DETERMINATION OF BRANCHED CHAIN FATTY ACIDS IN PLASMA OF TYPE 2 DIABETICS

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Abstract:

Insulin resistance in type 2 diabetic patients reduces activation of PPAR, which may lead to accumulation of branched chain fatty acids as well as saturated fatty acids. Natural sources of these fatty acids are dairy products. The aim of our study was to verify whether the accumulation of branched chain fatty acids takes place in type 2 diabetes and in positive case to specify the corresponding lipid fraction.

23 anonymized plasma samples of type 2 diabetic patients, which were subsequently divided by glycosidic haemoglobin levels into groups of 11 compensated and 12 decompensated; and plasma of 10 healthy blood donors were processed. At first the samples were divided into particular lipid classes using the thin layer chromatography. Then we set the content of individual fatty acids in all lipid classes using the gas chromatography. Results were calculated with statistical application SigmaStat 3.5.

The most abundant branched chain fatty acid is 14-methylhexadecanoic acid. Statistically significant increase of this acid was found both in compensated

diabetics (p≤0.001) and in the decompensated ones (p≤0.001) in comparison with controls. The 14-methylhexadecanoic acid was found in diacylglycerol fraction as well as in the free fatty acid fraction in compensated (p=0.008) and decompensated (p=0.007) diabetics.

Increase of the content of branched chain fatty acids in diabetic patients was proved. Accumulation of branched chain fatty acids in diabetics raises a question on the precise influence of these fatty acids on the human organism.

Key words: branched chain fatty acids, gas chromatography, type 2 diabetes mellitus, PPAR

Introduction

Information about intake, metabolism and physiological effect of branched chain fatty acids (BCFA) are inadequate, as well as detail knowledge about their favorable or adverse influence on the human organism [1]. One of the few published studies, using the rats, suggests that the increased concentration of BCFA in the gastrointestinal tract of unborn offsprings positively correlates with decreased incidence of necrotizing enterocolitis (a disease affecting premature babies) [2]. There is an increasing promotion of healthy lifestyle associated with consumption of dairy products, which contain relatively high amounts of BCFA. Ran-Ressler [3] described the content of BCFA in milk, supplied by American supermarkets, reaching up to 2 %.

Placement of Figure 1

It is known that straight-chain fatty acids are degraded only in the mitochondrial matrix, while BCFA and very long-chain fatty acids are primarily oxidized in peroxisomes and only then transported as acylcoenzyme A to mitochondria [4].

This process is controlled by PPAR (peroxisome proliferator-activated receptors), which represent the nuclear factors regulating production of lipogenic enzymes, which are necessary for implementing the aforesaid peroxisome and mitochondrial fatty acid oxidation. Several kinds of PPAR receptors exist. In humans, PPARα are expressed especially in the liver, skeletal muscles, kidney and vascular endothelium, wherein they regulate the expression of many genes encoding enzymes participating in the peroxisome proliferation and in fatty acid oxidation occurring in peroxisomes and mitochondria [5]. PPARβ / δ were found in various tissues, but knowledge about them is still limited; Michalik [6] suggests that they also interfere with lipid metabolism – these PPAR diminish serum triglyceride concentrations by stimulation of the elimination of VLDL (very low density lipoproteins). PPARγ has been the best-studied group of receptors so far. They are the most prominent in adipose tissue, where they occur in two isoforms - PPARγ1 and PPARγ2, in smaller quantities they are expressed in the spleen, cells of the hematopoietic system and skeletal muscles (predominantly PPARγ1 isoform) [7, 8]. Activation of PPAR is preferably implemented by polyunsaturated and essential fatty acids, while saturated fatty acids are less effective [9]. Effect of BCFA on PPAR has not been studied yet.

Experimental

Patients

The study has involved 23 anonymized plasma samples of type 2 diabetic (T2D) patients and 10 healthy blood donors as control group. The inclusion criteria of study were: All patients (1) above 30 years of age, (2) either gender,

(3) with diagnosed type 2 diabetes mellitus for up to 1 year, (4) who keep an antidiabetic low-calorie diet plus per-oral antidiabetic drugs.

