BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2017, **121**, 106–112

Doi: 10.1111/bcpt.12775

Decrease in Long-Chain Acylcarnitine Tissue Content Determines the Duration of and Correlates with the Cardioprotective Effect of Methyl-GBB

Janis Kuka[1], Marina Makrecka-Kuka[1], Helena Cirule[1], Solveiga Grinberga[1], Eduards Sevostjanovs[1], Maija Dambrova[1,2] and Edgars Liepinsh[1]

[1]Laboratory of Pharmaceutical Pharmacology, Latvian Institute of Organic Synthesis, Riga, Latvia and [2]Faculty of Pharmacy, Riga Stradins University, Riga, Latvia

(Received 25 November 2016; Accepted 22 February 2017)

Abstract: Ischaemia in the heart is accompanied by the accumulation of long-chain acylcarnitines (LCACs) which is one of the multiple factors that contribute to the ischaemia–reperfusion damage development. Long-term pre-treatment that decreases carnitine and LCAC contents also reduces ischaemia–reperfusion (IR) damage; however, the duration of the post-treatment effects is not known. The aim of the study was to assess the post-treatment effects of the carnitine transport (OCTN2) inhibitor, methyl-GBB, on LCAC content and the duration of its cardioprotective effect. Male Wistar rats received methyl-GBB (5 mg/kg for 28 days), and the anti-infarction effects on Langendorff-perfused hearts and the acylcarnitine profile in cardiac tissues were measured up to 28 days following the end of the treatment. Methyl-GBB pre-treatment for 28 days decreased LCAC heart tissue content by 87%, and the infarct size was decreased by 57%. Fourteen days post-treatment, the LCAC content was still decreased by 69%, and the infarct size was decreased by 32% compared to Control. A significant Pearson correlation ($r = 0.48$, $p = 0.026$) was found between infarct size and LCAC tissue content in the methyl-GBB-treated rat hearts. The addition of 2 mM carnitine to isolated heart perfusate significantly diminished the methyl-GBB-induced decrease in LCACs and infarct size. In conclusion, the anti-infarction effect of methyl-GBB continues for at least 2 weeks post-treatment. No less than a 70% decrease in LCAC content is required to protect ischaemic heart tissues, and the decrease in LCAC levels defines the duration of the post-treatment cardioprotective effect of the OCTN2 inhibitor, methyl-GBB.

Ischaemic conditions in the heart are accompanied by impaired mitochondrial oxidative metabolism [1] and the accumulation of non-metabolized fatty acid (FA) metabolism intermediates [2,3]. In particular, the accumulation of long-chain acylcarnitines (LCACs; esters of FAs and carnitine) further inhibits oxidative phosphorylation [4], impairs cell insulin signalling [5,6], modulates cell membrane ion channels, increases membrane permeability and activates cell stress pathways [7]. LCAC content can be effectively decreased using compounds that decrease the availability of carnitine, which is required for LCAC synthesis by CPT-1 [8,9], and inhibitors of the carnitine transporter, OCTN2, are far more effective in decreasing carnitine content than are inhibitors of γ-butyrobetaine dioxygenase [10], which is the final enzyme in the carnitine biosynthesis pathway. Methyl-GBB (4-[ethyl (dimethyl)ammonio]butanoate) is the most known potent compound to decrease the heart and muscle tissue content of carnitine and LCACs; this compound possesses cardioprotective properties without changing heart function [8,9,11] and improves insulin sensitivity in db/db mice with type 2 diabetes [12]. By decreasing carnitine-dependent long-chain fatty acid (LCFA) oxidation and LCAC content, methyl-GBB stimulates basal and insulin-stimulated glucose oxidation in the normoxic heart [8].

Similar to methyl-GBB in its mechanism of action, particularly regarding the inhibition of OCTN2, is the clinically used cardioprotective compound meldonium [13], which recently attracted increased attention from the World Anti-Doping Agency due to numerous findings of meldonium in athletes' blood and urine [14–16]. One finding of particular interest is that meldonium is found in blood samples in significant concentrations even 16 days after the administration of a single dose [16]. The authors assumed that meldonium is taken up by erythrocytes, whereas our findings have well documented that meldonium and methyl-GBB are both taken up by all tested tissues, creating significant depots of either of these substances [8,9,17] that will slowly release the compounds after the treatment has ended. Indeed, even 4 weeks after the end of treatment, plasma concentrations of meldonium in healthy volunteers are approximately 1 μM (approximately 20 times lower than the concentrations found during the treatment) [18]. It is known that meldonium competes with carnitine for transport via OCTN2 [19,20], and meat consumption (a natural source of dietary carnitine) is correlated with the carnitine-decreasing effect of meldonium [21], whereas co-treatment with carnitine can completely remove the cardioprotective effect of meldonium [22]. Methyl-GBB is a significantly more potent inhibitor of OCTN2-mediated

Author for correspondence: Janis Kuka, Laboratory of Pharmaceutical Pharmacology, Latvian Institute of Organic Synthesis, Aizkraukles 21, Riga, LV-1006, Latvia (e-mail janis.kuka@farm.osi.lv).

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

carnitine transport than meldonium and is well transported into tissues (OCTN2 IC_{50} is 3 μM for methyl-GBB [8] *versus* 21 μM for meldonium [20]), and it can be assumed that methyl-GBB might also be retained in significant amounts in tissues post-treatment, thus providing prolonged biochemical consequences and anti-infarction effect after the end of treatment.

