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Changes of testicular cholesteryl ester hydrolase activity in experimentally cryptorchid rats

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Summary. A simple and reliable method was developed to determine the neutral cholesteryl ester hydrolase (CEH) activity in rat testes, using cholesteryl-[1-^{14}C]-oleate as substrate. The activity was due to a soluble enzyme present in the cytoplasm of predominantly Sertoli cells, which could be shown after depleting the testes of Leydig cells with ethane dimethyl sulphonate. This treatment also revealed that the loss of CEH activity in abdominal testes of experimentally cryptorchid rats takes place in the Sertoli cells. In prepubertal rats made unilaterally cryptorchid at birth, the CEH activity was significantly higher in the abdominal than in the scrotal testes at 16 days of age. This is earlier than any previously described biochemical change and coincides with, or may even precede, the earliest morphological changes which are accumulation of lipid droplets in the Sertoli cells. The testicular CEH activity then decreased to 30 days of age in the abdominal testes, whereas the activity increased in the contralateral, scrotal testes. When adult rats were made unilaterally cryptorchid for 24 h, the CEH activity decreased rapidly in the abdominal testes. These results suggest that a derangement in cholesteryl ester metabolism is an early event in the pathogenesis of testicular degeneration in cryptorchidism.

Keywords: testis; Sertoli cell; cryptorchidism; enzyme activity; assay; ethane dimethyl sulphonate

Introduction

Several studies have shown that the levels of testicular lipids change after treatments that impair spermatogenesis (Davis & Coniglio, 1967; Fleeger *et al.*, 1968a, b). One such condition is cryptorchidism, which is generally thought to produce its degenerative effect by elevation of testicular temperature (VanDemark *et al.*, 1970; Blackshaw, 1977). However, the mechanisms by which temperature damages testicular function remain obscure.

Experimental cryptorchidism can be induced in newborn rats by preventing testicular descent (Bergh *et al.*, 1978). In this model, called primary experimental cryptorchidism, the earliest morphological change in the abdominal testes is an increased concentration of lipid droplets in the cytoplasm of Sertoli cells. This is noted at 16 days of age, the first time at which a temperature difference between the descending testis and the abdomen can be measured. At this age, no structural differences between germinal cells in the abdominal and scrotal testes are observed (Bergh, 1983). At 20 days the lipid accumulation in Sertoli cells is more pronounced, and degenerating germinal cells are noted (Bergh, 1983). With time, these differences become more pronounced, and the degeneration of the abdominal testes results in a difference in testicular weight, and decreased Leydig cell testosterone secretion (Bergh & Damber, 1978; Bergh & Helander, 1978; Bergh, 1983).

When cryptorchidism is induced in adult rats (secondary experimental cryptorchidism), an increased lipid concentration in the abdominal testes has been demonstrated by histochemical techniques (McEnery & Nelson, 1953). Direct determinations have shown that the cholesteryl ester

concentration is increased (Johnson *et al.*, 1971). By analogy, the lipid accumulation in primary experimental cryptorchidism may also be due, at least in part, to cholesteryl esters. Cholesteryl esters serve as a storage form of cholesterol, which can be drawn upon for steroid hormone biosynthesis (Bartke *et al.*, 1973; Moyle *et al.*, 1973; Saksena *et al.*, 1974). Cholesteryl ester hydrolase (EC 3.1.1.13) is therefore a potential regulator of steroidogenesis (Rommerts *et al.*, 1980) and of lipid composition in the testes. There have been reports that the activity of this enzyme is decreased in secondary cryptorchidism, and, moreover, that this enzyme is directly inactivated at abdominal temperatures (Durham & Grogan, 1982). The aim of this study was to develop a simple and reliable assay for cholesteryl ester hydrolase activity in the testes, and to follow the activity in prepubertal unilaterally cryptorchid rats.

