Polyunsaturated fatty acid biosynthesis in testis from rats fed a hypercholesterolemic diet

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The effect of 2% (wt/wt) cholesterol supplementation to an otherwise balanced diet on polyunsaturated fatty acid metabolism was studied in the total microsomes and isolated Leydig cells from rat testis. Microsomal Δ5 and Δ6 desaturation, as well as stearate, linoleate, α-linolenate, and eicosa-8,11,14-trienoate thioesterification were significantly depressed by cholesterol feeding. These changes were accompanied by increases in microsomal cholesterol levels and the cholesterol to phospholipid ratio, along with a consequential rigidification of the microsomal membrane, as judged by fluorescence polarization with the lipid probe 1,6-diphenyl-1,3,5-hexatriene. The dietary manipulation, however, neither affected the uptake and transformation of α-linolenate and eicosa-8,11,14-trienoate nor induced any compensatory changes in the fatty acid composition of the Leydig cells. These findings demonstrate that a high-cholesterol regime produces an overall impairment in polyunsaturated fatty acid biosynthesis in the testis and further suggests that the testicular germinal elements might be the cell population most severely compromised by this abnormality. (J. Nutr. Biochem. 5:15–20, 1994.)

Keywords: Leydig cells; Δ 5 and Δ 6 desaturases; long-chain fatty-acyl CoA synthetase; α-linolenic acid; eicosa-8,11,14-trienoic acid; cholesterol; fluorescence polarization

Introduction

Feeding cholesterol to animals with sub-optimal essential fatty acid (EFA) intake leads to a striking exacerbation of the signs of EFA deficiency.[1,2] This observation implies that cholesterol may interfere with EFA metabolism or increase the need for EFAs. These indirect indications of an effect of cholesterol on polyunsaturated fatty acid metabolism have been followed by direct measurements of the content of individual fatty acids in plasma and liver. Compared with a control diet, the cholesterol-containing regime produces a decrease in saturated fatty acids and an increase in monounsaturates, along with a significant decrease in 20:4 (n-6) and an increase in 18:2 (n-6) and 20:3 (n-6) acids.[3–6] These findings suggest that cholesterol may reduce the efficiency of the conversion of linoleic to arachidonic acid. This effect of dietary cholesterol was recently confirmed by a direct measurement of the microsomal activity of the desaturases. In accordance with the fatty acid patterns described previously, Δ9 desaturase activity was enhanced whereas Δ5 and Δ6 desaturation were depressed in hepatic microsomal membranes of rats fed a high-cholesterol diet.[5–9] Hepatic and adrenal long-chain fatty acyl CoA thioesterase activity also decreased under the same dietary conditions.[6]

These studies leave little doubt that dietary cholesterol has a profound effect on the biosynthesis of polyunsaturated fatty acids. Most investigations, however, have been centered on the liver, where it was suggested that cholesterol may reduce Δ6 desaturation activity and lower arachidonic acid levels by increasing their utilization in the formation of cholesterol esters.[3,10] This possibility becomes even more important in those tissues that require cholesterol not only for plasma mem-

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brane synthesis but also as a substrate for steroid production. In view of these considerations and given the fact that only very few studies have appeared in which the aforementioned effects have been evaluated in extrahepatic tissues, we were prompted to study the influence of excessive cholesterol levels in the testis.

It is well known that lipids are of fundamental importance in the development and functioning of testicular tissue. Testicular lipids contain a high concentration of polyenoic acids, which includes, in addition to linoleic and arachidonic acids, the 22-carbon members of those families.[11] It was accordingly suggested that any one of the numerous factors that adversely affect the level of polyenoate in the testis will also result in an impaired spermatogenesis.[12]

In view of all the foregoing considerations, the object of the experiments to be reported here was to elucidate the mechanism by which the cholesterol feeding of rats influences polyunsaturated fatty acid biosynthesis in both total testicular tissue and the Leydig cells isolated from the testis.