To determine the duration of the cardioprotective effect of methyl-GBB after the end of the compound administration, we treated rats for 28 days and assessed the myocardial infarct size immediately after the end of treatment until no cardioprotection was observed. In parallel, tissue concentrations of methyl-GBB, carnitine, its precursor GBB and the FA metabolites acylcarnitines were measured to better understand the pharmacokinetics of the strong OCTN2 inhibitor and to assess the role of various acylcarnitine, particularly LCAC, levels on the outcome of ischaemia–reperfusion damage. Previously, it has been shown that the acute addition of carnitine to the perfusion solution can worsen the outcome after global ischaemia by impairing glucose metabolism [23]. Because LCAC can effectively impair glucose metabolism [24], we studied the effects of methyl-GBB on changes in the LCAC content and cardioprotective efficacy after acute carnitine addition to the isolated heart perfusion solution.

Materials and Methods

Materials. Methyl-GBB was obtained from JSC Grindeks (Riga, Latvia). Krebs–Henseleit buffer components potassium dihydrogenphosphate, sodium chloride, calcium chloride dihydrate, potassium chloride, sodium bicarbonate and magnesium chloride hexahydrate were purchased from Acros Organics (Geel, Belgium). Ethylenediamine-tetraacetate sodium salt (EDTA) and triphenyltetrazolium chloride were purchased from Sigma-Aldrich (Schnelldorf, Germany). Sodium pentobarbital (Dorminal) solution was purchased from Alfasan (Woerden, Holland). Heparin sodium was purchased from Panpharma (Fougeres, France).

Animals and treatment. Male Wistar rats (n = 86) weighing 200–250 g (7–8 weeks old) were obtained from the Laboratory of Experimental Animals, Riga Stradins University (Riga, Latvia) and housed for 2 weeks prior to treatment under standard conditions (21–23°C, 12-hr light/dark cycle, relative humidity 45–65%) with unlimited access to food [R70 diet from Lantmännen, (Stockholm, Sweden)] and water. The experimental procedures were performed in accordance with the guidelines of the European Community and local laws and policies, and all of the procedures were approved by the Food and Veterinary Service, Riga, Latvia. All experiments were performed in a blinded manner. Studies involving animals are reported in accordance with the ARRIVE guidelines [25,26]. All experimental groups and animals per group are shown in Table S1. To determine post-treatment effects, animals were randomly separated into two experimental groups and given daily oral doses of water (Control group, n = 18) or 5 mg/kg methyl-GBB (n = 24) for 28 days. Methyl-GBB treated rats and age-matched Control group rats were used for myocardial infarction study right after the 28-day treatment and 14 and 28 days post-treatment. Additional infarction experiment [Control group n = 8, methyl-GBB treated n = 14 (n = 7 methyl-GBB and n = 7 methyl-GBB+2 mM carnitine)] was performed when 2 mM carnitine was added to the perfusion solution of methyl-GBB-treated rat isolated hearts. To determine changes in acylcarnitine profile when 2 mM carnitine is added to perfusion solution of methyl-GBB treated rat hearts, another experiment was performed where hearts were sectioned immediately after ischaemia and acylcarnitines measured in risk area [Control group, methyl-GBB treated group and methyl-GBB treated + 2 mM carnitine group (n = 6 per each group)]. Four rats were used to determine methyl-GBB plasma half-life after per oral administration of methyl-GBB at a single dose of 5 mg/kg. Non-fasting blood samples were collected from tail veins; samples were centrifuged, and the plasma was stored at −80°C for analysis at a later time.

Isolated rat heart infarction study. To determine the duration of anti-infarction effect of methyl-GBB, the first experiment was performed using hearts from methyl-GBB-treated animals immediately after 28-day treatment and 14 and 28 days post-treatment with the compound. For each time-point, data from methyl-GBB-treated rats were compared with data from age-matched control animals. The infarction was performed according to the Langendorff technique as described previously [22], with some modifications. 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 30 min. followed by 120 min. of reperfusion. Infarct size was determined as described previously [27]. 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, treated with triphenyltetrazolium chloride (TTC) and photographed. Unsliced part of the heart was not treated with TTC, but immediately frozen until further analysis of acylcarnitines, carnitine, GBB and methyl-GBB. Computerized planemetric analysis of stained left ventricle slice photographs was performed using Image-Pro Plus v6.3 software (Media Cybernetics Inc., Rockville, MD, USA) 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 $IS = AN/AR \times 100\%$. Two additional infarction experiments were performed to determine effects of acute carnitine addition to the perfusion solution on infarct size and LCAC content in methyl-GBB-treated rat hearts. For experiments with 2 mM carnitine in the perfusion solution, adaptation time was increased to 30 min. to allow carnitine to enter tissues. During ischaemia and reperfusion, hearts were perfused with normal KH perfusion solution. In one experiment, infarct size was determined as described above, while in the second experiment the contents of acylcarnitines were measured in tissues from risk area of hearts immediately after ischaemia without reperfusion.

Measurement of carnitine, GBB, methyl-GBB and acylcarnitines by UPLC/MS/MS. Determination of carnitine, GBB, methyl-GBB in heart tissues and plasma samples was performed by ultraperformance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS) using the positive ion electrospray mode [8]. Determination of the acylcarnitines in the heart tissue and plasma samples was performed by UPLC MS/MS method [24] and samples prepared as previously described [8].

