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Selective inhibition of OCTN2 is more effective than inhibition of gamma-butyrobetaine dioxygenase to decrease the availability of L-carnitine and to reduce myocardial infarct size

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ABSTRACT

L-Carnitine is a cofactor in the energy metabolism pathways where it drives the uptake and oxidation of long chain fatty acids (LCFA) by mitochondria. LCFA lipotoxicity causes mitochondrial damage and results in an insufficient energy supply and a decrease in L-carnitine content limits LCFA flux and protects mitochondria. Here, we tested whether the inhibition of GBB dioxygenase (BBOX) or organic cation transporter 2 (OCTN2) is the most effective strategy to decrease L-carnitine content.

The activity of 51 compounds was tested and we identified selective inhibitors of OCTN2. In contrast to selective inhibitors of BBOX, OCTN2 inhibitors induced a 10-fold decrease in L-carnitine content in the heart tissues and a significant 35% reduction of myocardial infarct size. In addition, OCTN2 inhibition correlated with the inhibitor content in the heart tissues, and OCTN2 could potentially be an efficient target to increase drug transport into tissues and to reduce drug elimination by urine.

In conclusion, the results of this study confirm that selective inhibition of OCTN2, compared to selective inhibition of BBOX, is a far more effective approach to decrease L-carnitine content and to induce cardioprotective effects. OCTN2 could potentially be an efficient tool to increase drug transport in tissues and to reduce drug elimination via urine.

1. Introduction

L-Carnitine is a cofactor in energy metabolism pathways where it drives carnitine palmitoyltransferase I (CPT I), which is the rate-limiting step in the uptake and oxidation of long chain fatty acids (LCFA) by mitochondria [1,2]. Acute or chronic overload of LCFA, in particular acyl-carnitines, induces disturbances in cell functions; LCFA lipotoxicity leads to mitochondrial damage, an insufficient energy supply, cell apoptosis and necrosis [3,4]. Decrease in L-carnitine content limits acyl-carnitines flux into the mitochondria [5,6], and the inhibition or redirection of acyl-carnitines flux protects mitochondria against LCFA overload-induced damage [7]. Management of heart ischemia represents an important clinical problem and, therefore, novel pharmacological approaches are under intensive investigation [8–11]. Currently, the only pharmacological agent in clinical use to decrease L-carnitine concentrations is meldonium (3-(2,2,2-trimethylhydrazinium)-propionate, PubChem CID 123868) [12,13]. More potent compounds that lower L-carnitine may present a novel class of drugs for the treatment of heart diseases, diabetes and atherosclerosis.

Convenient strategy to target L-carnitine content in tissues is the inhibition of L-carnitine biosynthesis [14,15]. L-Carnitine is synthesized from gamma-butyrobetaine (GBB) in the liver and kidneys by GBB dioxygenase (BBOX) [16]. BBOX catalyses the last step of L-carnitine biosynthesis from the amino acids lysine and methionine. Only a minor fraction of the total L-carnitine content is biosynthesized in the liver (rodents) or the kidneys (humans). The main

Abbreviations: AN, area of necrosis; AR, area at risk; BBOX, GBB dioxygenase; CPT I, carnitine palmitoyltransferase I; FBS, fetal bovine serum; HEK293, human embryonic kidney; KH, Krebs–Henseleit; LAD, left anterior descending coronary artery; LCFA, long chain fatty acids; LVDP, left ventricular developed pressure; OCTN2, organic cation transporter 2; PBS, phosphate buffered saline.

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source of L-carnitine is the dietary intake of meat and dairy products. Normally, in non-vegetarians, the intake of L-carnitine rich meat exceeds the amount of L-carnitine eliminated via the urine [12]. The L-carnitine concentration in tissues is generally 10–50-fold higher than the concentration in blood plasma [17]. Therefore, as only the kidneys and liver are able to synthesize considerable amounts of L-carnitine, other tissues depend on carnitine uptake from the blood via active transport.

Organic cation transporter 2 (OCTN2) is the most important L-carnitine transporter and mediates L-carnitine uptake in the intestines, transport in tissues and reabsorbtion in the kidneys [18–20]. Thus, in addition to the inhibition of L-carnitine biosynthesis, the inhibition of L-carnitine transport by OCTN2 could potentially serve as an effective strategy for the urinary elimination of L-carnitine. Several studies have confirmed that the inhibition of OCTN2 is an effective mechanism to decrease L-carnitine content [12,21,22]. In addition, there is evidence that GBB is transported by OCTN2 [21,23]. The cardioprotective drug meldonium inhibits both L-carnitine biosynthesis (BBOX IC_{50} 26 μM) and transport (OCTN2 IC_{50} 62 μM) [24]. A recent medicinal chemistry study disclosed novel potent inhibitors of BBOX [15]. To validate the importance of BBOX and OCTN2 as molecular targets to decrease the L-carnitine content, selective inhibitors of BBOX and OCTN2 are required.

