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Influence of lipid imbalance on butyrylcholinesterase activity and biotransformation efficiency

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Received August 1, 2011, accepted August 26, 2011

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Pharmazie 67: 345–350 (2012) *doi: 10.1691/ph.2012.1653*

Butyrylcholinesterase (EC 3.1.1.8, BChE) is highly active in plasma, skin and lung, the tissues that first contact xenobiotics, supporting a role for BChE in detoxication of xenobiotics including medicaments. A possible involvement of BChE in lipid metabolism has been suggested. Elevated BChE activity in obese individuals correlates with some parameters of lipid metabolism including increased levels of triacylglycerols (TAG) and cholesterol. The aim of this study was to estimate the BChE activity in rats on subcellular and inter-organ levels under the conditions of untreated and treated primary hypertriacylglycerolemia with the TAG lowering agent fenofibrate. No changes in BChE activity were observed in obese animals. However fenofibrate administration led to significant increase of BChE activity in all examined tissues (plasma, liver, white adipose tissue). The impact of lipid metabolic imbalance on BChE biotransformation ability was tested by measuring the rate of hydrolysis of 0,1 to 8 mM concentrations of the antimicrobial agent *N*-(2-benzoyloxyethyl)-ethyldimethylammonium bromide (BCH2). The results revealed a complete shift in the BChE kinetics in all studied models. In animals with hypertriacylglycerolemia the Km value of liver BChE rised 4,6-fold, but the total enzyme efficiency expressed as Vmax/Km dropped 40% comparing to control. In contrast, in animals treated with fenofibrate the BChE efficiency increased in liver 1,6-fold. We conclude here that BChE detoxification capacity is essentially altered under conditions of disturbed lipid metabolism. Clinically, this knowledge could be important in a view of xenobiotic elimination, especially when routinely prescribed medicaments are concerned.

1. Introduction

Butyrylcholinesterase (EC 3.1.1.8, BChE) is an enzyme classified as a serine hydrolase with a special target on cleavage of choline esters. BChE is often referred to as the sister enzyme of acetylcholinesterase (EC 3.1.1.7, AChE), playing an important role in synapses of the nervous system, breaking down the neurotransmitter acetylcholine. Despite this, BChE activity appeared to be vital for AChE knockout mice in supplementing cholinergic functions (Li et al. 2000). However, the physiological substrate for BChE has not yet been identified. Thus, we cannot ascribe to BChE with certainty a role only within the nervous system.

Since the physiological role of BChE is unknown, this enzyme remains the focus of many researchers. During the last two decades, many articles trying to elucidate the relationship between disorders of lipid metabolism and BChE activity have been published. For the most part, changes in BChE activity under disrupted lipid balance have been studied in conditions of obesity and diabetes mellitus, as well as other factors converging into metabolic syndrome. Correlations have been established between BChE activity and lipid metabolic parameters (triacylglycerols, cholesterol), degree of obesity, insulin resistance, or markers of inflammation (C-reactive protein, ferritin) (Alcantara et al. 2005; Annapurna et al. 1991; Cucuianu et al. 2002; Rustemeijer et al. 2001). It has even been speculated that a role for BChE exists in terms of regulation of lipid metabolism and recirculation of TAG between the liver and adipose tissue (Cucuianu et al. 2002; Iwasaki et al. 2007). Within this purview, interestingly BChE knockout mice turned out to be obese in comparison to its wild-type counterparts when fed on high-fat diet (Li et al. 2003). However, a clear explanation as to why BChE activity fluctuates during the above mentioned conditions remains missing. Even though the basis of this relationship has not yet been elucidated, the majority of studies carried out on humans have shown that BChE activity is elevated under the conditions of metabolic syndrome or some of its particular factors.

The extremely high BChE activity in tissues of the first contact (lung, skin, blood) and liver as well as the architecture of its active site predetermines BChE for splitting many molecules of different nature. Since the BChE active site allows for the accommodation of bulky molecules, BChE as a detoxifying enzyme is able to biotransform a wide spectrum of compounds ranging from organophosphates to routinely-used medications such as acetylsalicylic acid, tricyclic antidepressants, suxamethonium, bambuterol, etc. (Cokugras 2003). Mostly it metabolizes drugs by turning them into inactive residues, and in some cases, BChE activates a pre-drug form by deliberately cleaving an introduced bond in the drug molecule to produce pharmacologically active substances. These inactive compounds become pharmacologically active only after being metabolized. In general the knowledge of biotransformation pathways and identification of major metabolites is one of the conditions of a successful regis-

Table 1: Diet composition in particular experimental groups

GROUP	Number of animals in group	Standard food (g)	Pork fat (g)	Fenofibrate (mg)
C	6	20	–	–
T	8	20	4	–
F	8	20	–	15
TF	8	20	4	15

The amount of food, pork fat and fenofibrate is stated per day per rat. The fenofibrate dosage equals 60 mg/kg/day. Both pork fat and fenofibrate were incorporated into the standard diet before production of the pellets. C – control, T – hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F – fenofibrate. Fenofibrate was from Zentiva, Slovakia

tration of medications. On the other hand, one can take advantage of a well-known enzyme action as a tool for design of new prodrug forms. One way or the other for such purposes, an exact biochemical characterization of an enzyme's biotransformation pathways, as well as its kinetic properties, is desirable.

