Betaine increases the butyrylcholinesterase activity in rat plasma

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Short title: Betaine increases the butyrylcholinesterase activity

1

Summary

The physiological function of butyrylcholinesterase (EC 3.1.1.8., BChE) is not clearly understood, but a role was suggested in the fat utilization process, resulting in positive correlation between plasma triglyceride (TG) levels and BChE activity. Consequently we tested the hypothesis that regular intake of betaine, a natural compound intervening in the liver TG metabolism could influence the BChE activity. The BChE activity was estimated spectrophotometrically in plasma of rats fed with betaine enriched standard (B) or high-fat diet (HFB). The results confirmed decreased TG plasma levels after betaine treatment independently on the type of diet (0.15 ± 0.03 (B) vs. 0.27 ± 0.08 (control) mmol/L; $p=0.003$ and 0.13 ± 0.03 (HFB) vs. 0.27 ± 0.08 (control) mmol/L; $p=0.005$). The BChE activity increased significantly with betaine administration, however the change was more distinct in the HFB group (0.84 ± 0.34 (HFB) vs. 0.22 ± 0.04 (control) O.D./min/mg; $p<0.001$ and 0.41 ± 0.11 (B) vs. 0.22 ± 0.04 (control) O.D./min/mg; $p=0.001$).

In conclusion, betaine intake led to elevated BChE activity in plasma and this effect was potentiated by the HF diet. Since betaine is in general used as a supplement in the treatment of liver diseases accompanied by TG overload, its impact on the BChE activity in the role of the liver function marker should be taken into account.

Keywords: butyrylcholinesterase, betaine, high-fat diet, liver, lipid metabolism

Introduction

Unlike the vital function of acetylcholinesterase (EC 3.1.1.7.) in the process of acetylcholine breakdown, the accurate physiological role of the other enzyme of the cholinergic system - butyrylcholinesterase (EC 3.1.1.8, BChE) remains the research target for many scientists. The task would be easier, if the specific biological substrate for BChE was known. Therefore, BChE has been so far studied mainly in the context of different pathological conditions, where its activity appeared to have at least some relevance. This covers predominantly the field of neurological disorders (especially Alzheimer´s disease), intoxications with organophosphates and metabolic disorders (Cokugras 2003). Vigorous interest in the research of the relationship between BChE and the lipid metabolism was launched by observing that the BChE activity correlated positively with levels of triglycerides (TG) in plasma (Annapurna *et al*. 1991). The following studies concluded that BChE activity correlated also with other markers of the lipid metabolism (total cholesterol (TC), LDL-chlesterol, visceral fat area, subcutaneous fat area, body mass index) or even insulin resistance (Rustemeijer 2001 *et al.*, Alcantara *et al.* 2002, Iwasaki *et al.* 2007). The fact that the selective BChE inhibitor iso-OMPA (tetraisopropyl pyrophosphoramide) decreased TG levels in plasma of rats pointed out to a possible direct participation of BChE in TG metabolism (Annapurna *et al.* 1991). Also, the BChE *knockout* mouse had elevated TG plasma levels and became obese on high-fat diet, in contrast to the wild-type littermates (Li *et al*. 2008). This compelling evidence could predicate of BChE necessity for an appropriate lipolysis.

Involvement of BChE in the lipid metabolism is supported additionally by several genetic studies. Up to date, more than 60 mutations of the BChE gene have been identified,

including point mutations, small insertions and deletions (Parnas *et al.* 2001). Lower BChE activity originating from gene mutations was shown to be associated in human subjects with increased TG plasma levels and higher risk of obesity (Chaves *et al.* 2013, Lima *et al.* 2013). Despite the site at which BChE fits into the lipid metabolic pathways has not yet been identified, fluctuations of the BChE activity related to lipid metabolism might carry clinical consequences. BChE serves not only as one of the markers in the liver function test, but is also an important enzyme responsible for the biotransformation of many xenobiotics, including medicaments. Accordingly, the level of BChE activity influences potentially the overall pharmacokinetic profile of these medicaments (Pohanka 2013).