Methods and materials

Chemicals

[1-^{14}C]Palmitic acid (59 mCi/mmol), [1-^{14}C]stearic acid (56 mCi/mmol), [1-^{14}C]linoleic acid (56 mCi/mmol), [1-^{14}C]α-linolenic acid (56 mCi/mmol), and [2-^{14}C]eicosa-8,11,14-trienoic acid (47.7 mCi/mmol) were purchased from Amersham International (Buckinghamshire, UK). All these substrates were 98% to 99% radiochemically pure. Unlabeled palmitic, stearic, linoleic, α-linolenic, and eicosa-8,11,14-trienoic acids were supplied by Nu-Chek Prep. (Elysian, MN USA). Bovine albumin, essentially free of fatty acids; NADH; ATP; CoA; dithiothreitol; cholesterol; and cholic acid were provided by Sigma Chemical Co. (St. Louis, MO USA). The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI USA). All other chemicals used were of analytical grade.

Animal treatment

Weanling male Wistar rats were fed either a balanced diet free of cholesterol (control group, n = 9) or the same diet with the addition of 2 g% (wt/wt) cholesterol plus 1 g% (wt/wt) cholic acid (cholesterol group, n = 9). Rats were fed their respective diets for 2 months before use in experiments. The balanced diet contained (in calories) 55% starch, 20% casein, and 25% corn oil, supplemented with minerals and a vitamin mixture.[13] Diets were prepared weekly and stored at 4° C. Animals were housed in groups of two or three in stainless-steel cages in a temperature-controlled room at 24 ± 1° C with a 12-hour-light:dark cycle. Food and water were provided ad libitum. Animals were killed by decapitation and blood was drained and collected to determine glucose, triglyceride, cholesterol, and plasma fatty acid composition. Testicles were rapidly excised, weighed, and used both for the preparation of the microsomal fraction and for the isolation of Leydig cells.

Isolation of microsomes

For preparation of the microsomal fraction, the testes were decapsulated and homogenized in an ice-cold homogenizing solution (1:3 wt/vol) containing 0.15 M KCl, 0.25 M sucrose, 62 mM phosphate buffer pH 7.0, 5 mM $MgCl_2$, 100 μM EDTA, and 4 mM N-acetylcysteine. The microsomal fraction was separated by ultracentrifuging at 105,000*g* as described previously.[14] The pellets were resuspended in the fresh homogenizing solution (1:1 vol/vol) and protein was measured by the method of Lowry et al.[15]

Isolation of Leydig cells

Leydig cells were obtained from rats fed either a balanced diet or the diet supplemented with cholesterol as indicated above. The testes of each group were removed immediately after decapitation and kept in Krebs Ringer Bicarbonate solution supplemented with 0.1% (wt/vol) glucose plus 0.1% (wt/vol) albumin and buffered at pH 7.4 (KRBGA). The albuginea membrane was removed and the isolation of the Leydig cells was carried out by mechanical shaking in 25-mL flasks (Falcon, Division of Beckton Dickinson Co., Oxnard, CA USA) in the presence of 0.3 mg/mL collagenase according to the procedure of Suescun et al.[16]

The incubation of the cell suspensions and the measurement of cellular uptake and transformation of [1-^{14}C] α-linolenic acid and [2-^{14}C]eicosa-8,11,14-trienoic acid were carried out as described previously.[17] The cell viability, as estimated by trypan-blue exclusion,[18] was 85% to 90%. The medium used for cell incubations was KRBGA, pH 7.4.

Analytical procedures

Plasma cholesterol and triglycerides were assayed by a commercial enzymatic and colorimetric method, respectively (Wiener Lab. Test, Rosario, Argentina).

