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EFFECT OF HYPOTHYREOSIS ON THE CONTENT OF CERAMIDES IN RAT TISSUES.

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Ceramide is the second messenger in the sphingomyelin signalling pathway. A number of extracellular stimuli increase the content of ceramide in the cell. There are some data indicating that the content of ceramide may also be regulated by hormones. The aim of the present study was to examine the effect of hypothyreosis on the content and composition of ceramide in rat tissues. The rats were thyroidectomized and thereafter they received propylthiouracyl in drinking water. The control rats were sham operated. 30 days after thyroidectomy or sham operation the rats were anaesthetized and samples of the liver, white and red vastus lateralis and left ventricle were taken. One set of samples was frozen in liquid nitrogen for analysis of ceramide. Another set of samples was freshly homogenized in chloroform/methanol for further determination of the content of sphingomyelin phosphorous. The content and composition of ceramide-fatty acids was determined by means of gas-liquid chromatography. Twelve ceramides containing different fatty acid residues were identified in both groups. Hypothyreosis reduced the total content of ceramide in each tissue studied: in the heart by 50.9%, in the red vastus by 28.6%, in the white vastus by 29.4% and in the liver by 22%. Concomitantly, the content of individual ceramides was either reduced, stable or even elevated, depending on the tissue. The content of sphingomyelin was elevated in both sections of the vastus lateralis and remained stable in the heart and the liver. The ratio: total content of sphingomyelin to total content of ceramide was elevated in the muscles and remained stable in the liver. This indicates that the reduction in the content of ceramide in the tissues of hypothyroid rats may be a consequence either of a reduction in the formation of ceramide from sphingomyelin, its increased hydrolysis or both. It is concluded that normal thyroid function is needed to maintain the content and composition of ceramide in the tissues.

Key words: *ceramide, sphingomyelin, hypothyreosis, muscles, liver, rat.*

INTRODUCTION

Ceramide is the second messenger in the sphingomyelin transmembrane signalling pathway. In the process of signal transduction, sphingomyelin located in the plasma membrane and in the membranes of endosomes and lysosomes is hydrolysed by the enzymes neutral Mg^{++}-dependent sphingomyelinase and acidic sphingomyelinase, respectively. Ceramide exerts broad biological effects. The main ones are: inhibition of cell proliferation, activation of cell differentiation, induction of apoptosis and involvement in inflammatory reactions (1-3). The compound is also involved in regulation of insulin-induced glucose uptake in adipocytes and myocytes (4). Ceramide is hydrolysed to sphingosine and a fatty acid by the enzyme ceramidase. Sphingosine can be further converted to sphingosine-1-phosphate by the enzyme sphingoid base kinase. Both sphingosine and sphingosine-1-phosphate are also biologically active compounds. Their major function seems to be involvement in the regulation of intracellular calcium metabolism (2,5). Formation of ceramide is activated by a number of extracellular stimuli acting on the plasma membrane receptors. The stimuli were classified into four groups: inducers of apoptosis, inducers of differentiation, damaging agents and inflammatory cytokines (2). Hormones are among these factors. The content of ceramide was shown to be elevated by dexametasone in murine B lymphoma cells, by progesterone in Xenopus laevis oocytes, and by $1,25(OH)_2$vit. D_3 in human leukemia cells and human keratinocytes (3). In skeletal muscles of obese, insulin resistant Zucker rats (6) and in rats made diabetic by treatment with streptozotocin (7) the content of ceramide is elevated. This points to the role of insulin in the regulation of ceramide metabolism. Thyreoidectomy did not affect the total content of ceramide in the liver (8). No data on the involvement of thyroid hormones in the regulation of the content of ceramide in other tissues are available. The aim, therefore, of the present study was to examine the effect of hypothyreosis on the content and composition of ceramides in skeletal muscles, the heart muscle and in the liver. It has been shown that hypothyreosis results in a reduction in the total content of ceramide and changes in its composition in each of the examined tissues.