Materials and Methods

Animal and tissue preparations

Experimental cryptorchidism. The lower part of the gubernaculum testis was cut unilaterally in newborn Sprague–Dawley rats to prevent subsequent testicular descent and scrotal development, which normally start within 1 week of birth and are completed at about 35 days of age (Bergh & Helander, 1978). The operated rats were raised together with their mothers, and artificial daylight was provided for 12 h per day. The animals were used in experiments when 16, 20 or 30 days or 3 months old. Experimental unilateral cryptorchidism was also induced in 3-month-old rats by suturing one testis to the dorsolateral wall, as previously described (Bergh & Damber, 1984). The animals were used for experiments 24 h later.

Treatment with ethane dimethyl sulphonate (EDS). Adult, 3-month-old control and primary unilaterally cryptorchid rats were treated with 30 mg EDS/ml dimethyl sulphoxide–H_2O (1:3 v/v), administered by intraperitoneal injection (75 mg/kg body weight). Other rats were injected with the vehicle alone. After 3 days the animals were used in experiments. EDS treatment, by mechanisms unknown, leads to total depletion of Leydig cells from the testis (Jackson & Morris, 1977; Morris *et al.*, 1986). To verity that this was the case also in our intact and unilaterally cryptorchid rats, testes from EDS-treated animals were fixed in Bouin's solution and embedded in methacrylate resin (Histo-Resin, LKB, Stockholm, Sweden).

The animals were killed and both testes were removed, freed from the epididymis and the surrounding fat pads, and were immediately frozen and stored at −80°C until prepared for assay. Control experiments showed that freezing of the tissue did not change the activity of the cholesteryl ester hydrolase.

Each testis was rinsed and weighed. It was then homogenized in 10 parts ice-cold 20 mM-Tris–HCl buffer pH 7·4, 0·15 M-NaCl, 1 mM-EDTA, in a tissue grinder with a loose Teflon pestle. This preparation was centrifuged for 15 min in a Beckman Minifuge at 4°C to remove cell debris, or centrifuged at 100 000 *g* in a Beckman ultracentrifuge for 2 h at 4°C. The supernatant (S-100) was recovered, and extracted at 10°C with *n*-hexane (1:1 v/v) to remove endogenous lipids. Such fat-depleted S-100 preparations were used for enzyme characterization.

Protein content in the aqueous layer was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Enzyme assay and incubation procedure

The activity of cholesteryl ester hydrolase was measured as release of fatty acid from labelled cholesteryl ester. For this, cholesteryl oleate was chosen since it is the principal ester of cholesterol in the testis (Saksena *et al.*, 1974). To prepare a substrate emulsion, 35 µg cholesteryl-[^{14}C]-oleate in toluene were added to 1 ml 0·10 M-sodium phosphate buffer pH 7·4, 0·15 M-NaCl, 1 mM-EDTA. After evaporation of the toluene the mixture was sonicated for 10 min (5 min at room temperature, 5 min on ice) in a Branson Sonifier type B_{12}. To 100 µl of this mixture was added enzyme source and/or corresponding buffer to make a total volume of 200 µl. The final pH was 7·4, the salt concentration was 0·15 M.

The incubations were carried out with shaking in a waterbath at 25°C. The reaction was stopped and the labelled free fatty acids were isolated by a one-step liquid-liquid partition system as described by Belfrage & Vaughan (1969), but with 0·1 M-potassium carbonate buffer pH 10·5.

All incubations were carried out in triplicate. The enzymic activity is expressed in mU, equivalent to nmol cholesteryl oleate hydrolysed per min at 25°C unless otherwise indicated.

Chemicals

Cholesteryl-[^{14}C]-oleate (sp. act. 56 mCi/mmol) was obtained from Amersham International (Amersham, Bucks, UK). Bovine serum albumin (fraction V) and diethyl-*p*-nitrophenyl-phosphate (DNP) were from Sigma Chemical Co.

