Evaluation of cholesteryl ester transfer in the seminiferous tubule cells of immature rats *in vivo* and *in vitro*

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Sertoli cells and germ cells are separated from the interstitial blood capillaries by an extracellular matrix and the peritubular cells, which constitute a barrier to the movement of plasma lipoproteins. The present study was undertaken to evaluate *in vivo* and *in vitro* the high density lipoprotein (HDL) cholesteryl ester transfer from plasma to seminiferous tubule cells in the testis of 30-day-old rats. Firstly, the transfer of HDL cholesteryl oleate from plasma to testicular compartments was evaluated and, secondly, the role of apolipoproteins A-I and E in the uptake of cholesteryl ester by Sertoli cells was investigated. At 2 h after the administration of HDL reconstituted with [³H]cholesteryl ester, dimyristoyl phosphatidylcholine and apolipoproteins, the tissue space in the interstitial cells ($740 \pm 60 \mu\text{l g}^{-1}$ cell protein) was fourfold higher than that in the seminiferous tubule cells ($170 \pm 10 \mu\text{l g}^{-1}$). Sertoli cells were isolated and incubated with [³H]cholesteryl ester HDL reconstituted with apolipoprotein A-I or E to evaluate the mechanisms of cholesteryl ester influx. At the same apolipoprotein concentration ($50 \mu\text{g apolipoprotein ml}^{-1}$ medium), the uptake of [³H]cholesteryl oleate from phospholipid–apolipoprotein E vesicles was twofold higher than that with phospholipid–apolipoprotein A-I vesicles. The presence of heparin reduced the uptake of cholesteryl ester from apolipoprotein E vesicles but not with apolipoprotein A-I vesicles, indicating that uptake of apolipoprotein A-I vesicles via a secretion of apolipoprotein E by the cells themselves was not involved. These results demonstrate that plasma lipoprotein cholesterol is able to cross the testis lamina propria and that Sertoli cells take up cholesteryl ester for seminiferous tubule cell metabolism mainly via an apolipoprotein E pathway.

Introduction

The transport of hydrophobic constituents from plasma to interstitial cells is currently attributed to albumin and lipoproteins. Lipoproteins play a major role in the transport of cholesterol, glycerol esters, vitamins, steroid esters (Lavallée *et al.*, 1996), lipophilic xenobiotics such as carcinogens (Polyakov *et al.*, 1996) and drugs (Rifai *et al.*, 1996). Low density lipoproteins (LDL) via the apolipoprotein B-E receptor and high density lipoproteins (HDL) via the class B type I scavenger receptor (SR-BI) deliver cholesterol to peripheral tissues for membrane renewal and steroid hormone production (Schreiber *et al.*, 1982; Acton *et al.*, 1996). The relative contribution of lipoprotein cholesterol to steroid production varies according to steroidogenic cells. In adrenocortical cells, a system of fenestrated capillaries allows the easy passage of LDL and HDL and the high expression of SR-BI receptors supports the hypothesis that plasma lipoprotein is the preferential source of cholesterol for steroidogenesis (Landschulz *et al.*, 1996; Temel *et al.*, 1997; Azhar *et al.*, 1998).

In the testis, the capillary wall constitutes the first barrier for the transfer of plasma lipoproteins to the interstitium and the Leydig cells. Testicular capillaries are characterized by a continuous layer of endothelial cells, which are connected by tight junctions (Mayerhofer *et al.*, 1989). A system of plasmalemmal vesicles allows the passage of LDL and HDL to the interstitial compartment. Sertoli cells and germ cells are separated from the interstitial blood capillaries by an extracellular matrix and the peritubular cells, which constitute the lamina propria. Serum albumin administered to rats penetrates the lamina propria and accumulates in the Sertoli cells (Everett and Simmons, 1958; Christensen *et al.*, 1985). The transport of lipoprotein constituents from plasma to seminiferous tubule cells has not been demonstrated. In the ovary, which presents a similar basal membrane, the blood–follicle barrier behaves like a molecular sieve which allows the passage of HDL in inverse proportion to the molecular mass (Le Goff, 1994). Therefore, the ability of lipoproteins to penetrate the testis lamina propria should be similar.

