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Activation of PPARα by bezafibrate negatively affects *de novo* synthesis of sphingolipids in regenerating rat liver

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ABSTRACT

Serine palmitoyltransferase (SPT) is a key enzyme in *de novo* sphingolipid biosynthesis. SPT activity in liver is up-regulated by pro-inflammatory cytokines, which play an important role in initiation of liver regeneration after partial hepatectomy (PH). The aim of the study was to investigate the impact of a high-fat diet or PPARα activation by bezafibrate on the activity and protein expression of SPT in rat liver after PH. The animals were divided into three groups: those fed a standard chow (SD), those fed a high-fat diet (HFD), and those treated with bezafibrate (BF). It has been found that the expression and activity of SPT increased in regenerating liver. This was accompanied by the elevation of plasma NEFA concentration. Moreover, in both diet groups, the content of sphinganine increased. Bezafibrate decreased protein expression of SPT at the 4th and 12th hour, and inhibited SPT activity at the 4th hour after PH. Both, the plasma NEFA concentration and sphinganine content decreased in the groups treated with bezafibrate. We conclude that partial hepatectomy stimulates *de novo* sphingolipid synthesis. Activation of PPARα by bezafibrate negatively affects this process, presumably by decreasing the availability of plasma-borne fatty acids.

1. Introduction

Sphingolipids are widely recognized as not only inert building blocks of cellular membranes, but also as regulatory particles. Sphingolipid signaling molecules are released from biologically inactive sphingomyelin. Hydrolysis of sphingomyelin by signaling enzymes known as sphingomyelinases (SMases) liberates ceramide (CER). This secondary messenger plays a key role in the regulation of cell proliferation and apoptosis (reviewed in [1]). Subsequent degradation of ceramide by ceramidases (CDases) releases sphingosine (SPH), which can be phosphorylated by the enzyme sphingosine kinase (SPHK) to yield sphingosine-1-phospate (S1P) [2]. Sphingosine and sphingosine-1-phosphate influences intracellular calcium homeostasis [3] and regulates hepatocyte proliferation [4]. The catabolic pathway of signal transduction (e.g. hydrolysis of sphingomyelin and subsequent degradation of ceramide) was considered as the main source of biologically active sphingolipids. However, recent discoveries shed a new light on the role of *de novo* pathway in sphingolipid signaling. The activation of *de novo* sphingolipid synthesis is observed under the influence of various factors, such as cytokines [5], UV irradiation [6] and chemotherapeutics [7]. The above-mentioned factors stimulate synthesis of both the complex sphingolipids (e.g. glucosylceramides) and ceramide. The initial step in *de novo* sphingolipid synthesis is carried out by serine palmitoyltransferase (SPT) which catalyzes the condensation of palmitoyl-CoA and L-serine to yield 3-ketosphinganine. This reaction is considered to be the rate-limiting step in the *de novo* sphingolipids synthetic pathway [8]. The activity of SPT determines the availability of sphingoid backbones that are important for synthesis of the cellular membranes. In mammals, SPT is the product of two distinct genes SPTCL1 and SPTCL2 (the enzymatically active heterodimer is composed of both subunits) [9]. Upregulation of both SPT mRNA and protein content was observed in rat and mouse liver under the influence of bacterial endotoxins (LPS) [10] and pro-inflammatory cytokines [5]. So far, the increased activity of serine palmitoyltransferase was seen as a pro-apoptotic response, which precedes the production of ceramide and cell death, triggered by various pro-apoptotic factors of biological, chemical or physical origin. This phenomenon was observed in human Molt-4 lymphoma cell line [11], metastatic breast cancer cells [12], LNCaP or PC-3 prostate cancer cells [13], and C6 glioma cells [14]. However, serine palmitoyltransferase activity seems to be crucial for the proliferation of at least several cell lines. Inhibition of the enzyme activity by L-cycloserine [15] or mycotoxin myriocin [16,17] suppresses proliferation and induces apoptosis of the cytotoxic T cells. SPB-1 mutants of Chinese