(St Louis, MO, USA). Phenyl methyl sulphonyl fluoride (PMSF) was from Serva Feinbiochemica (Heidelberg, West Germany). EDS was kindly donated by Dr F. F. G. Rommerts (Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands).

Results

Characteristics of cholesteryl ester hydrolase activity

The enzyme activity showed a rather broad pH optimum centered around pH 7·4 (Fig. 1a). The reaction rate increased with the substrate concentration and plateaued at about 27 μM-cholesteryl oleate (Fig. 1b). Larger amounts of substrate did not inhibit the reaction. The data in Fig. 1(b) fit the Michaelis–Menten equation, and yield an apparent K_m of 13 μM. The rate of hydrolysis increased linearly with the amount of enzyme source at least up to 250 μg protein/200 μl incubation mixture (Fig. 1c), and the reaction was linear with time for at least 90 min (Fig. 1d).

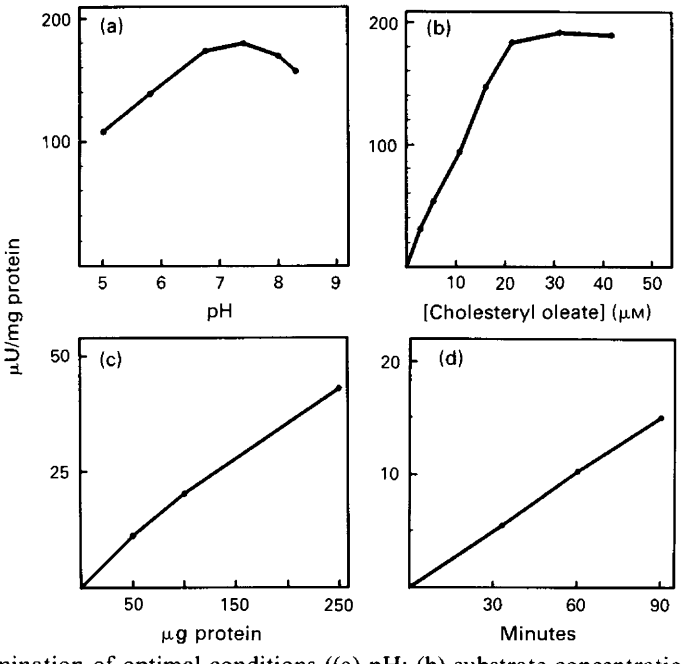

Fig. 1. Determination of optimal conditions ((a) pH; (b) substrate concentration; (c) protein concentration; (d) incubation time) for the assay of neutral cholesteryl ester hydrolase activity of rat testicular tissue. Except for the factors being varied, each incubation was carried out as described in 'Materials and Methods': 1 mU is equivalent to 1 nmol cholesteryl oleate hydrolysed/min at 25°C.

We therefore chose the following standard conditions for the assay: 5–10 μl of enzyme source, and 3·5 μg labelled cholesteryl oleate, in a total volume of 200 μl. Incubation was for 30 min at 25°C.

The activity of the enzyme was inhibited more than 98% by DNP and by PMSF. A 50% inhibition required a concentration of about 140 nM and 40 μM respectively (Fig. 2). PMSF is a serine hydrolase inhibitor that has previously been found to inhibit the activity of cholesteryl ester hydrolase in some other tissues (Fredrikson *et al.*, 1981).

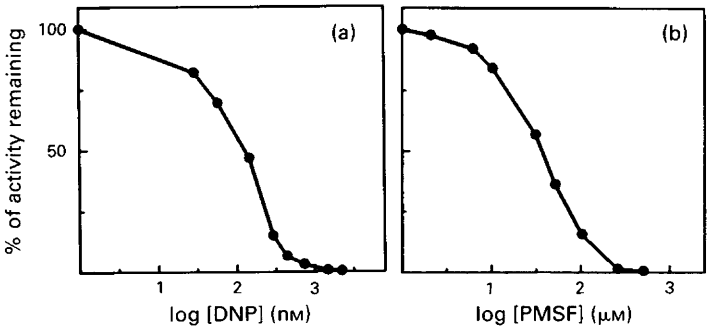

Fig. 2. Effect of serine-hydrolase inhibitors (a, DNP; b, PMSF) on testicular cholesteryl ester hydrolase activity. Incubations were carried out in triplicate at the indicated concentrations.

