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The influence of 5-fluorouracil on the α-ketoglutarate dehydrogenase complex in rat's cardiac muscle — a preliminary study

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Abstract: 5-fluorouracil (5-FU), which is a commonly used chemotherapy agent exerts undesired cardiac toxicity. Mitochondrial dysfunction is thought to be one of potentially important mechanisms of 5-FU-induced cardiotoxicity. α-ketoglutarate dehydrogenase (α-KGDHC) is the key regulatory enzyme of TCA cycle. The complex consists of multiple copies of three catalytic subunits: α-ketoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). α-KGDHC together with branched chain α-ketoacid dehydrogenase (BCKDHC) and pyruvate dehydrogenase (PDHC), are the members of 2-oxoacid dehydrogenases family that share some structural and functional similarities. Recently, it has been found that 5-FU stimulates BCKDHC in rat's cardiac muscle. Therefore, we hypothesize that 5-FU modifies α-KGDHC activity and affects cardiac muscle metabolism. The aim of this study was to determine the effect of 5-FU on α-KGDHC activity and protein levels of E1 and E2 subunits of the complex in rat's cardiac muscle. 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). α-KGDHC activity was assayed spectrophotometrically. The E1 and E2 proteins levels were quantified by Western blot. 5-FU administration resulted in stimulation of myocardial α-KGDHC activity in rats. In addition, E2 protein level increased in response to 5-FU treatment, while the E1 protein level remained unchanged. Up-regulation of α-KGDHC appears to result from change in E2 subunit protein level. However, the effect of 5-FU on factors modifying α-KGDHC activity at post-translational level cannot be excluded.

Keywords: 5-fluorouracil, α-ketoglutarate dehydrogenase, rats, cardiac muscle.

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Introduction

5-Fluorouracil (5-FU) and its prodrug capecitabine, are used widely in clinical practice as an anti-tumor treatment for cancers such as intestinal and liver cancers. Multiple studies have revealed that in addition to its beneficial anti-tumor effects, 5-FU also exerts undesired cardiac toxicity [1, 2]. Angina is one of the most common forms of cardiovascular toxicity induced by 5-FU. Others include myocardial infarction, arrhythmia, acute pulmonary edema, heart failure, and cardiac arrest and pericarditis [3].

The underlying mechanism of 5-FU cardiotoxicity still remains unclear. Several mechanisms have been proposed, including coronary artery vasospasm, direct toxicity to the myocardium, endothelial dysfunction, and a hypercoagulable state causing thrombosis [1, 4]. Mitochondrial damage is thought to be one of potentially important causes of 5-FU-induced cardiotoxicity. It has been found that 5-FU and/or its metabolites disturb mitochondria functions, induce mitochondrial swelling and decrease mitochondrial membrane potential leading to apoptosis of cardiomyocytes and cardiotoxicity [5–7]. In addition, some animal models have demonstrated specific metabolic changes in cardiomyocytes upon 5-FU treatment including increased oxygen consumption, depletion of high-energy phosphate compounds and impairment of mitochondrial energy metabolism. It has been shown that fluoroacetate, a catabolite of 5-FU inhibits aconitase, one of the enzymes of tricarboxylic acid cycle (TCA) [8].

Efficient energy transfer in cardiac muscle requires efficient TCA flux. The α-ketoglutarate dehydrogenase complex (α-KGDHC, known also as 2-oxoglutarate dehydrogenase complex; 2-OGDHC) represents a major control enzyme of TCA. Therefore, it plays the crucial role in overall cell metabolism. α-KGDHC together with pyruvate dehydrogenase complex (PDHC) and branched chain α-ketoacid dehydrogenase complex (BCKDHC) are members of the 2-oxoacid dehydrogenases family. These complexes have similar structures and catalyze the decarboxylation of their respective 2-ketoacids. Each complex comprises multiple copies of three different protein subunits: an α-keto acid specific dehydrogenase (E1), a less specific dihydrolipoamide acyltransferase (E2) and the common subunit dihydrolipoamide dehydrogenase (E3) [9].