Sertoli cells extend from the basement membrane to the tubular lumen. Tight junctions exclude the diffusion of HDL from interstitial medium to the germ cells and transfer of

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hydrophobic constituents such as cholesteryl ester involves an endocytic pathway through the Sertoli cell membrane. The aim of the present study was to investigate: (i) the *in vivo* transfer of HDL cholesteryl ester from plasma to testicular compartments in 30-day-old rats; and (ii) the role of apolipoproteins A-I and E in the uptake of cholesteryl ester by Sertoli cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and trypsin (USP grade) were from Gibco (Cergy Pontoise); collagenase dispase was from Boehringer-Mannheim (Meylan). Soybean trypsin inhibitor, bovine pancreas deoxyribonuclease (DNase), hyaluronidase (type I-S), dimyristoyl L- α -phosphatidylcholine and cholesteryl myristate were from Sigma (Saint-Quentin Fallavier). Centrisart I with 20 kDa cut-off ultrafilter was from Sartorius (Palaiseau). (1 α ,2 α (n)-³H)cholesteryl oleate (1.78 TBq mmol⁻¹) was from Amersham (Les Ulis) and all other reagents were of analytical grade.

[³H]cholesteryl ester reconstituted HDL

Labelled reconstituted HDLs were obtained after incubation of total HDL apolipoproteins (*in vivo* experiments) or purified apolipoproteins A-I or E (*in vitro* experiments) with dimyristoyl L- α -phosphatidylcholine, cholesteryl myristate and [³H]cholesteryl oleate. Briefly, HDL (density range 1.074–1.155 g ml⁻¹) from pooled rat sera were isolated by sequential ultracentrifugation (Havel *et al.*, 1955), dialysed against 1 mmol ammonium carbonate l⁻¹, lyophilized and lipids were removed according to the procedure of Brown *et al.* (1969). HDL apolipoproteins were subjected to chromatography on a heparin affinity column (heparin utrologel A4R) as described by Dyer and Curtiss (1988). Apolipoprotein E was again purified on a heparin affinity column. Apolipoprotein A-I was then purified on Superose 12HR (Pharmacia, Orsay). Reconstituted HDL were prepared by a modification of the sodium cholate dialysis method (Zorich *et al.*, 1987). Aliquots of dimyristoyl L- α -phosphatidylcholine, cholesteryl myristate and [³H]cholesteryl oleate were placed in glass tubes and dried under nitrogen. Apolipoproteins were dissolved in 20 mmol Tris-HCl l⁻¹, 150 mmol NaCl l⁻¹, 0.01% (w/v) sodium azide, 0.02% (w/v) EDTA, pH 7.4, and added to the dried lipids. Each reaction mixture contained dimyristoyl L- α -phosphatidylcholine:cholesterol:apolipoprotein (100:10:1 molar ratios). Sodium cholate was added and the mixture was shaken overnight. Reconstituted HDL were isolated by the density gradient ultracentrifugation procedure (density range 1.074–1.155 g ml⁻¹) according to Chapman *et al.* (1981), dialysed extensively against a solution containing 150 mmol NaCl l⁻¹, pH 7.4, concentrated with Centrisart I and sterilized through a 0.45 μ m micropore filter. Stereological analysis of lipoprotein particles was performed by means of a Leitz-Tas automatic image analysis system as described by Le Goff *et*

al. (1990). On electron photomicrographs, reconstituted HDL removed from the density gradient were found to have a spherical structure with an average diameter of 12 $\pm$ 2 nm. The size range of particles was highly homogeneous; however, a small number of particles with a discoidal structure were detected.

