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Stimulation of rat liver branched-chain alpha-keto acid dehydrogenase activity by low doses of bezafibrate

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ABSTRACT

Multienzyme branched-chain alpha-ketoacid dehydrogenase complex (BCKDH) catalyzes the regulatory step of oxidative catabolism of indispensable branched-chain amino acids (BCAA). The activity of the BCKDH complex is regulated by a reversible phosphorylation, end-product inhibition and by changes in the gene expression of BCKDH component enzymes. It has been shown previously that a high dose of bezafibrate (an agent added to rat chow at final concentration of 0.5%) changes mRNA levels of BCKDH-related enzymes and increases dephosphorylation of the complex leading to stimulation of liver BCKDH activity and the enhanced BCAA catabolism. The aim of the present study was to determine an in vivo effect of low, clinically relevant doses of bezafibrate on BCKDH activity in rat liver. Bezafibrate was administrated for 14 days by gastric gavage to Wistar male rats (fed low-protein chow; 8% protein) at one of the following daily doses of 5, 10 and 20 mg/kg b.wt. The control group was given the vehicle (0.3% methylcellulose) only. The actual BCKDH and total BCKDH activities were assayed spectrophotometrically before and after incubation with a broad-specificity phosphatase, respectively. The mRNA levels of the selected genes (BCKDH catalytic subunits and regulatory enzymes) were quantified by means of semi-quantitative RT-PCR. Current catalytic activity of BCKDH (described as BCKDH activity state – the proportion of the BCKDH complex in its active dephosphorylated form) increased by 2.1 ± 0.2, 2.3 ± 0.2 and 2.7 ± 0.2 fold ($p < 0.01$). Changes in BCKDH activity did not correspond with changes in mRNA levels of the complex catalytic subunits. Moreover, mRNA levels of regulatory enzymes remained unaltered. Initially bezafibrate caused a transient insignificant reduction in body weight, but it had no effect on the final body weight. The highest dose of bezafibrate induced hepatomegaly. In conclusion, these data indicate that under conditions of dietary protein restriction low, clinically relevant doses of bezafibrate have a similar adverse effect on rat liver BCKDH activity and BCAA degradation rate as the high experimental dose. Up-regulation of liver BCKDH activity by low doses of bezafibrate appears to result mainly from changes in phosphorylation status of the complex (increased dephosphorylation) and is not associated with elevations in mRNA levels of BCKDH enzymatic components.

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1. Introduction

Bezafibrate is a pharmacological agent, used in clinical practice to treat hyperlipidemia (Goldenberg et al., 2008). In addition to its well documented action on lipid metabolism there is growing evidence that bezafibrate improves glucose metabolism (Flory et al., 2009). Data on the effect of bezafibrate on amino acids metabolism, including essential branched-chain amino acid (BCAA – valine, isoleucine and leucine) metabolism, are scarcely available.

In mammals, BCAA cannot be synthesized and thus must be obtained from the diet. As food is the main source of BCAA, and they cannot be stored in any form other than proteins, the homeostasis of free BCAA is maintained by regulation of their degradation rate. BCAA are catabolized efficiently when their intake exceeds requirements of the body, and are conserved when dietary availability is limited.

A key step that commits carbon skeletons of BCAA to the degradation pathway and determines the overall level of BCAA is regulated by the mitochondrial multienzyme branched-chain α-ketoacid dehydrogenase (BCKDH) complex. It catalyses irreversible oxidative decarboxylation of branched-chain α-ketoacids (BCKA): α-ketoisovalerate, α-keto-β-methylvalerate and α-ketoisocaproate, which are, respectively, transamination products of valine, isoleucine and leucine. The complex contains multiple copies of three subunits: branched-chain ketoacid decarboxylase (E1, heterodimer α2β2), dihydrolipoyl transacylase (E2), and dihydrolipoyl dehydrogenase (E3). The E1 and E2 subunits are unique for the BCKDH complex, while the same E3 subunit is

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commonly shared between BCKDH, pyruvate dehydrogenase (PDH) and ketoglutarate dehydrogenase (KGDH). In addition to catalytic subunits, two regulatory enzymes, a specific kinase (BDK) and a specific phosphatase (BDP) are associated with the complex. BDK and its role in BCAA metabolism has been extensively studied, mostly in rat tissues (Harris et al., 1997, 2004). Lu et al. (2009) proved that mitochondrial protein phosphatase 2Cm is an endogenous phosphatase responsible for regulation of BCKDH activity. Here, protein phosphatase 2Cm is denoted as BDP.