The desaturation of fatty acids by testicular microsomes was measured by estimation of the percentage conversion of [1-^{14}C]α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid and of [2-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Three nmol of the labeled acid and 12 nmol of the corresponding unlabeled acid were incubated with 1.5 mg of testicular microsomal protein for 10 min at 32° C. The composition of the incubation medium and the isolation and identification of substrate and products have been detailed previously.[19]

The assay for measuring the long chain fatty acyl-CoA synthetase activity of testicular microsomes was similar to that described by Tanaka et al.[20] Amounts of 0.1 mg of microsomal protein and 0.2 μmol (1 mM) of substrate were incubated in a final volume of 0.2 mL for 5 min at 32° C. The fatty acid substrates used in this assay were 18:0, 18:2 (n-6), 18:3 (n-3), and 20:3 (n-6). Reactions were stopped with 2.5 mL of isopropanol/heptane/1 M H_2SO_4 (40/10/1, vol/vol/vol), and unreacted fatty acids were removed by a three-fold extraction with 2 mL of heptane. The aqueous phase containing the thioesterified fatty acids was then assayed for radioactivity. Reaction products were identified by thin-layer chromatography on silica gel G and compared with authentic standards after development in isopropanol/pyridine/acetic acid/water (60/15/1/25, vol/vol/vol/vol).

The microsomal fluorescence anisotropy measurements (at 342 nm excitation and 435 nm emission) were performed at 37° C following the procedure of Shinitzky and Barenholz[21] in an Aminco-Bowman Spectrofluorometer equipped with two Glan prism polarizers (American Instrument Company, Silver Springs, MD USA). Light scattering was less than 5% and fluorescence values were corrected accordingly. The phospholipid to DPH ratio was always maintained at more than 200:1 (mol:mol) to minimize probe-probe interactions

and perturbations in the membrane bilayer. The results were calculated as indicated before.[22]

Chloroform:methanol (2:1 vol/vol) extraction of the lipids from testicular microsomes were performed by the method of Folch et al.[23] Aliquots of the extract were removed to measure the amount of cholesterol[24] and total phospholipids.[25] From the remainder of the extracted sample the total phospholipids were separated by chromatography on a silicic acid column after eluting the polar lipids with methanol.[26] The fatty acids were converted to their corresponding methyl esters and, as such, subsequently analyzed in a Hewlett-Packard model 5840-A gas-liquid chromatograph (Hewlett-Packard, Palo Alto, CA USA) equipped with a flame-ionization detector, as described previously.[19] Total lipids from isolated Leydig cells were extracted with chloroform-methanol and their corresponding fatty acids analyzed as indicated above.

All results were tested for statistical significance by the Student's t test relative to the respective control.

Table 1 Lipid composition of testicular microsomes after cholesterol feeding

Lipids	Balanced diet	+ Cholesterol
Cholesterol (% weight)	17.1 ± 0.7	20.3 ± 0.3**
Total phospholipids (% weight)	82.9 ± 2.1	79.7 ± 3.1
Cholesterol/phospholipids (mol/mol)	0.425 ± 0.028	0.526 ± 0.030*
Lipid/protein (wt/wt)	0.337 ± 0.031	0.546 ± 0.060*

Results are the mean + SEM of four animals analyzed separately. Significantly different from the control diet at $*P < 0.05$ and $**P < 0.01$. Other lipids, such as triacylglycerols and cholesterol esters, were not considered.

Results

Body weight and plasma cholesterol levels

Body weight was unaffected by dietary cholesterol supplementation because the growth increment at the end of the diet was similar in both groups, the percent change of body weight was 432 ± 28 in the control rats and 395 ± 15 in those fed cholesterol. The latter diet, however, substantially elevated plasma cholesterol levels compared with those of the animals fed a balanced regimen: 461 ± 50 mg/dL versus 59 ± 5, respectively; values that were significantly different from those of the control group at $P < 0.001$.