We have repeatedly demonstrated that the overall BChE biotransformation activity depends essentially on inter-species, inter-tissue, and subcellular localization (Pauliková et al. 2006, 2009). However, it is still unclear whether there are some alterations in BChE biotransformation capacity in individuals with disturbed lipid metabolism. For this purpose, this study has been designed to investigate BChE activity on the inter-tissue and subcellular level in hypertriacylglycerolemic rats with and without hypolipidemic drug (fenofibrate) intervention. In addition, we performed a kinetic the study using the *N*-alkylderivate of benzoylcholine as a substrate to verify alterations in BChE kinetic properties in the liver, the main organ of biotransformation, where BChE is purportedly synthesized.

2. Investigations, results and discussion

Alterations in lipid metabolism were measured in the animals after 30 days on various diets (Table 1). The consumption of high-fat diet containing pork fat led to 36% increase of TAG and 32% increase of whole cholesterol levels, as well as causing obesity. In Fig. 1, obesity in the high-fat diet T group is indicated by the fact that animals in the T group gained 78 g in body weight, whereas animals in the C group gained 65 g in 30 days. Fenofibrate treatment in the animals on the high-fat diet prevented an increase in TAG levels and obesity and caused whole cholesterol levels to drop to 53% of control levels. In Fig. 1, the effect of fenofibrate on blocking development of obesity in the high-fat diet group is indicated by a gain in body weight of 72 g by the TF group, compared to a gain in body weight of 78 g by the T group. Fenofibrate also reduced weight gain in animals on a standard diet. Fig. 1 shows that animals fed a standard diet that included fenofibrate but no pork fat (F group) gained 54 g in body weight, whereas animals fed a standard diet without fenofibrate and without pork fat (C group) gained 65 g in 30 days. Fenofibrate lowers plasma TAG by activating fatty acid oxidation in the liver. It is known that fenofibrate decreases body weight gain in rats and increases lipolysis of WAT (Ferreira et al. 2006). Our results confirm the lipolysis effect of fenofibrate, as shown by the respective 60% and 20% increases in the levels of plasma glycerol in the F and TF groups and the C and T groups, as shown in Table 2.

BChE activity has been repeatedly reported to be elevated in obese individuals with elevated TAG levels, when measured on the commonly-used substrate butyrythiocholine (Alcantara et al. 2006; Rustemeijer et al. 2001; Iwasaki et al. 2007). The correlations carried out between BChE activity and TAG levels in plasma led to speculations about a possible role of BChE on lipid metabolism. It was suggested that BChE might be involved

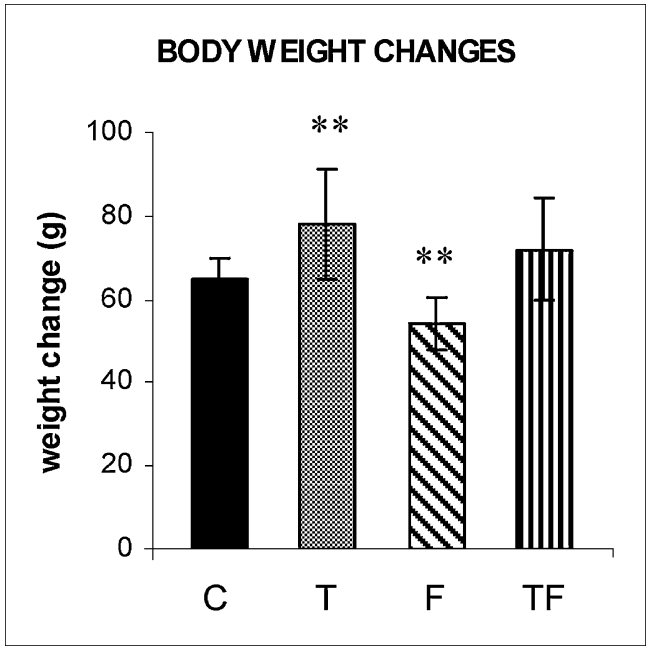


Fig. 1: Changes in animal weights after 30 days on diet. C – control, T – hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F – fenofibrate. Values are expressed as means ± SD, p was preset at < 0,01 (Student's t-test)

