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Changes in lipids and membrane anisotropy in human spermatozoa during epididymal maturation

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Previously it was demonstrated that immature and immotile human spermatozoa from the caput epididymides developed a good progressive motility after in-vitro stimulation with phosphatidylcholine (PC). In order to define the role of PC and membrane anisotropy in epididymal maturation and to determine the exact lipid composition of human spermatozoa during epididymal maturation, spermatozoa from seven epididymides from patients who underwent orchiectomy because of prostatic cancer were investigated. Lipids were determined by high-performance thin-layer chromatography and gas chromatography. Membrane anisotropy was measured by fluorescence polarization. The ratio between PC and phosphatidylserine (PS) plus phosphatidyl ethanolamine (PE) plus sphingomyelin (SM) was significantly higher in spermatozoa from the cauda compared to those from the caput and corpus. This was due to an increase of PC and a decrease of the concentration of PS plus PE plus SM. With regard to fatty acids, those with saturated chains predominated in caput spermatozoa while the highest concentration of unsaturated long-chain fatty acids was in cauda spermatozoa. A lower membrane anisotropy of cauda spermatozoa compared with caput or corpus spermatozoa was found. In conclusion, during epididymal maturation human spermatozoa integrate lipids, particularly PC, which is strongly associated with the induction of progressive motility. A change in the pattern of fatty acids and a decrease in the cholesterol/phospholipid molar ratio cause a decrease in membrane anisotropy in cauda spermatozoa.

Key words: epididymal maturation/fatty acids/membrane anisotropy/phospholipids/spermatozoa

Introduction

During epididymal maturation, membranes of spermatozoa are subject to numerous alterations, which are associated with the induction of progressive motility and further functions important with regard to fertilizing capacity (Cooper, 1986). Apart from the modification of the sperm surface by integration of proteins or glycoproteins (Moore, 1996), a decrease in membrane anisotropy is prerequisite for capacitation, acrosome reaction and subsequent sperm–egg binding (Hall *et al*., 1991). The fluidity of the membrane depends on its lipid composition. A high fluidity is achieved by a low cholesterol/phospholipid ratio as well as a high percentage of unsaturated long-chain fatty acids (Hall *et al*., 1991). Changes in the membrane lipids during epididymal transit have been reported on several animal species (Hall *et al*., 1991; Rana *et al*., 1993). Previously we demonstrated that immature and immotile human spermatozoa from the caput epididymides can be stimulated by phosphatidylcholine *in vitro* (Haidl *et al*., 1993). This points to the significance of lipids for epididymal maturation of human spermatozoa. In this paper, further studies on changes of lipids and membrane fluidity of human spermatozoa during epididymal maturation and their potential clinical impact are presented.

Material and methods

Materials

TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, and DPH (1,6-diphenyl-1,3,5-hexatrien) were obtained from Molecular Probes, Eugene, USA. Phospholipids and fatty acids were from Sigma, Taufkirchen, FRG and Percoll was from Deutsche Pharmacia, Freiburg, FRG. All other chemicals were commercially available and of analytical grade.

Preparation of epididymal spermatozoa

Spermatozoa were obtained from 10 patients (62–74 years old) in whom both testes had been removed because of carcinoma of the prostate. Immediately after operation, the epididymides were dissected in Biggers–Whitten–Whittingham (BWW) medium and divided into caput, corpus and cauda at the macroscopic level. Thereafter, the tissue of the different sections was suspended in BWW medium and centrifuged for 10 min at 80 *g*. The pellet was discarded, and the supernatant containing spermatozoa was centrifuged again for 10 min at 400 *g* and resuspended in BWW medium. Contamination by erythrocytes was markedly reduced by repeated washing steps, and a clean preparation could be achieved by the final Percoll centrifugation. The number of spermatozoa collected by this procedure varied considerably (on average 29×10^6/ml from the caput epididymis, 22×10^6/ml from the corpus, and 14×10^6/ml from the cauda). In seven epididymides spermatozoa were obtained from all sections.

Membrane anisotropy

Steady-state anisotropy studies were carried out in sperm preparations obtained as described above, adjusted to 5×10^5 cells/μl (final concentration). Fluorescence polarization according to the method of Kuhry *et al*. (1983) was measured with a luminescence spectrometer (Perkin–Elmer LS 50) using TMA-DPH and DPH as fluorescence probes (Figure 1). The anisotropy value (r) was calculated according to the equation:

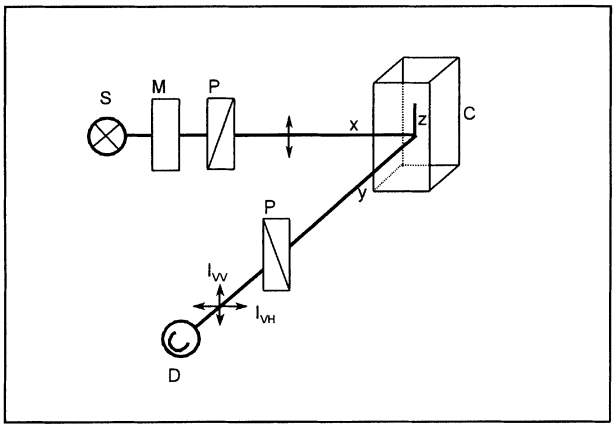

Figure 1. Scheme of the measurement of fluorescence polarization. S, light source; D, detector; M, monochromator; P, polarizer; C, cuvette; *x, y, z*, coordinate axes; I_{VV}, light intensity parallel to *y*-axis; I_{VH}, light intensity vertical to *x*-axis.

r = (I – GI)/(I + 2GI), where I = intensity and G = correction factor (Raha *et al*., 1989).

Lipid analysis

Lyophilized sperm samples were extracted by the Folch procedure (Folch *et al*., 1957). Phospholipid and cholesterol content was determined by high-performance thin-layer chromatography (HPTLC) on silica gel 60 (Merck, Darmstadt, FRG) using methylacetate–chloroform–*n*-propanol–methanol $CaCl_2$ (25% w/v)–acetic acid (25:25:25:10:9:3 v/v). The same solvent was used to isolate phospholipid fractions for fatty acid analysis. Lipids were quantified by a scanning method (Shimadzu CS 9 scanner) after staining by a method described elsewhere (Vitiello and Zanetta, 1987).

Fatty acids of the different phospholipid classes were transesterified to methyl fatty acids according to the method of Christie (1982) and analysed by gas chromatography (GLC Perkin–Elmer Model Autosystem, Supelcowax column Tm 10, 15 m×0.32 mm) with flame ionization detector detection. The amount of phospholipids was calculated in relation to sperm protein, which was determined according to the method by Lowry *et al*. (1951).

Statistical analyses

Significant differences between the mean values were calculated by analysis of variance (ANOVA) followed by the Duncan multiple range test. Results are expressed as means ± SD.

Results

Cholesterol/phospholipid molar ratio

The highest molar content of cholesterol/phospholipids was observed in spermatozoa from the caput epididymides, the lowest in the cauda preparations (Figure 2).

Distribution of phospholipids

The pattern of distribution of different classes of phospholipids showed particular differences in the amounts of phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) between the different steps of maturation with a decrease from the caput region to the cauda region (Figure 3b). The concentration of phosphatidylcholine (PC) increased from the caput to cauda (Figure 3a), as did the ratio between PC and PE plus PS plus SM (Figure 3c).

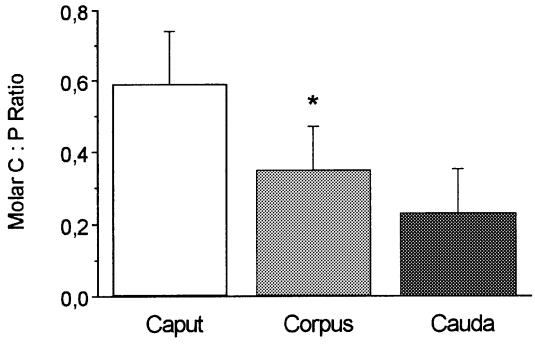

Figure 2. Cholesterol (C)/phospholipid (P)/molar ratio of human epididymal spermatozoa from the caput, corpus and cauda regions. Data are means ± SD of seven epididymal preparations (*ANOVA: $P < 0.05$, cauda and caput preparations compared).

Fatty acids

Saturated fatty acid chains predominated in caput spermatozoa, whereas the highest concentration of unsaturated fatty acid chains was in cauda spermatozoa (Table I). This suggests a lower membrane anisotropy of cauda sperm compared with spermatozoa from the caput and corpus epididymides.

Membrane anisotropy

Membrane anisotropy was measured with the fluorescence probes TMA-DPH and DPH was highest in caput spermatozoa compared with the corpus and cauda spermatozoa (Figure 4).