Subcellular localization of the cholesteryl ester hydrolase activity, and effects of physical treatments

More than 90% of the activity in testicular homogenates remained in the supernatant after centrifugation for 2 h at 100 000 *g*. Centrifugation in a Beckman Minifuge for 15 min was sufficient to remove cell debris, and was therefore used for the studies of changes of enzyme activity in prepubertal cryptorchidism. Homogenates prepared in Tris or in phosphate buffer gave the same activity. When homogenates prepared in phosphate buffer were frozen, much of their cholesteryl ester hydrolase activity was lost. Homogenates in Tris buffer, or supernatants prepared from these homogenates could, however, be frozen at −80°C without loss of enzyme activity.

Effects of cryptorchidism on cholesteryl ester hydrolase activity

Secondary unilateral cryptorchidism in adult rats resulted in loss of about 30% of the enzyme activity from the abdominal testis in 24 h (Table 1), but testicular weight was unaffected. Rats made unilaterally cryptorchid at birth (primary cryptorchidism) had up to 75% lower activity in the abdominal testes compared to the contralateral scrotal testes, when examined at adult age (Table 1; Fig. 3).

Table 1. Cholesteryl ester hydrolase activity (μU/mg protein) in scrotal and abdominal testes from adult, primary and secondary unilaterally cryptorchid rats

	Secondary cryptorchidism (N = 3)	Primary cryptorchidism (N = 3)	Primary cryptorchidism EDS-treated (N = 5)
Scrotal testis	166 ± 13·3	167 ± 14·9	151 ± 18·4
Abdominal testis	119 ± 14·9	36·4 ± 5·1	3·8 ± 0·5

Values are mean ± s.e.m.

Treatment with EDS resulted in complete loss of Leydig cells, but no apparent morphological changes were noted in the tubules. In scrotal testis more than 90% of the hydrolase activity remained, indicating that the activity was located mainly in the Sertoli cells (Table 1). In contrast, treatment of adult (primary) unilaterally cryptorchid rats with EDS resulted in almost total disappearance of the hydrolase activity of the abdominal testes (Table 1).

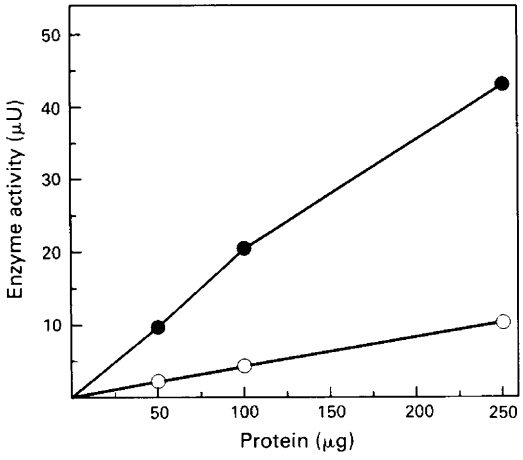

Fig. 3. Effect of primary experimental cryptorchidism on activity of cholesteryl ester hydrolase. Incubations were carried out in triplicate, and the activity is expressed in μU.

When cholesteryl ester hydrolase activity was studied in primary unilaterally cryptorchid rats of increasing age, at 16 days the activity was significantly higher in the abdominal testes than in the corresponding scrotal testes. At 20 days the activity in the abdominal testes was about 30% lower than in the contralateral scrotal testes, and at 30 days it was 36% lower (Fig. 4a; Table 2).

