*, 1988; Vihko et Huhtaniemi, 1989; Zwain *et al.*, 1991). Only recently, it has been suggested that a factor present in rat seminiferous tubular culture medium inhibits P450scc and P450c17 enzyme activities in mature rat Leydig cells (Zwain and Cheng, 1994). However, it was not investigated whether this inhibition was due to a decrease in the cellular amount of steroidogenic enzymes.
The observation that media obtained from a variety of different cell types mimic the action of SCCM indicates that inhibitory factor(s) present in SCCM are not specific. Verhoeven and Cailleau (1985) have also reported that media of different cell lines modulate Leydig cell steroidogenesis similarly as observed with Sertoli cell spent medium. However, since they measured the production of androgens (C_{19}-steroids) and their immediate precursors (C_{21}-steroids), no conclusions could be made which specific enzyme(s) (*e.g.* CSCC enzyme) of the steroidogenic cascade were involved. It seems possible that generally occurring (growth) factors, such as EGF or TNFα, have contributed to the observed inhibitory effects of SCCM. Previously, TNFα has been described to inhibit the hCG or dbcAMP induced expression of P450scc mRNA in rat and mouse Leydig cells (Xiong and Hales, 1993; Lin *et al.*, 1994b). Since our interstitial cell culture contains only 20-30% Leydig cells, it can not be excluded that the inhibitory action of SCCM is mediated by factors produced by non-Leydig cells present in the cell preparation.
In contrast with the inhibitory action of SCCM on the CSCC enzyme activity during long-term incubations, Sertoli cell medium stimulates the short-term (4 h) endogenous pregnenolone production (Grootenhuis *et al.*, 1990a; Murai *et al.*, 1992). This indicates that SCCM can either inhibit or stimulate steroid production in Leydig cells, depending on the time of exposure of the Leydig cells to SCCM. The short-term stimulatory effect of SCCM is most likely mediated by an enhanced trafficking of cholesterol to the CSCC enzyme, localized inside the mitochondria (Van der Vusse *et al.*, 1973; Moyle *et al.*, 1973). The long-term inhibitory action of SCCM which requires 48 h, is probably mediated by a genomic action or increase of protein degradation, as demonstrated by the decrease in the cellular immunoreactive P450scc content. This implicates that products in SCCM regulate

at least two different events in the steroidogenic cascade. The transition from stimulation to inhibition of Leydig cell steroidogenesis by Sertoli cell factors during prolonged incubations was observed as casual observation amongst many other effects of Sertoli cell factors on steroid production (Verhoeven and Cailleau, 1985). This reversal in biological action of SCCM could be one of the reasons why so many conflicting data on the paracrine regulation of the Leydig cell activities have been reported.

The observed inhibition of the LH induction of the CSCC enzyme by SCCM in cultured immature rat Leydig cells is in contrast with the stimulatory activity of Sertoli cells on Leydig cell function *in vivo*. However, it should be emphasised that the positive effects of Sertoli cells *in vivo* are observed only after FSH administration (Odell and Swerdloff, 1976; Teerds *et al.*, 1989; Vihko *et al.*, 1991). Our experiments have been carried out with isolated interstitial cells, cultured for 7 days in a chemical defined medium, containing 0.1% albumin only. In addition, the Sertoli cell medium used contains at least 50 different proteins and this pattern of secreted proteins changes during culture (Grootegoed *et al.*, 1978). Therefore these specific *in vitro* conditions may be responsible for the unexpected response of the Leydig cells. Since the effects of combinations of growth factors may depend more on the composition of the different factors than on the properties of the individual molecules (Sporn and Roberts, 1988), differences between *in vivo* and *in vitro* Leydig cell responses can be understood. Furthermore, the identification of (one) active component(s) in Sertoli cell medium affecting Leydig cells *in vitro* might be complicated by the fact that the biological activity *in vivo* can be influenced by the presence of many (unknown) paracrine factors in the testis.

Chapter 6

General Discussion

6.1. Introduction

The aim of the studies described in this thesis was to gain further insight in the regulation of steroidogenesis in rat Leydig cells at the level of the CSCC enzyme, since CSCC enzyme activity can be considered as the rate-determining step in steroidogenesis (Chapter 3). The conversion rate of cholesterol into pregnenolone by the CSCC enzyme depends on intracellular transport of cholesterol to the CSCC enzyme, and it depends on the activity of the CSCC enzyme itself.
These two topics were investigated, and described in this thesis. First, the role of SCP_2 in transporting intracellular cholesterol to the CSCC enzyme was evaluated. Second, the regulation of the CSCC enzyme activity by LH and local factors was studied. For both purposes, conditions for measuring steroidogenesis and enzyme induction were evaluated and improved.

6.2. Measurement of CSCC enzyme activity

Measurement of endogenous and 22R-hydroxycholesterol-supported steroid production by Leydig cells was performed in the presence of inhibitors of pregnenolone metabolism, in order to reduce the number of different steroids produced. Specific and complete inhibition of pregnenolone metabolism in rat Leydig cells, without affecting CSCC enzyme activity, could be accomplished with a combination of SU-10603, which is an inhibitor of P450c17 enzyme, and epostane (WIN 32729), an inhibitor of 3ßHSD. Using these compounds, pregnenolone metabolism could also be inhibited completely in the transplantable rat Leydig cell tumour H-540, but not in mouse Leydig cells, suggesting that specificity and effectiveness of the inhibitors are species dependent (Chapter 3).
With the use of these inhibitors of pregnenolone metabolism, the rate-limiting step of the steroidogenic cascade could be identified, by comparing the production of pregnenolone and testosterone at various rates of steroid production (Chapter 3). No differences were found between pregnenolone and testosterone production rates in isolated rat Leydig cells from mature rats, under basal conditions and in the presence of LH. This indicates that, under these circumstances, the capacity of the enzymes in the endoplasmic reticulum in rat Leydig cells is sufficient to convert all endogenous pregnenolone into testosterone, and is not rate-limiting for testosterone production. However, when a high rate of steroidogenesis was obtained using 22R-hydroxycholesterol, 10-fold higher than the rate obtained upon LH stimulation with endogenous substrate, the capacity for conversion of pregnenolone was much lower than the rate of pregnenolone production. Hence, in the presence of exogenous 22R-

hydroxycholesterol the conversion of pregnenolone into testosterone is rate-limiting for biosynthesis of testosterone. Several studies have shown that microsomal enzyme activities are rate-limiting also *in vivo*, before and after treatment with hCG. High levels of C_{21} precursor steroids have been detected in testis tissue of rats after administration of a high dose of hCG (Quinn and Payne, 1985). Appreciable amounts of C_{21} steroids have also been measured in testis tissue from untreated rats (Punjabi *et al.*, 1983; Grizard *et al.*, 1987) and from normal men (De la Torre *et al.*, 1982). Thus, *in vivo*, the microsomal enzyme activities appear to be the rate-limiting step for testosterone production.