in the regulation process of TAG circulation between the liver and adipose tissue, thus playing a role in adiposity (Iwasaki et al. 2007). However, it should be mentioned that BChE activity has been most intensively studied in humans and animals with secondary hypertriacylglycerolemia, predominantly as a consequence of diabetes mellitus (Alcantara et al. 2005; Annapurna et al. 2001). To the best of our knowledge, it has not been studied in rats with fatty diet-induced primary hypertriacylglycerolemia. Therefore, to investigate a possible correlation between BChE and TAG levels, we measured the BChE activity in the subcellular fractions of the liver (cytosol and microsomes), WAT, and plasma. Although fenofibrate treatment reduced TAG levels, it significantly increased BChE activity in both F and TF groups. On the other hand, we did not observe major changes in BChE activities in the studied tissues obtained from the animals with hypertriacylglycerolemia (Fig. 2, A–D). We hypothesized that the elevated BChE activity in fenofibrate treated groups could be caused by the increased BChE expression, since fenofibrate acts via stimulation of PPARα (peroxisome proliferator-activated receptor alpha), which is a well-known transcription factor. However, the results of real-time PCR reaction performed in the liver did not show any significant differences between the BChE gene expressions in neither of the experimental groups (Fig. 3). Since the animals were treated with fenofibrate daily, it is unlikely that the elevated BChE gene expression occurred in early days of the experiment and was decreased by the day when animals were sacrificed. Thus, we propose that the BChE activity changes are rather caused by qualitative alterations of the enzyme, triggered probably by modifications in its macromolecular structure. Another explanation is that the elevated BChE activity was caused by lipid metabolic changes as hypothesized previously, not on the basis of PPARα stimulation, but rather as a consequence of fenofibrate intervention in lipid metabolic pathways. Thus, the results of our study do not confirm the correlation between increased BChE activity and elevated TAG levels. In our experiments, BChE activity was elevated in the F and TF groups both in the liver and also in adipose tissue. The plasma BChE activity in particular models (Fig. 2A) matched rather the liver (Fig. 2C, D) in terms of BChE activity being more elevated

Table 2: Basic parameters of lipid metabolism mesured in plasma of rats after 30 days on diet

GROUP	GLU (mmol/l)	W-CH (mmol/l)	LDL-CH (mmol/l)	HDL-CH (mmol/l)	TAG (mmol/l)	GLY (mmol/l)
C	7.46 ± 0.89	1.48 ± 0.09	0.22 ± 0.01	1.23 ± 0.08	0.30 ± 0.02	0.28 ± 0.03
T	$9.87 \pm 0.32^{*}$	$1.96 \pm 0.13^{*}$	$0.33 \pm 0.05^{*}$	$1.55 \pm 0.09^{*}$	$0.41 \pm 0.03^{*}$	0.29 ± 0.05
F	8.44 ± 0.38	$0.70 \pm 0.06^{***}$	$0.46 \pm 0.04^{***}$	$0.52 \pm 0.04^{***}$	0.31 ± 0.03	$0.45 \pm 0.02^{***}$
TF	9.17 ± 0.29	$0.78 \pm 0.05^{***}$	$0.57 \pm 0.02^{***}$	$0.56 \pm 0.03^{***}$	$0.25 \pm 0.06^{*}$	$0.35 \pm 0.05^{*}$

C – control, T – hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F – fenofibrate, GLU – glucose, W-CH – whole cholesterol, LDL-CH – LDL cholesterol, HDL-CH – HDL cholesterol, TAG – triacylglycerols, GLY – glycerol. Values are expressed as means ± SD. Results were statistically evaluated using Student's t-test, with the p value preset at < 0,01 for statistically significant differences.

in the TF group than in the F group, in contrast to WAT. Therefore, we performed further studies with subsequent sequence analysis of the PCR product in the WAT to confirm the possibility that WAT can synthesize the BChE enzyme by itself. BChE enzyme is believed to be synthesized mainly within the liver and afterwards transported by the plasma into other tissues, however it has not yet been proven that the liver is the only source of the plasma BChE. The fact that BChE gene expression in the WAT proceeds independently of the liver (Fig. 6) indicates that the WAT is able to regulate BChE activity on its own accord. Thus, this finding supports the hypothesis that BChE may be involved in the regulation of lipid metabolic pathways. The independent expression of BChE in the WAT may be of a huge importance in the biotransformation of xenobiotics. In particular, it would potentially serve to hydrolyze molecules with bulky structures, which are good substrates for BChE, since they tend to accumulate within the adipose tissue. Whether the WAT has the ability to respond on the accumulation of such molecules by the elevated BChE expression and activity needs to be further elucidated.