The α-KGDHC catalyzes the oxidative decarboxylation of α-ketoglutarate (α-KG) into succinyl-CoA, generating NADH by the sequential operation of three enzymes: α-ketoglutarate dehydrogenase (α-KGDH; E1 subunit), dihydrolipoamide succinyltransferase (DLST; E2 subunit) and dihydrolipoamide dehydrogenase (DLD; E3 subunit), with the consecutive action of several cofactors, such as thiamine pyrophosphate (TPP), Mg^{2+}, lipoic acid and FAD^{+} [9, 10].

Recently it has been found that 5-FU treatment increases cardiac BCKDHC activity, a key enzyme of branched-chain amino acids metabolism [11]. We hypothesize

that 5-FU has an influence on α-KGDHC and thus, on cardiomyocyte mitochondrial metabolism. The current study was aimed at the investigation of the effect of 5-FU on α-KGDHC activity and protein levels of E1 and E2 subunits of the complex in rat's cardiac muscle.

Material and Methods

Experimental design

Adult male albino Wistar rats (n = 10) weighing 170–200 g (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 (CT; 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.

5-FU application

Before injection selected part of abdomen was gently shaved and disinfected. 5-FU (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 experiments (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 α-KGDHC activity

All chemicals used for α-KGDHC activity determination were purchased from Merck Life Science (Poland). Tissue extracts for the assay of cardiac α-KGDHC were prepared as described previously [12]. Briefly, a portion of frozen cardiac muscle was

powdered in liquid nitrogen, weighed and homogenized in an extraction buffer. α-KGDHC was then concentrated from the whole tissue extracts by precipitation with 9% polyethylene glycol. α-KGDHC activity was determined spectrophotometrically at 30°C by measuring the rate of NADH generation from NAD^+ (340 nm) using the saturating concentration of α-KG, a substrate for α-KGDHC (UV-VIS Cary 100 spectrophotometer Varian Medical Systems, Australia). One unit of α-KGDHC activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH/min.

Western blot analysis

Frozen cardiac muscle samples were ground into a fine powder using a nitrogen-cooled mortar and pestle and homogenized using manual homogenizers in ice-cold buffer containing 20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Homogenates were incubated on ice for 1 h under gentle agitation and then centrifuged for 15 min at 15000 rpm. The supernatants were transferred into fresh tubes. The protein concentration was measured using UV-VIS Cary 100 spectrophotometer (Varian Medical Systems, Australia) and Total Protein Kit (BioMaxima, Poland). Equal amounts of protein (30 μg total protein) with addition of 2.5% 2-mercaptoethanol were loaded into 12% SDS-polyacrylamide gels and separated. After electrophoresis, proteins were transferred into nitrocellulose membranes (Amersham Hybond™, GE™ Healthcare, Pittsburgh, PA, USA), at a constant voltage (25 V) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, with 20% methanol). Blots were washed with TBST (150 mM NaCl, 50 mM Tris, 0.05% Tween 20), blocked with 5% skimmed milk in TBST and incubated overnight at 4°C with the appropriate primary antibodies against E1 α-KGDHC (cat. #PA5-88571, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), E2 (cat. CSB-PA861526LA01HU, Cusabio Technology LLC, Houston, TX, USA), and β-actin (cat. #PA1-183, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as reference protein. After incubation with primary antibodies, membranes were washed with TBST and incubated with secondary antibodies, goat anti-rabbit IgG-H&L (HRP) (cat. ab6721, Abcam, UK). The protein bands were visualized using an enhanced chemiluminescence method (Clarity Western ECL Substrate BioRad, CA, USA) with GeneSys G:Box Chemi-XR5 (Syngene, UK). The values of optical density determined for E1 and E2 subunits were normalized to β-actin (reference protein).

Statistics

Statistical calculations and graphs were done using the GraphPad Prism version 5.0 (GraphPad Software, Inc., USA). Data are presented as mean ± SD (from two independent experiments). Statistical differences were analyzed using Student's t-test (α-KGDHC activity) or Mann–Whitney test (E1 and E2 protein levels). Acceptable significance level was set at $p < 0.05$.