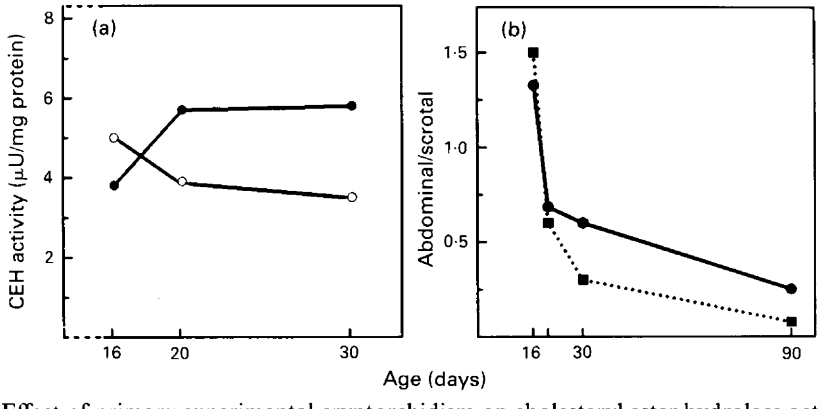

Fig. 4. Effect of primary experimental cryptorchidism on cholesteryl ester hydrolase activity in prepubertal rats. (a) Enzyme activity in scrotal (●) and abdominal (○) testes; (b) enzyme activity in the scrotal testes divided by the activity in the abdominal testes. The ratio is plotted both for the activities per mg protein (●), and for the activities per whole tissue (■).

The testicular weight also changed as a function of time (Table 3). At 16 days the weight of the abdominal testes was slightly higher (see also Bergh *et al.*, 1987), whereas at 20 days, or later, it was lower than for the scrotal testes. These differences in weight compound the differences in enzyme activity per g tissue (Fig. 4a), and differences in total organ activity were even more pronounced (Fig. 4b).

Table 2. Cholesteryl ester hydrolase activity (μU/mg protein) in scrotal and abdominal testes in unilaterally cryptorchid prepubertal rats

	16 days (N = 5)	20 days (N = 5)	30 days (N = 5)
Scrotal testis	3·8 ± 0·38	5·7 ± 0·50	5·8 ± 0·26
Abdominal testis	5·0 ± 0·54*	3·9 ± 0·24*	3·5 ± 0·30*

Values are mean ± s.d.
*Significantly different from scrotal testis ($P < 0·05$).

Table 3. Testicular weight (mg) for scrotal and abdominal testes in prepubertal rats

	16 days (N = 5)	20 days (N = 5)	30 days (N = 5)
Scrotal testis	57 ± 3	145 ± 15	569 ± 40
Abdominal testis	64 ± 4	129 ± 20	274 ± 15*

Mean ± s.d.
*Significantly lower than value for the scrotal testis ($P < 0·05$) according to Wilcoxon's signed value test for paired observations.

Discussion

The most obvious function for a testicular cholesteryl ester hydrolase would be to mobilize cholesterol from deposits in lipid droplets for steroid hormone biosynthesis. Hormone-sensitive hydrolases with this function are present in the adrenals and the ovaries (Mori & Christensen, 1980; Cook *et al.*, 1981, 1983), and preliminary studies suggest that testicular cholesteryl ester hydrolases share several properties with those enzymes (A. M. Hoffmann & G. Fredriksson, unpublished). Previous studies have shown that Leydig cells and Sertoli cells have cholesteryl ester hydrolase activity (Durham & Grogan, 1982), and in the present study we observe a marked increase in enzyme activity during sexual maturation, possibly due to gonadotrophin stimulation since both LH and FSH may stimulate this enzyme activity (Durham & Grogan, 1984).

In this study the measured activity could have been in Leydig and Sertoli cells. Treatment with EDS resulted in loss of less than 10% of the total activity in scrotal testes. It is therefore likely that most of the activity measured in normal testis represents Sertoli cells. This is in accord with the fact that Leydig cells in normal testes constitute only 2–4% of the total testicular mass (Bergh & Damber, 1978; Mori & Christensen, 1980), and large changes in Leydig cells are therefore needed to affect the total cholesteryl ester hydrolase activity.