In order to find out whether the changes in BChE activity triggered by alterations in lipid metabolism influence also the capacity to cleave molecules with bulkier structures and thus its detoxification properties, we performed a kinetic study with the substrate *N*-(2-benzoyloxyethyl)-*N*, *N*-dimethyl-*N*-ethylammonium bromide in the liver of all experimental animals. BCH2 from the homologic row of substituted *N*-alkyl

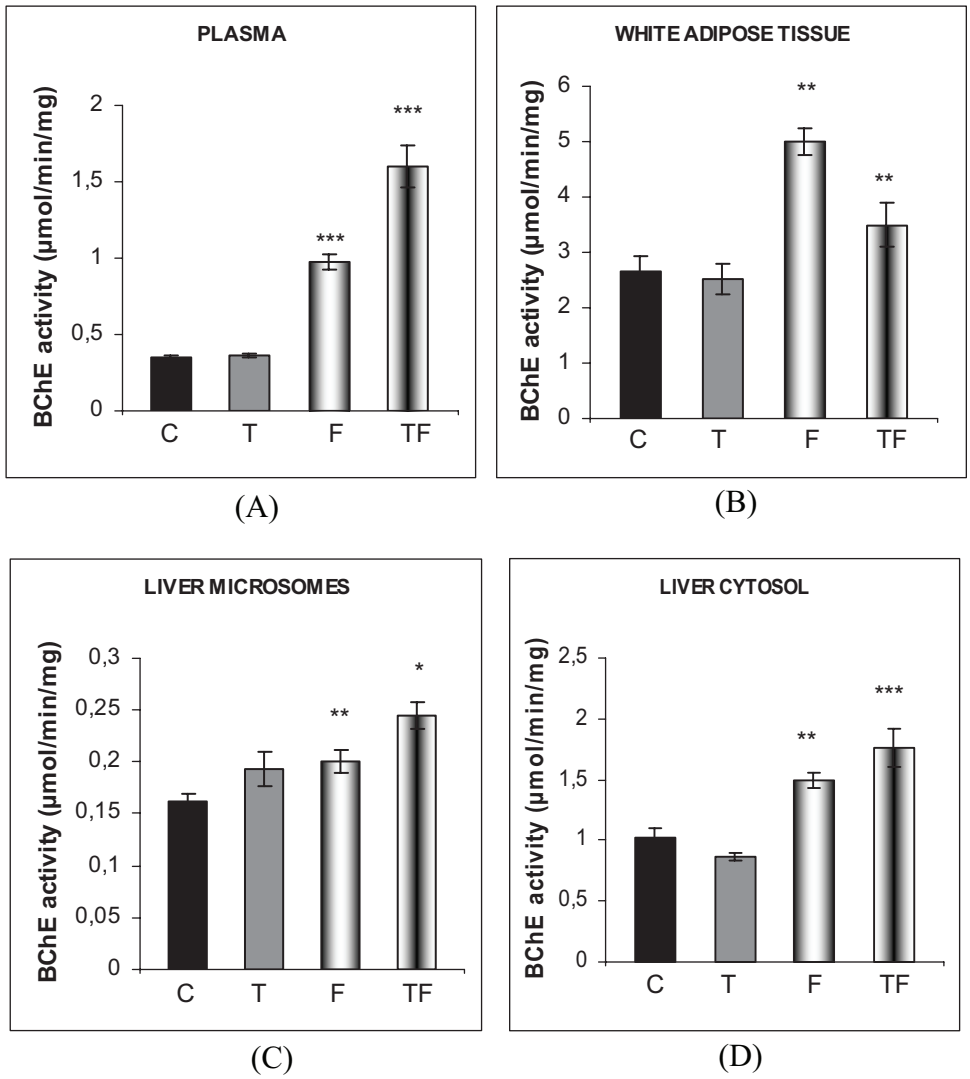
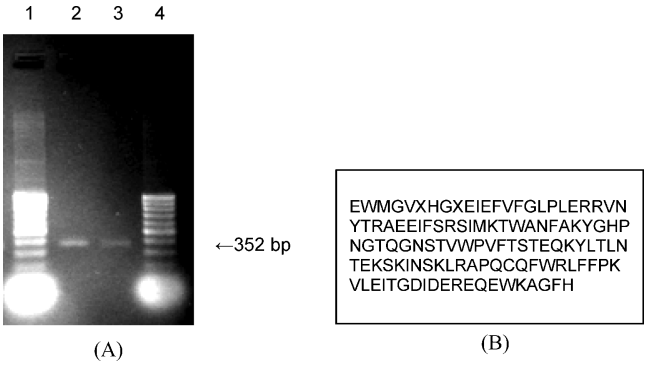


Fig. 2: BChE activities in different tissues (A-plasma, B- white adipose tissue, C – liver microsomes, D-liver cytosol). C – control, T – hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F – fenofibrate. The BChE activity is expressed in µmol of the reaction product 2-nitro-5-thiobenzoate formed in 1 min per 1 mg of protein in tissue fraction. Values are expressed as means ± SD, p was preset at < 0,01 (Student's t-test)



(A) (B)