Conversion of cholesterol into pregnenolone has often been defined as the rate-limiting step of steroidogenesis, since conversion of cholesterol into pregnenolone is the most important step for hormonal control of steroidogenesis (Hall, 1994; Saez, 1994). However, in view of the data discussed above, it seems preferable to point to this step as the rate-determining step of steroidogenesis. Moreover, the net amount of testosterone produced by the testis is not only the result of the rate-determining and rate-limiting steps of steroidogenesis, but also depends on metabolism of testosterone.

An enzymatic assay for measurement of CSCC enzyme activity, independent of the supply of endogenous cholesterol, was developed by measuring the conversion rate of exogenously added 22R-hydroxycholesterol into pregnenolone (Chapter 4). 22R-hydroxycholesterol was used as substrate for the CSCC enzyme since this hydroxylated sterol is more water soluble than cholesterol and can cross the aqueous space between the outer and inner mitochondrial membranes without hormone-dependent transport facilities (Sinensky, 1981; Toaff *et al.*, 1982). A good correlation was found between the rate of the 22R-hydroxycholesterol-supported pregnenolone production and the cellular P450scc content, the latter estimated by immunoblotting (Chapter 4). Thus, the rate of 22R-hydroxycholesterol conversion into pregnenolone reflects the amount of CSCC enzyme. After incubating Leydig cells for several days in the absence of LH, no immunoreactive P450scc could be detected, whereas CSCC enzyme activity was low but still detectable. This implies that estimation of the amount of P450scc by Western blotting is less sensitive than measurement of the enzyme activity. On basis of these data, we preferred the enzymatic assay to estimate the amount of CSCC enzyme, in experiments on induction of CSCC enzyme by LH and/or paracrine factors.

6.3. Short-term regulation by LH: any role for SCP_2?

SCP_2 stimulates mitochondrial production of steroids from cholesterol (Chanderbhan *et al.*, 1982; Van Noort *et al.*, 1988; McNamara and Jefcoate, 1989; Yamamoto *et al.*, 1991). A relationship between cellular content of SCP_2 in steroidogenic tissues and rate of steroid production has been observed (Trzeciak *et al.*, 1987a; McLean *et al.*, 1989, 1995). A similar

correlation between the steady-state SCP_2 mRNA level and steroidogenic capacity in the gonadotropin-primed ovary has also been reported (Billheimer *et al.*, 1990). The observation that fusion of adrenocortical cells with liposomes containing anti-SCP_2 antibody reduced the rate of ACTH-stimulated steroidogenesis indicates that SCP_2 plays some role, possibly an important role, in activated steroidogenesis (Chanderbhan *et al.*,1986). Moreover, co-transfection of COS cells with cDNAs encoding human SCP_2, cytochrome P450scc, and adrenodoxin, resulted in a 2.5-fold enhancement of steroid production as compared to the rate of synthesis obtained with expression of the steroidogenic enzyme system alone (Yamamoto *et al.*, 1991).

Although there is a correlation between the level of SCP_2 and the rate of steroidogenesis, several observations do not favour an important role of SCP_2 in short-term regulation of steroidogenesis. The predominant localization of SCP_2 in membranous fractions of Leydig cells was unaltered upon stimulation with LH (Chapter 2). Moreover, while synthesis of SCP_2 in adrenal cells is stimulated by ACTH, this regulation only occurred after many hours (Trzeciak *et al.*, 1987a). In the testis, SCP_2 was found not only in Leydig cells but also in Sertoli cells, whereas germ cells (spermatocytes and spermatids) did not contain SCP_2 (Chapter 2). The absence of SCP_2 in spermatocytes and spermatids is of interest, since these cells, although not active in steroidogenesis, probably require cholesterol to serve as a component of new cell membrane. The cell diameter (Bellvé *et al.*, 1977) and surface area (Millette *et al.*, 1976) of differentiating spermatocytes increase, and this is associated with an elevated rate of cholesterol synthesis (Potter *et al.*, 1981). This implies that cholesterol trafficking in these cells may occur by transport mechanisms not involving SCP_2, such as vesicular transport, and transport mediated by proteins other than SCP_2. Support for this hypothesis comes from a study by Johnson and Reinhart (1994), who showed that intracellular trafficking of lysosomal cholesterol to the plasma membrane and the rough endoplasmic reticulum can occur in SCP_2-deficient cells (Zellweger-syndrome fibroblasts). Van Heusden *et al.* (1992) also suggested that SCP_2 is not always required for efficient trafficking of intracellular cholesterol. They showed that SCP_2-deficient Chinese hamster ovary cells are essentially normal with respect to uptake and degradation of LDL and the release of free cholesterol.

In summary, the importance of SCP_2 in cholesterol trafficking in steroidogenic cells is not yet known, and, since there are other transport systems for cholesterol, SCP_2 may play an important role only when cholesterol is required in large amounts that cannot be delivered by other transport systems.

6.4. Clinical disorders associated with defects in the steroidogenic cascade

Interruption of production of testosterone or dihydrotestosterone can give rise to a variety of disorders in males. Most of these disorders will affect male sexual development (Fauser and Hsueh, 1995). Defects in the androgen receptor can also cause partial or complete androgen resistance, with phenotypes varying from mildly undervirilized males to external sex reversal. This topic will not be discussed in this thesis (see Brinkmann *et al.*, 1992).

Sex differentiation in the mammalian embryo depends on three sequential processes:

1) The establishment of genetic sex by the sex chromosomes, at fertilization.
2) Differentiation of the indifferent gonad into either an ovary or a testis. Ovarian differentiation can be considered the default pathway, which is "overruled" in males by protein encoded by the testis-determining gene (SRY) located on the Y chromosome.
3) Translation of gonadal sex into phenotypic sex, that is, formation of internal and external genitalia.

