Effect of 5-fluorouracil on branched-chain α-keto acid dehydrogenase (BCKDH) complex in rat's heart

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Abstract: Undisturbed branched-chain amino acids (BCAA) catabolism is necessary for normal heart function. The key enzyme in BCAA catabolism is a multienzyme branched-chain α-keto acid dehydrogenase complex (BCKDH). BCKDH activity is regulated mainly by reversible dephosphorylation (activation)/phosphorylation (inactivation) cycle catalyzed by regulatory enzymes, a specific phosphatase (PPM1K) and kinase (BDK). 5-fluorouracil (5-FU) is widely used in the treatment of different types of cancer. 5-FU has the potential to cause a wide spectrum of cardiotoxicity, ranging from asymptomatic electrocardiographic changes to cardiomyopathy and subsequent cardiac failure. We hypothesize that 5-FU modifies BCKDH activity and affects cardiac muscle metabolism. The current study was aimed at the investigation of the in vivo effect of 5-FU on BCKDH activity and mRNA levels for E1, PPM1K and BDK. Wistar male rats were administered with 4 doses of 5-FU, 150 mg/kg b.wt. each (study group) or 0.3% methylcellulose (control group). BCKDH activity was assayed spectrophotometrically. The mRNA levels were quantified by real-time PCR. 5-FU treatment caused an increase in BCKDH activity that appears to result mainly from increased dephosphorylation of the complex and is associated with an increase of PPM1K mRNA level and reduction of BDK and E1 mRNA levels. It is conceivable that 5-FU stimulation of BCKDH is an adaptive reaction with the purpose of enhancing the BCAA catabolism and protecting from toxic effect caused by excessive accumulation of these amino acids in heart.

Key words: 5-fluorouracil (5-FU), branched-chain amino acids (BCAA), branched-chain α-keto acid dehydrogenase (BCKDH), rat, heart.

Introduction

5-fluorouracil (5-FU) is widely used alone or in combination regimens in the treatment of gastrointestinal, breast, head and neck tumors and its oral prodrug, capecitabine, is approved for treatment of colorectal cancer and metastatic breast cancer. 5-FU has the potential to cause a wide spectrum of cardiotoxicity, ranging from asymptomatic electrocardiographic changes to cardiomyopathy and subsequent cardiac failure [1, 2]. The pathophysiological and molecular mechanisms of cardiotoxicity associated with 5-FU treatment are still not fully investigated. Several mechanisms have been proposed, including vascular endothelial damage followed by coagulation, ischemia secondary to coronary artery spasm, direct toxicity on the myocardium and thrombogenicity [3]. Some animal models have demonstrated specific metabolic changes in cardiomyocytes upon 5-FU treatment, including increased oxygen consumption, depletion of high-energy phosphate compounds, citrate accumulation and oxidation of macromolecules caused by free radicals [3].

Branched-chain amino acids (BCAA), leucine, isoleucine and valine are essential amino acids derived from food. Unlike other amino acids, BCAA are primarily catabolized in the extrahepatic tissues, including the cardiac muscle. The first two steps in BCAA catabolism are similar for the three BCAA. In the initial step branched-chain amino acid transaminase (BCAT) catalyzes transamination of BCAA, which is a readily reversible reaction that yields the corresponding branched-chain α-keto acid (BCKA), α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV) and α-ketoisovalerate (KIV), transamination products of leucine, isoleucine and valine, respectively. Next, rate limiting step in the BCAA catabolism is catalyzed by the mitochondrial branched chain α-keto acid dehydrogenase complex (BCKDH). The complex is responsible for the irreversible oxidative decarboxylation of BCKA derived from BCAA. Subsequently, each amino acid follows a unique catabolic pathway. Finally, valine is degraded to succinyl-CoA, isoleucine to propionyl-CoA (which can be converted to succinyl-CoA) and acetyl-CoA and leucine exclusively to acetyl-CoA. All products of BCAA catabolism can be used as energy source by oxidation to CO_2 and H_2O in TCA cycle. In some tissues, succinyl-CoA can be used for glucose synthesis, while acetyl-CoA for ketone bodies production.