The exclusion criteria of study were: (1) type-1 diabetes, (2) renal failure, hepatic, oncologic or thyroid disease (3) regularly consumption of alcohol, (4) insulin treatment. None of study participants suffered from apparent cardiovascular disease, diabetic nephropathy and retinopathy. None of control persons was aware of metabolic disorder such as diabetes mellitus or hyperlipidemia. They did not follow any specific dietary recommendation. They had not been taking any long term medication. A written informed consent was obtained from all the participants.

Patients with glycosidic haemoglobin levels from 43 to 53 mmol/mol were taken, for purposes of the study, as a group of compensated diabetics, while T2D patients with higher levels of glycosidic haemoglobin were considered as decompensated diabetics. Control group consists of plasma of 10 healthy blood donors. Both diabetic and control samples were provided by the Regional Hospital of Pardubice. Samples was approved by the Hospital Ethical Committee.

Placement of Table I

Laboratory methods

Venous blood was obtained under standard conditions, from 7 to 8 a.m. after fasting for at least 12 hours. Blood was collected in tubes with EDTA, plasma was obtained by centrifugation at 1500 g for 20 min and immediately stored at -80 °C.

Extraction

EDTA plasma was cleared from protein using solution of 2-propanol, n-heptane and 2 M phosphoric acid (v/v/v 40:20:1) (Merck, KGaA, Darmstadt, Germany). The solution was mixed by vortex and conditioned 10 min. Methanol/toluene mixture (1:4) (Merck, KGaA, Darmstadt, Germany) and distilled water were added to the test tube. After 10 min centrifugation at 8 175 g, the upper organic layer was transferred into a clean tube and evaporated under nitrogen at room temperature.

Thin layer chromatography

The lipids were dissolved in chloroform/methanol (2:1) and applied to a silica gel chromatography plate (20x20 cm Kieselgel 60, Merck, KGaA, Darmstadt, Germany). The phospholipids (PL), diacylglycerols (DAG), free fatty acids (FFA), triacylglycerols (TAG) and cholesterol esters (CE) were separated using mixture of n-hexane, diethylether and acetic acid (160:40:6) as a mobile phase. To identify the individual fractions, pooled control plasma was also separated on each plate and lipid fractions were visualized by 2,7-dichlor-fluoresceine under ultraviolet light. The fractions were scraped off the TLC plate, transferred to screw-capped vials.

Transesterification

Internal standard (cis-13, 16, 19-docosatrienoic acid, 10 μg/mL) and methanol/toluene mixture were added to the samples in the Pyrex glass. Acetyl chloride was added to the sample in a thermal block. The mixture was heated for 1 hour under 100 °C in closed Pyrex glass, cooled down and neutralized by K_2CO_3, 6 %. Fatty acids (FA) were changed to their corresponding methyl

esters. The upper phase was concentrated to 80 µl under the nitrogen atmosphere.

Gas chromatography

FA methyl esters were quantified by gas chromatograph (GC System 7890A, Agilent Technologies, USA) with autosampler and a flame ionization detector, using a chromatographic fused column HP-88 (length 100 m, internal diameter 0.25 mm, film thickness 0.2 µm). Helium was used as a carrier gas at a flow rate of 3 mL/min. The gas chromatograph oven temperature was initially held at 130 °C for 1 min; then the temperature was programmed up to 176 °C at 2 °C/min and held for 2 min; then the temperature was programmed to 186 °C at 1 °C/min and held for 1 min; then at 0.1 °C/min to 190 °C and held for 1 min; then at 1 °C/min to 220 °C and held for 4 min. The samples were injected in Split mode (split ratio 10:1). The temperature of injector was 250 °C and the temperature of detector was set to 280 °C [10].

Placement of Table II

Statistical analysis

All statistical analyses were computed using SigmaStat 3.5. Differences in variables between the groups were evaluated using Mann Whitney test. P value less than 0.05 was considered statistically significant. The results are expressed as absolute concentrations of the relevant fatty acids in µmol/L.

