Studies on the metabolism of essential fatty acids in isolated human testicular cells

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The essential fatty acid 22:6(n-3) is a minor component of the Western diet, but a major fatty acid in human testis and semen. In mature spermatozoa, the physical and fusogenic properties of the plasma membrane are probably influenced by its particular fatty acid composition. In this study, the synthesis of 22:6(n-3) and 22:5(n-6) was investigated in isolated human testicular cells. [1-¹⁴C]20:4(n-6), [1-¹⁴C]20:5(n-3), [1-¹⁴C]22:4(n-6) and [1-¹⁴C]22:5(n-3) were incubated in a 'crude' cell suspension (consisting of a mixture of the cells in the seminiferous tubule), and in fractionated pachytene spermatocytes and round spermatids. The esterification of fatty acids in lipid and phospholipid classes and the fatty acid chain elongation and desaturation were measured. The crude cell suspension metabolized the fatty acids more actively than did the fractionated germ cell suspension, indicating that types of cell other than the germ cells are important for fatty acid

elongation and desaturation and thus the production of 22:6(n-3). This finding is in agreement with previous results in rats that indicated that the Sertoli cells are the most important type of cell for the metabolism of essential fatty acids in the testis. Some [1-¹⁴C]20:5(n-3) was elongated to [1-¹⁴C]22:5(n-3) in the fractionated germ cells, but very little was elongated further to [1-¹⁴C]24:5(n-3), possibly restricting the formation of [1-¹⁴C]22:6(n-3). In the fractionated germ cells, the fatty acid substrates were recovered primarily in the phospholipid fraction, indicating an incorporation in the membranes, whereas in the crude cells, more substrates were esterified in the triacylglycerol fraction. In the phospholipids, more radioactivity was recovered in phosphatidylcholine than in phosphatidylethanolamine and more radioactivity was recovered in phosphatidylethanolamine than in phosphatidylinositol or phosphatidylserine.

Introduction

In cell membranes, fatty acids are both structural elements and bioactive compounds. It has long been known that (n-6) fatty acids are essential for testis development and fertility (Burr and Burr, 1929; Evans *et al.*, 1934). The testis has a high content of 20 and 22 carbon atom polyunsaturated fatty acids (PUFA) and the PUFA further accumulate in semen. In testis of rats (Chanmugam *et al.*, 1991), rabbits and other rodents (Ahluwalia and Holman, 1969), the (n-6) PUFA dominate, and the testes from these species have a high content of 22:5(n-6). However, in testis of humans (Ahluwalia and Holman, 1969; Coniglio *et al.*, 1975; Nissen *et al.*, 1978), monkeys (Connor *et al.*, 1997), rams (Scott, 1973), bulls (Scott, 1973) and pigs (Paulenz *et al.*, 1995) the (n-3) PUFA dominates. In these species, the amount of 22:6(n-3) is high.

In whole human testis, 8–24% of the fatty acids are 22:6(n-3) (Bieri and Prival, 1965) and a low concentration of PUFA in semen has been associated with poor sperm quality. In normal human semen, up to 58% of the fatty acids are 22:6(n-3) (Nissen and Kreysel, 1983), and in the

phospholipids of spermatozoa recovered from 47% Percoll fractions, the content of 22:6(n-3) in normozoospermic semen was 22% compared with 16% in oligozoospermic semen (Zalata *et al.*, 1998). Speculation about the function of PUFA in testis has been related to their possible effect on the fluidity of the sperm membrane and the packing of membrane-bound receptors. 22:6(n-3) increases the membrane fluidity, and this may also contribute to the motility of the spermatozoa (Connor *et al.*, 1998).

Studies have been performed on the metabolism of essential fatty acids in the testis of rats, but little is known about it in human testis. Fatty acid synthesis has been demonstrated in human whole testis tissue incubated with [1-¹⁴C]acetate (Fisher and Coniglio, 1983). Furthermore, both human whole testis tissue and microsomal preparations can synthesize arachidonic acid from labelled precursors (Coniglio *et al.*, 1977; Albert *et al.*, 1979). The pathway for the synthesis of 22:6(n-3) (Voss *et al.*, 1991) and 22:5(n-6) (Mohammed *et al.*, 1995) has been reported but little is known about its regulation. The present study investigated this revised pathway in human testis. To the authors' knowledge, this is the first study on the synthesis and metabolism of 22:6(n-3) and 22:5(n-6) in human testicular cells.