Three different hormones are involved in the formation of the male phenotype: anti-müllerian hormone (AMH), testosterone and dihydrotestosterone. AMH is expressed by Sertoli cells early after the onset of sex determination and inhibits development of the müllerian ducts, and thereby to prevent formation of fallopian tubes and uterus in males (George and Wilson, 1994). In this thesis, the first two processes and defects in AMH are not discussed (see thesis Baarends, 1995). Testosterone stimulates development of the wolffian ducts into epididymes, vasa deferentia, and seminal vesicles (internal genitalia), whereas dihydrotestosterone promotes formation of the prostate and external genitalia (male urethra, penis, and scrotum). The female phenotype can again be considered as the default pathway, that occurs in the absence of SRY, AMH, and androgens. Reduced production of androgens in the fetal testes may result in various forms of male pseudohermaphroditism (incomplete masculinization of genital duct and/or external genitalia). Androgen production can be disregulated at different levels of the steroidogenic cascade:

LH - LH receptor interaction - Absence of LH, abnormal LH with diminished biological activity, or defects in the LH receptor, will cause impairment of Leydig cell development and steroid production due to insufficient gonadal stimulation. In rare cases of hypogonadal function it has been demonstrated that the LH molecules were devoid of biological activity (Park *et al.*, 1976; Axelrod *et al.*, 1979; Beitins *et al.*, 1981; Weiss *et al.*, 1992). In adolescence, these males present with elevated serum immunoreactive LH and low testosterone concentration. A normal rise in serum testosterone concentration is observed following the administration of exogenous gonadotropins.

In other patients, mutations of the LH receptor gene have been described, causing a constitutive activation of the LH receptor resulting in an elevated testosterone concentration

despite a low pre-puberal concentration of serum LH, which can cause gonadotropin-independent precocious puberty (Kremer *et al.*, 1993; Shenker *et al.*, 1993; Laue *et al.*, 1995a; Kraaij, 1996). Also mutational loss of function of the LH receptor has been described, resulting in impaired testosterone production and male pseudohermaphroditism (Kremer *et al.*, 1995; Laue *et al.*, 1995b).

Cholesterol transport to the CSCC enzyme - In the liver of infants with cerebro-hepatorenal (Zellweger) syndrome, SCP_2 is absent due to disturbed processing of the precursor molecule of SCP_2 (Van Amerongen *et al.*, 1987; Walton *et al.*, 1992). The absence of SCP_2 in these patients may contribute to the observed accumulation of cholesterol esters (Goldfischer *et al.*, 1983) and to impaired synthesis of cortisol in the adrenal cortex after ACTH stimulation (Govaerts *et al.*, 1984). A mutational defect in the protein StAR can be responsible for impaired synthesis of all adrenal and gonadal steroids in patients with congenital lipoid adrenal hyperplasia (Lin *et al.*, 1995). Affected infants die from salt loss, hyperkalemic acidosis, and dehydration, unless treated with steroid hormone replacement therapy. The boys with this syndrome show pseudohermaphroditism, as a result of the absence of testicular testosterone synthesis.

Conversion of cholesterol into testosterone - The activity of different steroidogenic enzymes can be disrupted, resulting in diminished or absent production of androgens. Enzyme defects involved in early steps of the cascade (P450scc, 3βHSD and P450c17) affect not only gonadal steroid production, but also adrenal steroid production (glucocorticoids and mineralocorticoids). Genetic defects have been suggested and/or identified in the following steroidogenic enzymes:

1) ***P450scc***. A genetic defect in the P450scc enzyme was thought to be responsible for a deficiency of adrenal and gonadal hormones in congenital lipoid adrenal hyperplasia patients, because mitochondria from affected adrenal glands and gonads failed to convert cholesterol to pregnenolone (Hauffa *et al.*, 1985). However, molecular genetic analysis of affected individuals indicated that the gene encoding P450scc enzyme was completely intact (Lin *et al.*, 1991; Sakai *et al.*, 1994). Recently, it has been demonstrated that the defect in steroidogenesis in these patients resides in a defect in the protein StAR (Lin *et al.*, 1995).
2) ***3ßHSD***. This enzyme is also needed for the synthesis of steroid hormones in both adrenal glands and gonads. Two 3ßHSD genes have been identified; type II is expressed only in the gonads and the adrenals, whereas type I is expressed in skin, kidney and placenta. Signs and severity of adrenal hyperplasia vary in newborns with a partial deficiency of type II enzyme activity (Pang *et al.*, 1983). Incomplete androgen production causes deficient masculinization and pseudohermaphroditism in males (Grumbach and Conte, 1992; Mébarki *et al.*, 1995).

3) ***P450c17***. 17α-Hydroxylase deficiency can occur independently, or in combination with $C_{17\text{-}20}$ lyase deficiency. In patients with a selective $C_{17\text{-}20}$ lyase deficiency the adrenal cortisol production is normal (Pellicer *et al.*, 1991). 17α-Hydroxylase deficiency is rare; it leads to male pseudohermaphroditism and poor virilization at puberty in males (Yanase *et al.*, 1991; Rumsby *et al.*, 1993).
4) ***P450arom***. This enzyme catalyzes the conversion of androgens to estrogens. A mutational defect in this enzyme is extremely rare. Recently, a female patient has been described which showed primary amenorrhoea, elevated serum gonadotropin concentrations, and a history of ambiguous external genitalia at birth (Conte *et al.*, 1994).
5) ***17βHSD***. Three 17βHSD genes have been cloned, and one of these genes (type 3) shows testis-specific expression. More than 90% of the affected genetic males with mutations in the 17βHSD3 gene (encoding the 17βHSD type 3 isoenzyme) have male internal and female external genitalia at birth (Geissler *et al.*, 1994). At puberty, these patients fail to menstruate and instead virilize, probably caused by peripheral conversion of the high circulating level of androstenedione into testosterone and DHT, by the 17βHSD type I or type II isoenzymes (Thigpen *et al.*, 1993; Andersson *et al.*, 1996).
6) ***5α-reductase***. Deficiency of this enzyme impairs the conversion of testosterone into dihydrotestosterone. The 5α-reductase enzyme is encoded by two genes in human, and mutations in the type II gene are associated with 5α-reductase deficiency (Wilson *et al.*, 1993). At birth, individuals with a severe 5α-reductase deficiency have a predominantly female phenotype, showing signs of virilization at puberty (Imperato-McGinley, 1980; Akgun *et al.*, 1986).