BCKDH is composed of multiple copies of three catalytic subunits: branched-chain dehydrogenase (E1 component), dihydrolipoamide acyltransferase (E2 component) and dihydrolipoamide dehydrogenase (E3 component) [4]. E1 is responsible for the rate-limiting step of the overall reaction catalyzed by the BCKDH complex. In addition to catalytic subunits, two regulatory enzymes, a specific kinase (BDK) and a specific phosphatase (PPM1K) can be associated with BCKDH, depending on the metabolic conditions [5, 6].

The activity of the BCKDH complex is regulated by different mechanisms: by a covalent modification, end-products and by changes in the gene expression of

BCKDH component enzymes. It is generally believed that the reversible phosphorylation of E1 subunit (at E1 Ser 293) is a major mechanism involved in BCKDH activity modification [7, 8]. Phosphorylation of E1 subunit mediated by BDK inactivates BCKDH, while dephosphorylation catalyzed by PPM1K activates the complex [9]. Therefore, current catalytic activity of BCKDH (described as BCKDH activity state) and thus BCAA catabolism rate depends directly on the portion of BCKDH complex occurring in its active dephosphorylated form.

It has also been demonstrated that undisturbed BCAA catabolism is necessary for normal heart function [10]. Compared with fatty acids and glucose, BCAA quantitatively contribute to a lesser degree to ATP generation in the heart. However, BCAA, especially leucine plays essential roles in myocardial function that extend beyond energy metabolism, including regulation of protein synthesis and acting as signaling molecules [11]. It has been found that impaired BCAA catabolism which results in accumulation of BCKA/BCAA is an integral part of the metabolic reprogramming in stressed hearts [12–14].

Taking into account the BCAA and BCKDH roles in cardiomyocytes we can suspect that 5-FU modifies BCKDH activity and affects cardiac muscle metabolism. The current study was aimed at the investigation of the in vivo effect of 5-FU on BCKDH complex activity and mRNA levels for E1, PPM1K and BDK.

Material and Methods

Experimental design

Adult male albino Wistar rats (n = 10) weighting 170-200g (from the Jagiellonian University Medical College Animal Laboratory, Krakow, Poland) were included in the experiment. During the whole experiment rats were housed under standard conditions at temperature (20 ± 4°C), humidity (55 ± 10%), 12-h light/dark cycle and access to standard rat chow (protein 25%, fat 8%, carbohydrates 67%, metabolizable energy 2.86 kcal/g — Labofeed B, Kcynia, Poland) and tap water *ad libitum*. Acclimatization to housing rooms and handling were performed daily for one week before including into study. After an initial acclimatization period rats were randomized into study group (5-FU; n = 5) or control group (C; n = 5). Study group received intraperitoneal injections of 5-fluorouracil and control group received 0.3% methylcellulose. This study was carried out in accordance with ethical, regulatory and scientific principles with the approval of the local Jagiellonian University Ethical Committee (protocol numbers 355/2019, 64/2018).

5-fluorouracil application

Before injection selected part of abdomen was gently shaved and disinfected. 5-fluorouracil (MEDAC, 50 mg/ml) was given intraperitoneally 4 times (150 mg/kg) every 2 weeks (4 doses in total). After each injection rats were returned to their housing cages and were closely observed for potential side effects of chemotherapeutic agent.

Samples preparation

At the end of the experiment (2 weeks after the last injection) animals were sacrificed using intraperitoneal injection with lethal dose of pentobarbital (Morbital, Biowet, Puławy, Poland). The whole hearts were immediately removed, washed with saline, dried, frozen and stored at –86°C for future analyses.

Determination of BCKDH activity

All chemicals used for BCKDH activity determination were purchased from Sigma–Aldrich Chemical Company (Germany). E3 (recombinant human dihydrolipoamide dehydrogenase) was bought from Bio-Techne (USA). Extracts of tissues for determination of BCKDH activity were prepared as described previously [15]. 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 (KIV), a substrate for BCKDH complex (Cary 100 Bio UV-VIS spectrophotometer — Varian, Australia). 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 a partially active/dephosphorylated state (actual activity) or completely active/dephosphorylated state (total activity) were determined before and after incubation with lambda protein phosphatase (BioLabs, England), respectively. Percent of BCKDH complex in its 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.