Fig. 3: BChE PCR product in liver and WAT. A – electrophoretic analysis of the PCR product, lane 1 and 4: 100 bp ladder, 2: liver, 3: white adipose tissue. B - The nucleotide sequence was translated and compared with sequences in GenBank (www.ncbi.nlm.gov/BLAST). The sequence is identical with part of the Rattus norvegicus butyrylcholinesterase sequence, which is registered under No. AAF44713.1, where the two X are M and Y.

benzoylcholines was claimed to be one of the best substrates for rat BChE for *in vitro* studies and docking analysis (Pauliková et al. 2006; Hrabovská et al. 2006). Using BCH2 as a substrate we observed a 1,8-fold increase of BChE activity in the F and 2,8-fold increase in the TF groups respectively (Fig. 4). Table 3 shows the kinetic parameters Km and Vmax determined in all studied groups. In the T model the BChE affinity to BCH2 dropped comparing to control, as well as the total efficiency of the enzyme performance expressed by Vmax/Km. In contrast in both groups treated with fenofibrate the BChE efficiency rised 1,6-fold. Moreover in T and TF groups the saturation concentration shifted to 6 mM and 4 mM, respectively, in comparison to 1 mM in control and F group (Fig. 5).

The involvement of BChE into lipid metabolism has already been discussed intensively. Several hypotheses have been proposed and tested in order to clarify how BChE could participate in lipid metabolic pathways and thus correlate with different parameters of lipid metabolism. No doubt, the elucidation of the essence of this relationship would be interesting from the view of lipid metabolism. This knowledge can also contribute to a more intensive characterization of the BChE enzyme and thus may provide more evidence about its physiological function. Additionally, it would also be very important to consider the characterization of BChE as a detoxification agent and to point out how the changes in lipid metabolism may influence this function in organisms. Based on our results, it seems quite evident that all diets affect BChE kinetics to different extents, which in turn may be of clinical importance for biotransformation of medications eliminated or, as mentioned earlier, activated by this enzyme.

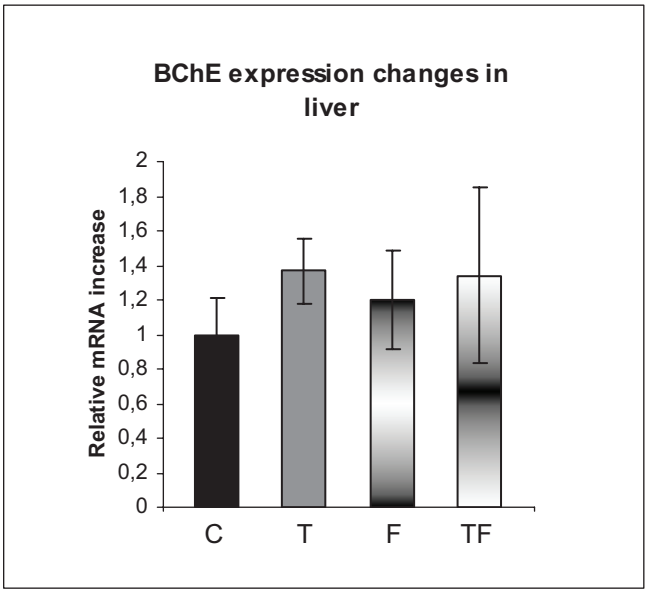


Fig. 4: BChE mRNA levels in rat livers under imbalanced lipid metabolism conditions. C – control, T-hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F-fenofibrate. Values are expressed as means ± SD, p was preset at < 0,01 (Student's t-test)

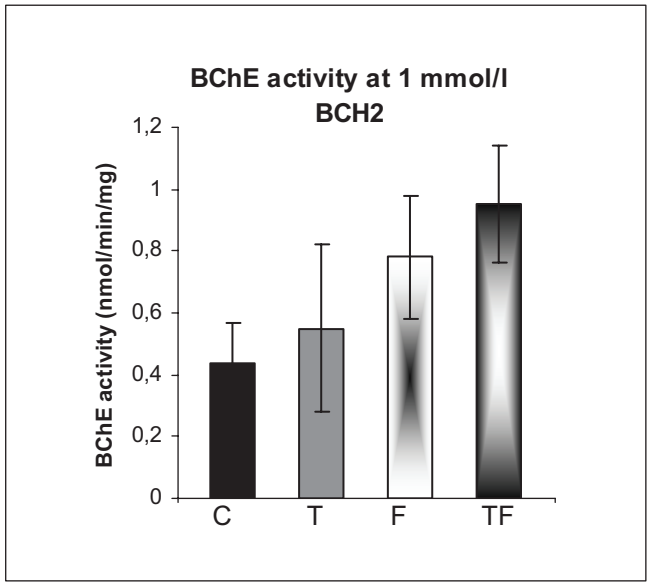


Fig. 5: BChE activity in 15000 rpm liver homogenate in different experimental rat models. The activity of BChE was measured at 1 mmol/l concentration of *N*-(2-benzoyloxyethyl)-*N*, *N*-dimethyl-*N*-ethylammonium bromide (BCH2). Values are expressed as means ± SD, p was preset at < 0,01 (Student's t-test)