Results

α-KGDHC activity increased by 42% upon 5-FU treatment ($p < 0.0001$) (Fig. 1). E1 subunit protein level of α-KGDHC was 4% lower, while E2 protein level was 30% higher in 5-FU administered rats in comparison with the control group (Fig. 2 and Fig.3, respectively). The difference was statistically significant only for E2 subunit ($p < 0.05$).

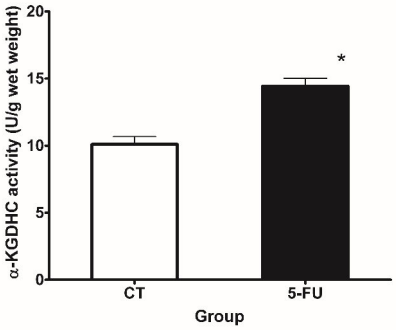

Fig. 1. Effect of 5-FU on α-KGDHC activity in rat's cardiac muscle. Data are presented as mean ± SD. CT — control group (n = 5), 5-FU — 5-FU-treated group (n = 5). *p <0.0001.

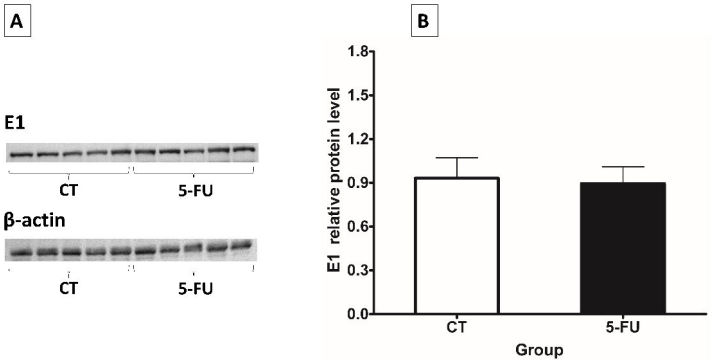

Fig. 2. Effect of 5-FU on E1 subunit protein level in rat's cardiac muscle. The protein level was analyzed by Western blot and expressed as relative E1 protein level. Data are presented as mean ± SD. CT — control group (n = 5), 5-FU — 5-FU-treated group (n = 5). **A**: representative immunoblot; **B**: bar graph displaying the mean values ± SD.

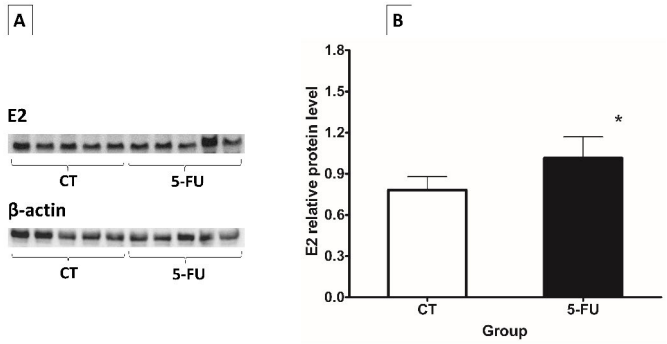

Fig. 3. Effect of 5-FU on E2 subunit protein level in rat's cardiac muscle. The protein level was analyzed by Western blot and expressed as relative E2 protein level. Data are presented as mean ± SD. CT — control group (n = 5), 5-FU — 5-FU-treated group (n = 5). **A**: representative immunoblot; **B**: bar graph displaying the mean values ± SD. *p <0.05.

Discussion

We have found that 5-FU administration results in stimulation of myocardial α-KGDHC activity in rats. In addition, E2 protein level increases in response to 5-FU treatment while the E1 protein level remains unchanged.

α-KGDHC exists in mitochondria as a polymeric structure that comprises an E2 core, to which are attached E1 and E3 homodimers [9, 10]. Although E2 is the structural core of the α-KGDHC, the first and rate-limiting reaction is catalyzed by E1 component of the complex. Therefore, we suspect that stimulation of α-KGDHC by 5-FU resulted not solely from the increase in E2 protein level but also from up-regulation of α-KGDHC activity by factors acting at post-translational level.