Results and Discussion

The fatty acid analysis revealed that the most represented BCFA in all measured lipid fractions was 14-methylhexadecanoic acid (an average 57 % of BCFA content across all lipid fractions). Therefore this compound was

considered as a representative of total BCFA level. Statistically significant increase of this acid was found in DAG and FFA fraction in T2D patients compared to the controls (see Fig. 1). Median of BCFA sum in control fractions was in DAG 3.25 μmol/L and in FFA 4.53 μmol/L. The fractions richest in the incidence of BCFA were TAG (median 19.02 μmol/L in controls) and CE (median 14.86 μmol/L in controls). No significant changes in BCFA content were found in PL fraction.

Placement of Figure 2

The T2D patients exhibited in comparison with controls, increased content of BCFA in three plasma lipid fractions (DAG - Figure 1A, FFA - Figure 1B and TAG - Figure 1C). No significant differences were found in phospholipid fraction. Noticeable decrease in the concentration of these acids was detected in cholesterol ester fraction (Figure 1D).

This finding points to the fact that reduced activation of PPAR receptors occurs in type 2 diabetics. In consequence, accumulation of both saturated and branched chain fatty acids takes place. We assume that the overall increase in BCFA content, related to insulin resistance of adipose tissue in T2D, has been caused by reduced activation of PPAR receptors. This results in an increased deposition of TAG in the liver and formation of hepatic steatosis. This fact is in accordance with our finding of increased content of BCFA in TAG fraction, which is formed in the liver. BCFA are not oxidized in liver peroxisomes in diabetics, but they are incorporated as undesirable components into triglycerides and secreted by the liver in the form of VLDL, which are primarily designed as a source of energy for muscle and other organ cells [9]. The fate of

BCFA has not been studied in detail so far. The question therefore arises - which organ is responsible for the removal these branched chain fatty acids from the circulation?

We have found reduced content of BCFA in cholesterol ester fraction, which may indicate their oxidation in peroxisomes of muscle cells. Cholesterol ester fraction is present in LDL (low density lipoproteins) and HDL (high density lipoproteins). In type 2 diabetics LDL concentration is increased and HDL is decreased [11]. From our results it is obvious that the content of BCFA in cholesterol esters is generally diminished The oxidation of BCFA in extrahepatic organs can be deduced from the fact that they do not return in the form of cholesterol esters to liver and their content is even lower than in the healthy control group.

Regulation of muscle peroxisomes (containing mainly PPARα and γ) via PPAR is less probable than in case of liver ones. Simple oxidation of present fatty acids seems to occur in muscle peroxisomes, function of which is apparently controlled only by a lack of energy. It is known that diabetics have an abundance of energy sources, but the cells process these sources insufficiently and by different metabolic pathways compared to the healthy population. Activation of PPARα receptors up-regulates catabolism of fatty acids and reduces the formation of Apo C-III, and thereby decreases the secretion of FFA, TAG and consequently VLDL into the blood [12, 13, 14, 15]. Further activation of these receptors enhances capture of FFA in cells by an increase of the activity of the transport protein - fatty acid translocase (FATP) and Acyl-CoA synthetase at the transcriptional level [12, 16, 17]. Main target organ for FATP

and Acyl-CoA synthetase is the liver that reduces the amount of fatty acids used in the production and secretion of TAG and VLDL [12]. The situation is different for PPARγ. Our assumption that the BCFA are oxidized predominantly in muscle peroxisomes is confirmed by the analysis of biopsy samples of human muscle and adipose tissue, which reflect the different tissues distribution of PPARγ [18].

Conclusion

Our analyses proved that diet containing BCFA of bacterial origin is not completely suitable for type 2 diabetics. It is evident that the BCFA are captured in the liver and subsequently secreted only in the form of TAG, part of VLDL, designed for the energy source. This conclusion is supported by the fact that elevated concentration of these BCFA in other liver lipid fractions - phospholipids and cholesterol esters would lead to their recycling and accumulation in diabetics. But this does not occur, and therefore BCFA must be oxidized in extrahepatic organs mainly in the muscle tissue, which is regulated by PPAR receptors, less than for example liver or adipose tissue. To our knowledge, this finding has not been published so far, and it definitely deserves further examination.

With change in lifestyle to a healthier one, people eat more dairy products containing BCFA of bacterial origin and this leads to accumulation of these compounds in diabetics compared to the healthy population. We have not found, however, significant differences between compensated and decompensated group of diabetic patients. Our results draw the attention to the

question of the influence of BCFA on human organism; which would require the development of extensive studies on a larger group of patients.