Since the function of Sertoli cells but not that of Leydig cells is influenced during the earliest phase of experimental cryptorchidism (Bergh & Damber, 1978; Bergh, 1983), it is likely that the changes observed in this study primarily reflect changes in Sertoli cell hydrolase activity. In fact, treatment of primary cryptorchid rats with EDS resulted in an almost complete loss of cholesteryl ester hydrolase activity in the abdominal testis. This suggests that the enzyme activity in these testis

was located primarily in the Leydig cells and that cryptorchidism had decreased this activity in Sertoli cells by more than 90%.

Spermatogenesis cannot take place at normal body temperature, but the cellular mechanisms behind this remain unknown. Some investigators claim that the primary reason is that the function of some types of germ cells, particularly early spermatids and early primary spermatocytes, is inhibited at abdominal temperature (Lee & Fritz, 1972; Nakamura & Hall, 1978; Hall *et al.*, 1985). Other investigators suggest that the primary target cell is the Sertoli cell, since Sertoli cell protein secretion *in vivo* and *in vitro* is inhibited by abdominal temperatures (Hagenäs *et al.*, 1978; Rommerts *et al.*, 1980; Steinberger, 1981). One early change that can be observed in cryptorchid testes is accumulation of lipid droplets in the cytoplasm of Sertoli cells, when experimental cryptorchidism is induced in immature or in adult rats (Bergh, 1981, 1983; Bergh & Damber, 1984). This lipid accumulation is probably caused by a direct effect of cryptorchidism on Sertoli cell function since lipid accumulation is also noted early in experimentally cryptorchid testes that do not contain germ cells, e.g. because of irradiation *in utero* (Bergh, 1981).

Durham & Grogan (1982) reported that Sertoli cells contain a cholesteryl ester hydrolase which is unstable at abdominal temperatures, and that the activity is decreased in the abdominal testes in secondary cryptorchidism. The present observation of a highly reduced hydrolase activity in experimentally cryptorchid testes is in agreement with their results, and with our observation of an increased lipid content in abdominal Sertoli cells (Bergh, 1981, 1983). However, the earliest change noted in this study was an increased cholesteryl ester hydrolase activity in the abdominal testes at 16 days of age. At this age Sertoli cells in some stages of the spermatogenic cycle contain an increased amount of lipid droplets, but apart from this, there are no morphological signs of Sertoli or Leydig cell malfunction (Bergh, 1983). On the contrary, tubule lumen formation, which is initiated by Sertoli cell fluid secretion, occurs earlier in the abdominal than in the scrotal testes, and the weight of the abdominal testis is slightly increased at this age (Bergh, 1983; Bergh *et al.*, 1987). From 4 days later, degenerating germ cells are noted. The endoplasmic reticulum is dilated and the secretion of oestrogen, androgen binding protein and probably also inhibin are decreased (Bergh, 1983; Bergh *et al.*, 1984). Moreover, as shown in this study the cholesteryl ester hydrolase activity is subnormal.

It appears that the earliest changes noted in abdominal testes could be an increased rather than decreased Sertoli cell function, raising the interesting possibility that the primary abnormality in abdominal testes could be some type of overactivity, which subsequently leads to degenerative changes. Our observations suggest that the initial increase and later decrease in cholesteryl ester hydrolase activity may not be a direct effect of temperature on this enzyme but probably secondary to other yet unknown mechanisms. Perhaps the synthesis or uptake of lipids is increased in the Sertoli cells of abdominal testes at 16 days of age and the increased enzyme activity could be a secondary response to this phenomenon. The increased enzyme activity at 16 days could also reflect an increase in Leydig cell activity, overshadowing the early decrease in Sertoli cells.

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