Although we do not know exactly whether the changes in biotransformation capacity of BChE seen in the animals treated with fenofibrate are caused by this drug directly or through its intervention in lipid metabolism, it is clear that this lipid lowering agent is able to profoundly increase the rate of BChE biotransformation activity. Since the lipid lowering drugs are largely prescribed during the past years as a combination of fenofibrate with other drugs cleaved by BChE (aspirin, tricyclic antidepressants and others), this seems to be a point of interest for many clinical pharmacologists. In fact, it can have a great impact on dosage and steady-state kinetics.

Although the BChE detoxification ability is altered under conditions of disturbed lipid metabolism and fenofibrate treatment the expression of BChE is not changed. Thus, the basis of these alterations and their impact on the enzyme structure and kinetics needs to be further elucidated.

3. Experimental

3.1. Animals

Thirty male Wistar rats (Dobrá Voda, Slovak Republic), weighing 200–265 g were housed in cages one by one. After a 6-day adapting period, the animals were divided into 4 groups and fed different types of diet (Table 1). Each day, an exact portion of a defined diet (according to the group) was allocated to each animal to assure an equal food intake. During the whole experiment the animals were supplied with water *ad libitum*. 24 hours before sacrifice, animals underwent a period of starvation. The performance of the experiment was approved by The State veterinary and food administration of the Slovak republic (1706/08–221).

After 30 days on different diets, the animals were anaestethized with pentobarbiturate (i.p. 60 mg/kg, 2,5% solution), and the blood was taken into S-monovette syringes containing EDTA gel to prepare the plasma samples, (15000 rpm at 4 °C). The organs (liver, epididymal white adipose tissue (WAT)) were repeatedly rinsed in saline and immediately frozen in liquid nitrogen. Tissues were stored at −80 °C until sample analysis.