Determination of cholesterol transfer *in vivo*

A total of 27 male Sprague–Dawley rats aged of 20–30 days were obtained from our colony. Rats were anaesthetized with sodium pentobarbital. Labelled reconstituted HDL were injected through the femoral vein. After 2 h, the rats were killed, exsanguinated and the testes were removed. Interstitial cells, peritubular cells and Sertoli–germ cell enriched aggregates were isolated by sequential enzymatic digestions at 32°C as described by Tung *et al.* (1984). Briefly, the capsules were removed from the testes and they were treated with trypsin (1.0 mg ml⁻¹) and DNase (0.02 mg ml⁻¹) in DMEM for 20 min at 32°C. After addition of 0.1 mg soybean trypsin inhibitor ml⁻¹, interstitial cells were recovered by unit gravity sedimentation. The remaining seminiferous tubules were washed twice, subjected to enzymatic digestion using a mixture of collagenase dispase (0.5 mg ml⁻¹) and testicular hyaluronidase (1 mg ml⁻¹), and the peritubular cells and Sertoli–germ cell aggregates were collected by unit gravity sedimentation. The protein content of samples was determined by the bicinchoninic acid procedure (Smith *et al.*, 1985). Cell lipids were extracted by the procedure of Hara and Radin (1978). Lipids were separated by thin layer chromatography and [³H]cholesterol spots were counted. The tissue space was expressed in terms of the plasma volume that would contain the same amount of radiolabelled compounds (Koelz *et al.*, 1982). The tissue space was calculated as follows: (d.p.m. of cell [³H]cholesterol g⁻¹ cell protein) $\times$ (μ l plasma per d.p.m. of plasma [³H]cholesterol); values are expressed as μ l plasma g⁻¹ cell protein. Data from these experiments are presented as the equivalent plasma space attained by cholesterol in each type of cell after 2 h. The evaluation of cholesterol transfer from plasma HDL to seminiferous tubule cells was calculated as the ratio of seminiferous tubule cell d.p.m. to the mean of plasma HDL specific radioactivity.

Sertoli cell culture

Sertoli cells were obtained from the testes of 40 rats (20 days old, Sprague–Dawley) by sequential enzymatic digestions at 32°C as described by Maboundou *et al.* (1995). Approximately 250 000 cells cm⁻² were plated onto 25 cm² plastic flasks to obtain confluent cultures. Cells were maintained in a humidified atmosphere containing 3% CO₂ at 32°C. After 48 h, the culture medium was replaced with fresh medium without ultrosor SF (steroid-free serum substitute). On day 3, germ cells were removed by hypotonic treatment as described by Galdieri *et al.* (1981).

Determination of cholesterol influx

On day 4, confluent monolayers were incubated with [^3H]cholesteryl ester reconstituted HDL for 4 h at 32°C in the presence or absence of 5 mg heparin ml^{-1} medium. At the end of incubation, the medium was aspirated and the cells were washed twice with cold BSA medium (2.5 mg BSA ml^{-1} cold DMEM) and once with cold PBS. Cell lipids were extracted using the procedure of Hara and Radin (1978). Cell proteins were dissolved in 0.2 mol NaOH l^{-1} (Brown *et al.*, 1980). The protein content of samples was determined by the bicinchoninic acid procedure (Smith *et al.*, 1985). An aliquot of the extracted lipids was counted to determine the total radioactivity incorporated into the cells. The influx was calculated as the apparent uptake of apolipoprotein and expressed as μg apolipoprotein per mg cell protein per 4 h incubation, as reported by Pittman *et al.* (1987).

Statistical analysis

Student's *t* test was used to compare the effect of heparin on uptake of cholesteryl ester. Significance was assumed at $P < 0.001$, $P < 0.01$ or $P < 0.05$.

Results

Distribution of labelled cholesterol

The plasma decay of radioactivity after administration of [^3H]cholesteryl ester reconstituted HDL is shown (Fig. 1). A high proportion of the labelled HDL remained within the plasma: $81 \pm 3\%$ and $66 \pm 5\%$ at 1 and 2 h after injection, respectively.