Statistical methods. Data are presented as the mean ± S.E.M. Statistically significant differences in the mean values were evaluated using Student's t-test and one-way ANOVA followed by Tukey's test. Student's t-test was used to compare results of methyl-GBB treated group to respective Control group at the same time-point. One-way ANOVA followed by Tukey's test was used to compare methyl-GBB tissue concentration changes at different post-treatment time-points. One-way ANOVA followed by Tukey's test was also used to compare

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

anti-infarction effect of methyl-GBB in the presence or absence of carnitine in the perfusion solution. Correlation was tested using the Pearson method. The differences were considered significant when $p < 0.05$. The data were analysed using GraphPad Prism statistical software (Graph Pad Inc., La Jolla, CA, USA).

Results

Isolated rat heart infarction.

A heart infarction study was performed after 4 weeks of treatment with methyl-GBB at a dose of 5 mg/kg and also at 14 and 28 days after the last administration of the drug to determine the time at which the cardioprotective effect ends. Treatment with methyl-GBB significantly decreased (by 57%) myocardial infarct size after 28 days of treatment compared to the Control group. Significantly decreased myocardial infarct size (by 32%) was also observed 14 days after the end of methyl-GBB administration; however, 28 days after the end of the treatment, no significant cardioprotection was observed (fig. 1A). A significant Pearson correlation was observed between infarct size and tissue content sum of LCAC ($r = 0.48$, $p = 0.026$), sum of medium-chain acylcarnitines (MCAC) ($r = 0.47$, $p = 0.029$), sum of short-chain acylcarnitines (SCAC) ($r = 0.63$, $p = 0.0021$) and carnitine ($r = 0.66$, $p = 0.001$). Contents of individual acylcarnitines C2, C14, C16, C18 and C18:2 correlated with the infarct size, while no significant correlation was for tissue contents of individual acylcarnitines C4, C6, C8, C10 and C12 (figs 2A and 2B, Table S2).

An additional experiment in which 2 mM carnitine was added to the perfusion buffer was performed to test the role of free carnitine and LCAC decrease on the cardioprotective effect of methyl-GBB. In this experiment, methyl-GBB administration resulted in significantly decreased infarct size, whereas the addition of carnitine to the perfusion solution diminished the cardioprotective effect of methyl-GBB (fig. 1B).

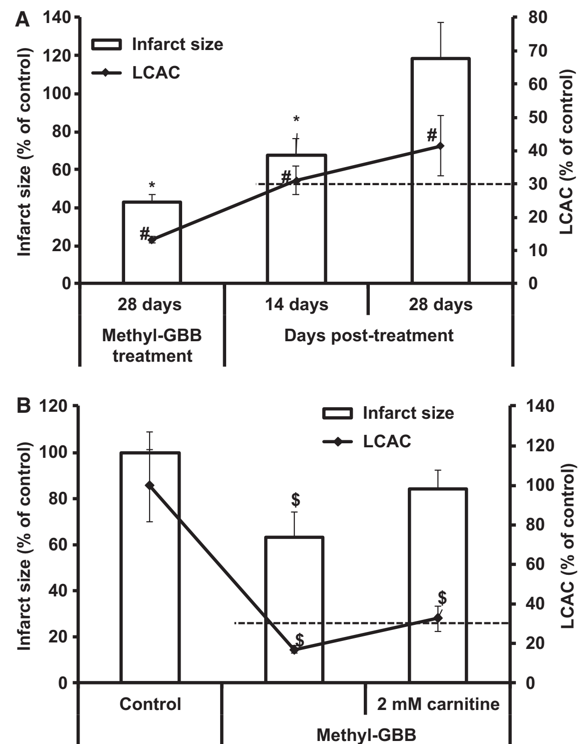

Fig. 1. Per cent values of infarct size and corresponding per cent values of LCACs found in ischaemic tissues. For Panel A, infarct size and LCAC values represent the mean ± S.E.M. of seven hearts in methyl-GBB-treated groups and are calculated as the percentage of the average respective age-matched Control group values. For panel B, infarct size and LCAC values represent the mean ± S.E.M. of six hearts in Control and methyl-GBB groups and seven hearts in methyl-GBB+2 mM carnitine group and are calculated as the percentage of the average respective Control group values. $^{*}p < 0.05$ infarct size in methyl-GBB group *versus* respective age-matched control (Student's t-test), $^{\#}p < 0.05$ LCAC level in methyl-GBB group *versus* respective age-matched control (Student's t-test), $^{\$}p < 0.05$ significantly different from respective Control group (ANOVA followed by Tukey's test). Dashed line represents 30% of LCAC remaining (70% decrease in LCAC content).

Plasma concentration of methyl-GBB and heart tissue concentrations of methyl-GBB, GBB and carnitine.

Methyl-GBB concentration in plasma reached 24 ± 1.8 μM at the end of the 28-day treatment was significantly lower 14 days post-treatment (14.8 ± 1.3 μM), and at 28 days after the last administration of the drug, the concentration of methyl-GBB was 10.1 ± 0.5 μM. The elimination of methyl-GBB from plasma followed the one-compartment model (Figure S1A and B). The plasma half-life of methyl-GBB, as determined by the single dose (5 mg/kg) administration, was found to be 27 hr; the C_{max} of methyl-GBB in the plasma was reached in 4.4 hr. The elimination rate constants after acute and chronic administration were 0.894 and 0.0304 $days^{-1}$, respectively, indicating that plasma clearance is approximately 29 times faster than tissue clearance. The plasma half-life of methyl-GBB after chronic administration largely reflected elimination from all tissues and was determined to be 32.9 days (Figure S1B).