In the future, better understanding of the different steps involved in synthesis and action of androgens, will establish a firm basis for diagnosis and clinical management of disorders of steroid hormone production by Leydig cells.

6.5. LH induction of the CSCC enzyme: no role for IGF-I?

The cAMP-dependent signal transduction pathway, activated by tropic hormones, plays a key role in the induction of P450scc, as demonstrated by the cAMP responsiveness of the promoters of human, rodent, and bovine P450scc genes (Moore *et al.*, 1990; Oonk *et al.*, 1990; Rice *et al.*, 1990; Ahlgren *et al.*, 1990; Inoue *et al.*, 1988, 1991). However, evidence is growing that P450scc induction can also be regulated in part by cAMP-independent pathways, activated by growth factors and cytokines (Waterman, 1994). In this thesis, we have investigated whether and how IGF-I can modulate induction of CSCC enzyme by LH.

Interstitial cells obtained from immature rat testes and cultured in a chemically defined medium were used to study effects of LH and IGF-I on CSCC enzyme induction (Chapter 4). It was found that during the first days of the culture period the CSCC enzyme content of the Leydig cells diminished continuously, which could not be prevented by addition of LH. Only after a culture period of more than 3 days, CSCC enzyme content could be enhanced by LH. The short-term stimulatory action of LH on cAMP production and endogenous pregnenolone production was sustained during the culture period. These data show a discrepancy between short-term and long-term actions of LH during the first days of the culture, and it seems that cAMP is not always capable to induce CSCC enzyme. This could indicate that other, non-cAMP dependent signal transduction pathways might operate in the control of CSCC enzyme induction. Studies on adrenal cells have shown that cAMP does not function as the only intracellular mediator of tropic hormone induction of steroidogenic enzymes. Exposure of adrenal cortex cells to a short or long pulse of ACTH resulted in increased levels of mRNA and enzyme (Hanukoglu *et al.*, 1990). In contrast, even a prolonged pulse of dbcAMP or stimulation of adenylyl cyclase by forskolin, was ineffective in inducing steroidogenic enzymes. It seems possible that induction of P450scc by LH in immature rat Leydig cells requires other paracrine factors that were not present in sufficient amounts during the first days of the culture period. In this connection, we have investigated whether IGF-I might influence P450scc induction. Indications for an autocrine loop mechanism were initially provided by Cailleau *et al.* (1990) who found that immature rat Leydig cells do release IGF-I into the incubation medium upon hormonal stimulation. Surprisingly, and in apparent disagreement, Lin and co-workers (1994a) showed that the IGF-I mRNA level in Leydig cells is down-regulated by hCG. We found no evidence that IGF-I plays any role in LH induction of CSCC enzyme; neither addition of exogenous IGF-I or analogs that do not bind to IGF-I binding proteins (IGFBPs), nor inactivation of endogenous IGF-I action (through binding to IGFBP and antibodies to IGF-I, or using antibodies targeting the IGF-I receptor) did influence the LH-induced CSCC enzyme activity (Chapter 4).

Our conclusion, therefore, is that IGF-I is not involved in CSCC enzyme induction under our experimental conditions. This is in contradiction with several reports which provide evidence for a stimulatory action of IGF-I on Leydig cell steroidogenesis, and studies on ovarian cells, which show induction of P450scc enzyme synthesis by IGF-I (Veldhuis *et al.*, 1986; Magoffin *et al.*, 1990; Magoffin and Weitsman, 1993; Urban *et al.*, 1990, 1994). Patients with isolated GH deficiency have delayed puberty and show a poor response to exogenous hCG. This can be (partly) abolished by administration of GH during childhood (Sheikholislan and Stempfel, 1972; Kulin *et al.*, 1981). In normal children, the circulating IGF-I concentration increases at the time of puberty (Olivié *et al.*, 1995). The roles of IGF-I and GH in the regulation of testicular function have also been studied in Snell dwarf mice. These

mice have an anterior pituitary defect, resulting in production of no or only small amounts of GH and prolactin. In addition, these mice show delayed puberty and a low serum testosterone level, probably related to an IGF-I deficiency. Treatment of these dwarf mice with GH or IGF-I increased the number of testicular LH receptors and the acute steroidogenic response to hCG (Chatelain *et al.*, 1991). In the testis, IGF-I is produced by Leydig, Sertoli, and peritubular cells (Cailleau *et al.*, 1990; Naville *et al.*, 1990; Lin *et al.*, 1990). An enhanced LH-stimulated steroid production by IGF-I, via specific IGF-I receptors present on Leydig cells (Lin *et al.*, 1986a,b), has been reported by several investigators (Adashi *et al.*, 1982; Kasson and Hsueh, 1987; Benahmed *et al.*, 1987). Verhoeven and Cailleau (1990) were able to show a stimulatory effect of IGF-I on endogenous steroid production, using a similar Leydig cell culture system as described by us. The following points might be important to try to explain the discrepancy between these results and our findings:

1) As mentioned previously, a stimulatory effect of IGF-I on Leydig cell steroidogenesis has been demonstrated by measuring an increase in endogenous steroid production. Since endogenous production of steroids not only depends on CSCC enzyme activity but also on the available amount of cholesterol, an effect of IGF-I may be explained by increased substrate availability. Although we found with our Leydig cell culture system no evidence for such a stimulatory effect of IGF-I (Chapter 4), substrate availability may constitute an important target for action of growth factors on steroidogenic cells. A good example of this is an inhibitory action of TNFα on LH stimulated testosterone production in immature porcine Leydig cells, which is caused by a decreased cholesterol substrate availability in mitochondria (Mauduit *et al.*, 1991).
2) Changes in testosterone production could also be caused by changes of enzymatic activities in the endoplasmic reticulum necessary for conversion of pregnenolone into androgens (Chapter 3).
3) Differences in Leydig cell preparations, such as the percentage of contaminating cells, may also underlie discrepancies in experimental data. Although the method to obtain an interstitial cell preparation as used by Verhoeven and Cailleau (1990) is very similar to the method used by us, differences in cellular composition cannot be excluded. A stimulatory action of IGF-I might be masked by the presence of inhibitory factors from unknown cellular origin, or induced by some unknown aspect of the cell culture conditions.