Fig. 1. Plasma decay of [^3H]cholesterol in 30-day-old rats after i.v. administration of [^3H]cholesteryl oleate reconstituted high density lipoprotein (HDL). The results are expressed as the percentage of radioactivity recovered in the plasma. Values are the mean $\pm$ SD of four experiments ($n = 3$ in each experiment).

At 2 h after administration of [^3H]cholesterol oleate reconstituted HDL, the tissue spaces were 740 ± 60 , 150 ± 20 and $170 \pm 10 \mu\text{l g}^{-1}$ cell protein in the interstitial cells, the peritubular cells and the seminiferous tubule cells, respectively (Fig. 2). In the interstitial cells, the tissue space was fourfold higher than that in the peritubular and seminiferous tubule cells. Transfer of cholesteryl ester between plasma and seminiferous tubule cells was estimated at approximately 25 ng mg^{-1} cell protein in 2 h.

Role of apolipoproteins A-I and E in the uptake of cholesteryl ester

Sertoli cells apparently took up appreciable amounts of cholesteryl ester from phospholipid vesicles with apolipoprotein (Table 1). Uptake of cholesteryl ester is expressed in terms of the amount of apolipoprotein E that would be delivered to the cells if the uptake was via internalization of HDL particles. At the same apolipoprotein concentration (50 μg apolipoprotein ml^{-1} medium), the uptake of cholesteryl oleate with phospholipid–apolipoprotein E vesicles was twofold higher than that with phospholipid–apolipoprotein A-I vesicles. The presence of heparin reduced the uptake of cholesteryl ester from apolipoprotein E vesicles but not apolipoprotein A-I vesicles, indicating that the uptake of apolipoprotein A-I vesicles via secretion of apolipoprotein E by the cells themselves was not involved. The cell content of [^3H]cholesterol was determined after incubation with increasing concentrations of apolipoprotein E reconstituted HDL (Fig. 3). The shape of the concentration curve indicated that uptake of [^3H]cholesteryl oleate from phospholipid–apolipoprotein E vesicles occurred through saturable processes.

Discussion

In previous studies, transfer of plasma lipoprotein cholesterol has been estimated in the whole testes without separation of the interstitial compartment from the seminiferous tubules (Bravo *et al.*, 1994; Woollett and Spady,

Table 1. Effect of heparin on uptake by rat Sertoli cells of [^3H]cholesteryl oleate from reconstituted high density lipoprotein (HDL) containing apolipoproteins A-I or E

Type of vesicle	Apparent uptake of HDL (μg apolipoprotein per mg cell protein per 4 h)	
	Without heparin	With heparin
Phospholipid–apolipoprotein A-I	1.06 ± 0.18^a	1.07 ± 0.09^c
Phospholipid–apolipoprotein E	2.00 ± 0.20^b	1.32 ± 0.30^d

Cells were incubated for 4 h with reconstituted HDL apolipoproteins A-I or E (50 μg apolipoprotein ml^{-1} medium) in the presence or absence of 5 mg heparin ml^{-1} . Superscripts indicate significant differences: a versus b: $P < 0.001$; b versus d: $P < 0.05$; c versus d: not significantly different.

Fig. 2. Tissue space of [³H]cholesterol in rat testicular cells at 2 h after i.v. administration of [³H]cholesteryl oleate reconstituted high density lipoprotein (HDL). Each bar represents the mean $\pm$ SD of five experiments ($n = 3$ in each experiment).

Fig. 3. Uptake of cholesteryl ester from apolipoprotein E (apo E) reconstituted high density lipoprotein (HDL) by rat Sertoli cells. Sertoli cells were obtained from the testes of 20 rats per experiment. Values are the mean $\pm$ SD of two experiments.