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hamster ovary cells (possessing thermolabile SPT enzyme) cease to grow at a non-permissive temperatures, as a result of a reduction in the *de novo* sphingolipid synthesis. Both sphingosine and sphinganine (but not phosphatidylcholine) were effective in the restoration of cell ability to grow and divide in sphingolipid-deficient culture medium [18]. Cytokines which activate *de novo* sphingolipid synthesis in liver through upregulation of SPT are also crucial in the initiation of liver regeneration after partial hepatectomy (PH) [19]. The loss of the liver cells either through surgical resection, chemical injury or viral injury triggers proliferation of hepatocytes (reviewed in [20]). We have previously shown, that PH stimulates sphingolipid signaling in the rat liver and increases production of pro-mitotic sphingolipids such as sphingosine-1-phosphate and sphinganine-1-phosphate [21]. Subsequently, we have shown that bezafibrate (a PPARα agonist) inhibits [22], whereas high-fat diet activates [23], production pro-mitotic sphingosine-1-phosphate in rat liver after PH. Data is scarce on the role of serine palmitoyltransferase and *de novo* sphingolipid synthesis in the proliferation of hepatocytes during liver regeneration. The aim of the present study was to investigate the activity and the expression of serine palmitoyltransferase in the rat liver, at 4th, 12th and 24th hour after 70% PH. Above-mentioned time points correspond to the early signaling events (G_0 phase to G_1 phase transition), induction of mitosis (S and G_2 phases) and mitotic division (the M phase), respectively. We also examined the influence of high-fat diet and activation of PPARα receptor by bezafibrate on the expression and activity of SPT in the regenerating rat liver.

2. Materials and methods

Animals and study design. The investigation was approved by the Ethical Committee for Animal Experiments at the Medical University of Bialystok. Male Wistar rats (weighting between 210 and 230 g of body weight) were divided into three experimental groups: those fed on a standard laboratory rodent chow (SD); those fed for 7 days on high-fat diet based on sunflower oil, rich in oleic (18:1) and linoleic (18:2) fatty acids (HF); those treated with bezafibrate, a PPARα agonist (BF). The bezafibrate was administrated at a dose of 7.5 mg/100 g of body weight/day for 7 days by oral gavage, as described previously [24]. The high-fat diet was given isocalorically compared to standard diet. The standard diet composition (g/100 g of diet, w/w) was: 17 g casein, 12.5 g maltodextrin, 62 g starch, 2.8 g saturated fat, 4.2 g mineral mix, 1.3 g vitamin mix, 0.2 g methionine. The high-fat diet composition (g/100 g of diet, w/w) was: 34.9 g (63% – linoleic, 23% – oleic fatty acids) sunflower oil, 25 g corn starch, 25 g casein, 1.9 g gelatin, 5 g wheat bran, 6 g mineral mix, 1.3 g vitamin mix, 0.3 g methionine. The SD chow was composed of 2.8% total fat, while the HF chow was 39%. Rats from each experimental group were subjected to 70% partial hepatectomy. Removal of two-thirds of the liver was performed under pentobarbital anesthesia, as described earlier [21]. Samples of the regenerating liver were taken 4, 12 and 24 h respectively post-surgery ($n = 8$ per each time point) which corresponds to the early signaling events, the cell cycle progression and the cellular division in rat liver after PH [25]. Moreover, samples of the normal liver were taken from sham-operated rats, (untreated, sham control group; C-SD; $n = 8$), from sham-operated rats treated with bezafibrate (sham control-bezafibrate group; C-BF; $n = 8$) and sham-operated rats fed high-fat diet (sham control, high-fat diet; C-HD; $n = 8$). The samples of the liver and plasma were frozen in liquid nitrogen and stored (at −80 °C) until further analysis.

Determination of the plasma non-esterified fatty acids. The concentration of plasma non-esterified fatty acids (NEFA) was measured by an enzymatic microcolorimetric assay using a Wako NEFA C kit (Wako Chemicals GmbH, Neus, Germany).