The average content of carnitine in the heart was 703 ± 55 nmol/g tissue (622–732 nmol/g tissue) in the Control group. Treatment with methyl-GBB for 28 days decreased the heart tissue content of carnitine by an average of 94% (table 1). Carnitine content in the heart tissues gradually recovered over time but was still decreased by 54% 28 days after the last administration of methyl-GBB. The content of methyl-GBB in the heart tissues after 28 days of treatment was 217 nmol/g tissue. After the last administration, the content of methyl-GBB started to decrease; however, even after 28 days, 114 nmol/g tissue or 53% of the maximal content of methyl-GBB remained present in the heart tissues (table 1); methyl-GBB elimination from the heart tissue was linear (Figure S2). The content of GBB after 28 days of treatment with methyl-GBB was increased two times (table 1) and gradually decreased over the following 28 days.

The acute addition of 2 mM carnitine to the isolated heart perfusion solution had no significant effect on carnitine levels in methyl-GBB-treated hearts (117 ± 16 nmol/g tissue in the methyl-GBB group *versus* 88 ± 11 nmol/g tissue in the

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

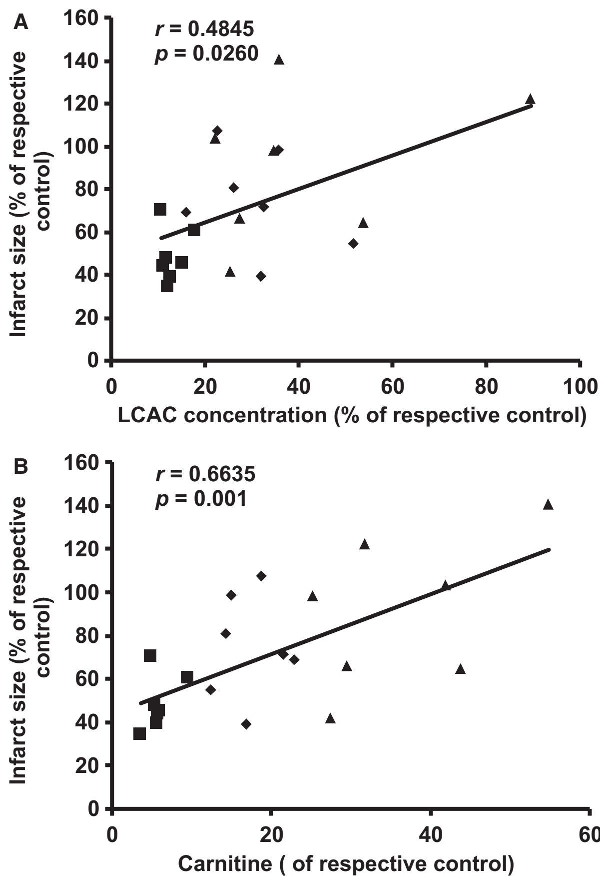

Fig. 2. Pearson correlation of infarct size and LCAC (A) and carnitine (B) contents in methyl-GBB-treated rat hearts. Each % value was calculated as the percentage of the average respective age-matched Control group values. Square markers indicate 28-day treatment, rhomb markers indicate 14 days post-treatment and triangle markers indicate 28 days post-treatment.

Table 1.
The effect of methyl-GBB administration on carnitine, methyl-GBB and GBB concentrations in heart tissues after 28 days of treatment and at 14 and 28 days after the last day of treatment.

	28 days treatment	14 days post-treatment	28 days post-treatment
Carnitine, nmol/g			
Control	622 ± 66	756 ± 39	732 ± 60
Methyl-GBB	36 ± 4*	129 ± 10*	337 ± 75*
Methyl-GBB, nmol/g			
Control	nd	nd	nd
Methyl-GBB	217 ± 9	157 ± 10**	114 ± 6**,***
GBB, nmol/g			
Control	31.7 ± 1.3	28.2 ± 1.8	25.5 ± 1.8
Methyl-GBB	61.5 ± 2.7*	61.9 ± 3.7*	49.9 ± 3.7*

Each value represents the mean ± S.E.M. of six rats in Control groups and seven rats in methyl-GBB treatment groups.
*Significantly different from the respective age-matched Control group (Student's t-test, $p < 0.05$).
**Significantly different from the 28-day methyl-GBB treatment group (Tukey's test; $p < 0.05$).
***Significantly different from the 14-day post-treatment group (Tukey's test; $p < 0.05$). nd – not detectable.

Table 2.
The effect of methyl-GBB administration on short-chain (SCAC), medium-chain (MCAC) and long-chain (LCAC) acylcarnitine levels in the plasma after 28 days of treatment and at 14 and 28 days after the last day of treatment.

	28-day treatment	14 days post-treatment	28 days post-treatment
SCAC, μM			
Control	15 ± 1.4	28 ± 1.6	31 ± 2.3
Methyl-GBB	3.8 ± 0.3*	9.3 ± 0.7*	15 ± 0.8*
MCAC, nM			
Control	29 ± 1.5	43 ± 3.8	44 ± 5.3
Methyl-GBB	11.2 ± 1.0*	21 ± 1.5*	24 ± 1.0*
LCAC, nM			
Control	173 ± 19	227 ± 22	232 ± 19
Methyl-GBB	83 ± 5*	142 ± 15*	154 ± 13*

Each value calculated as the mean ± S.E.M. of six rats in Control groups and seven rats in methyl-GBB treatment group.
*Significantly different from the respective age-matched Control group (Student`s t-test, $p < 0.05$).

methyl-GBB+carnitine group). Similarly, the acute addition of 2 mM carnitine had no significant effect on GBB levels in methyl-GBB-treated hearts (58 ± 8 nmol/g tissue in the methyl-GBB group *versus* 70 ± 6 nmol/g tissue in the methyl-GBB+carnitine group).