The activity of the BCKDH complex is regulated by a covalent modification, end-product inhibition and by changes in the gene expression of BCKDH component enzymes. Reversible phosphorylation of the E1α subunit at serine 293 is thought to be the most important mechanism determining catalytic activity of the BCKDH complex and BCAA oxidative disposal. Phosphorylation catalyzed by BDK inactivates BCKDH, while dephosphorylation, mediated by BDP activates the complex. Current catalytic activity of BCKDH (described as BCKDH activity state) in particular tissues and under specific metabolic conditions depends directly on the proportion of BCKDH occurring in its active dephosphorylated form and is determined primarily by the relative activities of BDK and BDP.

An excess of BCAA and their metabolites can be toxic, especially to central nervous system, as manifested in patients with maple syrup urine disease (Mitsubuchi et al., 2005). On the other hand, low BCAA level is also detrimental (Watford, 2007). BCAA (especially leucine) serve not only as substrates for protein synthesis but also function as nutritional signals regulating different metabolic processes in various tissues. For example BCAA modulate protein and carbohydrate metabolism, energy balance and hormone secretion (Li et al., 2003, 2011; McAllan et al., 2012). Additional regulatory roles for leucine require plasma and intracellular levels above the minimum necessary to synthesize proteins. Therefore, factors stimulating BCKDH activity and BCAA degradation may decrease BCAA level and affect multiple metabolic processes regulated by these amino acids.

BCKDH activity is tightly regulated under various metabolic conditions to maintain proper BCAA homeostasis. For example, in rats subjected to protein malnutrition and limited dietary BCAA availability, liver BCKDH activity decreases as a result of diminished expression of the E1 and E2 genes as well as increased E1 phosphorylation (Harris et al., 1986; Solomon et al., 1987; Zhao et al., 1994). Such a modification of BCKDH activity prevents essential BCAA from degradation. Restriction of protein intake by feeding rats low-protein chow provides an experimental model for examining factors that cause up-regulation of BCKDH activity.

It was also shown that liver BCKDH activity is modified by fibrates such as clofibrate, bezafibrate and fenofibrate (Knapik-Czajka et al., 2002; Kobayashi et al., 2002; Ono et al., 1990). Fibrates constitute a group of hypolipidemic agents that are commonly used in the treatment of dyslipidemias characterized by elevated concentrations of TG (Chapman et al., 2010). At the molecular level fibrates bind and activate peroxisome proliferator-activated receptors (PPAR) which belong to a group of nuclear receptor proteins that function as transcription factors (Fruchart et al., 1999). PPAR activation by fibrates results in change of transcription rate of the target genes encoding different proteins.

As we have shown previously, bezafibrate (and other fibrates) have an adverse effect on BCAA metabolism. In rats subjected to protein malnutrition bezafibrate promoted BCAA degradation by increasing the activity of the liver BCKDH complex. Activation of the BCKDH was due to up-regulation of E1 and E2 gene expression and increased dephosphorylation of the complex (Knapik-Czajka et al., 2002; Knapik-Czajka and Jaskiewicz, 2003).