Discussion

Although there is general agreement that epididymal maturation is prerequisite for spermatozoa to gain fertilizing capability, at least for natural reproduction, many of the mechanisms of this process have not been clarified. This is particularly true for the induction of progressive motility. In this context an increase during epididymal transit in the S–S-dependent stability of various sperm tail structures such as fibrous sheath and outer dense fibres is important (Bedford *et al*., 1973). An early candidate for the key regulator of sperm motility was AMP (Amann *et al*., 1982). Subsequent studies have shown that intracellular pH and calcium are further parameters for consideration (Hoskins and Vijayaraghavan, 1990). This was confirmed by recent studies on rat spermatozoa demonstrating that induction of motility is regulated by Ca^{2+} and the guanylate cyclase and adenylate cyclase pathways but not through the phospholipase C and phospholipase A2 (PLA2) pathways (Armstrong *et al*., 1994). PLA2 has been attributed a role in membrane fusion events during the fertilization process (Fry *et al*., 1992). On the other hand, in ejaculated boar spermatozoa phospholipids were demonstrated by means of the PLA2–colloidal gold technique not only at the acrosomal and postacrosomal region but also over cross-fractioned mitochondria, dense fibres and fibrous sheath of the tail (Kan and Lin, 1996). Therefore, a role of PLC and PLA2 for sperm motility cannot be excluded. Phospholipids may serve as substrates for

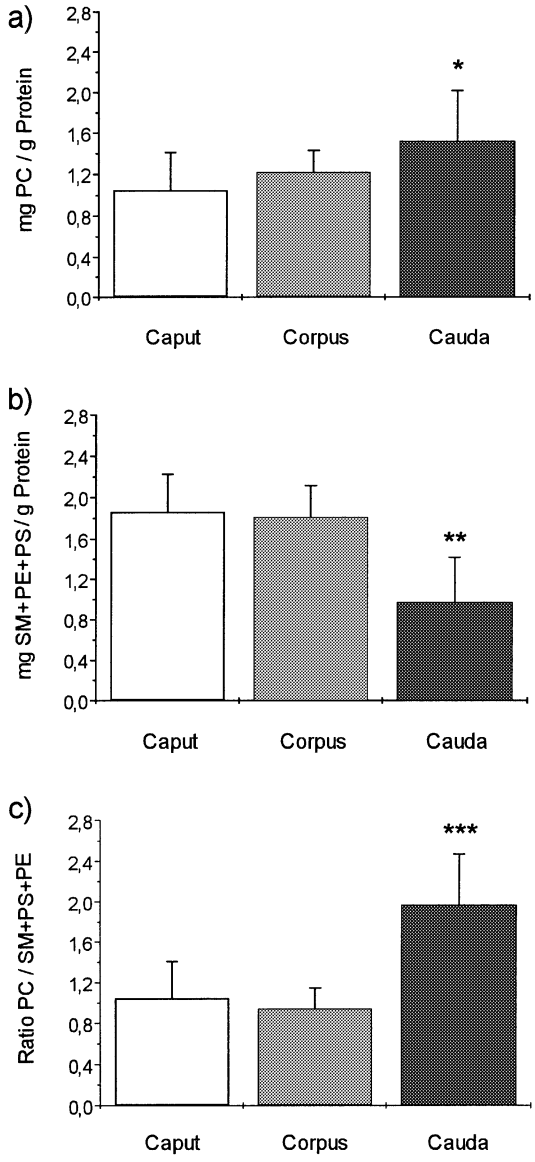

Figure 3. Phospholipid and protein distribution of human epididymal spermatozoa from the caput, corpus and cauda regions. Data are means ± SD of seven preparations. (**a**) Phosphatidylcholine (PC) content calculated according to sperm protein (*ANOVA: $P < 0.05$, cauda and caput preparations compared). (**b**) Content of phosphatidylethanolamine (PE) plus phosphatidylserine (PS) plus sphingomyelin (SM) plus other phospholipids (phosphatidylinositol and lysophosphatidylcholine) calculated according to sperm protein (**ANOVA: $P < 0.01$, cauda and caput preparations compared). (**c**) PC/PE+PS+SM ratio (***ANOVA: $P < 0.001$, cauda and preparations compared).

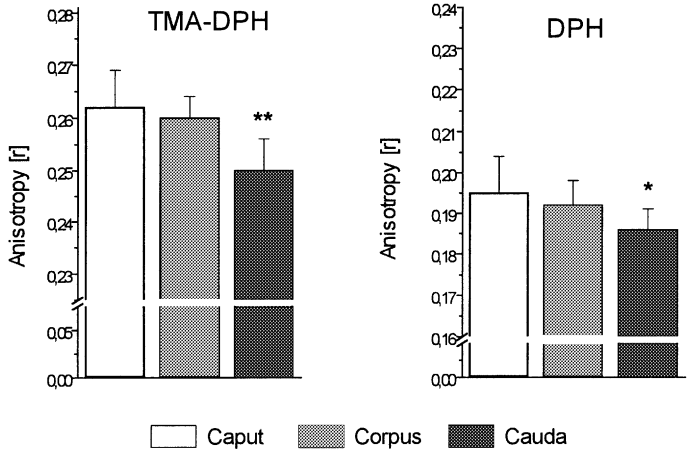

Figure 4. Fluorescence polarization measurement of human epididymal spermatozoa from the caput, corpus and cauda regions. Data are means ± SD of five epididymal preparations. (**a**) Fluorescence label was TMA-DPH (**ANOVA: $P < 0.01$, cauda and caput preparations compared). (**b**) Fluorescence label was DPH (*ANOVA: $P < 0.05$, cauda and caput preparations compared). For experimental details see text.