In conclusion, we have found that, under our experimental *in vitro* conditions, IGF-I is not involved in LH induction of P450scc in immature rat Leydig cells. However, a stimulatory effect of IGF-I on other steps of the steroidogenic cascade cannot be excluded. The

importance of the composition of the cell preparation and culture conditions for cellular responses will be discussed in Section 6.6.

6.6. Regulation of LH induction of CSCC enzyme by paracrine factors (other than IGF-I)

The list of paracrine factors which take part in regulation of P450scc gene expression in steroidogenic tissues is continuously growing. TNFα has been described to inhibit P450scc mRNA induction in mouse Leydig cells and mature rat Leydig cells (Xiong and Hales, 1993; Lin *et al.*, 1994b). In contrast, epidermal growth factor (EGF) stimulates rat and ovine P450scc gene expression (Trzeciak *et al.*, 1987b; Pestell *et al.*, 1995). Observed effects of activin on P450scc induction are ambiguous. In rat granulosa cells, regulation of P450scc induction by activin has been demonstrated to be dependent on the stage of follicular development. Initially activin is stimulatory in preovulatory nondifferentiated granulosa cells. However, in differentiated granulosa cells, after treatment of rats with recombinant FSH, activin becomes completely inhibitory (Miro *et al.*, 1995).

We have chosen to study long-term effects of Sertoli cell-conditioned medium (SCCM) on CSCC enzyme induction, since SCCM has previously been described to stimulate endogenous pregnenolone production in immature Leydig cells during short-term incubations (Grootenhuis *et al.*, 1990a). We have shown that this conditioned medium from Sertoli cells strongly inhibited LH induction of CSCC enzyme in cultured rat Leydig cells. This inhibitory effect, which was not due to cytotoxic effects on the interstitial cells, was dose-dependent and occurred at steps both upstream and downstream of the adenylyl cyclase complex in the signal transduction pathway (Chapter 5). A similar observation has been made by Zwain and Cheng (1994), who showed that a 21 kDa protein present in rat seminiferous tubule culture medium inhibited P450scc and P450c17 enzyme activities in mature rat Leydig cells. Since we observed that media obtained from different cell types also inhibited CSCC enzyme induction by LH, it appears that inhibitory factor(s) present in SCCM are not cell specific. It is possible that quite generally occurring (growth) factors such as TNFα and/or EGF have contributed to the observed inhibitory effect of SCCM (Chapter 5).

In this connection, it is important to put forward that specific biological effects of growth factors on cells depend very much on previous exposure to, and presence of other signalling molecules in the culture medium (Sporn and Roberts, 1988). For example, in rat 3T3 fibroblasts, transforming growth factor ß (TGFß) synergizes with platelet-derived growth factor (PDGF) to stimulate growth but inhibits growth induced by EGF *in vitro* (Roberts *et al.*, 1985). Evidence for a "context dependent" effect of exogenous factors has also been

provided for steroidogenic cells. In immature rat granulosa cells, EGF either inhibits or stimulates DNA synthesis, depending upon the presence or absence of FSH (Bendell and Dorrington, 1990). In immature rat Leydig cells, LH alone had little effect on DNA synthesis; however, there was a synergistic action of LH with insulin plus TGFα (Khan *et al.*, 1992). Thus, studies on the actions of growth factors on Leydig cell function *in vitro* are complicated by the fact that these actions depend on the presence of other signalling molecules in the culture medium, and factors, such as the interaction of the cultured Leydig cells with other cell types and extracellular matrix components. The presence and activities of these different factors, in turn, depend on the culture conditions chosen by the investigator. Leydig cell density can also affect the basal and hCG stimulated testosterone production (Murphy and Moger, 1982; Hedger and Eddy, 1990), which is probably also caused by different levels of various factors in the cell-conditioned medium.

Since the action of a given growth factor may depend on the presence of other (often unknown) growth factors in the culture medium, it appears that the interpretation of results from *in vitro* experiments, and the extrapolation of such data to Leydig cell function *in vivo*, is an error-prone process. In Chapter 5 we have been faced with the limits of extrapolation. The observed inhibition of LH induction of CSCC enzyme by SCCM in cultured immature rat Leydig cells is in sharp contrast with the data on stimulatory activities of Sertoli cells on Leydig cell function *in vivo*. Since our experiments have been carried out with isolated interstitial cells, cultured for 7 days in a chemically defined medium, containing 0.1% albumin as the only protein supplement, this environment of Leydig cells *in vitro* is totally different from the environment *in vivo*, where Leydig cells are engaged in a completely different context. *In vivo*, Leydig cells are subject to cell-cell and cell-matrix interactions which can affect their function (Skinner, 1991). For example, *in vivo* Leydig cells are organized in clusters and can communicate by gap junctions, which are able to transmit informative molecules including cAMP (Saez, 1994). Furthermore, the Leydig cells show close interaction with endothelial cells, and the interstitial extracellular matrix may also influence Leydig cell function. Altering the composition of the extracellular substratum affects shape and gene expression of 3βHSD in Leydig cells *in vitro* (Vernon *et al.*, 1991). To establish the real importance of a certain growth factor in the *in vivo* situation, specific gene knock-out experiments are indispensable. IGF-I has been shown to play a vital role in reproduction, since mice carrying a null mutation of the IGF-I gene exhibit a marked reduction in the size of reproductive organs, and are infertile (Baker *et al.*, 1993). In the future, observations on such knock-out mouse models will contribute to try to identify the role of other specific growth factors in reproductive function *in vivo*. The outcome of these studies, together with results obtained using *in vitro* experiments, will further elucidate the importance of growth factors in the regulation of the steroidogenic cascade in Leydig cells.

