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

Petr LAŠTOVIČKA[1a], Tomáš ČERMÁK[a], Filip HÁJEK[a],
Martina LÍBALOVÁ[a], Vladimíra MUŽÁKOVÁ[a], Jiří SKALICKÝ[b],
and Alexander ČEGAN[a]
[a]Department of Biological and Biochemical Sciences,
The University of Pardubice, CZ–532 10 Pardubice,
[b]Department of Clinical Biochemistry and Diagnostics,
Regional Hospital of Pardubice, CZ–530 03 Pardubice

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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, when natural sources of these fatty acids are dairy products. The aim of our study was to verify whether or not the accumulation of the branched chain fatty acids takes place in type 2 diabetes and, if so, to specify the corresponding lipid fraction. Totally 23 anonymised plasma samples of type 2 diabetic patients had been collected and subsequently divided by glycosidic haemoglobin levels into two groups of 11 compensated and 12 decompensated; the plasma of 10 healthy blood donors being further processed. At first, the samples were divided into particular lipid classes using thin layer chromatography. Then, we set the content of individual fatty acids in all lipid classes using gas chromatography. The results

[1] To whom correspondence should be addressed.

were calculated and evaluated statistically by applying the SigmaStat 3.5 program. It has been found that the most abundant branched chain fatty acid is 14-methylhexadecanoic acid as statistically significant increase of this acid was found in both compensated diabetics ($p \leq 0.001$) and decompensated ones ($p \leq 0.001$) in comparison with controls. The 14-methylhexadecanoic acid was ascertained in the diacylglycerol fraction, as well as in free fatty acid fraction of the compensated ($p = 0.008$) and decompensated ($p = 0.007$) diabetics.

Furthermore, an increase in the content of branched-chain fatty acids in diabetic patients has been proved. An accumulation of these substances in diabetics thus raises a question on their actual effect on the human organism.

Introduction

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

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), representing the nuclear factors regulating production of lipogenic enzymes being necessary for implementing the aforesaid peroxisome and mitochondrial fatty acid oxidation. There exist several kinds of PPAR receptors and, 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 that participate in the peroxisome proliferation and in fatty acid oxidation that occur in peroxisomes and mitochondria [5]. Other two forms, PPARβ / δ, were found in various tissues, but knowledge about them is still quite limited; according to Michalik [6] they also interfere with lipid metabolism — these PPAR lower the concentrations of serum triglyceride 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, occurring there in two isoforms — PPARγ1 and PPARγ2; in smaller quantities being 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]. Regarding the effect of BCFA on PPAR, it has not been yet studied.

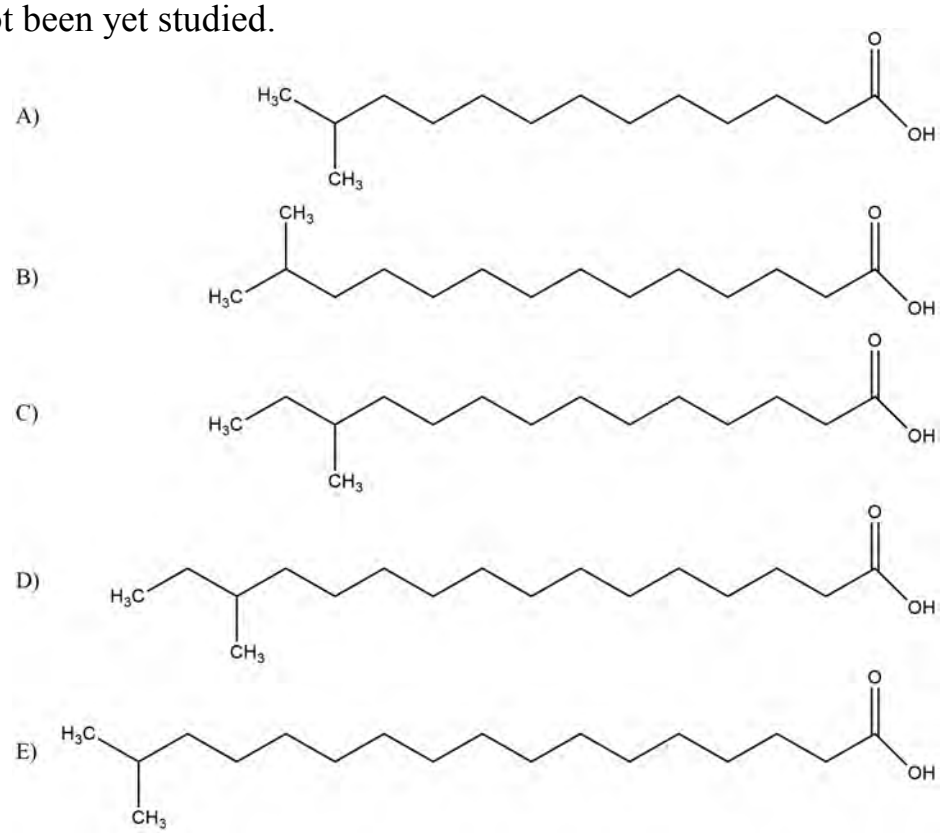

Fig. 1 Branched-chain fatty acids A) 12-methyltridecanoic acid, B) 13-methyltetra-decanoic acid, C) 12-methyltetradecanoic acid, D) 14-methylhexadecanoic acid, E) 16-methylhepta-decanoic acid