RNA isolation and real-time PCR

Frozen heart muscle samples were ground into fine powder using a nitrogen-cooled mortar and pestle, homogenized in RNA Extracol (EURx, Poland) and total RNA was extracted according to the manufacturer's protocol. Concentration and the quality of RNA was measured using Cary 100 Bio UV-VIS spectrophotometer (Varian, Aus-

tralia). Reverse transcription reaction was performed with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The qPCR reaction was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, USA) and appropriate TaqMan probes (E1-Bckdha Rn01457724_m1; PPM1K Rn00667869_m1; BDK-Bckdk Rn00709396_g1). Expression of the reference gene, β-actin (Actb Rn01410038_m1), was quantified to control for variation in cDNA amounts. The real-time PCR (qPCR) tests were performed in a StepOnePlus Real-Time PCR System instrument (Thermo Fisher Scientific, USA). Approximately 25 ng of cDNA was used for each reaction and prepared in triplicate. The relative expression level of particular mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Statistical calculations and graphs were done using the GraphPad Prism version 5.0 (GraphPad Software, Inc., USA). Data are presented as mean ± SEM (from two independent experiments). Data were analyzed using Student's t-test for unpaired samples. Acceptable significance level was set at $p < 0.05$.

Results

BCKDH activity

Both actual and total BCKDH complex activities increased in response to 5-FU treatment (by 90% and 42%, respectively; $p < 0.001$ and $p < 0.01$) (Fig. 1A and 1B). Heart BCKDH activity state in rats exposed to 5-FU was 33% higher as compared to the control animals ($p < 0.01$) (Fig. 1C).
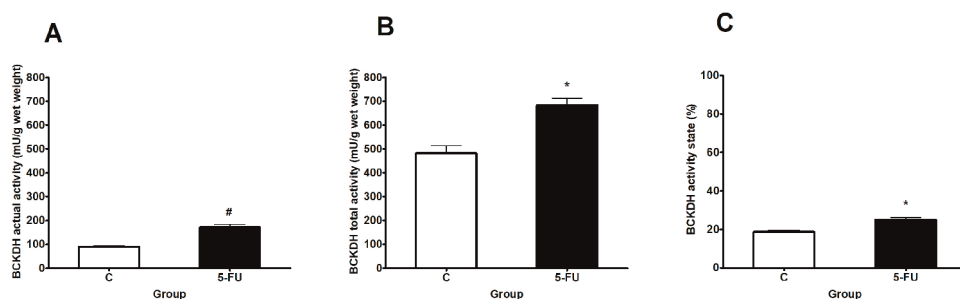

Fig. 1. Effect of 5-FU on BCKDH actual activity (1A), BCKDH total activity (1B) and BCKDH activity state (1C) in rat's heart.
BCKDH actual activity was determined before complete dephosphorylation of BCKDH complex with a lambda phosphatase. BCKDH total activity was determined after complete dephosphorylation of BCKDH complex with a lambda phosphatase. Activity state means percentage of the BCKDH complex in the active dephosphorylated form. Data are presented as mean ± SEM. 5-FU — study group (n = 5), C — control group (n = 5). *p <0.01; #p <0.001

BCKDH enzymes mRNA level

Both E1 subunit and BDK mRNA levels were lower in 5-FU administered rats than in the control group, by 55% and 56%, respectively (p <0.001 and p <0.01) (Fig. 2A and 2B). In contrast, PPM1K mRNA level increased by 86% in response to 5-FU (p <0.01) (Fig. 2C).
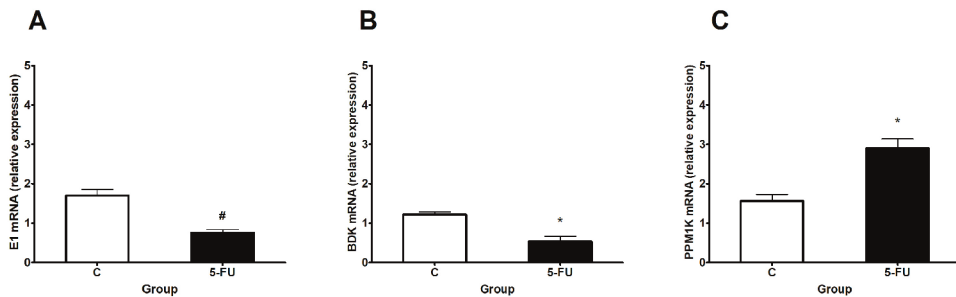