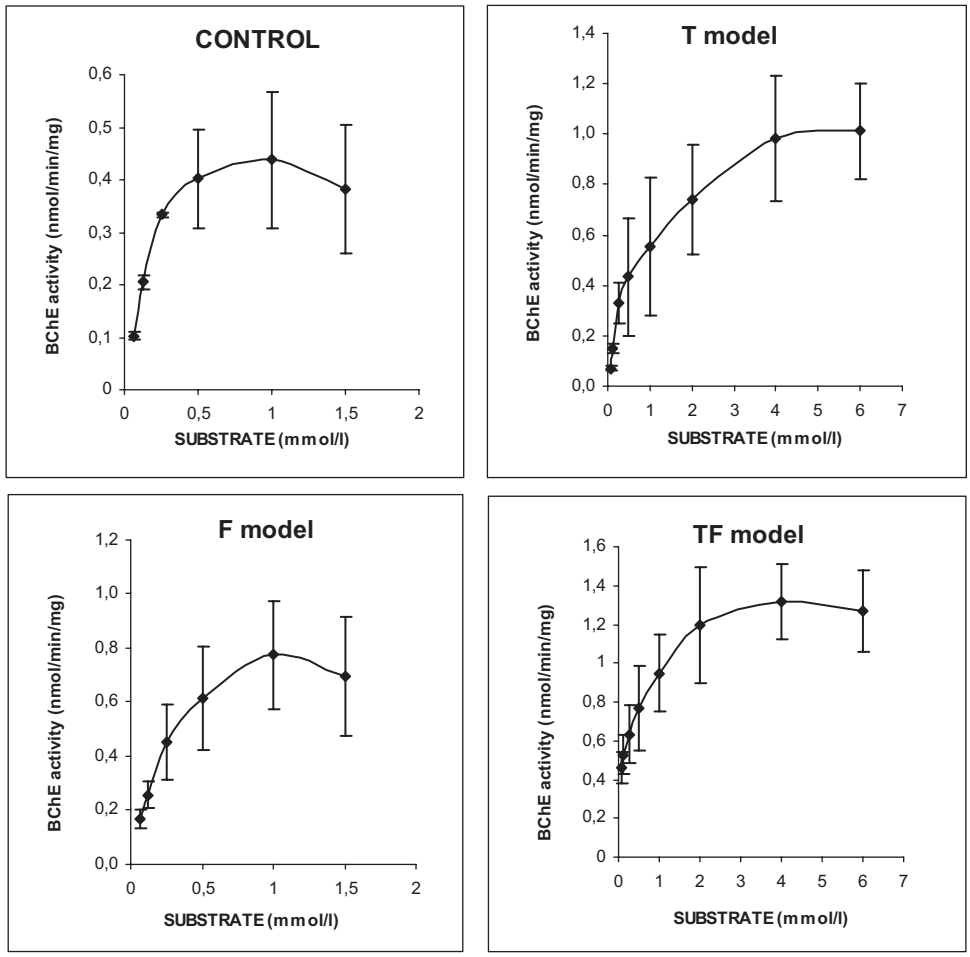


Fig. 6: Kinetic study of *N*-(2-benzoyloxyethyl)-*N,N*-dimethyl-*N*-ethylammonium bromide hydrolysis in rat liver under diverse lipid imbalance conditions. The BChE activity is expressed in nmol of benzoic acid produced in one minute per 1 mg of protein in reaction. T model – hypertriacylglycerolemia, F model – fenofibrate treated animals, TF model - hypertriacylglycerolemic animals treated with fenofibrate. Values are expressed as means ± SD, p was preset at < 0,01 (Student's t-test)

3.2. Preparation of tissue homogenates

10% liver homogenates were prepared in a medium consisting of 0,25 M saccharose solution, adjusted by 0,15 mM Tris-HCl buffer to pH 7,4. Subcellular liver fractions (microsomes and cytosol) were obtained by differential centrifugation of the crude organ homogenate according to Cinti et al. (1972).

For biotransformation studies, liver was homogenized in potassium phosphate buffer (0,1 M, pH 7,4) and centrifuged at 15,000 rpm for 20 min. WAT was homogenized in phosphate buffer, pH 7,4 with addition of Brij 96, to give a final 1% solution (0.2 g tissue/1 ml solution). After 15 min centrifugation (4 °C, 2800 rpm), the postnuclear supernatant, which was finally tested, was obtained. Protein content in fractions was determined according to Bradford protocol (Bradford 1976).

3.3. BChE enzyme activity

BChE activity was determined by the method of Ellman et al. (1961) in a total volume of 1.5 ml. The activity of acetylcholinesterase was inhibited by 20-minute preincubation of 30 µl of tissue homogenate in 0.1 M phosphate buffer, pH 7.4 with 0.1 ml of specific AChE inhibitor (bw284c51; 0.02 mM) at 25 °C. After the following 30-minute incubation with 0.1 ml of 7.5 mM 5.5′-dithiobis(2-nitrobenzoic) acid at 25 °C, the substrate – 0.1 ml of 14.5 mM butyrylthiocholine iodide was added. The absorbance was read at 412 nm for 5 min in 30-second intervals. The exclusive participation of BChE and AChE in butyrylthiocholine cleavage was confirmed by 5-minute preincubation of the tissue homogenate with common AChE and BChE inhibitor (0.1 ml of 1.5 mM eserine solution). Enzyme activity was expressed in µmol of 2-nitro-5-thiobenzoate formed per minute, calculated per 1 mg of whole protein. All chemicals were purchased from Sigma, Slovakia.

3.4. Total RNA isolation

The total RNA was isolated from 100 mg of tissue (liver and WAT) using Total RNA Isolation Kit (SBS Genetech, China). Isolated RNA was treated with DNase by DNA Free Kit (Ambion, USA) and RNA yield was determined by A_{260} reading. 1 µg of total RNA was reversely transcribed using First Strand cDNA Synthesis Kit (SBS Genetech, China).

3.5. Polymerase chain reaction

Polymerase chain reaction was performed in order to verify BChE gene expression in WAT, using BChE specific primers (forward 5′-AGAATGGATGGGAGTAATGCATGG-3′, reverse 5′-GATGGAATCCTG CCTTCCACTCTTGC-3′, (Mis et al. 2001)) located in exons 2 and 4. Reaction mixtures were prepared by adding of 1 µl of cDNA, 2 µl of each primer (working solution 50 nmol/ml) and 13 µl of water to Easy-Do^{TM}PCR Pre-Mixes (SBS Genetech, China). After 15 min of DNA predenaturation at 94 °C, 40 cycles were performed with 10 min final extension at 72 °C. Each cycle consisted of denaturation (94 °C; 60 s), annealing (61 °C; 60 s) and extension (72 °C; 60 s). PCR products were examined for their size by electrophoresis in 1.6% agarose gel using 100 bp DNA Ladder Marker (SBS Genetech, China). A 352 bp long PCR product was excised from the gel and extracted from agarose using QIAquick Gel Extraction Kit (QIAGEN, USA). Subsequently the PCR product was subjected to sequence analysis. The nucleotide sequence was compared with sequences in GenBank (www.ncbi.nlm.nih.gov/BLAST).

3.6. Real-time polymerase chain reaction

Real-time PCR was performed using GoTaq qPCR Master Mix (Promega, USA). BChE specific primers (sense: CCGGGCTCC-CCAGTGCCAATT; anti-sense: GCGATGGAATCCTGCCTTCCACT; PCR product 106 bp) were designed using *Rattus norvegicus* butyrylcholinesterase sequence NM_022942.1 with help of software Primer3 (http://frodo.wi.mit.edu/primer3/). The primers are located in exons 3 and 4, thus ensuring the amplification of cDNA. The reaction was performed in a total volume of 25 µl containing 0.2 µl of sample cDNA, 5 pmol of each primer, 0.25 µl of CXR Reference Dye, 12.5 µl of 2X GoTaq qPCR Master Mix (Nuclease-Free water to a final volume of 25 µl).