To determine whether the inhibition of BBOX or OCTN2 is the most effective strategy to decrease L-carnitine content and protect the heart against acute ischemia–reperfusion injury, we compared the ability of selective inhibitors of BBOX or OCTN2 to decrease L-carnitine content in blood plasma and heart tissues in the present study. In addition, the cardioprotective properties of the most potent selected compounds were evaluated in ex vivo isolated rat hearts.

2. Materials and methods

2.1. Animals and treatment

Eighty male Wistar rats weighing 250–300 g were housed under standard conditions (21–23 °C, 12 h light–dark cycle) with unlimited access to food (R70 diet, Lantmännen, Sweden) and water. The experimental procedures were carried out in accordance with the guidelines of the European Community and the local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia.

Synthesis of all compounds was performed according to methods described previously [15].

The rats were adapted to local conditions for 2 weeks before the start of treatment. In order to compare the effects of selected inhibitors on L-carnitine content in this study we administrated compounds perorally at a dose of 20 mg/kg. Single administration and long-term administration (14 days) of compounds were performed. A pilot experiment was undertaken to find the optimal dose. After the administration of a dose of 20 mg/kg, the blood plasma and tissue concentrations of inhibitors reached values which are sufficient to inhibit BBOX or OCTN2. Control rats received water. The rats were used in the experiment 24 h after the last administration.

2.2. The inhibition of OCTN2-mediated transport of L-carnitine

The transport of L-carnitine was measured as L-[N-methyl-^{3}H] carnitine hydrochloride (specific activity, 85 Ci/mmol, Biotrend, USA) uptake by human embryonic kidney (HEK293, ATCC collection code CRL-1573) cells. HEK293 cells used for the study express human organic cation transporter 2 and do not synthesize L-carnitine. The cells were cultured in 24-well plates in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, Germany) until approximately 90% confluence was reached. Prior the assay, the cells were washed twice with 0.5 ml of DMEM/F-12 without FBS; the assay was performed in 300 μl of medium. The cells were pre-incubated with the tested compounds (10 or 100 μM) for 15 min at 37 °C. The uptake was initiated by the simultaneous addition of unlabeled L-carnitine (10 μM) and L-[N-methyl-^{3}H]-carnitine (4 nM, 12 kBq/ml). After incubation for 60 min at 37 °C, the medium was removed and the cells were washed 3 times with 300 μl of ice-cold PBS. The cells were then lysed directly in the plate with 100 μl 0.1% SDS in 1 M NaOH. A 200-μl aliquot of the scintillation cocktail was added to 50 μl of the cell lysate, and the radioactivity was measured using Wallac MicroBeta Trilux scintillation counter (PerkinElmer, Inc., Waltham, USA). The data were normalized to the protein content (determined using the Lowry method). The control measurement of L-carnitine uptake in the absence of inhibitor was taken as 100%. The tested compounds that decreased L-carnitine transport by at least 50% at the 100 μM concentration compared to the control were tested further to determine the IC_{50} value. The measurements were performed in triplicate for each concentration of the tested compounds. The IC_{50} value was calculated using GraphPad Prism 3.0 software.

2.3. Measurement of levels of L-carnitine by UPLC/MS/MS

Determination of L-carnitine amounts in heart tissues, plasma and urine samples were performed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) using the positive ion electrospray mode as described previously [25].

2.4. Isolated rat heart infarction study

The infarction was performed according to the Langendorff technique as described previously [5], with some modifications. Twenty-four hours after the last drug administration, rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). For the infarction studies the hearts were perfused with Krebs–Henseleit (KH) buffer solution at a constant perfusion pressure of 60 mmHg. The isolated rat hearts were adapted for 20 min, and the left anterior descending coronary artery (LAD) was subsequently occluded for 40 min followed by 120 min of reperfusion. Infarct size was determined as described previously [26]. Briefly, at the end of reperfusion LAD was re-occluded and heart was perfused with 0.1% methylene blue dissolved in KH buffer solution. Afterwards the ventricles of the heart were transversely cut into 2 mm thick slices and photographed. Computerized planemetric analysis of stained left-ventricle slice photographs was performed using Image-Pro Plus v6.3 software to determine the area at risk (AR) and the area of necrosis (AN), and each area was expressed as a percentage of the left ventricle area. The obtained values were then used to calculate the infarct size (IS) as a percentage of the risk area, according to the formula $IS = AN/AR \times 100\%$.

2.5. Statistical methods

The data are presented as the mean ± S.E.M. Statistically significant differences in the mean values were evaluated using a one-way ANOVA with Tukey's test. Pearson's correlation analysis was used to examine the relationship between the OCTN2 inhibitor IC_{50} value and the L-carnitine content in heart tissues. The differences were considered significant when $p < 0.05$. The data were analyzed using Graph Pad Prism 3 statistical software (Graph Pad, Inc., USA).