Plasma and heart tissue concentrations of acylcarnitines. The average concentration of SCACs (C2 and C4) in the plasma was 25.1 μM (15–31 μM) in the Control group rats, and treatment with methyl-GBB for 28 days decreased the acylcarnitine plasma concentration by 75% (table 2). The concentration of SCACs gradually recovered over time but was 52% lower than that in the Control group even 28 days after the end of the treatment. The average concentration of MCACs (C6-C12) was 39 nM (29–44 nM) in the Control group and was similarly decreased by 61% after treatment with methyl-GBB. The concentration of MCACs was 46% lower than that in the Control group 28 days after the end of the treatment (table 2). The average concentration of LCACs (C14-C18:2) in the plasma was 213 nM (173–232 nM) in the Control group and was similarly decreased by 52% after treatment with methyl-GBB. The concentration of LCACs was 34% lower at 28 days after the end of the treatment compared to the Control group (table 2). The recovery of LCACs was statistically significantly more pronounced than the recovery of MCACs in the plasma of rats treated with methyl-GBB. The entire acylcarnitine profile in plasma is included in the Table S3.

The average content of SCACs in the heart was 712 nmol/g tissue (638–811 nmol/g tissue) in the Control group, and treatment with methyl-GBB for 28 days decreased the acylcarnitine heart tissue content by 92% (fig. 3A). Overall, the recovery of acylcarnitine levels in the heart was similar to the changes in plasma. No significant differences among the recoveries of various chain length acylcarnitines in the heart tissues could be observed in the hearts of methyl-GBB-treated animals. The content of SCACs gradually recovered but was 68% lower than that in the Control group even at 28 days

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

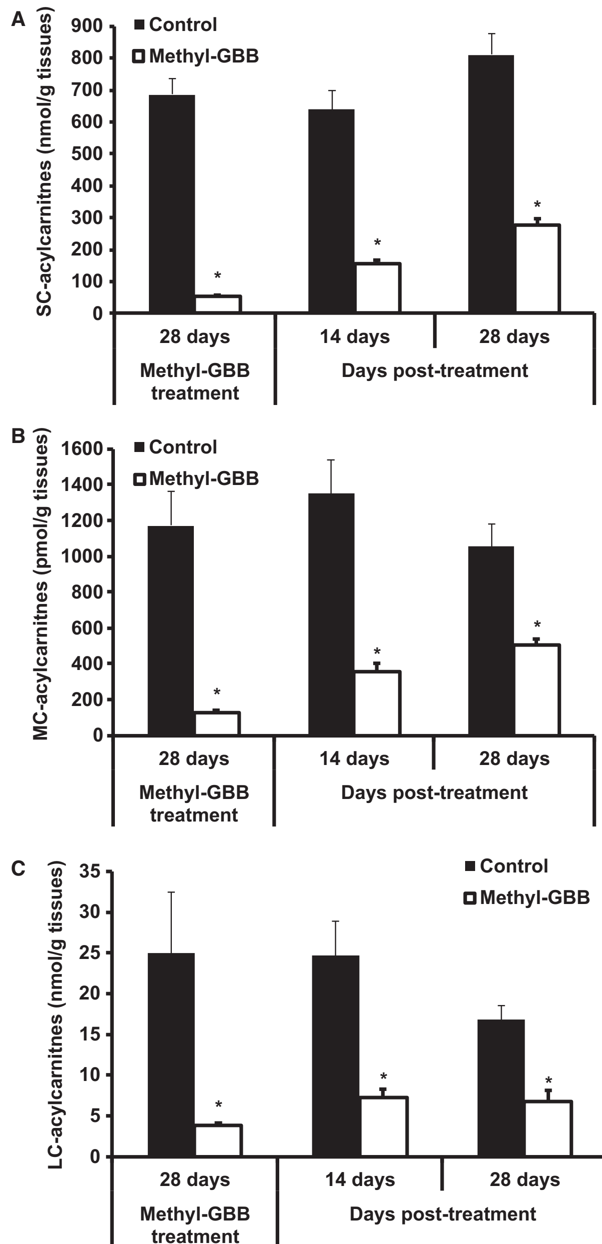

Fig. 3. The effect of methyl-GBB administration on short-chain (A), medium-chain (B) and long-chain (C) acylcarnitine contents in heart tissues after 28 days of treatment and at 14 and 28 days after the last day of treatment. Each value was calculated as the mean ± S.E.M. of six hearts in Control groups and seven hearts in methyl-GBB treated groups. *Significantly different from the respective age-matched Control group (Student's t-test; $p < 0.05$).

after the end of the treatment. The average content of MCACs was 1193 pmol/g tissue (1055–1350 pmol/g tissue) in the Control group and was decreased by 90% after treatment with methyl-GBB. The content of MCACs also recovered gradually (fig. 3B) and was 51% lower than that in the Control group even at 28 days after the end of the treatment. The average content of LCACs was 22.1 nmol/g tissue (17–25 nmol/g tissue) in the Control group and was decreased by 87% after treatment with methyl-GBB (fig. 3C). The content of LCACs was 69% lower than that in the Control group 14 days post-treatment (anti-infarction effect present) and 59% lower 28 days post-treatment when anti-infarction effect was not present (fig. 1A). The entire acylcarnitine profile in heart tissues is included in Table S4.