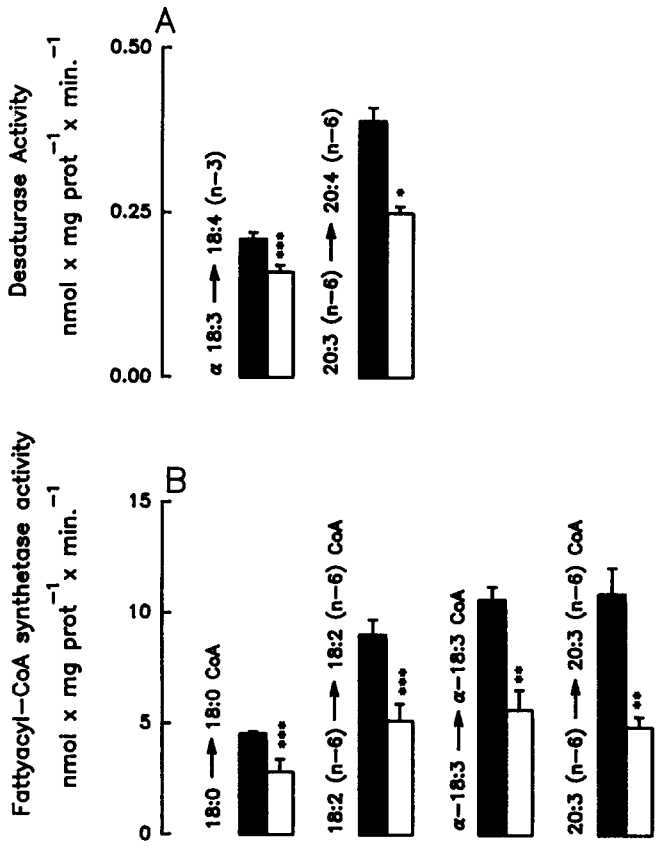

Figure 1 Fatty acid desaturase (A) and fatty acyl-CoA synthetase (B) activities in testicular microsomes of rats fed the balanced (filled columns) or the cholesterol-supplemented (open columns) diet. Results are the means ± SEM from four animals, with each having been analyzed in triplicate. Significant difference from control values at $*P < 0.01$, $**P < 0.02$, $***P < 0.05$.

Microsomal desaturase and long chain fatty acyl-CoA synthetase activities

The effect of dietary cholesterol on fatty acid desaturation was studied by measuring microsomal Δ6 and Δ5 desaturase activities. The reactions were α-18:3 → 18:4(n-3) and 20:3(n-6) → 20:4(n-6), respectively *(Figure 1A)*. After 2 months on the experimental diet, the animals fed cholesterol exhibited significantly lower values for both of these desaturases than the control rats. The dimunition in Δ6 desaturase activity was 23.8%, while that in Δ5 desaturase activity was 35.9%.

The thioesterification of labeled stearic, linoleic, α-linolenic or eicosa-8,11,14-trienoic acid was depressed in the microsomes of the rats fed cholesterol as compared with the controls *(Figure 1B)*. The inhibition of the enzyme was especially remarkable in the examples of α-linolenyl-CoA and eicosatrienoyl-CoA synthesis, where the decreases were about 50%.

Lipid composition and total fatty acid profile of phospholipids from testicular microsomes

Dietary cholesterol induced changes in the testicular microsomal lipid composition: in the sterol-fed animals, there was an increase in the abundance of cholesterol as well as an increment in both the cholesterol to phospholipid and the lipid to protein ratios in this subcellular compartment relative to control values *(Table 1)*.

By contrast, the fatty-acid composition of this same phospholipid fraction was unaffected by dietary cholesterol intake, with the pattern observed in the animals on either diet being comparable with the profiles reported previously by other authors for rats fed a balanced diet.[27,28] Briefly stated, palmitic acid was the major saturated component at 32%, while oleic and linoleic acids were notable among the 18-carbon unsaturates at 12%

to 13% and 5.5% to 6.5%, respectively; arachidonic and docosapentaenoic were the principal representatives of the long chain polynosaturates, being at an abundance of 15% and 15.5% to 16.5%, respectively.

Steady-state fluorescence anisotropy of DPH

As compared with the microsomes obtained from the animals maintained on a balanced diet, those from the cholesterol-fed animals manifested a significant increase in the DPH steady-state fluorescence anisotropy of their lipid phase, as evidenced by a decrease in the rotational mobility of the probe in the membrane samples. The values obtained were 0.140 ± 0.001 for the control animals versus 0.158 ± 0.001 for the cholesterol-fed rats, with the two sets of data being significantly different at $P < 0.001$.