MATERIAL AND METHODS

The experiments were carried out on male Wistar rats, 140-150 g of initial body mass. The animals had free access to a commercial pellet diet for rodents and tap water. A 12h light/ dark schedule was maintained in the animal quarters. The animals were divided into two groups: 1-control, euthyroid, sham operated. 2-hypothyroid. Rats in this group were subjected to thyroidectomy under ether anaesthesia. Thereafter, they received propylthiouracyl (Prosst, Canada) dissolved in drinking water (0.04% solution) for 30 days. The rats were then anaesthetized with pentobarbital sodium (80mg/100g) and samples of the white and red sections of the vastus lateralis, liver, and left heart ventricle were taken, weighed and quickly frozen in liquid nitrogen for further analysis of ceramide content and composition. Then, further samples were taken, weighed and homogenized in chloroform/methanol (2:1) in a glass homogenizer. Lipids were extracted

according to Folch *et al.* (9). The lipids were fractionated on silica plates (Kieselgel 60, 0.25mm, Merck) using a developing solvent composed of chloroform, methanol, acetic acid and water (50:37.5:3.5:2 v/v/v/v) (10). The phospholipid bands were visualized under an ultraviolet lamp after spraying the plates with 0.5% solution of 2'7'-dichlorofluoresceine in absolute methanol and exposure to ammonium vapours. The band corresponding to sphingomyelin was identified according to standard (Sphingomyelin from bovine brain, Sigma) which was run along with the samples. Next, it was scraped off the plate and analyzed for the content of phosphorus (11). The samples frozen in liquid nitrogen were pulverized in an aluminum mortar with a stainless steel pestle precooled in liquid nitrogen. The powder was transferred to glass tubes containing methanol at a temperature of -21°C (12). Methanol contained butylated hydroxytoluene (Sigma) as an antioxidant at a concentration of 30mg/100ml. Lipids were extracted as above. To isolate ceramide, the samples were spotted on silica plates (as above) and developed to one-third of the total length of the plate in chloroform/methanol/25% NH_3 (20:5:0.2 v/v/v)(13). Next, they were dried and rechromatographed in heptane/isopropyl ether/acetic acid (60:40:3 v/v/v) (14). The standard of ceramide (Non-hydroxy fatty acid ceramide, Sigma) was run along with the samples. Lipid bands were visualized as above. The band corresponding to ceramide was scraped off the plate and transferred to screw tubes containing methylpentadecanoic acid (Sigma) as an internal standard. Fatty acids were transmethylated along with the gel in the presence of 1 ml of 14% boron fluoride in methanol at temperature of 100°C for 90min (15). Thereafter, the samples were cooled to room temperature, 1 ml of pentane and 0.5 ml of water were added and the resulting phases were separated by centrifugation. The upper pentane phase was transferred into new tubes and evaporated under nitrogen. The methyl esters were dissolved in 40µl of hexane and analyzed by gas-liquid chromatography using a Hewlett-Packard 5890 Series II chromatograph and a fused Hp-INNOWax (50m) capillary column. The chromatograph was equipped with a double flame ionization detector. Injector and detector temperatures were set at 250°C each. The oven temperature was increased linearly from 160 to 230°C at a rate of 5°C/min. Individual fatty acid methyl esters were quantified using the area corresponding to the internal standards. The standards of long chain fatty acids were purchased from Sigma. The results are presented as means ± SD. N=10 rats in each group. The data were evaluated statistically using the Student t-test for unpaired data.

RESULTS

Ceramide

Heart (*Table 1*). The total content of ceramide in hypothyroid rats was lower than in the control group. In the hypothyroid group, the content of ceramide containing myristic, palmitic, stearic, oleic, linoleic and arachidonic acid was reduced, the content of ceramide containing nervonic acid was elevated and the content of other ceramides remained stable.

Red vastus (*Table 2*). Hypothyreosis resulted in a reduction in the total content of ceramide. This was accounted for by a reduction in the content of ceramide containing palmitic, oleic and linoleic acid. The content of other ceramides was stable.

White vastus (*Table 3*). Hypothyreosis decreased the total content of ceramide. This was accounted for by a reduction in the content of ceramide containing

Table 1. Effect of hypothyreosis on the content and percentage composition of ceramide-fatty acids in the heart

Acid	Control		Hypothyreosis	
	Content (nmol/g of tissue)	% Composition	Content (nmol/g of tissue)	% Composition
14:0	3.64 ± 0.46	3.09 ± 0.53	2.70 ± 0.69^{c}	4.54 ± 0.25^{d}
16:0	34.09 ± 11.96	27.72 ± 5.85	13.17 ± 3.10^{d}	22.24 ± 1.73^{b}
16:1	2.93 ± 0.86	1.48 ± 0.77	3.39 ± 1.51	5.54 ± 1.36^{d}
18:0	35.37 ± 7.45	29.56 ± 3.92	12.12 ± 1.92^{d}	20.73 ± 2.28^{d}
18:1	24.78 ± 5.05	20.90 ± 3.96	15.53 ± 3.69^{d}	26.19 ± 1.64^{c}
18:2	7.02 ± 1.30	5.91 ± 1.00	2.75 ± 0.69^{d}	4.66 ± 0.62^{c}
18:3	2.05 ± 1.08	1.64 ± 0.61	1.17 ± 0.54	1.93 ± 0.61
20:4	5.50 ± 2.87	4.40 ± 1.90	3.10 ± 1.37^{a}	5.37 ± 2.68
20:5	1.66 ± 0.31	1.41 ± 0.28	1.37 ± 0.25	2.37 ± 0.50^{d}
22:6	1.36 ± 0.55	1.17 ± 0.49	1.04 ± 0.28	1.77 ± 0.40^{d}
24:1	2.00 ± 0.52	1.74 ± 0.62	2.77 ± 0.69^{b}	4.67 ± 0.46^{b}
Total	120.40 ± 22.88		59.11 ± 12.46^{d}	