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Summary

In the male, androgens are responsible for initiation and maintenance of spermatogenesis and for development of male sex characteristics. Androgen production takes place in the Leydig cells, which are located in the interstitium of the testis. The rate at which testosterone is produced by Leydig cells is determined by different steps involved in the steroidogenic cascade:

1) cholesterol transport to the cholesterol side-chain cleavage (CSCC) enzyme, located at the inner mitochondrial membrane of Leydig cells;
2) the rate of conversion of cholesterol to pregnenolone, catalyzed by the CSCC enzyme;
3) conversion of pregnenolone into testosterone.

Finally, metabolism of testosterone is an additional factor that plays a role in determining the net amount of androgens that is produced.

The gonadotropic hormone luteinizing hormone (LH), produced by the pituitary gland, is an important regulator of Leydig cell steroidogenesis. This hormone stimulates androgen production via two different mechanisms: a rapid (non-genomic) effect and a slower (genomic) action. The short-term effect is mediated by enhanced cholesterol transport from the outer to the inner mitochondrial membranes. This occurs probably through a rapidly-turning-over protein, named StAR, which is thought to bring outer and inner mitochondrial membranes in contact and thereby facilitates cholesterol transport. The long-term effect is mediated by enhanced synthesis of the P450 enzymes involved in testosterone production. The induction of steroidogenic enzymes does not only depend on LH, but also paracrine factors can influence gene expression in these cells.

The aim of the experiments described in this thesis was to study regulation of Leydig cell steroidogenesis by endocrine and paracrine mechanisms, at the level of the CSCC enzyme, which is the rate-determining step for steroid production. The conversion rate of cholesterol into pregnenolone by the CSCC enzyme depends on the amount of substrate (cholesterol) available to the enzyme, and the amount of CSCC enzyme. These aspects are addressed in this thesis:

1) the role of sterol carrier protein 2 (SCP_2) in transporting cholesterol to the CSCC enzyme in short-term LH action, and
2) the role of LH and paracrine factors in CSCC enzyme induction.

SCP_2 is a protein which has been found in liver and in various steroidogenic tissues. SCP_2 enhances the transfer of cholesterol between isolated membranes and stimulates production of steroids by isolated mitochondria. From measurements of SCP_2 in cytosol fractions it has

been suggested that rapid stimulation of steroid production by LH is associated with intracellular redistribution of SCP_2 and enhanced cholesterol trafficking to the mitochondria. To further investigate this hypothesis, the cellular and subcellular localization of SCP_2 in testicular cells was investigated. Analysis of subcellular fractions of Leydig cells showed that SCP_2 is mainly membrane bound, whereas the distribution between membranes and cytosol was unaltered by LH. The absence of a correlation between changes in the intracellular distribution of SCP_2 and the rate of steroidogenesis, is not in support of an important role for SCP_2 in short-term regulation of steroidogenesis by LH. Moreover, although SCP_2 could be detected in Leydig and Sertoli cells, SCP_2 was found to be absent in germ cells. This absence is of interest, since germ cells, although not active in steroidogenesis, require cholesterol for membrane synthesis during development. This demonstrates that other mechanisms for cholesterol transport exist, and that SCP_2 may not be essential for intracellular cholesterol transport (Chapter 6).

Conditions for measurement of endogenous CSCC enzyme activity and CSCC enzyme induction were established in experiments described in Chapters 3 and 4. For this purpose, inhibitors of pregnenolone metabolism have been used. In the absence of such inhibitors, it would be required to measure different steroids, to determine total steroidogenic activity. With the use of the inhibitors, the rate-limiting step of the steroidogenic cascade could be identified, by comparing production of pregnenolone and testosterone at various rates of total steroid production. Under basal conditions and after LH stimulation, no difference was found between pregnenolone and testosterone production, indicating that the capacity of the enzymes in the endoplasmic reticulum under *in vitro* conditions is sufficient to convert all pregnenolone into testosterone. This implies that the rate-determining step, at the level of the CSCC enzyme, also is the rate-limiting step. However, at a high rate of steroidogenesis, in the presence of 22R-hydroxycholesterol as substrate for the CSCC enzyme, the capacity for conversion of pregnenolone was much lower than the rate of pregnenolone production, so that under these conditions the rate-limiting step for testosterone production is to be found at the conversion of pregnenolone into testosterone.

An assay for measurement of CSCC enzyme activity was developed, using inhibitors of pregnenolone metabolism and an excess of 22R-hydroxycholesterol as substrate to saturate the enzyme. A good correlation was found between 22R-hydroxycholesterol-supported pregnenolone production and the cellular cytochrome P450scc (P450scc) content, the latter estimated by Western blotting. Thus, the rate of sterol conversion into pregnenolone under these conditions of substrate saturation, reflects the amount of CSCC enzyme. Since a low amount of enzyme as measured using the enzymatic assay could not be detected with the Western blotting procedure, the enzymatic assay for quantification of CSCC enzyme was used to study induction of CSCC enzyme.

Synthesis of P450scc is regulated at the level of gene expression, and the cAMP-dependent signal transduction pathway, activated by LH, plays a key role in this process. However, paracrine factors are also important for CSCC enzyme induction. In this connection, we have investigated whether IGF-I and Sertoli cell-conditioned medium (SCCM) can modulate induction of CSCC enzyme by LH. The action of IGF-I was studied, since several reports have suggested an important stimulatory action of IGF-I on steroid production in testis and ovary. However, from our *in vitro* experiments, no evidence could be provided that IGF-I plays any role in LH induction of CSCC enzyme; neither the addition of IGF-I or analogs that do not bind to IGF-I binding proteins, nor immuno-inactivation of endogenous IGF-I action, were found to exert an effect on LH-induced CSCC enzyme activity. A stimulatory role of IGF-I on other steps of the steroidogenic cascade could not be excluded (Chapter 4). Addition of factors from Sertoli cells (SCCM) to cultured Leydig cells, caused almost complete inhibition of LH induction of CSCC enzyme (Chapter 5). This inhibition was not specific for Sertoli cell products, since conditioned media from other cells evoked similar effects. Part of the inhibitory effect obtained with SCCM can be caused by generally occurring (growth) factors. This inhibitory effect of Sertoli cell products *in vitro* is in contrast with a long-term stimulatory effect of Sertoli cells on Leydig cell steroidogenesis *in vivo*.