1997). Everett and Simmons (1958) reported that, in rats, the transfer of [¹³¹I]-labelled albumin from plasma into the tissue spaces begins immediately after injection and continues for many hours at different rates in various tissues. The amount of [¹³¹I] albumin in the liver and adrenal gland, which represent a system of fenestrated capillaries, was highest at 3 min after i.v. injection and decreased progressively after 30 and 60 min. In contrast, many tissues (intestine, skin, muscle and testis) showed an increase in [¹³¹I]-labelled albumin

content over time. Among these tissues, the testis displayed the greatest increase at 60 min. It has been suggested that these observed differences can be explained, at least in part, by the testicular capillaries which are characterized by a continuous layer of endothelial cells (Mayerhofer *et al.*, 1988). In view of these observations, an incubation time of 2 h was used in the present study, which allows diffusion of cholesterol from plasma into the interstitial spaces and eventually into the seminiferous tubule spaces of the rat testicular tissue.

The data related to the tissue space values are the result of (i) the capacity of lipoprotein particles to cross the endothelial cells probably via the plasmalemmal vesicles; and (ii) the capacity of testicular cells to take up cholesterol through lipoprotein receptors. The results of the present study show that the cholesteryl esters pass through the sieve constituted by the extracellular matrix that is secreted by peritubular and Sertoli cells. The transfer of cholesteryl ester from plasma to the seminiferous tubule cells is fourfold lower compared with the interstitial cells and, therefore, may be involved in membrane renewal by Sertoli cells and putatively by germ cells (Levallet *et al.*, 1998). The inability of Sertoli cells to convert cholesterol to androgens excludes the participation of exogenous cholesterol in steroid production (Gregory and DePhilip, 1998). Rat Sertoli cells can convert testosterone into oestradiol, but such steroid production requires exogenous steroid precursors and not cholesterol (Wiebe *et al.*, 1980).

HDL are known to exchange free cholesterol between the surface of HDL and the external sheet of cell plasma membrane without internalization of cholesterol (Johnson *et al.*, 1991). In contrast, cholesteryl ester is a poorly exchangeable fraction and its transfer into the cell requires the internalization of lipoprotein particles by the LDL receptor, LRP receptor or binding of HDL to the SR-BI receptor. The present study was conducted to determine whether Sertoli cells take up cholesteryl esters of HDL reconstituted with apolipoprotein A-I or apolipoprotein E. Rat HDL contain apolipoprotein A-I that can mediate the uptake of HDL cholesterol ester via the SR-BI receptor (Acton *et al.*, 1996; Temel *et al.*, 1997). In rats, adrenal cells use the HDL cholesterol rather than LDL cholesterol (Hammami *et al.*, 1991). In addition, a significant fraction of rat HDL contains apolipoprotein E that can modulate the uptake of free and esterified cholesterol through internalization of HDL via the LDL receptor family. In the whole testes, significant numbers of VLDL receptors (Oka *et al.*, 1994) and LDL receptors (Yamazaki *et al.*, 1996) have been detected. In addition, the presence of LRP receptors has been shown by immunocytochemical analysis in Sertoli cells (Igdoura *et al.*, 1997).

The present study demonstrated that rat Sertoli cells have the capacity to take up HDL cholesterol for cell metabolism, mainly by an apolipoprotein E pathway. In humans, HDL contain mainly apolipoprotein A-I and low concentrations of apolipoprotein E. Although SR-BI receptors are present in Sertoli cells (Shiratsuchi *et al.*, 1999), internalization of HDL containing mainly apolipoprotein A-I could be promoted by uptake of apolipoprotein E during its passage through the testicular interstitial fluid. Leydig cells and testicular

macrophages produce apolipoprotein E (Schleicher *et al.*, 1993) and HDL rapidly picks up apolipoprotein E (Murdoch and Breckenridge, 1995).

In summary, the findings of the present study show that the plasma lipoprotein cholesterol is able to cross the lamina propria of the immature rat testis and that the seminiferous tubule cells take up cholesteryl ester for cell metabolism *in vivo* and *in vitro*, mainly by an apolipoprotein E pathway.

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