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Studies on rats indicate that Sertoli cells are more important than germ cells for the formation of PUFA (Beckman and Coniglio, 1980; Retterstøl *et al.*, 1998, 2000). In the present study, various cell types of seminiferous tubules were used to examine the origin of PUFA in human testis.

Materials and Methods

Chemicals

[1-¹⁴C]20:4(n-6), [1-¹⁴C]20:5(n-3), [1-¹⁴C]22:4(n-6) and [1-¹⁴C]22:5(n-3) were obtained from American Radio-labeled Chemicals Inc. (St Louis, MO), and the unlabelled fatty acids were from Sigma Chemicals Co. (St Louis, MO).

Preparation of cell suspensions

Testicular material was obtained from four organ donors (aged 45–61) in connection with donation of multiple organs after brain death according to a procedure approved by the local ethical committee. Each testicle was studied separately. Data about the organ donors are spare, but the testis had a normal macroscopic appearance and a normal weight (11–28 g after decapsulation). Furthermore, acceptance as an organ donor requires good health before death. A 'crude' germ cell suspension, consisting of a mixture of the cells in the seminiferous tubule, was prepared by treatment with trypsin and DNase (Meistrich, 1977). The germ cells were fractionated further by centrifugal elutriation (Blanchard *et al.*, 1991) to 75–83% (range) purity of haploid cells (1300–450 g and 22.8–45.5 ml min⁻¹). Elutriation is a counterflow centrifugation which means that the sedimentation of cells in a centrifugal field is offset by a liquid flowing in the opposite direction. The cell densities in the isolated germ cell and the testis crude suspensions were 0.77–0.97 and 2.7–11.0 × 10⁶ cells ml⁻¹, respectively, and the corresponding mean protein concentrations were 0.40–0.43 and 1.2–4.7 mg ml⁻¹, respectively. Five millilitres of this preparation was used for incubation in NUNC 50 ml cell culture flasks. The suspensions contained 1.5% (w/v) essentially fatty acid free BSA. Twenty nanomoles of radiolabelled fatty acid

with a specific activity of approximately 55 mCi mmol⁻¹ was added to each flask (the fatty acid concentration was 4 μmol l⁻¹).

Analytical methods

Lipids were extracted (Folch *et al.*, 1957) and aliquots were separated on silicic acid thin-layer plates (Stahl H+) with hexane–diethylether–acetic acid (70:29:1, v/v) and counted in an imager scanner from Packard. The phospholipids were isolated using the same thin liquid chromatography (TLC) system and eluted with 2 × 2 ml CHCl₃–MeOH–acetic acid–water (50:39:1:10, v/v). The phospholipid was separated further into phosphatidyl-inositol (PtdIns), phosphatidylserine (PtdSer), phosphatidyl-choline (PtdCho) and phosphatidylethanolamine (PtdEtn) on Whatman LK5 thin layer plates with CHCl₃–MeOH–40% methylamine (120:40:10, v/v) as solvent. The Whatman LK5 thin layer plates were scanned and thereafter the phospholipid classes were eluted with 2 × 2 ml CHCl₃–MeOH–acetic acid–water (50:39:1:10, v/v).

Aliquots of the total lipids of the cell suspensions, the isolated cells and the isolated media, as well as the phospholipid and triacylglycerol (TAG) fractions were transmethyated (BF₃–methanol) and the fatty acids were separated with primary reverse phase HPLC (isocratically) on a Supelcosil LC-18 (250 mm length × 4.6 mm diameter) reversed phase column (Supelco, Bellefonte, PA). The mobile phase was acetonitrile–water (80:20, v/v) 2.0 ml min⁻¹ or acetonitrile–water (90:10, v/v) 0.75 ml min⁻¹ delivered by a LC-9A Shimadzu apparatus (Kyoto). Radioactivity was measured in a flow detector A-100 (Radiomatic Instruments and Chemical, Tampa, FL). The fatty acids were identified by comparing their retention times with those from [1-¹⁴C]-labelled fatty acid standards and with retention times from previous experiments.