In prophylaxis and supportive treatment of liver disorders related to excessive TG accumulation often the natural lipotropic compound betaine is used in praxis (Purohit *et al.* 2007). Betaine is produced *in vivo* via the oxidation of choline, but it is also present in many foods, such as spinach, sugar beet or whole wheat breads and grains. Betaine serves as a methyl donor and affects in this way the methionine - homocysteine equilibrium in favour of methionine, leading to increased formation of S-adenosylmethionine, one of the most important methyl transferring agents in cells (Kathirvel *et al.* 2010). On the other hand, long - term consumption of high-fat diet evokes methyl donor deficiency and hyperhomocysteinemia, a state which is linked to decreased secretion of TG – rich VLDL (very low-density lipoproteins) into circulation and development of steatosis (Craig *et al.* 2004, Chang *et al.* 2010). Although the exact mechanism coupling the hypomethylation state with steatosis has not yet been elucidated, it is supposed that impaired activities of methylation - dependent enzymes (Kharbanda *et al.* 2007) and methylation - controlled genes playing a role in the TG metabolism are involved (Wang *et al.* 2013). An example could be the rectification of the paradoxical PPARα (peroxisome proliferator-activated receptor α)

4

gene promoter hypermethylation, caused by the overall hypomethylation state in the cell. PPARα oversees the expression of several genes taking part in the TG breakdown and its expression is typically supressed in steatosis (Kohjima *et al*. 2007). It was shown, that betaine administration to mice suffering from steatosis led to decrease of the PPARα gene promoter hypermethylation and subsequently increased expression in the liver (Wang *et al.* 2013).

From the above mentioned, it is clear that betaine interferes with the TG metabolism and its intake leads to steatosis elimination, which was proven by several animal (Kharbanda *et al.* 2007, Kharbanda *et al.* 2011, Wang *et al.* 2010) and human (Miglio *et al.* 2000, Abdelmalek *et al.* 2001) studies. As BChE activity is hypothetized to be coupled to TG metabolism, the goal of this study was to evaluate the clinical implications of betaine treatment on BChE activity in plasma and liver of rats under the conditions of high-fat diet.

Methods

Animals, diets, chemicals and biochemicals

Animals and diets were from The Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dobrá Voda, Slovak Republic. Reagents and chemicals used in all experiments were purchased from Sigma Aldrich (Slovak Republic), unless specified otherwise.

Experimental design

40 male Wistar rats weighing 215 - 295 g were used in the experiment. After 7-day adapting period, the animals were randomly divided into four groups by 10 and each group was fed for the following 4 weeks a different type of diet. The control group (C) was fed the standard diet. The high-fat diet group (HF) was fed the same diet with addition of pork lard (16%, w/w). The standard chow of the betaine group (B) was enriched with betaine (2%, w/w). The HFB group was fed the standard diet containing both pork lard (16%, w/w) and betaine (2%, w/w). Each day an equal chow portion was allocated to each animal cage in order to minimize the differences in the amounts of consumed chow.

Rats were sacrificed in pentobarbital anaesthesia (i.p. 60 mg/kg, 2.5% solution) after overnight starvation. Whole blood was collected from the stomach vein into heparinized syringes and plasma was prepared by centrifugation after applying the blood samples on Ficoll-Paque (15000 rpm at 4∘C, 20 min). Livers were partially perfused with saline and immediately frozen in liquid nitrogen. Tissues were stored at −80∘C until sample analysis.

In the course of the experiment the animals were supplied with water *ad libitum*. The care, use and procedures performed on these rats were approved by The State veterinary and food administration of the Slovak Republic (Ro-4295/12-221, from 20th December 2012).

Preparation of tissue homogenates

20% liver homogenates were prepared in potassium phosphate buffer (0.1 mmol/l, pH 7.4) with 0.5% Triton X-100 and centrifuged at 15 000 rpm, 4 °C, for 20 min. Protein content in the samples was determined according to Bradford protocol (Bradford 1976).

Biochemical laboratory analysis

Determination of standard biochemical parameters in plasma – glucose, triglycerides, cholesterol, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and C-reactive protein (CRP) was carried out using the appropriate kits from Beckman Coulter on AU 400 analyzer (Beckman Coulter). All measurements were performed and evaluated by following the manufacturer's instructions. Thiobarbituric acid reactive substances (TBARS) were determined after extraction with *n*-butanol by spectrofluorimetric method (excitation wavelength 528 nm, emission wavelength 558 nm, spectrofluorometer Cary Eclipse, Varian; (Solichová *et al.* 2003)).

For determination of GSH/GSSG ratio, ragent kit BIOXYTECH® GSH/GSSG-412[TM] (*Oxis*Research, a division of OXIS Health Products, Inc.) was used; absorbance was read on spectrophotometer ECOM 6122 (Eppendorf).