Total – the sum of individual ceramides. Values are mean ± SD, N=10. The acids are: myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4), eicosapentaenoic (20:5), docosahexaenoic (22:6), nervonic (24:1). a-$p < 0.05$, b-$p<0.02$, c-$p < 0.01$, d-$p<0.001$, vs. the respective control value;

Table 2. Effect of hypothyreosis on the content and percentage composition of ceramide-fatty acids in the red vastus.

Acid	Control		Hypothyreosis	
	Content (nmol/g of tissue)	% Composition	Content (nmol/g of tissue)	% Composition
14:0	3.85 ± 1.38	3.09 ± 1.23	3.37 ± 0.62	3.92 ± 1.15
16:0	31.11 ± 9.78	24.01 ± 6.00	18.62 ± 6.87^{c}	20.15 ± 3.06
16:1	3.60 ± 1.44	2.72 ± 0.68	4.21 ± 0.93	4.70 ± 0.71^{d}
18:0	26.15 ± 2.54	20.72 ± 2.60	25.60 ± 15.42	26.49 ± 6.23^{a}
18:1	41.02 ± 11.89	31.77 ± 5.12	27.72 ± 8.26^{b}	30.24 ± 2.51
18:2	10.71 ± 6.18	7.85 ± 3.29	2.13 ± 1.44^{c}	3.55 ± 1.87^{c}
18:3	1.69 ± 0.74	1.28 ± 0.41	1.11 ± 0.51	1.25 ± 0.62
20:4	2.99 ± 1.15	2.31 ± 0.58	2.27 ± 0.71	2.72 ± 1.22
20:5	2.83 ± 1.34	2.23 ± 0.95	1.79 ± 0.75	2.12 ± 0.65
22:6	1.48 ± 0.57	1.16 ± 0.40	1.09 ± 0.36	1.28 ± 0.56
24:1	3.62 ± 1.45	2.85 ± 1.00	3.20 ± 0.86	3.58 ± 0.79
Total	129.05 ± 28.94		92.11 ± 29.29^{b}	

Legend as in the *Table 1*.

palmitic, palmitoleic, oleic and linoleic acid. The content of ceramide containing eicosapentaenoic acid increased and the content of other ceramides remained stable.

Table 3. Effect of hypothyreosis on the content and percentage composition of ceramide-fatty acids in the white vastus.

Acid	Control		Hypothyreosis	
	Content (nmol/g of tissue)	% Composition	Content (nmol/g of tissue)	% Composition
14:0	3.19 ± 1.37	3.36 ± 0.95	2.55 ± 0.73	3.88 ± 0.58
16:0	25.09 ± 4.11	27.08 ± 1.76	13.23 ± 4.78[d]	19.69 ± 3.29[d]
16:1	2.11 ± 0.40	2.31 ± 0.57	3.01 ± 0.83[b]	4.59 ± 1.02[d]
18:0	18.47 ± 3.85	19.89 ± 2.89	19.96 ± 3.71	30.63 ± 2.08[d]
18:1	24.76 ± 1.03	27.01 ± 3.04	17.61 ± 3.17[d]	27.06 ± 2.09
18:2	12.47 ± 4.54	13.16 ± 2.78	1.95 ± 0.52[d]	3.05 ± 0.89[d]
18:3	1.10 ± 0.25	1.18 ± 0.12	1.07 ± 0.56	1.63 ± 0.79
20:4	1.91 ± 0.99	1.99 ± 0.85	1.95 ± 1.01	2.91 ± 1.40
20:5	1.08 ± 0.30	1.20 ± 0.45	1.69 ± 0.53[c]	2.72 ± 1.08[c]
22:6	1.02 ± 0.33	1.16 ± 0.51	0.79 ± 0.21	1.25 ± 0.44
24:1	1.52 ± 0.10	1.66 ± 0.19	1.68 ± 0.46	2.59 ± 0.61[c]
Total	92.74 ± 14.32		65.51 ± 13.23[d]	

Legend as in the *Table 1*.