Western Blot analysis of SPTLC2 subunit. The amount of SPTLC2 protein was determined by a Western Blot technique as described previously [26]. Equal amounts of protein (50 μg) were separated by 10% SDS-PAGE. Protein bands were transferred on to nitrocellulose membranes (BioRad). The membranes were probed with primary anti-SPTLC2 specific antibody (Cayman Chemicals) and bound antibody was detected after incubation with alkaline phosphatase conjugated secondary antibody (Sigma–Aldrich). Protein bands were visualized and densitometrically quantified using a Gel Doc EQ system (BioRad).

Determination of SPT activity. The activity of serine palmitoyltransferase was measured in isolated rat liver microsomes according to a method by Dickson et al. [27]. The liver microsomal fraction was obtained by ultracentrifugation at 150,000 × *g* for 40 min at 4 °C. Up to 200 μg of microsomal protein was incubated in the reaction buffer (100 mM HEPES, pH = 8.3, 5 mM DTT, 2.5 mM EDTA, 50 μM pyridoxal phosphate (PLP), 200 mM palmitoyl-CoA and 2 mM radiolabeled ^{3}H-L-serine specific activity 100 DPM/pmol) for 10 min at 37 °C with shaking. The reaction was terminated by the addition of 1.5 ml of chloroform/methanol (1:2, v/v). The radiolabeled enzymatic product, 3-ketosphinganine, was extracted with $CHCl_3$/methanol (1:2, v/v) and the radioactivity measured by scintillation counting.

Protein determination. The protein content in homogenates and liver microsomes was measured with a BCA-1 kit (Sigma–Aldrich, St. Louis, MO, USA) and a Thermo Labsystems Multiskan EX ELISA reader.

The content of sphinganine. The content of sphinganine was measured according to the method by Min et al. [28]. Sphingoid bases were extracted, converted to their OPA-derivatives and quantified on the Varian ProStar liquid chromatograph equipped with a Varian OmniSpher 5 C18 column and fluorescence detector. The mobile phase was composed of acetonitrile/H_2O (9:1, v/v). Quantitative and qualitative analysis was based on standard curves prepared using commercially available standards.

Statistical analysis. Results are shown as means ± standard deviation ($n = 8$ per group). Statistical differences between groups were determined by ANOVA and subsequent post hoc tests. Homogeneity of variances was checked by the Brown-Forsyth test. In the case of different variances, Welch ANOVA was used in conjunction with the Dunnet T3 post hoc test. If the condition of homogeneity of variance had been met the standard ANOVA was used together with the Tukey post hoc test. The significance level was set to $p \leq 0.05$.

3. Results

The concentration of plasma non-esterified fatty acids (NEFA). Partial hepatectomy strongly increased the concentration of plasma NEFA in the standard and high-fat diet groups. In PH-SD groups, the concentration of NEFA at the 4th hour after the operation was fivefold higher as compared to the standard diet control (C-SD, $p < 0.001$) (Fig. 1). The concentration of plasma NEFA decreased in the subsequent time points. However, 24 h after the operation it was still 2.5-fold higher than the C-SD control ($p < 0.01$). There were no statistical differences between the PH-SD and PH-HF groups, except the sham-operated controls (C-SD and C-HF). In the C-HF group, plasma NEFA concentration increased by 85% ($p < 0.001$), whereas bezafibrate treatment in C-BF group increased it by 35% ($p < 0.05$) as compared to the values in C-SD group (Fig. 1). Bezafibrate treatment strongly decreased the concentration of plasma NEFA in the partially hepatectomized animals. In the PH-BF groups, the NEFA concentration after 4th, 12th and 24th hours post-surgery decreased by 65% ($p < 0.001$), 54% ($p < 0.001$) and 31% ($p < 0.05$) respectively, as compared to PH-SD groups (Fig. 1). In contrast to both the SD and HF groups, the postoperative increase