Spectrophotometric estimation of butyrylcholinesterase activity

BChE activity was determined spectrophotometrically by the Ellman method (Ellman *et al.* 1961) in a total volume of 0.2 ml on 96-well plates. Samples were preincubated for 30 min.

7

with the specific acetylcholinesterase inhibitor (bw284c51; 0.02 mmol/l) and 5,5-dithiobis(2-nitrobenzoic) acid (0.625 mmol/l) at 25 °C in dark. The reaction was started by addition of the substrate - butyrylthiocholine iodide in the final concentration (0.625 mmol/l). BChE enzyme activity was expressed in ΔO.D. per miligram of protein in the sample per minute.

Total RNA isolation and cDNA synthesis

The total RNA was isolated from 100 mg of liver tissue using RNAzol-RT (Molecular Research Center, Inc., USA) following the instructions of the manufacturer. 500 ng of total RNA was reversely transcribed using the PrimeScriptTM RT reagent kit (Takara Bio Inc., Japan).

Real-time polymerase chain reaction

Real-time PCR was performed with 5xHot FIREPol EvaGreen qPCR Mix Plus ROX kit (Solis Biodyne, Estonia). To determine the PPARα gene expression specific primers were used (forward 5´ → 3´: AAGGGCTTCTTTCGGCGAAC; reverse 5´ → 3´: TGACCTTGTTCATGTTGAAGTTCTTCA; 21, (Qu *et al*. 2007)) to give a 304 bp product. The reaction was performed in a total volume of 20 µl containing 100 ng of sample cDNA (2 µl), 4 pmol of each primer (0.4 µl), 4 µl of EvaGreen with ROX and nuclease-free water to a final volume of 20 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 15 min followed with 40 cycles of 95 °C for 30 s, 60 °C for 30s and 72 °C for 30 s. The reaction was finished with the dissociation step. Transcription efficiency was analysed with help of ΔΔCt value method (Winer *et al.* 1999), whereas the target gene Ct value was normalised by ß-actin expression (forward: CCGCGAGTACAACCTTCTTG, reverse: GCAGCGATATCGTCATCCA, (Mackovičová *et al.* 2011)), 81 bp PCR product in the sample was analysed under the same reaction conditions. 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.

Statistics

Data were analysed by one-way analysis of variance (ANOVA) using OriginPro 7.5, SR0, v7.5714 (B714), OriginLab Corporation, USA, followed by the Tukey´s multiple comparision. Results are expressed as mean ± standard deviation. Statistical significance was assumed for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Results

The results of plasma biochemistry are summarized in Tab. 1. Consumption of high-fat diet induced increased glucose levels in plasma (6.96±1.41 (HF) vs. 4.52± 0.43 (C) mmol/l; p=0.002), suppressed by concomitant betaine supplementation (3.24±1.47 (HFB) vs. 6.96±1.41 (HF) mmol/l; p=0.0012), even if rats were fed control diet (3.55±0.28 (B) vs. 4.52± 0.43 (C) mmol/l; p=<0.001). TG were not significantly elevated in the high-fat diet group (0.30±0.17 (HF) vs. 0.27±0.08 (C) mmol/l; p=0.70), but betaine income decreased their levels approximately by half in case of both diets (0.13±0.03 (HFB) vs. 0.30±0.17 (HF) mmol/l; p=0.005 and 0.15±0.03 (B) vs. 0.27±0.08 (C) mmol/l; p=0.003). Betaine had no impact on the total cholesterol (TC) in rats fed standard diet (0.78±0.06 (B) vs. 0.84±0.09 (C) mmol/l; p=0.19), but its consumption prevented the significant TC increase recorded in the HF group (0.84± 0.09 (HFB) vs. 1.18±0.15 (HF); mmol/l; p=0.015).

Betaine intake affected also one of the two general markers of hepatocellular injury - AST. Whereas the concentrations of AST remained unchanged in the HF group (1.53± 0.26 (HF) vs. 1.60±0.30 (C); mmol/l; p=0.64), betaine caused a significant drop of this parameter regardless the type of diet betaine was added to (1.24± 0.26 (B) vs. 1.60±0.30 (C); mmol/l; p=0.031 and 0.91± 0.41 (HFB) vs. 1.53±0.26 (HF); mmol/l; p=0.005). By contrast, betaine had no impact on ALT, which was significantly increased in plasma of animals on high-fat diet (0.44±0.10 (HF) vs. 0.28±0.03 (C) mmol/l; p<0.001).