Table 4. Effect of hypothyreosis on the content and percentage composition of ceramide-fatty acids in the liver.

Acid	Control		Hypothyreosis	
	Content (nmol/g of tissue)	% Composition	Content (nmol/g of tissue)	% Composition
14:0	4.65 ± 0.67	3.09 ± 0.53	3.04 ± 0.63[d]	4.54 ± 0.25[d]
16:0	47.85 ± 5.53	27.72 ± 5.85	49.50 ± 9.95	22.24 ± 1.73[b]
16:1	3.70 ± 0.37	2.48 ± 0.77	5.42 ± 0.68[d]	5.54 ± 1.36[d]
18:0	38.07 ± 3.76	29.56 ± 3.92	31.89 ± 6.60[b]	20.73 ± 2.28[d]
18:1	97.77 ± 15.46	20.90 ± 3.96	48.89 ± 31.88[d]	26.19 ± 1.64[c]
18:2	41.67 ± 10.06	5.61 ± 1.75	33.78 ± 13.20	4.66 ± 0.62
18:3	1.93 ± 0.78	1.64 ± 0.61	2.07 ± 0.56	1.93 ± 0.61
20:4	8.67 ± 0.79	4.40 ± 1.90	8.66 ± 5.56	5.37 ± 2.68
20:5	2.22 ± 0.19	1.41 ± 0.28	3.44 ± 1.10[c]	2.37 ± 0.50[d]
22:6	1.28 ± 0.21	1.17 ± 0.49	1.05 ± 0.37	1.77 ± 0.69
24:1	18.69 ± 2.72	1.74 ± 0.62	18.57 ± 4.75	4.67 ± 0.46[d]
Total	263.78 ± 28.10		205.96 ± 59.29[b]	

Legend as in the *Table 1*.

Liver (*Table 4*). As in the case of the muscles, the total content of ceramide in the liver in hypothyroidism was reduced. This was accounted for by a reduction in the content of ceramide containing myristic, stearic, and oleic acid. Simultaneously, the content of ceramide containing palmitoleic and eicosapentaenoic acid decreased whereas the content of other acids did not change.

Sphingomyelin (*Table 5*). In the hypothyroid group, the content of sphingomyelin in the red and white section of vastus lateralis increased whereas in the heart and in the liver it did not change.

Table 5. Effect of hypothyreosis on the content of sphingomyelin in different tissues of the rat

Tissue	Control	Hypothyreosis
Heart	487.35 ± 67.17	471.15 ± 185.37
Red Vastus	462.41 ± 54.84	633.63 ± 126.21**
White Vastus	385.67 ± 52.44	469.41 ± 91.35*
Liver	438.28 ± 42.21	448.27 ± 193.91

Sphingomyelin content is expressed in nmol/gram of wet tissue.
* $p<0.05$, ** $p<0.001$ vs. the respective control value.

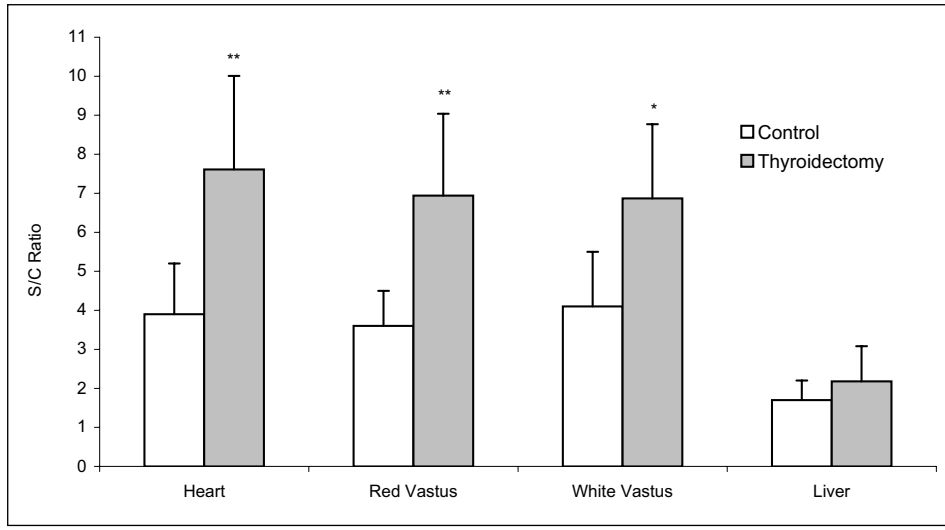

Figure 1. Ratio of the total content of sphingomyelin to the total content of ceramide (S/C ratio) in rat tissues. * $p<0.01$; ** $p<0.001$ vs. the respective control value.