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Abbreviations

BCFA	branched chain fatty acids
CE	cholesterol esters
DAG	diacylglycerols
EDTA	ethylenediaminetetraacetic acid
FA	fatty acids
FATP	fatty acid translocase
FFA	free fatty acids
HDL	high density lipoproteins
IQR	interquartile range
LDL	low density lipoproteins
PL	phospholipids
PPAR	peroxisome proliferator-activated receptors
T2D	type 2 diabetics
TAG	triacylglycerols
VLDL	very low density lipoproteins

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Table I Characterization of the groups, data are presented as mean ± standard deviation.

Group	Age	Glycosidic haemoglobin	Sex (male/female)
Control group	33.8±2.6	29.6±5.4	5/5
Compensated diabetics	36.5±3.4	46.0±3.3	5/6
Non-compensated diabetics	37.8±3.3	77.8±14.7	8/4

Table II Precision of the method implemented in FFA fraction, n = 10.

BCFA	Mean [μmol/L]	Standard deviation [μmol/L]	Coefficient of variation [%]
12-MeC13:0	0.78	0.08	10.67
13-MeC14:0	0.65	0.03	5.27
12-MeC14:0	0.67	0.04	6.58
14-MeC16:0	3.86	0.23	5.87
16-MeC17:0	1.28	0.05	3.95

Fig. 1 Branched chain fatty acids A) 12-methyltridecanoic acid, B) 13-methyltetradecanoic acid, C) 12-methyltetradecanoic acid, D) 14-methylhexadecanoic acid, E) 16-methylheptadecanoic acid