It is known that the influence of bezafibrate on different metabolic processes may vary with dosage, with agent showing an effect when used in a high experimental dose, and having no effect when used in a low dose. In many rodent studies, aimed at determining the effects of bezafibrate on different metabolic processes, 100 mg/kg b.wt dose was considered high, while 10 mg/kg b.wt a low, clinically relevant dose (Mori et al., 2004; Nakajima et al., 2009). Our previous study evaluated the effect of a single high experimental dose of bezafibrate in rats (low protein diet containing 0.5% bezafibrate that corresponds roughly to dose exceeding 200 mg/kg b.wt). Such a dose is several times higher than clinical dose (≤10 mg/kg b.wt). At present, we wanted to determine the effect of bezafibrate on rat liver BCKDH complex in a more controlled environment and using more clinically relevant doses (≤20 mg/kg b.wt). The aim of this study was to investigate the effect of low doses of bezafibrate (5, 10 and 20 mg/kg b.wt) on liver BCKDH activity, as well as E1α, E1β, E2, BDK and BDP mRNA levels in rats fed with low-protein chow.

2. Materials and methods

2.1. Reagents

Bezafibrate (2-[4-[2-(4-chlorobenzamido)ethyl]phenoxy]-2-methylpropanoic acid) and other reagents necessary for assay of enzyme activities were purchased from Sigma–Aldrich Chemical Company (Germany). Low-protein (8% protein) chow was bought in ICN Biomedicals (USA).

2.2. Animals and experimental treatment

Animal experiments were conducted in accordance with the guidelines for animal experiments of Animal Research Committee and were approved by the Jagiellonian University Ethic Committee.

Male Wistar rats (from inbred strain) were purchased from the breeding facility of the Jagiellonian University Faculty of Pharmacy. They were housed four per cage and maintained under standardized conditions of artificial 12-h light/dark cycle and constant room temperature (21–23 °C). Rats were fed low-protein chow (8% protein content) ad libitum and allowed free access to water. After acclimatization to low-protein chow and the oral administration of 0.3% dimethylcellulose (agent's vehicle) rats were randomized into 4 treatment groups ($n = 4$). Bezafibrate was administrated by gastric gavage to consecutive groups at one of the daily doses: 5, 10 and 20 mg/kg b.wt. The control group was given only a vehicle. During the treatment body weight and food intake were monitored daily. After 14 days (the study duration was chosen based on prior reports by Paul et al. (1996)) rats were sacrificed. Liver tissues were excised, weighted and immediately freeze-clamped with aluminium tongs precooled in liquid nitrogen and then stored at −80 °C until analysis.

2.3. Determination of BCKDH activities

Extracts of tissues for the BCKDH were prepared as described previously (Goodwin et al., 1988). BCKDH was concentrated from the whole tissue extracts prior to assay by precipitation with 9% polyethylene glycol. BCKDH complex activity was determined spectrophotometrically at 30 °C by measuring the rate of NADH generation from NAD^+ in the presence of the saturating concentration of α-ketoisovaleric acid, a substrate for BCKDH complex (Cary 100 spectrophotometer – Varian). One unit of BCKDH complex activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH/min. The activity of the BCKDH complex occurring in partly dephosphorylated form (actual activity) or completely dephosphorylated form (total activity) were determined before and after incubation with a broad-specificity phosphatase, respectively. The broad-specificity phosphatase necessary to complete dephosphorylation of BCKDH complex was isolated from bovine heart, according to the procedure described by Goodwin et al. (1988). Percentage of BCKDH complex in active dephosphorylated form to totally dephosphorylated form was calculated from values of actual and total activities of BCKDH complex and expressed as the activity state of BCKDH complex.

2.4. Determination of mRNA levels

Relative levels of specific mRNAs were assessed by semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR). Each gene was amplified together with a housekeeping gene β-actin (internal control). Total RNA was isolated from the liver using TRI reagent (Sigma–Aldrich, Germany) and cDNA was subsequently synthesized using 1 μg total RNA, reverse-transcriptase and oligo dT primers (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas, Germany). cDNA was amplified with OptiTaq DNA polymerase (Eurx, Poland) following manufacturer's instructions. Each PCR reaction was performed with 1.2 μl cDNA and 1.6 μl of rat-specific primers that were designed for the genes of interest using Primer-BLAST software (NCBI) (Table 1). After an initial denaturation at 95 °C, PCR was carried out for 35 (E1α, E1β, E2, BDK, β-actin) or 33 (BDP) cycles. Each cycle

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