Fig. 2. Effect of 5-FU on mRNA levels for E1 (2A), BDK (2B) and PPM1K (2C) in rat's heart. The mRNA levels for E1, BDK and PPM1K were quantitated by qPCR then normalized to β-actin mRNA (in the same sample) and expressed relative to the control rats (relative expression). Data are presented as mean ± SEM. 5-FU — study group (n = 5), C — control group (n = 5). *p <0.01; #p <0.001

Discussion

Catabolism of BCAA-derived BCKA in particular tissues, including heart, is determined mainly by the BCKDH activity state reflecting phosphorylation status of BCKDH complex. We have found that 5-FU administration caused an increase in myocardial BCKDH activity state. Up-regulation of BCKDH by 5-FU appears to result mainly from increased dephosphorylation of the complex. The enhanced dephosphorylation of the BCKDH complex and its activation may be a consequence of increased action of PPM1K and/or diminished action of BDK, which in turn reflect the levels of expression and distinct regulatory properties of each of these two regulatory enzymes.

Stimulation of BCKDH activity could be potentially caused by a reduction of BDK expression and/or stimulation of PPM1K expression. We have demonstrated that in 5-FU treated rats mRNA level for BDK decreased, while mRNA level for PPM1K increased. Such effect of 5-FU treatment could be associated with changes in different factors regulating PPM1K and BDK expression. It seems likely that 5-FU increases BCKDH activity state (dephosphorylation) affecting adiponectin level. It has been demonstrated that 5-FU-based chemotherapy significantly increases plasma levels of adiponectin during treatment of some types of cancer [16]. Lian *et al.* [17] have found that in different tissues, including skeletal muscles, adiponectin increases PPM1K

expression and decreases BDK expression. In addition, adiponectin deficiency contributes to decreased BCKDH activity.

It appears likely that a decrease in BDK mRNA level and an increase in BCKDH activity state upon 5-FU treatment are associated with changes in insulin level. It has been found that insulin increases BDK mRNA and protein levels, and suppresses BCKDH activity [18]. Feng *et al.* [19] have demonstrated that 5-FU administration diminishes plasma insulin level.

The effect of 5-FU on BDK mRNA level may also be related to the acidification of cardiomyocytes. It has been found that 5-FU treatment may confer the risk of lactic acidosis [20–22]. Results of in vitro study performed by Wang and Price [23] have indicated that acidification decreases the amount of BDK mRNA in renal tubule cells by affecting its stability.

We have shown that E1 mRNA level decreased in 5-FU-treated rats. However, BCKDH total activity was higher than in the control group. Cook *et al.* [24] revealed that the affinity of E1 to other catalytic subunits of BCKDH complex is higher in a dephosphorylated form. 5-FU promoting dephosphorylation of BCKDH may stimulate E1 association with other catalytic subunits and the whole complex formation. This could explain an increase in BCKDH total activity in 5-FU-treated rats.

The increased BCKDH activity state would indicate a significantly stimulated catabolism of BCKA derived from BCAA. Recent studies have investigated the role of altered cardiac BCAA metabolism in cardiovascular disease models [13, 14]. It has been found that decreased BCAA catabolism resulting from downregulation of BCKDH complex contributes to the accumulation of BCKA and BCAA that leads to deterioration of cardiac functions [25]. For example, BCKA/BCAA accumulation can impair insulin signaling and contribute to cardiac insulin resistance [26]. On the other hand, pharmacological activation of BCAA catabolism improved cardiac contractility in heart failure patients [27].

In view of these findings 5-FU stimulation of BCKDH complex seems to be an adaptive reaction with the purpose of enhancing the BCKA oxidation and protecting from toxic effect caused by excessive accumulation of BCKA/BCAA in cardiac muscle. Further studies are needed to recognize the molecular mechanism and metabolic consequences of 5-FU effect on BCAA catabolism in cardiac muscle.

Conclusion

In this study, we demonstrated mRNA and post-translational changes in myocardial BCKDH complex in rats subjected to 5-FU treatment. Up-regulation of BCKDH appears to result mainly from changes in phosphorylation status of the complex (increased dephosphorylation) and is associated with an increase in PPM1K mRNA level and reduction of BDK and E1 mRNA levels.

Acknowledgments

Conflict of interest

The authors declare that they have no conflicts of interest.

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