Determination of radioactive acid soluble products and of radioactive CO₂ (as a measure of the rate of oxidation of carbon one) was performed as described by Christiansen *et al.* (1976). Flasks in which the formation of radioactive CO₂ was to be measured had a centre well containing 0.2 ml of 10% NaOH and a folded filter paper, and a side arm containing 0.2 ml of 5 mol HClO₄ l⁻¹. At the end of the

Table 1. Amounts of [1-¹⁴C] fatty acid esterified and elongated in the lipid extract of cells plus medium after 24 h of incubation in a 'crude' cell suspension

Substrate	Nanomoles of fatty acid esterified (%)	Nanomoles of fatty acid elongated (%)	Number of incubations
20:4(n-6)	9.6 ± 2.1 (51)	0.6 ± 0.3 (6)*	8
20:5(n-3)	8.6 ± 1.4 (56)	2.7 ± 1.7 (31)	7
22:4(n-6)	11.8 ± 2.7 (69)	1.2 ± 0.3 (10)	8
22:5(n-3)	12.1 ± 3.7 (71)	1.9 ± 1.1 (16)	8

Values are means ± 1 SD.

*20:4(n-6) was elongated less than 20:5(n-3), *P* < 0.05.

Table 2. Amounts of [$1\text{-}^{14}\text{C}$] fatty acid esterified and elongated in the lipid extract of cells plus medium after 24 h of incubation in a suspension of isolated pachytene spermatocytes (PS) or round spermatids (RST)

Substrate	Nanomoles of fatty acid esterified (%)	Nanomoles of fatty acid elongated (%)	Number of incubations
PS			
20:4(n-6)	3.8 ± 0.1 (20)*	0.8 ± 0.4 (21)	5
20:5(n-3)	3.4 ± 0.2 (17)*	0.9 ± 0.3 (26)	5
RST			
20:4(n-6)	1.8 ± 0.1 (10)	0.2 ± 0.2 (11)	5
20:5(n-3)	1.7 ± 0.1 (9)	0.1 ± 0.1 (6)	5

Values are means $\pm$ 1 SD.

*Esterification in PS > RST, $P < 0.05$.

Fig. 1. (a) Nanomoles of [$1\text{-}^{14}\text{C}$]20:4(n-6), [$1\text{-}^{14}\text{C}$]20:5(n-3), [$1\text{-}^{14}\text{C}$]22:4(n-6) and [$1\text{-}^{14}\text{C}$]22:5(n-3) esterified in the phospholipid (PL; ■) and triacylglycerol (TAG; □) fractions after 24 h of incubation in 'crude' cell suspension. (b,c) Nanomoles of [$1\text{-}^{14}\text{C}$]20:4(n-6) and [$1\text{-}^{14}\text{C}$]20:5(n-3) esterified in pachytene spermatocytes (b) and round spermatids (c). Mean values from 5–9 incubations $\pm$ 1 SD. ^bPL > TAG ($P < 0.05$); ^c'crude' > the two germ cell fractions ($P < 0.05$).

incubation, the HClO_4 was tipped in and the shaking was continued for 60 min to trap all $^{14}\text{CO}_2$ in the filter paper. Thereafter, the filter paper was counted in a Packard Tri-Carb 1500 liquid analyser. Analyses on lipids and acid-

soluble products were performed on an identical pair of incubation flasks. The acid soluble radioactivity was measured in the HClO_4 extracts. Labelled oxidation products were also measured in the water phase of the lipid

extraction. Cellular protein was determined according to the method of Lowry *et al.* (1951).

Statistical analyses

An unpaired, two-sided *t* test was used. When multiple comparisons were performed the *P* values were Bonferroni-corrected.

Results

In the crude suspension, 6–31% of the esterified radio-labelled fatty acids were elongated (Table 1). More labelled fatty acid substrates were esterified and elongated in the crude cell suspension than in the fractionated germ cell suspensions ($P < 0.05$, Table 1 versus Table 2; and pachytene spermatocytes > round spermatids, $P < 0.05$, Table 2).