Experimental

Patients

The study has involved 23 anonymous plasma samples of type 2 diabetic (T2D) patients and 10 healthy blood donors as control group. The inclusion criteria of study were as follows: all patients (1) aged above 30 years, (2) either gender, (3) with diagnosed type 2 diabetes mellitus for up to 1 year, (4) the fact if the respective patient is on antidiabetic low-calorie diet plus per-oral antidiabetic drugs.

The exclusion criteria of study then were: (1) type-1 diabetes, (2) renal failure, hepatic, oncologic or thyroid disease (3) regularly consumption of alcohol, (4) insulin treatment. None of participants under study suffered from apparent

cardiovascular disease, diabetic nephropathy, and retinopathy. Also, none of persons observed 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. After the selection, a signed agreement was obtained from all the participants.

Patients with the glycosidic haemoglobin level from 43 to 53 mmol mol^{-1} were purposely taken, as a group of the compensated diabetics, while T2D patients with a higher level of glycosidic haemoglobin had been considered as decompensated diabetics. Control group consisted of plasma of 10 healthy blood donors. Both diabetic and control samples were provided by the Regional Hospital of Pardubice; all samples being approved by the Hospital Ethical Committee.

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

Laboratory Methods

Venous blood was drawn 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 a solution of 2-propanol, *n*-heptane, and 2 M phosphoric acid (40:20:1, v/v/v; Merck). The solution was mixed by vortex and conditioned for 10 min. Methanol / toluene mixture (1:4) (Merck) and distilled water were added to the test tube; after 10 min centrifugation at 8 175 g, the upper organic layer was withdrawn, 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 chromatographic plate (20×20 cm Kieselgel 60, Merck). 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 visualised by 2,7-dichlorfluorescein under ultraviolet light. The fractions were scraped off the TLC plate and transferred to screw-capped vials for further analysis.

Transesterification

Internal standard (cis-13, 16, 19-docosatrienoic acid; c = 10 μg ml^{-1}) and methanol/toluene mixture were added to the samples in the Pyrex® glass. Acetylchloride was added to the sample in a thermal block, the mixture heated for 1 hour at 100 °C in closed Pyrex glass, cooled down and neutralised with 6 % K_2CO_3. Fatty acids (FA) were changed to their corresponding methyl esters. The upper phase was concentrated to 80 μl under the nitrogen atmosphere.

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

BCFA	Mean μmol l^{-1}	Standard deviation μmol l^{-1}	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

Gas Chromatography

FA methyl esters were quantified by a gas chromatograph (GC System 7890A, Agilent Technologies, USA) with autosampler and a flame ionisation 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^{-1}. 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^{-1}

and held for 2 min; then the temperature re-programmed to 186 °C at 1 °C min^{-1} and held for 1 min; at 0.1 °C min^{-1} to 190 °C for 1 min, and, finally, at 1 °C min^{-1} to 220 °C for 4 min. The samples were injected in a split mode (split ratio 10:1). The temperature of injector was 250 °C, whereas temperature of detector was set to 280 °C [10].

Statistical Analysis

All statistical analyses were computed using SigmaStat 3.5 program. Differences in variables between the groups were evaluated using Mann Whitney test; the *P* value less than 0.05 being considered statistically significant. The results are expressed as absolute concentrations of the relevant fatty acids in μmol l^{-1}.

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 BCFAs content across all lipid fractions). Therefore, this compound was considered as a representative of the total BCFA level. Statistically significant increase of this acid was found in DAG and FFA fractions in T2D patients compared to the control ones (see Fig. 1). Median of BCFAs sum in the control fractions was in DAG 3.25 μmol l^{-1} and in FFA 4.53 μmol l^{-1}. The fractions richest in the incidence of BCFA were TAG (median 19.02 μmol l^{-1} in controls) and CE (median 14.86 μmol l^{-1} in controls). Regarding the PL fraction, no significant changes in BCFA content were found.

In comparison with control values, the T2D patients exhibited the increased content of BCFA in three plasma lipid fractions (DAG – Fig. 1A, FFA – Fig. 1B and TAG – Fig. 1C). No significant differences were found in phospholipid fraction. In contrast to this, noticeable decrease in the concentration of these acids was detected in the cholesterol ester fraction (Fig. 1D).