In Chapter 6, it is discussed that studies on the action of growth factors on Leydig cell function *in vitro* are complicated by the fact, that such *in vitro* effects depend very much on exposure of the cells to other signalling molecules in the culture medium, even when a chemically defined culture medium is used (context dependent effects of growth factors). In addition, cell-cell and cell-matrix interactions play a role. The presence of different factors depends on the culture conditions chosen by the investigator. Even relatively small differences in culture conditions may influence the sensitivity of Leydig cells to particular growth factors. A difference in the context of Leydig cells *in vitro* and *in vivo*, explains the difficulty of extrapolation of *in vitro* results to *in vivo* Leydig cell function.

Samenvatting

Het mannelijk geslachtshormoon testosteron speelt een belangrijke rol bij de spermatogenese en de ontwikkeling van de overige mannelijke geslachtskenmerken. De Leydig cellen in de testikels produceren dit hormoon. De hoeveelheid geproduceerd testosteron is onder meer afhankelijk van de hoeveelheid substraat (cholesterol) en de activiteit van een aantal enzymen. De serie verschillende reacties die betrokken zijn bij de omzetting van cholesterol in testosteron wordt ook wel de steroïdogene cascade genoemd:

1) cholesterol transport naar het cholesterol zijketen-splitsend (CSCC) enzym, dat zich bevindt in het binnenmembraan van de mitochondriën;
2) omzetting van cholesterol in pregnenolon door het CSCC enzym;
3) omzetting van pregnenolon in testosteron in het endoplasmatisch reticulum.

Uiteindelijk wordt de totale hoeveelheid androgenen die door Leydig cellen wordt geproduceerd ook bepaald door de omzetting van testosteron in testosteron-metabolieten.

Luteiniserend hormoon (LH), dat geproduceerd wordt door de hypofyse, stimuleert de steroïdhormoon produktie in Leydig cellen via een snel en een langzaam mechanisme. Het snelle mechanisme berust op een verhoogd cholesterol transport naar het binnenmembraan van de mitochondriën. Het langzame mechanisme wordt bereikt door een toename van de hoeveelheid van steroïdogene enzymen.

Doel van dit onderzoek was om op het niveau van het CSCC enzym de regulatie van de steroïdhormoon produktie in Leydig cellen nader te bestuderen, met name:

1) de rol van het eiwit sterol carrier protein 2 (SCP_2) in het transport van cholesterol naar het CSCC enzym;
2) het effect van paracriene factoren op de produktie van het CSCC enzym.

In Hoofdstuk 1 wordt achtergrondinformatie gegeven over de steroïdogene cascade, mechanismen van cholesterol transport, en de effecten van LH en paracriene factoren op regulatie van de CSCC enzymactiviteit in Leydig cellen.

In vitro stimuleert SCP_2 transport van cholesterol tussen membranen en de mitochondriale steroïdproduktie. Een bestaande hypothese was dat stimulatie van steroïdproduktie door LH veroorzaakt wordt door een verandering in de intracellulaire localisatie van SCP_2, waardoor het cholesterol transport naar de mitochondriën zou worden versneld. In Hoofdstuk 2 wordt beschreven dat SCP_2 in Leydig cellen hoofdzakelijk in membraan-fracties en niet in de cytosol is aangetoond. Omdat deze verdeling niet werd beïnvloed door de activering van Leydig cellen door LH, is het onwaarschijnlijk dat SCP_2 een belangrijke rol speelt in de snelle stimulatie van de steroïdproduktie door LH. SCP_2 werd gevonden in Leydig en Sertoli

cellen, maar niet in germinale cellen. De afwezigheid van SCP_2 in germinale cellen is interessant omdat deze cellen tijdens hun ontwikkeling waarschijnlijk veel cholesterol gebruiken voor de vorming van membranen. Kennelijk is SCP_2 niet essentieel voor transport van cholesterol, en kan cholesterol ook via andere transportsystemen in de cel worden verplaatst.

De CSCC enzymactiviteit in geïsoleerde Leydig cellen kan eenvoudig gemeten worden door gebruik te maken van verbindingen die het pregnenolon metabolisme volledig remmen (Hoofdstuk 3). Hierdoor wordt de produktie van verschillende steroïden gereduceerd tot de produktie van uitsluitend pregnenolon. De geproduceerde hoeveelheid pregnenolon is dan een directe maat is voor de enzymactiviteit. Door de hoeveelheid pregnenolon in aanwezigheid van, en de hoeveelheid testosteron in afwezigheid van de remmers te vergelijken kon de snelheidsbeperkende stap in de steroïdogene cascade geïdentificeerd worden. Het bleek dat deze stap zowel op het niveau van het CSCC enzym kan liggen, als verderop in het traject. Dit laatste was het geval bij maximale stimulatie (na toevoeging van het exogene substraat 22R-hydroxycholesterol) waardoor de enzym capaciteit in het endoplasmatisch reticulum beperkend wordt voor de produktie van testosteron. Geconcludeerd werd dat de CSCC enzymactiviteit beter aangeduid kan worden als de snelheidsbepalende stap (in plaats van snelheidsbeperkende stap), aangezien de omzetting van cholesterol in pregnenolon bepalend is voor de snelheid waarmee steroïden kunnen worden gevormd.

De produktie van het CSCC enzym wordt gereguleerd op het niveau van genexpressie en er bestaat geen twijfel over dat cyclisch AMP, de tweede boodschapper geïnduceerd door LH, hierin een sleutelrol vervult. Er zijn echter vele aanwijzingen dat paracriene factoren ook belangrijk zijn voor inductie van het CSCC enzym. In Hoofdstuk 4 zijn effecten van IGF-I en Sertoli cel-geconditioneerd medium (SCCM) onderzocht. IGF-I werd gekozen omdat veel studies een stimulerend effect van IGF-I op steroïdhormoonproduktie laten zien. De inductie van CSCC enzymactiviteit werd gemeten met een CSCC enzym assay, waarbij een overmaat van 22R-hydroxycholesterol werd gebruikt, samen met remmers van het pregnenolon metabolisme. De pregnenolon produktie correleerde goed met de hoeveelheid CSCC enzym in de cel. Het bleek dat IGF-I in gekweekte Leydig cellen van immature ratten niet betrokken was bij inductie van CSCC enzym door LH. SCCM remde de inductie van het CSCC enzym door LH bijna volledig (Hoofdstuk 5). Dit effect was niet specifiek voor Sertoli cel factoren, aangezien medium van andere cellen dezelfde remming teweeg bracht. Dit suggereert dat de remming van SCCM gedeeltelijk veroorzaakt wordt door veel voorkomende (groei)factoren. Onderzoek naar effecten van groeifactoren op het functioneren van Leydig cellen is gecompliceerd (Hoofdstuk 6). De werking van groeifactoren is afhankelijk van eerdere blootstelling aan groeifactoren, en de aanwezigheid van andere signaalmoleculen. Bij *in vitro* onderzoek kunnen kleine verschillen in kweekcondities het effect van een bepaalde