Control of α-KGDHC activity is achieved mainly via allosteric interactions. ADP, Pi, and Ca^{2+} are positive effectors increasing the affinity of the E1 subunit for its substrate (α-KG), whereas ATP, NADH and succinyl-CoA are inhibitors of α-KGDHC [13]. It has been demonstrated that administration of 5-FU triggers an increase in ADP, AMP and Pi levels [14]. Moreover, the intracellular free Ca^{2+} concentration increases with 5-FU treatment [15]. Furthermore, 5-FU appears to decrease peripheral thiamine levels by increasing cellular accumulation of this compound and promoting thiamine conversion to its active form (TPP) which is a cofactor for E1 subunit of α-KGDHC [16].

It has been found that 5-FU decreases the level of mitochondrial ATP in guinea pig's cardiac muscle [14]. Moreover, 5-FU administration reduces mitochondrial fatty acid oxidation [17]. Fatty acids are the main energy substrates for cardiomyocytes that provide the majority of NADH necessary for mitochondrial oxidative phosphorylation. Therefore, it is conceivable that up-regulation of α-KGDHC by 5-FU is associated with an increase in E2 protein amount as well as with increasing the levels of

positive allosteric effectors and decreasing the levels of inhibitory allosteric effectors. The effect of 5-FU on α-KGDHC activity may also be associated with the acidification of cardiomyocytes. Lowering mitochondrial pH stimulates α-KGDHC [18]. It has been revealed that 5-FU treatment is associated with accumulation of lactate in myocardium [14] and confer the risk of lactic acidosis [19, 20].

Up-regulation of the α-KGDHC activity in response to different stressors has been demonstrated in numerous *in vivo* systems. Graf *et al.* [21] have shown that metabolic stress imposed by different toxicants, for example ethanol or $MnCl_2$, induces up-regulation of α-KGDHC in heart and brain. They suggest that the increase of α-KGDHC activity may be one of the mechanisms to alleviate general perturbation of cellular homeostasis occurring in response to metabolic stress. In view of Graf *et al.* [21] findings, it cannot be excluded that α-KGDHC up-regulation by 5-FU is a part of an adaptation mechanism that is aimed at the prevention of disturbances of mitochondrial function. However, it should be emphasized that with prolonged exposition to the toxicants the adaptive mechanism may become ineffective.

Some experimental models revealed increased levels of reactive oxygen species (ROS) such as superoxide anions and depletion of antioxidants in rat's cardiomyocytes after the treatment with 5-FU [3, 22]. Oxidative stress induced by 5-FU results in oxidation of proteins, lipids and other macromolecules, and leads to disturbed cellular function. It has been found that in cardiac and liver tissues α-KGDHC is a major source of mitochondrial ROS [23, 24]. Hence, the up-regulation of α-KGDHC could result in promotion of oxidative stress and cardiomyocyte dysfunction.

α-KGDHC activity is one of the factors determining intracellular α-KG level [25]. Recent studies have indicated the role of α-KG as a signaling molecule that control cell growth and functions [26, 27]. For example, α-KG is an obligatory co-substrate for 2-oxoglutarate-dependent dioxygenases, a group of enzymes that play the key role in physiologically important processes, such as response to hypoxia and chromatin modifications [28]. Therefore, up-regulation of α-KGDHC activity may result in reduced α-KG level and therefore, affect cardiomyocyte metabolism.

Our study has some limitations. Because we intended to perform a preliminary study we used a small number of rats and did not evaluate the metabolic consequences of increased α-KGDHC activity. We plan to perform further studies to fully elucidate the effect of 5-FU administration on α-KGDHC and cardiomiocyte metabolism.

Conclusion

In this study, we have demonstrated an increase in α-KGDHC activity in cardiac muscle of rats subjected to 5-FU treatment. Up-regulation of α-KGDHC appears to be associated with the increase in E2 subunit protein level. However, the effect of 5-FU on factors modifying α-KGDHC activity at post-translational level cannot be excluded.

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Conflict of interest

None declared.

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