HF diet consumption resulted in increased CRP levels, signaling pro-inflammatory state. Despite we observed lower CRP in the HFB group, this decrease was not significant due to the high SD values in both groups. Compared to control, increased levels of TBARS representing lipid associated oxidative stress were observed in all groups, the most

10

significant increase was caused by betaine consumption with the standard diet (0.51±0.04 (B) vs. 0.34±0.04 (C) mg/l; p<0.001). Although statistically uric acid (UA) levels in the HF group equaled to those in the C group, betaine intervention was clear in terms of decreased levels, when used in both groups (41.38±5.07(B) vs. 64.00±10.15 (C) µmol/l; p<0.001 and 34.50±6.29 (HFB) vs. 52.29±10.39 (HF) µmol/l; p=0.004). Another marker of oxidative stress, the GSH/GSSG ratio was slightly decreased in the HF group (5.14±0.47 (HF) vs. 6.92±1.41 (C); p=0.01) and recovered to control value along with betaine treatment (7.82±0.51 (HFB) vs. 5.14±0.47 (HF); p<0.001). Betaine raised the GSH/GSSG ratio even in the group of animals on standard diet (8.91±1.35 (B) vs. 6.92±1.41 (C); p=0.04).

The butyrylcholinesterase activity was measured in rat plasma and liver homogenates. In plasma, both high-fat diet and betaine intake led to significantly elevated BChE activity (0.36±0.12 (HF) vs. 0.22±0.04 (C) O.D./min/mg; p=0.014 and 0.41±0.12 (B) vs. 0.22±0.04 (C) O.D./min/mg; p=0.0012), which was even potentiated, when betaine was combined with high-fat diet (0.84±0.34 (HFB) vs. 0.22±0.04 (C) O.D./min/mg; p<0.001, Fig. 1). The same pattern of BChE activity was observed in the liver (Fig. 2), with even more significant BChE activity rise in HF and B group, compared to control. But similarly to plasma, the combination of high-fat diet and betaine eventuated in more than fivefold of the BChE activity in the control group (1.00±0.60 (HFB) vs. 0.17±0.03 (C) O.D./min/mg; p=0.0011).
The PPARα gene expression in the liver turned out to be suppressed as a result of high-fat diet as well as betaine intake. Surprisingly the same effect was not recorded in the HFB group with parallel high-fat diet and betaine consumption, where the PPARα mRNA levels did not differ substantially from those in the control group (Fig. 3).

Discussion

The enzyme butyrylcholinesterase is expressed in several tissues (brain, lungs, liver, gut, adipose tissue, heart and others), but the BChE activity in plasma reflects primarily the level of its synthesis in the liver. The knowledge that BChE is secreted into plasma from liver at a constant rate is relevant mainly to the clinical practice, where estimation of the enzyme´s activity serves as one of the markers in the liver function test. Altered BChE activity is generally considered to be the consequence of severe chronic liver disorders. While decreased BChE activity is ascribed to distinct liver damages profoundly reducing its function, such as steatosis, fibrosis, cirrhosis or hepatitis, increased BChE activity occurs rather rarely as the outcome of hepatocellular carcinoma (Pohanka 2013). There are only a few other pathological conditions influencing the BChE activity, including renal diseases, burns or intoxications (Soliday *et al.* 2010).

In the last two decades BChE activity changes have been often discussed as the consequence of its possible integration in the lipid metabolic pathways, particularly TG turnover, whereby a certain mechanism has not been elucidated so far. Positive correlations between BChE activity and TG levels in plasma were proven repeatedly in subjects suffering from obesity, diabetes or metabolic syndrome (Rustemeijer *et al.* 2001, Alcantara *et al.* 2002, Iwasaki *et al.* 2007), whereas inhibition of BChE activity was found to be accompanied by decreased TG plasma levels (Annapurna *et al.* 1991, Iwasaki *et al.* 2007). We hypothesized, that the natural compound betaine, used for the treatment of liver disorders (Purohit *et al*. 2007), which was also shown to lead to TG decrease in plasma (Hayesa *et al.* 2003, Kawakami *et al.* 2012) might evoke modifications of BChE activity. Betaine did not alter the animal´s weight gain after one month, no matter whether on standard, or high fat diet. However its intake was