In the crude cell suspension, more [^{14}C]22:4(n-6) and [^{14}C]22:5(n-3) was esterified in the TAG than in the phospholipid fraction ($P < 0.05$, Fig. 1a). In the fractionated germ cells, more [^{14}C]20:5(n-3) and [^{14}C]20:4(n-6) was esterified in the phospholipid than in the TAG fraction ($P < 0.05$, Fig. 1b,c). [^{14}C]20:4(n-6) was the best substrate for esterification in phospholipids (Fig. 1), and little [^{14}C]20:4(n-6) was oxidized compared with [^{14}C]20:5(n-3) (0.6 ± 0.5 versus 1.8 ± 0.4 nmoles). In the crude cell suspension and the fractionated germ cell suspension, 0.3–0.5 nmoles and 0.1–0.2 nmoles, respectively, were esterified in the diacylglycerol fraction, and 0.08–0.15 nmoles and 0.01–0.02 nmoles, respectively, were esterified in the cholesterol ester fraction.

More fatty acid substrates were esterified in PtdCho than in PtdEtn, PtdSer and PtdIns in all cell fractions (Fig. 2).

The most active elongation activity was found to be from [^{14}C]20:5(n-3) to [^{14}C]22:5(n-3) in the TAG fraction of the crude cell suspension (Fig. 3b). In the fractionated germ cells, the degree of elongated fatty acid substrates seemed to be highest in the TAG fraction, although little was esterified in this lipid class (Fig. 4). Very little C_{24} fatty acid and [^{14}C]22:5(n-6) or [^{14}C]22:6(n-3) were formed in either the fractionated germ cells or the crude cell suspensions (Figs 3 and 4), indicating a low capacity both for the elongation reaction from 22 to 24 carbon fatty acids and for the $\Delta 6$ desaturation.

Discussion

The present results are from experiments performed on separate testicular material from four men. In spite of the differences among these testes, large differences in fractionated cells were not expected.

Data from studies in rats indicate that the synthesis of 22:5(n-6) and 22:6(n-3) is very efficient in Sertoli cells, while germ cells are considerably less active (Beckman and Coniglio, 1980; Retterstøl *et al.*, 2000), in agreement with the hypothesis of a transport of PUFA from the Sertoli cells

to the germ cells (Beckman *et al.*, 1978). In the present study, almost no 22:5(n-6) and 22:6(n-3) were formed by fractionated human germ cells indicating that, in humans also, the Sertoli cell is important for the conversion of essential fatty acids in the testis. In human germ cells, the content of 22:6(n-3) increased with increasing maturity (Lenzi *et al.*, 2000). A considerable lipid remodelling of rat spermatozoa has been shown to occur during their journey through the epididymis (Hall *et al.*, 1991; Avelandano *et al.*, 1992), and an active role of the epididymis in humans is indicated by the high concentration of carnitine in human epididymis, as discussed by Lenzi *et al.* (2000).

Fatty acid metabolic activity in the human testicular cells was low compared with that found in rats (Retterstøl *et al.*, 1998). Therefore, it is possible that human cells have a lower capacity for metabolizing fatty acid substrates than rat cells, which would not be surprising since the testis of adult rats accounts for approximately 0.7% of the body weight compared with approximately 0.04% of the body weight in men (Fredricsson and Pousette, 1994). Furthermore, the daily sperm production per gram testis is 5–6 times greater in rats than it is in men (Sharpe, 1994).

[^{14}C]20:4(n-6) was esterified efficiently in phospholipids, which may reflect a stable incorporation in the membranes, and this fatty acid is also normally present in relatively high quantities in human testis *in vivo* (Coniglio *et al.*, 1975). However, [^{14}C]22:4(n-6) and [^{14}C]22:5(n-3) were primarily esterified in the TAG fraction, and little of these fatty acids is found in human testis *in vivo*, reflecting the lesser degree of incorporation in the cell membranes. The same differences between these fatty acids have been demonstrated in isolated rat hepatocytes (Retterstøl *et al.*, 1995).

Although the testicular cells of rats seemed to be more active than those of men, many similarities between the two species were found. In the fractionated human germ cells only, a higher proportion of the substrate was esterified in the phospholipid than in the TAG fraction. These results resemble those reported in studies of rat testicular cells. In the phospholipids of the human testicular cells, the fatty acid substrates were mainly esterified in PtdCho, which is similar to the situation in rats. In the germ cells from men, pachytene spermatocytes were more active in metabolizing the fatty acids than were the round spermatids, and this was also similar to the situation in rats.