Table I. Fatty acids of human epididymal spermatozoa

	Caput	Corpus	Cauda
C16:0	31.8 ± 2.6	30.8 ± 3.2	30.1 ± 3.1
C18:0	32.1 ± 2.9	31.7 ± 3.0	29.1 ± 3.2
C22:0	2.4 ± 0.2	2.4 ± 0.2	1.7 ± 0.1
C24:0	2.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1
C18:1, 18:2 12.8 ± 1.0	15.1 ± 1.2	14.1 ± 0.1	
C20:4	5.6 ± 0.7	5.3 ± 0.6	6.7 ± 0.8
C22:2, 22:3, 22:5	9.7 ± 0.8	10.5 ± 0.9	11.5 ± 1.5
C22:4	3.3 ± 0.2	2.9 ± 0.4	4.5 ± 0.5
C(saturated) 16:0, 18:0, 22:0, 24:0	68.5 ± 5.8	66.09 ± 6.6	61.8 ± 6.5*
C(unsaturated) 18:1, 18:2, 20:4, 22:2, 22:3, 22:4, 22:5	31.4 ± 2.8	33.8 ± 3.2	36.8 ± 3.0**
C(saturated)/C(unsaturated)	2.18	1.96	1.68

Data are means ± SD of seven epididymal preparations (ANOVA: *$P < 0.05$, and **$P < 0.01$, cauda preparation compared with caput preparation).

phospholipases that generate second messengers, inducing intracellular response (Hinkovskagalchev and Srivastava, 1992). Moreover, induction of sperm motility is associated with modifications of the plasma membrane. In this regard, changes involving acquisition of proteins and glycoproteins during epididymal transit have been reported (Kirchhoff, 1996; Moore, 1996). In a previous study we have pointed out that human spermatozoa from the caput epididymides may be stimulated *in vitro* by addition of phosphatidylcholine (Haidl *et al*., 1993), which emphasizes the significance of membrane lipids for epididymal maturation. This is not only important with regard to induction of progressive motility but also for subsequent functions and processes including capacitation, induction of acrosome reaction and sperm–egg binding (Fleming and Yanagimachi, 1981; Benoff *et al*., 1993). The change in the phospholipid/ cholesterol ratio during epididymal transit with an increase in the amount of PC, as demonstrated in this study, may be indicative of a substantial role of PC in the induction of progressive motility. In addition, the molar cholesterol/phospholipid ratio and fatty acid profile determine membrane anisotropy (Verkeiji, 1995). A lower membrane anisotropy is essential for sperm motility as well as prerequisite for acrosome reaction and capacitation (Hoshi *et al*., 1990). A larger amount of unsaturated fatty acid chains in cauda spermatozoa compared to spermatozoa from the caput and corpus was demonstrated in our study, leading to a lower membrane anisotropy in cauda spermatozoa.

There have been several reports on the modification of the lipid pattern in animal models. In rat epididymal spermatozoa

a decrease in the cholesterol/phospholipid ratio from caput to cauda was also determined; this was, however, due to a reduction in both cholesterol and phospholipids but with a more pronounced decline in cholesterol (Hall *et al*., 1991). This phenomenon has also been observed in boar epididymal spermatozoa (Nikolopoulou *et al*., 1985). A shift from saturated to unsaturated fatty acids in sperm membranes during epididymal transit, which also contributes to a lower membrane anistropy, is comparable between different species (Hall *et al*., 1991).

These results and the present observations are in agreement with a previous study demonstrating an increased anisotropy in human ejaculated spermatozoa which were completely immotile due to the absence of epididymal maturation (Wiese *et al*., 1996). As described previously such spermatozoa are characterized by a different staining behaviour following the Shorr technique which can be explained by missing or abnormally composed membrane lipids (Haidl *et al*., 1993). In contrast to the animal studies, an increase of PC was demonstrated in experiments on human spermatozoa. For future research work, it will, therefore, be interesting to study the role of PC for induction of progressive motility and the mechanisms of lipid integration into the membranes as well as the interaction of lipids and proteins for signal transduction. These questions are also of clinical relevance, not least because it has been demonstrated that in cases of asthenozoospermia where an improvement could be achieved by kallikrein treatment, a decrease in the incidence of atypically stained sperm tails was observed (Haidl and Schill, 1993).

Thus, new treatment modalities for a distinct group of asthenozoospermia may be developed, once the role of lipids in this condition is better understood.

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