Amplification and detection were done with a detection system Applied Biosystems ABI Prism 7300 Real Time PCR System with the profile of 95 °C for 10 min and then 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Transcription efficiency was analysed with help of $\Delta\Delta C_t$ value method (Winer et al. 1999), whereas the target gene Ct value was normalised by ß-actin (sense: CCGCGAGTACAACCTTCTTG, anti-sense: GCAGCGATATCGTCATCCA, PCR product 81 bp (Mackovičová et al. 2011)) expression in the same sample. PCR products were also evaluated by gel electrophoresis and melting curve analysis to confirm the specific amplification. To confirm gene DNA decontamination, reactions with RNA and water instead of cDNA were performed.

3.7. Biochemical laboratory analysis

Determination of lipid metabolic parameters – triacylglycerols, whole cholesterol (W-CHOL), HDL (HDL-CH) and LDL cholesterol (LDL-CH) and glucose (GLU) were carried out using the appropriate kits from Biosystems, Spain. Glycerol was measured with a kit from Randox, UK. All experiments were performed following the manufacturer's instructions.

3.8. Kinetic study with benzoylcholines

3.8.1. Substrates

The own kinetic study of BChE in the rat liver was carried out with a substrate of benzoylcholine type. This compound is *N*-alkylderivate of benzoylcholines, with following general formula: *N*-(2-benzoyloxyethyl)ethyldimetylammonium bromide (BCH2). The substrate is a quaternary ammonium salt with antimicrobial activity, and it was synthesized by The Department of Organic Chemistry at The Pharmaceutical Faculty, Comenius University (Csiba et al. 1986).

3.8.2. Incubation

The enzyme reaction was performed under aerobic conditions, at 37 °C for 30 min in a final volume of 3.5 ml. 0.5 ml of tissue homogenate was preincubated for 5 min at 37 °C with 0.1 M potassium phosphate buffer; pH 7.4 and 0.1 ml of 5 mM magnesium chloride. The substrate BCH2 was dissolved in water immediately before each experiment in water (35 mM solution) and added to incubation mixtures in order to initiate the reaction. The volumes of BCH2 in incubation mixtures ranged from 0.0125 to 0.8 ml depending on the desired final substrate concentrations as determined by the type of experiment (0.125 to 8 mM). The reaction was terminated by lowering the pH value to 2.5 with 1 M HCl.
To confirm the exclusive participation of BChE on BCH2 biotransformation process, BChE was preincubated for 5 min with 0.1 ml of 3.5 mM eserine solution.

3.8.3. Extraction and HPLC analysis

After incubation of the internal standard, p-iodobenzoic acid in methanol was added to all incubation mixtures in concentrations according to the supposed yield of benzoic acid as the product of BCH2 hydrolysis. Incubation mixtures were extracted 3 times into chloroform, using 10 ml for each extraction. The extracts were combined, filtered, then vacuum dried. Dried samples were re-dissolved in 10 ml of methanol and analyzed by the means of HPLC (Helia et al. 1975). Enzyme activity was expressed in nmol of benzoic acid formed per minute, calculated per 1 mg of the whole protein.

3.9. Calculations and statistical analysis

The kinetic parameters Km and Vmax were calculated by a linear regression analysis (Lineweaver–Burk) from the linear parts of the saturation curves.

Table 3: Kinetic parameters of BChE measured on the substrate *N*-(2-benzoyloxyethyl)-*N,N*-dimethyl-*N*-ethylammonium bromide in liver

GROUP	Km (mmol/l)	Vmax (nmol/min/mg)	Vmax/Km	c_S (mmol/l)
C	0.28	0.58	2.07	1
T	1.28	1.54	1.20	6
F	0.24	0.80	3.33	1
TF	0.42	1.43	3.40	4

Km – Michaelis – Menten constant, Vmax – maximal reaction rate, cs – saturation concentration, C – control, T – hypertriacylglycerolemia, TF – hypertriacylglycerolemia + fenofibrate, F – fenofibrate. Km and Vmax were calculated using the average velocity values as shown in Fig. 5.

Results were statistically evaluated using Student's t-test. Data are shown as the mean plus standard deviation (SD). All tests were two tailed whereas the P value was preset at < 0.01 for statistically significant differences and correlations. The T and F group were compared to control, whereas the TF group was compared to T group.

Acknowledgement: Authors give thanks to Dr. T. Szemes from Comenius University, Faculty of Science for sequence analysis of isolated PCR product.

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