The acute addition of 2 mM carnitine to the isolated heart perfusion solution had no significant effect on the levels of short- and medium-chain acylcarnitines compared to the methyl-GBB-treated hearts. The contents of short- and medium-chain acylcarnitines in the methyl-GBB group (103 ± 14 nmol/g tissue for SCACs and 335 ± 46 pmol/g tissue for MCACs) and in the methyl-GBB+carnitine group (102 ± 9 nmol/g tissue for SCACs and 288 ± 8 pmol/g tissue for MCACs) were significantly lower than those in the Control group (520 ± 35 nmol/g tissue for SCACs and 636 ± 52 pmol/g tissue for MCACs in the Control group). LCAC content was significantly decreased in the methyl-GBB group (0.9 ± 0.1 nmol/g tissues) compared to the Control group (5.1 ± 0.8 nmol/g tissues); however, when 2 mM carnitine were added to the perfusion solution, the LCAC content in the methyl-GBB+carnitine group was significantly higher (1.7 ± 0.3 nmol/g tissues) than that in the methyl-GBB group (fig. 1B). Content of LCAC in the methyl-GBB+carnitine group remained significantly lower (by 67%) than that in the Control group, but no anti-infarction effect could be observed (fig. 1B).

Discussion

The present study demonstrates that methyl-GBB can effectively compete with carnitine for OCTN2-mediated transport and is effectively retained in the tissues over a prolonged period of time. The prolonged presence of methyl-GBB is concordant with lowered carnitine levels and a significantly decreased content of acylcarnitines, in particular, LCACs, which contribute to the severity of ischaemia–reperfusion-induced mitochondrial and cardiomyocyte damage. In this study, based on rat isolated heart infarction model, we further highlight the detrimental contribution that long-chain acylcarnitines have in the outcome of heart ischaemia–reperfusion-induced damage.

Previously, it has been shown that OCTN2 is highly specific towards carnitine and structurally related organic cations and is a key transporter that ensures the uptake of the cardioprotective compound, meldonium, in tissues [19]. The results of the present study provide evidence that the tissue distribution of methyl-GBB follows a similar pattern. However, methyl-GBB at a 20 times lower dose has better anti-infarction effect in the isolated rat heart model compared to meldonium [8]; this can at least partially be explained by the greater affinity of methyl-GBB for OCTN2, which results in lower carnitine level and subsequent LCAC content compared to meldonium-induced effects. Previously, we found that the co-treatment of meldonium with carnitine prevents the decrease in carnitine content, and no cardioprotective effect was observed when the carnitine content was decreased by less than approximately 60% [22]. Methyl-GBB decreases carnitine levels significantly better than meldonium, and the contents of carnitine (54% decrease) and LCACs (59% decrease) in the

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

tissues were decreased even at 28 days post-treatment with methyl-GBB; however, no anti-infarction effect was observed (fig. 1A). When an infarction study was performed at 14 days post-treatment, the levels of carnitine were decreased by 83% and levels of LCACs by 69%. A similar decrease in carnitine content was observed immediately after 14 days of methyl-GBB treatment [8]. The decrease in infarct size that was observed at 14 days post-treatment is comparable to the meldonium (14 days, 100 mg/kg)-induced cardioprotective effect [22] and the methyl-GBB anti-infarction effect after only 7 days of treatment [8]. Altogether, these findings indicate that carnitine content in the heart should be decreased by 60–70% so that LCAC content could be decreased by 70% to significantly reduce the infarct size by 20%. Apparently, if the LCAC content in rat heart tissues is decreased by at least 84–87% as observed in the present study and previously by our group [8], only then can one expect a robust cardioprotective effect like a reduction in infarct size of greater than 50%.

Previously, it has been noted that the supplementation of carnitine to the perfusion solution worsens global ischaemia outcome by impairing glucose metabolism [23]. This can be explained by the ability of LCACs at physiological levels to decrease glucose metabolism both in an isolated heart setup and *in vivo* [24], and acylcarnitine levels were increased after supplementation with carnitine [28]. In this study, when 2 mM carnitine were added to the perfusion buffer solution, no significant increase in carnitine tissue content was observed because all supplemented carnitine was used for LCAC synthesis, resulting in an almost two times increase in LCAC levels (67% decrease) compared to those observed in the methyl-GBB group (83% decrease), and the anti-infarction effect of methyl-GBB was not present (fig. 1B). After 28 days of treatment, the anti-infarction effect of methyl-GBB was correlated with a decrease in the contents of all chain length acylcarnitines and free carnitine. However, the acute addition of 2 mM carnitine significantly increased only LCAC content. Moreover, it has been shown that, unlike LCAC [24], SCAC and MCAC cannot significantly impair mitochondrial energy metabolism [29]. Altogether, these findings indicate that under acute ischaemic conditions, mitochondrial LCAC content is far more important than the contents of carnitine or short- and medium-chain acylcarnitines.