Ratio: the content of sphingomyelin to the content of ceramide (*Fig. 1*). Hypothyroidism resulted in an elevation of the ratio in each muscle studied. The ratio remained stable in the liver.

DISCUSSION

The results obtained clearly show that hypothyroidism results in a reduction in the total content of ceramide in each tissue studied. However, the magnitude of the reduction depends on the tissue: in the heart, the content of ceramide is reduced by 50.9%, in the red vastus by 28.9%, in the white vastus by 29.4% and in the liver by 22%. The results in the heart and skeletal muscles in hypothyroidism are reported for the first time and therefore they cannot be compared to data obtained by others. In the liver, Babenko *et al.* (8) did not observe any significant change in the content of ceramide in hypothyroidism and thus our data are different in this respect. This discrepancy may be due to a different protocol in the production of hypothyroidism. In Babenko *at al.* (8) study, the hypothyroidism was induced by a daily injection of a compound called mercasolil for 16 days whereas we sampled the rats 30 days after removal of the thyroid and daily treatment with propylthiouracyl. Our data indicate that thyroid hormones are needed to maintain the content of ceramide in the examined tissues. They also show that the heart muscle is the most susceptible in this respect.

As mentioned in the introduction, in the sphingomyelin signalling pathway, ceramide is formed as a result of hydrolysis of sphingomyelin present in the plasma membrane or/and the membranes of endosomes and lysosomes. A reduction in the content of ceramide in hypothyroidism was most likely a consequence of the decreased rate of sphingomyelin hydrolysis. Indeed, the content of sphingomyelin remained constant in the heart and in the liver and was even elevated in the skeletal muscles and this supports this possibility. Also, a reduction in the ratio: total content of sphingomyelin to total content of ceramide in the heart and skeletal muscles provides indirect evidence for reduced activity of the pathway in hypothyroidism. However, it must be kept in mind that we took samples 30 days after the removal of the thyroid gland. Therefore, not only changes in functioning of the sphingomyelin signalling pathway but also other pathways of ceramide metabolism might have contributed to the reduction in the content of this compound in the tissues. It should be added that regulation of ceramide metabolism is a complex issue. It can also be formed on routes other than hydrolysis of sphingomyelin and moreover, these routes are reversible. Ceramide is catabolyzed to sphingosine by the action of the enzyme ceramidase. This reaction is also reversible since sphingosine can be acylated to ceramide by the enzyme ceramide synthase. The reactions catalysed by sphingomyelinase and ceramidase, i.e. ceramide liberation from sphingomyelin and ceramide deacylation are considered to play a key role in the regulation of the content of ceramide (1-3). Other routes of ceramide metabolism are slow and there are no data available indicating that they may contribute to any considerable extent to the content of ceramide in the tissues. It may, therefore, be presumed that in hypothyroidism, the content of ceramide in the tissues is reduced, most likely

because of a reduction in the rate of its formation from sphingomyelin. It is also likely that the rate of hydrolysis of the compound is augmented.

Though the total content of ceramides was reduced in hypothyreosis, the content of the individual ceramides behaved differently: it was reduced, stable or even elevated depending on the tissue examined. Similar diversity in the behavior of the content of individual ceramides was previously observed in different skeletal muscles after prolonged exercise (14). The reason for this phenomenon has not been elucidated so far. It is likely that it depends on the availability of particular sphingomyelins for the action of sphingomyelinase. Also, any difference in the biological action of particular ceramides has not been proved, as yet. Therefore, any consequences of changes in the composition of ceramides in the tissues, if indeed they exist, remains to be elucidated.

The present paper is the second one to report on a reduction in the content of ceramide in the tissues. It has previously been shown that prolonged exercise reduces the content of the compound in skeletal muscles (14). As far as we are aware, the biological effects of a reduction in the content of ceramide in the tissues has not been studied. Ceramide reduces insulin stimulated glucose uptake by isolated myocytes and adipocytes (4). Its content in skeletal muscles is inversely related to 2-deoxyglucose uptake by the muscles (14). Hypothyroidism may reduce requirements for insulin and cause a tendency to hypoglycemia in diabetic patients (16). It remains to be established whether the reduction in the content of ceramide in the tissues in hypothyreosis contributes to the phenomenon.

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