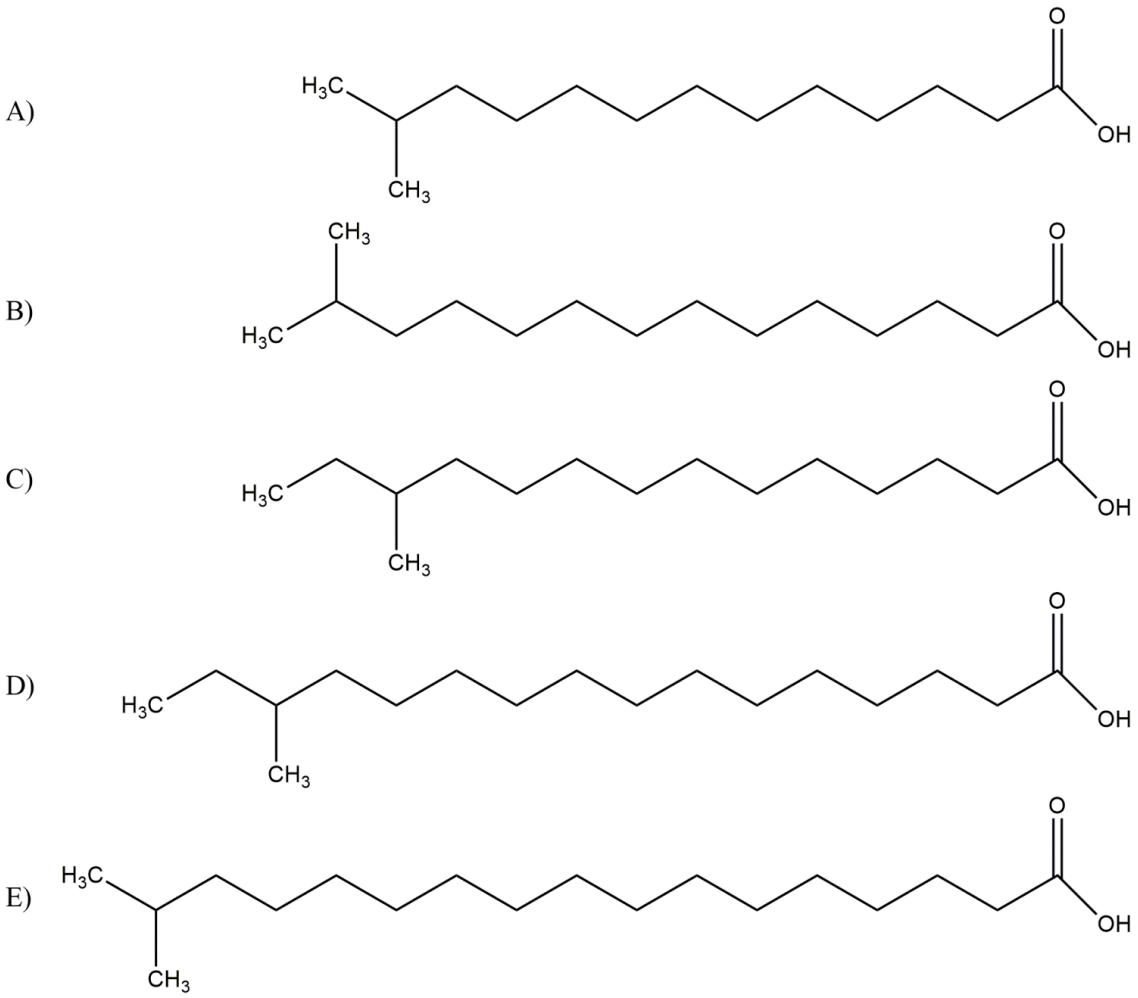
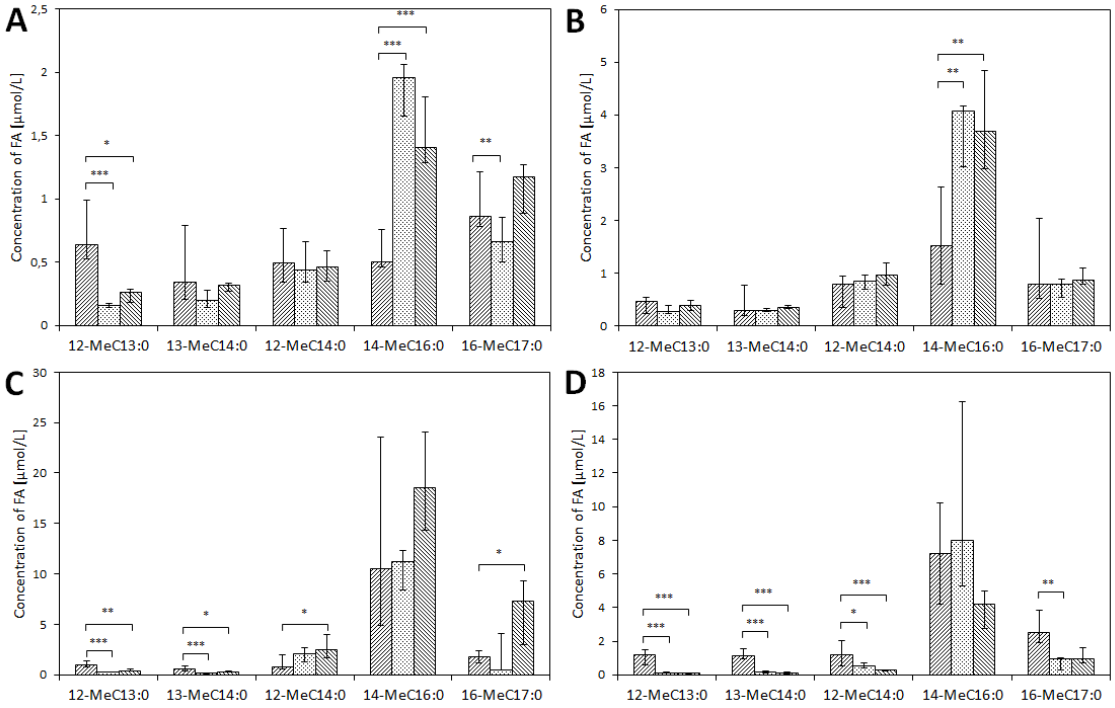


Fig. 2 Branched chain fatty acid content in lipid fractions A) DAG fraction, B) FFA fraction, C) TAG fraction and D) CE fraction. Control group, compensated diabetics, decompensated diabetics. Data are presented as median ± IQR. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.



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