Incomplete fatty acid oxidation may lead to accumulation of LCAC, long-chain acyl-coenzyme A esters, diacylglycerol, triacylglycerol and ceramide that can impair energy metabolism [2,3,30]. Long-chain acyl-coenzyme A esters are potentially highly toxic [31,32], however, during ischaemia LCAC, but not long-chain acyl-coenzyme A esters, accumulate at concentrations that are harmful to mitochondria [11]. Acylcarnitine accumulation is one of the multiple factors that contribute to the ischaemia–reperfusion damage by affecting cell insulin signalling [5,6], modulation of cell membrane ion channels, changes in membrane permeability and activation of cell stress pathways [7]. Current and previous findings have shown that compounds like meldonium and methyl-GBB that significantly decrease LCAC tissue content also decrease infarct size by 30–50%. This allows to speculate that detrimental effects of LCAC accumulation could account for an important part of necrotic damage in ischaemic tissues. The only currently known mechanism of anti-infarction effect of meldonium and methyl-GBB is related to the decrease in carnitine and LCAC contents. Results from experiments with meldonium [33] and methyl-GBB (unpublished data) indicate that decrease in carnitine tissue content has no significant effect on contents of free fatty acids and long-chain acyl-coenzyme A in the heart. Decreased acylcarnitine formation after treatment with methyl-GBB leads to lowered fatty acid oxidation in the heart [8]. As fatty acids and glucose compete for contribution in energy production, methyl-GBB treatment increases basal and insulin-stimulated glucose metabolism [8]. Nevertheless, competition of glucose and fatty acid metabolism pathways is effectively mediated by LCACs at both cellular and mitochondrial levels [5,6,24] and decrease in LCAC content improves insulin signalling and facilitates glucose metabolism, that is more energy efficient during hypoxic conditions [34]. Thus, decrease in carnitine and LCAC levels leads to changes in energy metabolism pathways and results in less severe ischaemia-induced damage in the heart.

Conclusions

The anti-infarction effect of methyl-GBB continues for at least 2 weeks post-treatment. LCAC tissue content decreased by 70% leads to moderate infarct size decrease, but maximum anti-infarction effect can be observed when LCAC content is decreased by 87%. The decrease in LCAC levels defines the duration of the post-treatment cardioprotective effect of the OCTN2 inhibitor, methyl-GBB.

Acknowledgements

This study was supported by the Latvian National Research Programme Biomedicine for Public Health (BIOMEDICINE) 2014–2017. E. Liepinsh was supported by the FP7 project InnovaBalt.

Conflicts of Interest

Methyl-GBB substance was received from JSC Grindeks. The authors declare that they have no other conflict of interest.

References

1 Fillmore N, Mori J, Lopaschuk GD. Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy. Br J Pharmacol 2014;**171**:2080–90.

2 Ford DA, Han X, Horner CC, Gross RW. Accumulation of unsaturated acylcarnitine molecular species during acute myocardial ischemia: metabolic compartmentalization of products of fatty acyl chain elongation in the acylcarnitine pool. Biochemistry 1996;**35**:7903–9.

3 Idell-Wenger JA, Grotyohann LW, Neely JR. Coenzyme A and carnitine distribution in normal and ischemic hearts. J Biol Chem 1978;**253**:4310–8.

4 Korge P, Honda HM, Weiss JN. Effects of fatty acids in isolated mitochondria: implications for ischemic injury and cardioprotection. Am J Physiol Heart Circ Physiol 2003;**285**:H259–69.

5 Aguer C, McCoin CS, Knotts TA, Thrush AB, Ono-Moore K, McPherson R *et al.* Acylcarnitines: potential implications for skeletal muscle insulin resistance. FASEB J 2015;**29**:336–45.

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)