groeifactor beïnvloeden. Deze "contextafhankelijkheid" heeft betekenis voor de interpretatie van *in vitro* resultaten en de extrapolatie van deze gegevens naar het functioneren van Leydig cellen *in vivo*, waar de omgeving van Leydig cellen sterk verschilt van de omgeving *in vitro*. Als de steroïdogenese in Leydig cellen wordt vergeleken met een zeilwedstrijd, dan zullen de prestaties van het schip (steroïdhormoonproduktie) afhankelijk zijn van de aanwijzingen van de kapitein (LH) maar ook van de vaardigheden van de bemanning (lokale factoren) en vooral van de weersomstandigheden (context). Windsnelheid, windrichting, golfslag en koers bepalen mede de interacties tussen kapitein en bemanning. Het is belangrijk om vast te stellen in welke mate de context het functioneren van Leydig cellen beïnvloedt, teneinde extrapolatie van *in vitro* resultaten naar de *in vivo* situatie mogelijk te maken.

Dankwoord

Graag wil ik iedereen bedanken die een bijdrage aan dit proefschrift heeft geleverd. Allereerst mijn co-promotor Focko Rommerts, met wie ik vaak aan tafel heb gezeten. Met bewondering heb ik je enthousiasme gezien als we tijdens de labbabbels samen de resultaten bespraken. Zowel verwachte als onverwachte resultaten werden door jou van alle kanten belicht. Het schrijven van artikelen en dit proefschrift ging trager dan we wellicht hadden gewenst. Maar, ondanks onze lange discussies over de rode draad, "waar wil je naar toe", en "wat is je boodschap", is het proefschrift toch tot stand gekomen.
Anton Grootegoed, mijn promotor, wil ik hartelijk bedanken voor de snelle beoordeling van het manuscript, de correcties en suggesties, waardoor de inhoud zeer verbeterd is.
Professor Degenhart en Professor Smals wil ik bedanken voor hun bereidheid om het manuscript te beoordelen. Ook wil ik graag Frank de Jong bedanken, niet alleen als lid van de promotie-commissie, maar ook omdat hij een belangrijke rol gespeeld heeft in de afrondingsfase van dit boekje.
Francisca Flinterman, heel hartelijk bedankt voor de fantastische samenwerking. Moeiteloos konden wij van elkaar de experimenten overnemen, dankzij optimaal overleg. Als collega heb ik van jou geleerd dat 1+1 ook 3 kan betekenen op het werk. Tevens ben je een vriendin voor altijd, en daarom kan het niet anders dan dat jij paranimf bent.
Jos Hoogerbrugge, met name in het begin van het onderzoek was jij mijn steun en toeverlaat. Ik ben je dankbaar dat jij mij altijd hebt willen helpen.
Alle overige collega's van E&V, heel hartelijk bedankt voor de prettige samenwerking.
Karel Wirtz, Bernadette van Ossendorp, Paul van Heusden en José van Beckhoven, bedankt voor de fijne samenwerking bij de SCP_2-experimenten.
Katja Teerds, ook jij bedankt voor je hulp bij het SCP_2-werk en vooral dank voor het advies en de suggesties bij de uitvoering van de experimenten.
De dames-club: Ingrid, Cobie, Marianna, Ria, Petra, Francisca en Rosemarie. Vooral de lunch en de sigaret brachten ons samen. Heerlijke ontspanning in soms spannende tijden.
Ingrid, mijn maatje in het kwaad (van der Kleij, personal communication), bedankt voor alle opbeurende gesprekken, én voor het tekenen van de figuren in Hoofdstuk 1.
Mijn zussen wil ik bedanken, Katinka voor het schilderen van de omslag, Hanneke voor het paranimf zijn, en Corine voor het full-time moederen, hetgeen een uitkomst kan betekenen voor een gestresste promovendus-moeder.
Mamma, pappa, pa en ma Verseput, familie en vrienden. Een dochter, familie-lid of

vriendin hebben die al zo lang bezig is met het schrijven van een proefschrift, is lang niet altijd even gezellig. Verjaardagen, kraamvisites, feesten, of gewoon, zomaar even buurten, is er te vaak bij ingeschoten. Bedankt voor jullie geduld en voortdurende support, tot gauw!

Hubert, Maddy en Ivo, het einde van dit boekje is voor ons een nieuw begin.

Curriculum vitae

De schrijfster van dit proefschrift werd op 5 december 1962 geboren in Vught. Na het behalen van het Gymnasium ß diploma aan het Fons Vitae te Amsterdam in 1981 werd begonnen met de studie Scheikundige Technologie aan de Technische Universiteit te Delft, gevolgd door de studie Geneeskunde aan de Universiteit van Amsterdam in 1982. In 1986 werd het doctoraalexamen behaald en tevens gestart met een wetenschappelijke stage aan de Cambridge University U.K. (Dr. A.D. Tait), met als onderwerp de regulatie van de steroïd produktie in de gonaden.
Vanaf mei 1987 was zij werkzaam als assistente in opleiding (AIO) in de Vakgroep Endocrinologie & Voortplanting aan de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het onderzoek beschreven in dit proefschrift werd uitgevoerd. In november 1991 werd begonnen met de 2e fase van de studie Geneeskunde (co-assistentschappen) aan de Katholieke Universiteit Nijmegen en in mei 1994 werd het artsexamen behaald. In 1993 en in 1995 werden Maddy en Ivo geboren. Sinds juli 1995 werkt ze op de medische afdeling van Searle (Monsanto Nederland B.V.) te Maarssen, op het gebied van de female healthcare en de cardiologie.