12

beneficial for normalizing the levels of GLC, AST and CRP and it even increased the ratio

GSH/GSSG pointing to decreased oxidation stress burden (Tab. 1). The levels of plasma GLC

dropped in both groups treated with betaine (B and HFB) below the control levels. The effect

of betaine on GLC metabolism was studied by Kathirvel, who reported GLC decrease in mice

on high-fat diet after a long-term betaine intake along with improved hepatic insulin

resistance (Kathirvel *et al.* 2010). Inhibition of gluconeogenesis and increased use of glucose

in glycogen synthesis were proven to contribute to this effect of betaine, although

participation of other mechanisms was not excluded.

As expected, we recorded major changes in BChE plasma activity following betaine

treatment, but while betaine led to the expected decrease of plasma TG (Tab. 1), BChE

activity in plasma was elevated (Fig. 1). Compared to control, betaine treatment led to

approximately equal TG decrease in B and HFB groups (Tab. 1), but the BChE activity was

significantly higher in the HFB group compared to the B group (Fig. 1). In contrast, despite

there was no statistically significant rise of plasma TG in the HF group due to the extreme

variance of this parameter (Tab. 1), the BChE activity in plasma increased (Fig. 1). To sum up

the observed BChE activity fluctuations, based on our results we could not draw a general

rule stating positive nor negative correlations between BChE activity and TG levels in plasma.

The PPARα gene expression was decreased in animals on high-fat diet and this effect could

be reversed by betaine intake in the HFB group (Fig. 3). The same result was attained in mice

with steatosis after betaine administration (Wang *et al.*, 2013). In our previous rat

experiment we used the hypolipidemic drug fenofibrate, which acts as PPARα ligand to

decrease the plasma TG in order to determine whether the plasma BChE activity will

decrease. Similar to betaine treatment, the decrease of plasma TG was accompanied by

enhanced BChE activity, again observing a more prominent rise in the group consuming high-

13

fat diet (Šišková *et al.* 2012). Increased expression of PPARα along with elevated BChE activity support the hypothesis of BChE playing a role in the fat utilization process (Li *et al.* 2008).

In conclusion, even though the mechanism is unknown, the BChE activity in plasma is significantly elevated during betaine intake and this should be taken into account when BChE activity is interpreted as a marker in the liver function test.

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Conflict of Interests

The authors report no declarations of interest.

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19

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Table 1. Animal weights and plasma biochemistry (GLC - glucose, TG - triglycerides, TC – total cholesterol, ALT - alanine aminotransferase, ALT - aspartate aminotransferase, CRP - C-reactive protein, TBARS - thiobarbituric acid reactive substances, UA - uric acid, GSH – glutathione, GSSG – oxidized glutathione). Results are given as mean ± SD. Symbol * indicates statistic different value from C at P<0.05 (*),P<0.001 (**) and P<0.001 (***). Symbol # indicates statistic different value from HF at P<0.05 (#),P<0.001 (##) and P<0.001 (###).