This finding points to the fact that the reduced activation of PPAR receptors occurs in type 2 diabetics. As a consequence, accumulation of both saturated and branched chain fatty acids takes place. Thus, we assume that the overall increase in BCFAs content, related to the insulin resistance of adipose tissue in T2D, has been caused by the reduced activation of PPAR receptors, which results in an increased deposition of TAG in the liver and formation of hepatic steatosis. This is in accordance with our observation of the increased content of BCFA in the TAG fraction, which is formed in the liver. In diabetic patients, BCFA are not oxidised in liver peroxisomes, but they are incorporated into triglycerides as undesirable components and secreted by the liver in the form of VLDL that 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 and, therefore, there is a question — which organ is responsible for the removal these branched chain fatty acids from the circulation?

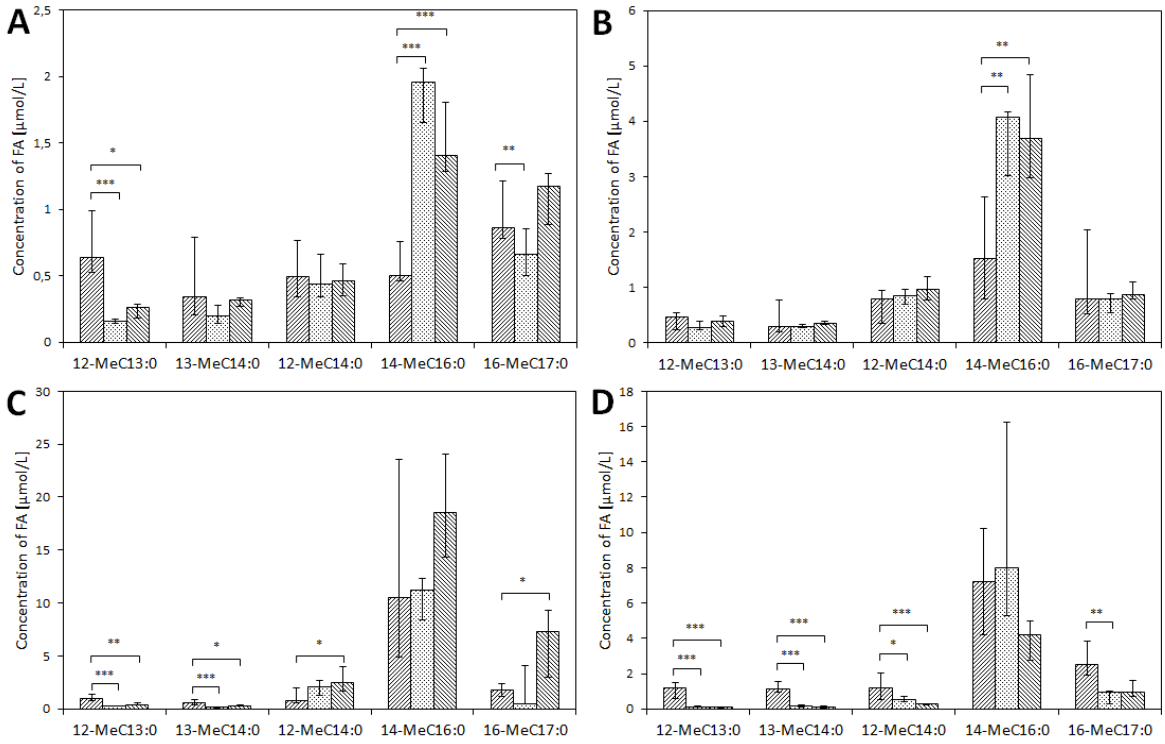

Fig. 2 Branched-chain fatty acid content in lipid fractions A) DAG fraction, B) FFA fraction, C) TAG fraction and D) CE fraction. Control group, compensated diabetics, decompensated diabetics. Data are presented as median ± *IQR*. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

We have found the reduced content of BCFA in the 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, the LDL concentration is increased and HDL decreased [11]. From our results, it is obvious that the content of BCFA in cholesterol esters is generally lowered. The oxidation of BCFA in extrahepatic organs can then be deduced from the fact that these substances do not return in the form of cholesterol esters to liver and their content is even lower than that of the healthy control group.

Regulation of muscle peroxisomes (containing mainly PPARα and γ) *via* PPAR is less probable than in the 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, thereby decreasing the secretion of FFA, TAG, and consequently VLDL into the blood [12-15]. 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 thus the liver reducing the amount of fatty acids used in the production and secretion of both TAG and VLDL [12]. The situation is different for PPARγ. Our assumption is that the BCFA are oxidised predominantly in muscle peroxisomes, which is confirmed by the analysis of biopsy samples of the human muscle and adipose tissue, reflecting the different tissues distribution of PPARγ [18].

Conclusion

Our analyses have 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 the 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 oxidised in the extrahepatic organs mainly in the muscle tissue, which is regulated by PPAR receptors, less than for example liver or adipose tissue. To our knowledge, such a finding has not been published yet, and it definitely deserves further attention and examination.

With change in lifestyle toward a healthier way, 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 imply an answer to the question about the influence of BCFA on human organism; nevertheless, such a hypothesis would require the continuing studies with a larger group of patients.

Acknowledgement

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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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