Uptake and desaturation of [1-^{14}C]α-linolenic and [2-^{14}C] eicosa-8,11,14-trienoic acids in isolated Leydig cells

Figure 2 shows that the uptake and transformation of both labeled acids in the Leydig cells of the experimental animals was not modified by the dietary treatment.

Fatty acid composition of total lipids in isolated testicular cells

The relative distribution of total fatty acids in isolated and non-incubated Leydig cells is shown in *Table 2*. The general pattern observed in the rats fed a balanced diet showed that the major components of the non-essential fatty acid series were the saturated acids while 18:2 (n-6), 20:4 (n-6), and 22:5 (n-6) were the most abundant among the polyunsaturated fatty acids.

No significant change in the fatty acid composition of Leydig cells was observed as a consequence of the addition of cholesterol to the diet.

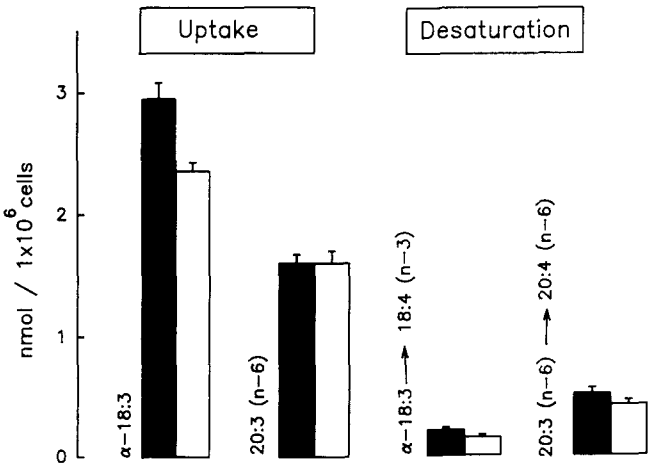

Figure 2 Uptake of [1-^{14}C]α-linolenic and [2-^{14}C] eicosa-8,11,14-trienoic acids (left) and their respective desaturations (right) in Leydig cells isolated from rats fed a balanced diet (filled bars) or from those fed the cholesterol-supplemented diet (open bars). The incubation conditions are described in Methods and materials. Values are the means ± SEM from five animals, with each having been analyzed in triplicate.

Table 2 Fatty acid composition of Leydig cells isolated from rats fed a balanced diet or a cholesterol-supplemented diet

Fatty acid	Balanced diet	+ Cholesterol
16:0	20.0 ± 1.3	25.6 ± 2.0
16:1	1.3 ± 0.1	1.9 ± 0.5
18:0	20.3 ± 0.0	17.2 ± 1.1
18:1	12.3 ± 0.3	12.2 ± 1.1
18:2 (n-6)	12.2 ± 0.3	11.7 ± 0.1
20:3 (n-6)	2.0 ± 0.0	2.2 ± 0.3
20:4 (n-6)	19.4 ± 0.8	16.5 ± 2.2
22:4 (n-6)	3.7 ± 0.0	3.1 ± 0.9
22:5 (n-6)	6.9 ± 0.7	5.7 ± 0.8
22:5 (n-3)	1.4 ± 0.0	3.0 ± 0.6
22:6 (n-3)	0.5 ± 0.0	0.9 ± 0.3

Results are expressed as μg% of the total fatty acids extracted from the cells under the conditions described in Methods and materials. Values are the means ± SEM from five animals, with each having been analyzed in triplicate.