	C		HF		B		HFB	
	mean	SD	mean	SD	mean	SD	mean	SD
Weight gain (g)	78.85	19.78	98.75*	6.94	76.00	18.84	98.89	6.52
GLC (mmol/l)	4.52	0.43	6.96**	1.41	3.55***	0.28	3.24 ##	1.47
TG (mmol/l)	0.27	0.08	0.30	0.17	0.15**	0.03	0.13**	0.03
TC (mmol/l)	0.84	0.09	1.18**	0.15	0.78	0.06	0.84#	0.24
ALT (µkat/l)	0.28	0.03	0.44***	0.10	0.31	0.06	0.39**	0.07
AST (µkat/l)	1.60	0.30	1.53	0.26	1.24*	0.26	0.91**/**	0.41
CRP (mg/l)	167.50	26.46	248.50**	60.36	176.90	28.34	205.40	39.45
TBARS (µmol/l)	0.34	0.04	0.49**	0.11	0.51***	0.04	0.48*	0.11
UA (µmol/l)	61.67	8.82	51.13	10.16	41.38***	5.07	34.50***/##	6.29
GSH/GSSG	6.92	1.41	5.14*	0.47	8.91*	1.35	7.82###	0.51
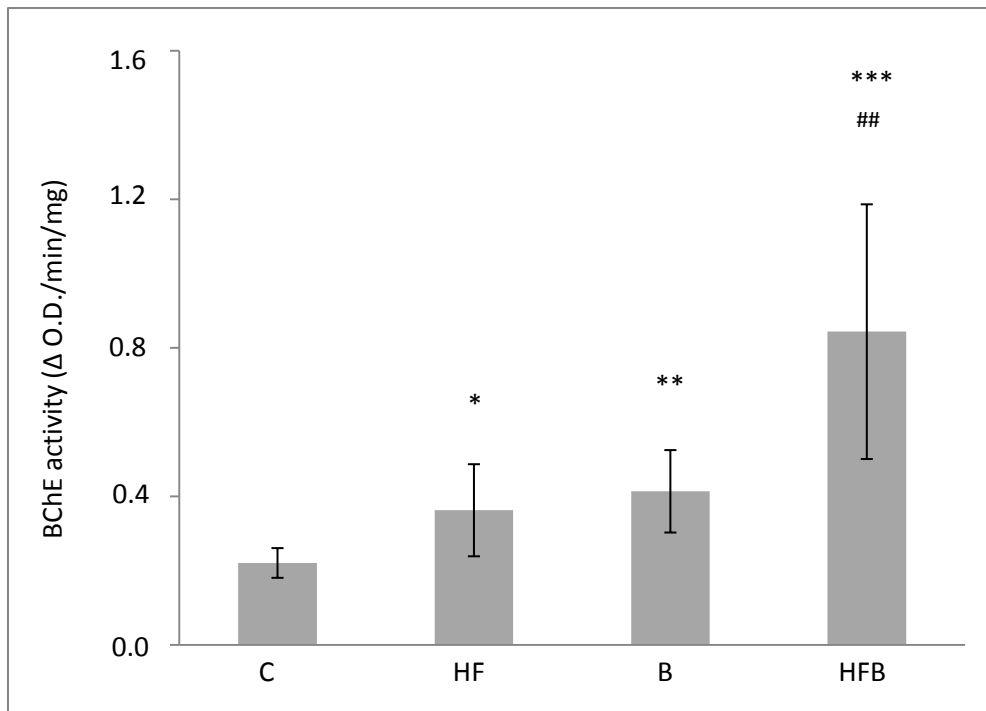
Figure 1. Butyrylcholinesterase activity in plasma of Wistar rats after 4 weeks on different diets. BChE activity was estimated on substrate butyrylthiocholine iodide, the results are given as mean values ± SD. C – control, HF – high fat diet, B – betaine, HFB – high fat diet + betaine; * is statistically different from C, $^{\#}$ is statistically different from HF. Symbol * indicates statistic different value from C at P<0.05 (*),P<0.001 (**) and P<0.001 (***). Symbol $^{\#}$ indicates statistic different value from HF at P<0.05 ($^{\#}$),P<0.001 ($^{\#\#}$) and P<0.001 ($^{\#\#\#}$).