Sprecher (1999) has shown that 22:4(n-6) and 22:5(n-3) are not desaturated at position 4 as was believed for many years, but elongated to 24-carbon fatty acid intermediates and then desaturated at position 6. Little is known about the regulation of this revised pathway from 20:5(n-3) $\rightarrow$ 22:5(n-3) $\rightarrow$ 24:5(n-3) $\rightarrow$ 24:6(n-3) $\rightarrow$ 22:6(n-3). The present data indicate that 20:5(n-3) is a better substrate for fatty acid elongation than 20:4(n-6) in human testis, facilitating the synthesis of 22:6(n-3) over that of 22:5(n-6). However, the next elongation, from 22:5(n-3) to 24:5(n-3), was inefficient, possibly restricting the formation of both 22:5(n-6) and 22:6(n-3). The next step in the pathway is the $\Delta 6$ desaturation, and this activity is lower in isolated rat

Fig. 2. (a) Nanomoles of $[1-^{14}\text{C}]20:4(\text{n-6})$, $[1-^{14}\text{C}]20:5(\text{n-3})$, $[1-^{14}\text{C}]22:4(\text{n-6})$ and $[1-^{14}\text{C}]22:5(\text{n-3})$ esterified in phosphatidylcholine (PtdCho; $\square$), phosphatidylethanolamine (PtdEtn; $\square$ with diagonal lines), phosphatidylinositol (PtdIns; $\blacksquare$) and phosphatidylserine (PtdSer; $\square$ with diagonal lines) after 24 h of incubation in 'crude' cell suspension. (b,c) Nanomoles of $[1-^{14}\text{C}]20:4(\text{n-6})$ and $[1-^{14}\text{C}]20:5(\text{n-3})$ esterified in the same phospholipid classes in pachytene spermatocytes (b) and round spermatids (c). Mean values from 5–9 incubations $\pm$ 1 SD. ^aPtdCho > PtdEtn ($P < 0.05$); ^bPtdEtn > PtdIns and PtdSer ($P < 0.05$).

Fig. 3. The elongation and desaturation of (a) $[1-^{14}\text{C}]20:4(\text{n-6})$, (b) $[1-^{14}\text{C}]20:5(\text{n-3})$, (c) $[1-^{14}\text{C}]22:4(\text{n-6})$, and (d) $[1-^{14}\text{C}]22:5(\text{n-3})$ incubated in the testis 'crude' cell suspension and esterified in the phospholipid ($\blacksquare$) or the triacylglycerol ($\square$) fractions. Mean values from 3–9 incubations $\pm$ 1 SD.

Fig. 4. The elongation and desaturation of (a) [1-¹⁴C]20:4(n-6), (b) [1-¹⁴C]20:5(n-3), (c) [1-¹⁴C]20:4(n-6) and (d) [1-¹⁴C]20:5(n-3) incubated in the isolated germ cell suspension (a,b: pachytene spermatocytes; c,d: round spermatids) and esterified in the phospholipid (■) or the triacylglycerol (□) fractions. Mean values from 4–5 incubations $\pm$ 1 SD.

germ cells than it is in Sertoli cells (Retterstøl *et al.*, 2000). The present results indicate a similar situation in men. The human $\Delta 6$ desaturase gene has recently been cloned (Cho *et al.*, 1999) and there is a low $\Delta 6$ mRNA concentration in isolated rat germinal cells compared with isolated rat Sertoli cells (T. N. Tran, unpublished).

In conclusion, almost no 22:6(n-3) was synthesized by the human germ cells. This finding is probably related to a low capacity for fatty acid elongation and possibly also the $\Delta 6$ desaturation. The high concentrations of 22:6(n-3) and other polyunsaturated fatty acids found in human germ cells *in vivo* may be strictly related to their normal function and morphology (Lenzi *et al.*, 2000), and the present results indicate that germ cells are dependent on a supply of this fatty acid, most likely from the surrounding Sertoli cells.

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