6 Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O *et al.* Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab 2008;**7**:45–56.
7 McCoin CS, Knotts TA, Adams SH. Acylcarnitines–old actors auditioning for new roles in metabolic physiology. Nat Rev Endocrinol 2015;**11**:617–25.
8 Liepinsh E, Makrecka-Kuka M, Kuka J, Vilskersts R, Makarova E, Cirule H *et al.* Inhibition of L-carnitine biosynthesis and transport by methyl-gamma-butyrobetaine decreases fatty acid oxidation and protects against myocardial infarction. Br J Pharmacol 2015;**172**:1319–32.
9 Vilskersts R, Kuka J, Liepinsh E, Makrecka-Kuka M, Volska K, Makarova E *et al.* Methyl-gamma-butyrobetaine decreases levels of acylcarnitines and attenuates the development of atherosclerosis. Vascul Pharmacol 2015;**72**:101–7.
10 Liepinsh E, Makrecka M, Kuka J, Cirule H, Makarova E, Sevostjanovs E *et al.* 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. Pharmacol Res 2014;**85**:33–8.
11 Liepinsh E, Makrecka-Kuka M, Volska K, Kuka J, Makarova E, Antone U *et al.* Long-chain acylcarnitines determine ischaemia/reperfusion-induced damage in heart mitochondria. Biochem J 2016;**473**:1191–202.
12 Liepinsh E, Makrecka-Kuka M, Makarova E, Volska K, Svalbe B, Sevostjanovs E *et al.* Decreased acylcarnitine content improves insulin sensitivity in experimental mice models of insulin resistance. Pharmacol Res 2016;**113**(Pt B):788–95.
13 Dambrova M, Makrecka-Kuka M, Vilskersts R, Makarova E, Kuka J, Liepinsh E. Pharmacological effects of meldonium: biochemical mechanisms and biomarkers of cardiometabolic activity. Pharmacol Res 2016;**113**(Pt B):771–80.
14 Gorgens C, Guddat S, Dib J, Geyer H, Schanzer W, Thevis M. Mildronate (Meldonium) in professional sports - monitoring doping control urine samples using hydrophilic interaction liquid chromatography - high resolution/high accuracy mass spectrometry. Drug Test Anal 2015;**7**:973–9.
15 Stuart M, Schneider C, Steinbach K. Meldonium use by athletes at the Baku 2015 European Games. Br J Sports Med 2016;**50**:694–8.
16 Tretzel L, Gorgens C, Geyer H, Thomas A, Dib J, Guddat S *et al.* Analyses of Meldonium (Mildronate) from blood, dried blood spots (DBS), and urine suggest drug incorporation into erythrocytes. Int J Sports Med 2016;**37**:500–2.
17 Liepinsh E, Kuka J, Svalbe B, Vilskersts R, Skapare E, Cirule H *et al.* Effects of long-term mildronate treatment on cardiac and liver functions in rats. Basic Clin Pharmacol Toxicol 2009;**105**:387–94.
18 Liepinsh E, Dambrova M. The unusual pharmacokinetics of meldonium: implications for doping. Pharmacol Res 2016;**111**:100.
19 Grigat S, Fork C, Bach M, Golz S, Geerts A, Schomig E *et al.* The carnitine transporter SLC22A5 is not a general drug transporter, but it efficiently translocates mildronate. Drug Metab Dispos 2009;**37**:330–7.
20 Grube M, Meyer zu Schwabedissen HE, Prager D, Haney J, Moritz KU, Meissner K *et al.* Uptake of cardiovascular drugs into the human heart: expression, regulation, and function of the carnitine transporter OCTN2 (SLC22A5). Circulation 2006;**113**:1114–22.
21 Liepinsh E, Konrade I, Skapare E, Pugovics O, Grinberga S, Kuka J *et al.* Mildronate treatment alters gamma-butyrobetaine and l-carnitine concentrations in healthy volunteers. J Pharm Pharmacol 2011;**63**:1195–201.
22 Kuka J, Vilskersts R, Cirule H, Makrecka M, Pugovics O, Kalvinsh I *et al.* The cardioprotective effect of mildronate is diminished after co-treatment with L-carnitine. J Cardiovasc Pharmacol Ther 2012;**17**:215–22.
23 Diaz R, Lorita J, Soley M, Ramirez I. Carnitine worsens both injury and recovery of contractile function after transient ischemia in perfused rat heart. J Physiol Biochem 2008;**64**:1–8.
24 Makrecka M, Kuka J, Volska K, Antone U, Sevostjanovs E, Cirule H *et al.* Long-chain acylcarnitine content determines the pattern of energy metabolism in cardiac mitochondria. Mol Cell Biochem 2014;**395**:1–10.
25 Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 2010;**160**:1577–9.
26 McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 2010;**160**:1573–6.
27 Liepinsh E, Kuka J, Dambrova M. Troubleshooting digital macro photography for image acquisition and the analysis of biological samples. J Pharmacol Toxicol Methods 2013;**67**:98–106.
28 Wessels B, van den Broek NM, Ciapaite J, Houten SM, Wanders RJ, Nicolay K *et al.* Carnitine supplementation in high-fat diet-fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction *in vivo*. Am J Physiol Endocrinol Metab 2015;**309**:E670–8.
29 Sauer SW, Okun JG, Hoffmann GF, Koelker S, Morath MA. Impact of short- and medium-chain organic acids, acylcarnitines, and acyl-CoAs on mitochondrial energy metabolism. Biochim Biophys Acta 2008;**1777**:1276–82.
30 Zhang L, Keung W, Samokhvalov V, Wang W, Lopaschuk GD. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. Biochim Biophys Acta 2010;**1801**:1–22.
31 Drosatos K, Schulze PC. Cardiac lipotoxicity: molecular pathways and therapeutic implications. Curr Heart Fail Rep 2013;**10**:109–21.
32 Li LO, Klett EL, Coleman RA. Acyl-CoA synthesis, lipid metabolism and lipotoxicity. Biochim Biophys Acta 2010;**1801**:246–51.
33 Liepinsh E, Skapare E, Kuka J, Makrecka M, Cirule H, Vavers E *et al.* Activated peroxisomal fatty acid metabolism improves cardiac recovery in ischemia-reperfusion. Naunyn Schmiedebergs Arch Pharmacol 2013;**386**:541–50.
34 Ussher JR, Lopaschuk GD. Targeting malonyl CoA inhibition of mitochondrial fatty acid uptake as an approach to treat cardiac ischemia/reperfusion. Basic Res Cardiol 2009;**104**:203–10.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Methyl-GBB elimination from plasma after both acute (A) and chronic (B) administration follows one compartment elimination type. Each value calculated as mean ± S.E.M. of 4 (acute) or 7 (chronic) independent measurements.

Figure S2. Linear elimination of methyl-GBB from heart tissues. Each value calculated as mean ± S.E.M. of 7 independent measurements.

Table S1. Animals used in each experimental setup and number of animals excluded from data analysis.

Table S2. Pearson correlation of infarct size and contents of different acylcarnitines in methyl-GBB treated rat hearts.

Table S3. The effect of methyl-GBB administration on acylcarnitine levels in the plasma after 28 days of treatment and at 14 and 28 days after the last day of treatment.

Table S4. The effect of methyl-GBB administration on acylcarnitine content in the heart after 28 days of treatment and at 14 and 28 days after the last day of treatment.

© 2017 Nordic Association for the Publication of BCPT (former Nordic Pharmacological Society)