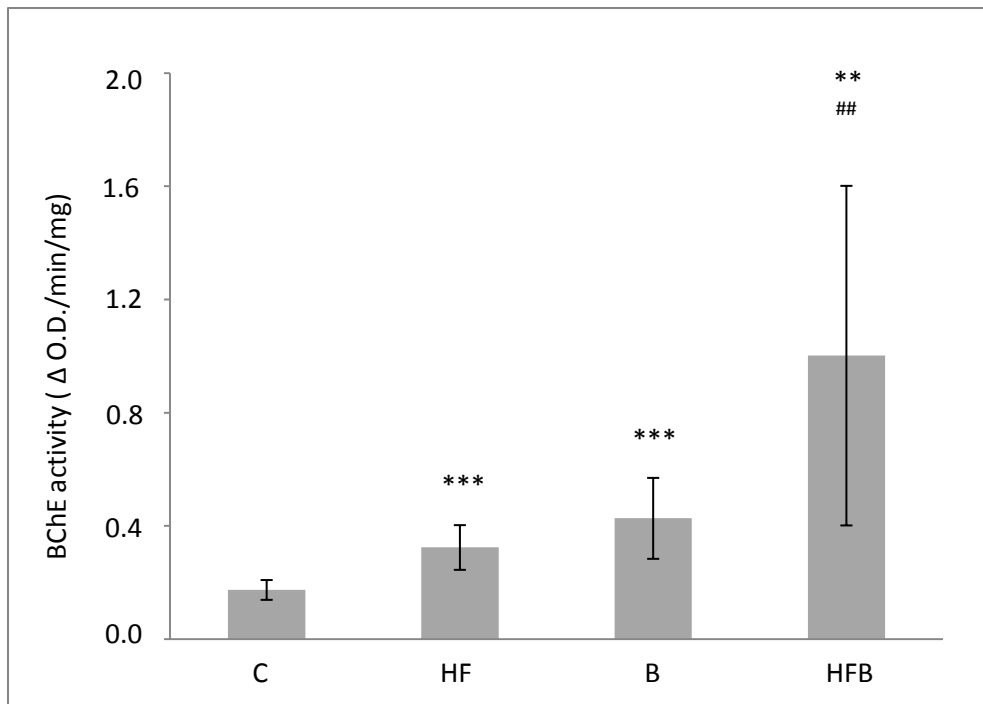
Figure 2. Butyrylcholinesterase activity in the liver homogenates of Wistar rats after 4 weeks on different diets. BChE activity was estimated on substrate butyrylthiocholine iodide, the results are given as mean values ± SD. C – control, HF – high fat diet, B – betaine, HFB – high fat diet + betaine; * is statistically different from C, # is statistically different from HF. Symbol * indicates statistic different value from C at P<0.05 (*),P<0.001 (**) and P<0.001 (***). Symbol # indicates statistic different value from HF at P<0.05 (#),P<0.001 (##) and P<0.001 (###).
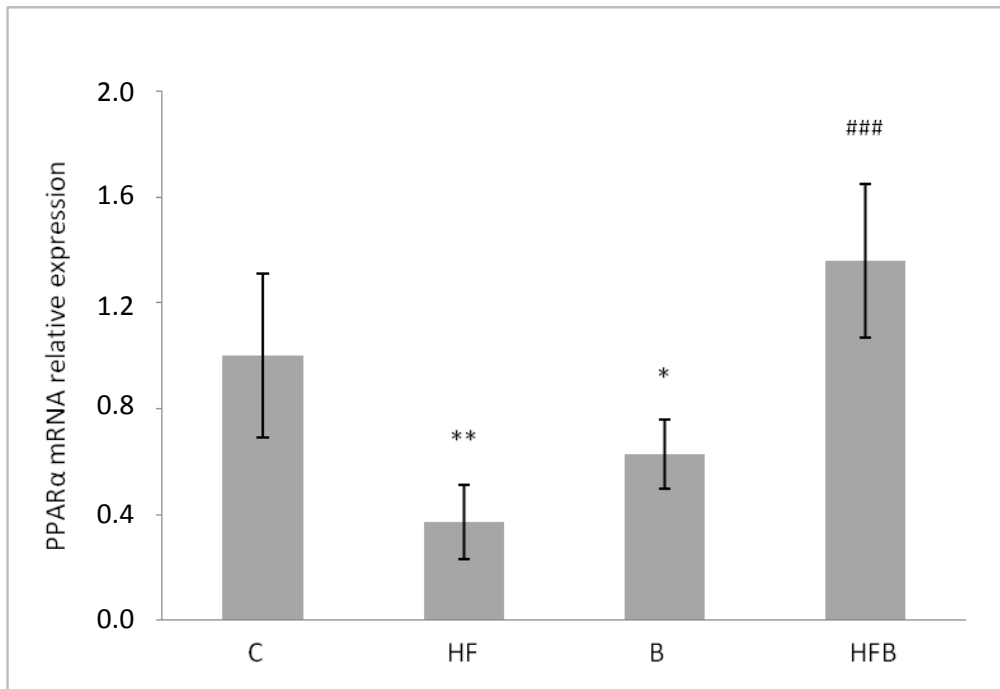
Figure 3. PPARα gene expression in the liver of Wistar rats after 4 weeks on different diets. Relative changes of PPARα gene expression are given as PPARα mRNA normalized to β-actine mRNA, whereas control was preset at 1. The results are given as mean values ± SD. C – control, HF – high fat diet, B – betaine, HFB – high-fat diet + betaine; * is statistically different from C, # is statistically different from HF. Symbol * indicates statistic different value from C at $P<0.05$ (*),$P<0.001$ (**) and $P<0.001$ (***). Symbol # indicates statistic different value from HF at $P<0.05$ (#),$P<0.001$ (##) and $P<0.001$ (###).