Discussion

It is well known that dietary cholesterol participates in the modulation of desaturase activity in liver and adrenal gland.[5-9] In the work described here, this effect was shown also to be extensive within the testicular microsomes, where Δ5 and Δ6 desaturase activities, as well as the long chain fatty acyl-CoA synthetase, were significantly reduced after the inclusion of cholesterol in the diet *(Figure 1)*. We assume that this depression of the desaturases was independent of the concomitant decrease in the fatty acyl-CoA synthetase because the amount of thioesterified fatty acids available for the desaturases was always many times higher than that necessary for the substrate saturation of those enzymes. Despite this dimunition in the desaturation capacity of the testicular microsomes, however, no changes were observed in the fatty acid composition of the phospholipids from this subcellular fraction after rats were fed the high cholesterol diet. One possible explanation for such a discrepancy would be that, instead of being synthesized in situ in the testes, most of the polyunsaturates present in the testicular membranes (e.g., 20:4 and 22:5) are first assembled in the liver or intestine and then transported to the testis. Such a putative form of testicular homeostasis would serve to protect the animal's reproductive capacity at the expense of only a modest exacerbation of the existing polyenoate deficiency in either of those far larger internal organs.

Considering that microsomes represent merely a subcellular fraction of the whole tissue and that individual testicular cells are highly specialized, we decided to evaluate the capacity of isolated Leydig cells for incorporating and transforming labeled α-18:3 and 20:3 (n-6) acids in short-term incubations. In this regard, the uptake and desaturation of 20:3 (n-6) by the cells in the control rats were comparable with those respective values from similar experiments recently reported by

us *(Figure 2)*.[17] In the present work, we demonstrate that these cells are also capable of incorporating and transforming α-18:3 acid to 18:4 (n-3), the degree of completion for this overall process being about 5% to 10%. Whereas this activity represents only a meager level of desaturation, such a result would not seem surprising in view of the fact that n-3 acids are present in sparing amounts in Leydig cells (2% to 4% of the total fatty acids). With both substrates, under similar experimental conditions, the extents of conversion by the Leydig cells were lower than had been obtained previously in isolated adrenocortical cells.[29] This difference is consistent with earlier findings with the microsomal fractions of both parental tissues, in which the activities of the Δ5 and Δ6 desaturases measured in either liver or adrenal microsomes[30] were higher than the values presently obtained in testicular microsomes *(Figure 1)*.

In contrast to these observations with the various microsomal fractions *(Figure 1)*,[30] a high cholesterol diet failed to inhibit either Δ5 or Δ6 desaturation in the isolated Leydig cells *(Figure 2)*; and this insensitivity to cholesterol feeding was in accord with the similarity in the fatty acid profiles obtained with these cells under both dietary conditions. Thus, to explain the cholesterol-induced reduction in polyenoate desaturation in total testicular microsomes, we can conclude provisionally that alterations occurring within testicular cells other than the Leydig were responsible for the changes observed within the overall tissue. Because of the two principal non-Leydig elements within the testis, the Sertoli and the germinal cells, the latter are present in greater number,[31] we would reason that the germinal cells were the more likely target for these adverse effects of the high cholesterol regime.

Finally, we found a significant enhancement in the fluorescence anisotropy of the lipid phase in the testicular membranes from the cholesterol-fed rats, relative to the data on parallel preparations from control animals. This decrease in the fluidity would be directly attributable to the higher cholesterol content of the testes from this group *(Table 1)* and could conceivably be responsible, at least indirectly, for the depression observed in the desaturases and the long chain fatty acyl-CoA synthetases as well. These results might have been anticipated from previously documented observations on the effect of hypercholesterolemia in other tissues,[5,6] because a positive correlation between membrane fluidity and microsomal membrane Δ5 and Δ6 desaturase activities had been previously established in liver.[5]

On the basis of the findings from these experiments, we can conclude that a high cholesterol regime carries adverse effects on the desaturation as well as on the thioesterification of essential fatty acids in testicular microsomes. In spite of these alterations, it is nonetheless interesting to note that the testes would appear to have a high resistance to changes in their fatty-acid composition. By contrast, both liver and adrenal gland evidenced significant alterations in their membrane total fatty acid profiles under comparable experimental conditions. The involvement of the enzymic changes observed in the present work on the testicular function is still unknown. Further studies are currently in progress to gain additional insight into these questions.

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