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DISCOVERY OF A NOVEL CARDIOPROTECTIVE DRUG METHYL-GBB: pharmacological potential for lowering acyl-carnitines

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ABBREVIATIONS

ADP – adenosine diphosphate
AR – area at risk
BBOX – γ-butyrobetaine dioxygenase
BSA – bovine serum albumin
Chloro-GBB – 4-((chloromethyl)dimethylammonio)butanoate
CoA – coenzyme A
CPT I – carnitine palmitoyltransferase I
CrAT – carnitine acetyltransferase
Dimethyl-GBB – 4-[diethyl(methyl)ammonio]butanoate
DMEM – Dulbecco's Modified Eagle Medium
ECG – electrocardiography
Et-Me-meldonium – 3-(1,1,2-trimethyl-1-propylhydrazin-1-ium-2-yl)propanoate
FA – fatty acids
GBB – γ-butyrobetaine
GBB-phoshinate – (3-(trimethylammonio)propyl)phosphinate
HEK293 – human embryonic kidney cell line
IC_{50} – half maximal inhibitory concentration
KH buffer – Krebs-Henseleit buffer
K_m – Michaelis constant
LAD – left anterior descending coronary artery
LEAK state – respiration state induced by inhibition of adenine nucleotide translocator
Methyl-GBB – 4-(ethyldimethylammonio)butanoate
OCTN 2 – organic cation transporter type 2
OXPHOS state – ADP-stimulated mitochondrial respiration
PDH – pyruvate dehydrogenase
UPLC MS/MS – ultra-performance liquid chromatography tandem mass-spectrometry

INTRODUCTION

Ischemic heart disease is the major cause of disability and death in millions of people annually (*Go*, 2014; *Moran*, 2014; *Nichols*, 2013; *Nowbar*, 2014). The important pathological consequences of ischemic heart disease arise from impaired cellular energy metabolism. The most important energy substrates in the heart are fatty acids (FAs) and glucose (*Stanley*, 2005). The hearts of healthy subjects possess exceptional metabolic flexibility and are able to switch rapidly between available energy substrates. Glucose and FAs compete with each other to enter oxidative metabolism in mitochondria (*Randle*, 1998). Growing evidence suggests that the pharmacological regulation of cardiac energy metabolism by the inhibition of FA oxidation and/or the stimulation of glucose oxidation can improve cardiac function in ischemic or failing hearts (*Fillmore*, 2014; *Jaswal*, 2011; *Wang*, 2007). Thus, a better understanding of the regulation of cardiac energy metabolism pathways can provide novel strategies for the treatment of ischemic heart disease.

L-carnitine, a cofactor of acyltransferases, participates in the regulation of mitochondrial energy metabolism. The most well-known function of L-carnitine is to facilitate the transport of long-chain FAs into mitochondria via carnitine palmitoyltransferase I (CPT I) for further β-oxidation. Another L-carnitine-dependent enzyme, carnitine acetyltransferase (CrAT), regulates the acetylCoA/free CoA ratio, which is essential for pyruvate dehydrogenase activity. In addition, changes in the L-carnitine concentration may also influence acyl-carnitine availability. However, the role of acyl-carnitine is currently limited to the transport of the acyl moiety, and the possible effects of acyl-carnitines in cardiac energy metabolic pathways remain unclear.

Despite previous studies indicating that a reduction in L-carnitine availability seems to be beneficial for the optimization of cardiac energy metabolism (*Kuka*, 2012; *Liepinsh*, 2008, 2011, 2013a), the effects of a

decrease in the L-carnitine concentration in cardiac tissues during ischemia-reperfusion injury are not fully understood. Further studies are necessary to clarify the roles of L-carnitine and particularly, of its metabolites, in the regulation of cardiac energy metabolism and to elucidate whether the L-carnitine system can be considered to be a drug target for the treatment of ischemic heart disease.

Aim of the study

To discover novel drug targets in the L-carnitine system for the regulation of energy metabolism to achieve cardioprotection in experimental animal models of cardiac ischemia-reperfusion injury.

Objectives of the study

1. To determine how changes in energy metabolism pattern affect the outcome of cardiac ischemia-reperfusion injury under different nutritional states.
2. To determine the effects of long-chain acyl-carnitines on glucose metabolism (pyruvate/lactate) in cardiac mitochondria under physiological conditions.
3. To study the effects of sodium pivalate, an L-carnitine-lowering agent, on cardiac functionality and mitochondrial energy metabolism.
4. To determine whether the inhibition of γ-butyrobetaine dioxygenase (BBOX) or organic cation transporter 2 (OCTN2) is the most effective strategy for decreasing the L-carnitine concentration and protect the heart against acute ischemia–reperfusion injury.
5. To determine whether pharmacologically decreasing the L-carnitine concentration is an effective strategy for decreasing the accumulation of long-chain acyl-carnitines to protect the heart against acute ischemia–reperfusion injury.

Hypotheses of the study

1. The accumulation of long-chain acyl-carnitines impairs glucose metabolism, thereby increasing the severity of cardiac ischemia-reperfusion injury.
2. The reduction in L-carnitine availability diminishes the accumulation of long-chain acyl-carnitines, stimulates glucose oxidation and protects the heart against ischemia-reperfusion injury.

Scientific novelty of the study

The roles of L-carnitine and its metabolites, long-chain acyl-carnitines, in the regulation of cardiac energy metabolism and the outcome of cardiac ischemia-reperfusion injury were studied, with the following findings:

1. The long-chain acyl-carnitine concentration determines the energy metabolism pattern in the heart. In addition, the accumulation of long-chain acyl-carnitines impairs glucose metabolism and increases the severity of cardiac ischemia-reperfusion injury.
2. For the first time, the anti-infarction activities of the BBOX and OCTN2 inhibitors were compared, demonstrating that OCTN2 is the most effective strategy for decreasing L-carnitine availability and protecting the heart against ischemia-reperfusion injury.
3. A novel inhibitor of OCTN2 and BBOX, the cardioprotective agent Methyl-GBB, was discovered. Its mechanism of action is based on reducing the long-chain acyl-carnitine concentrations in cardiac tissues and mitochondria by decreasing L-carnitine availability.

1. MATERIALS AND METHODS

1.1. Animals and treatment

Male *Wistar* rats (Laboratory of Experimental Animals, Riga Stradins University, Latvia), *Goto-Kakizaki* and *Wistar-Kyoto* rats (Taconic Farms, USA) and *ICR* mice (Harlan, Netherlands) were used for the experiments. The experimental procedures were performed in accordance with the European Community Directive 2010/63/EU guidelines and local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. For the energy metabolism studies Wistar and Goto-Kakizaki rats were randomly separated into two experimental groups. Rats in the 'fed' group had unlimited access to food, whereas those in the 'fasted' group were deprived of food for 18 h prior to the start of the experiment. Wistar rats received a 40 mM sodium pivalate pH 7.0 (approx. 500 mg/kg) as their drinking water to study the effects of L-carnitine lowering compounds on energy metabolism. In order to compare the effects of selective inhibitors of L-carnitine biosynthesis or transport the selected compounds (Chloro-GBB, Dimethyl-GBB, Et-Me-meldonium, GBB-phoshinate) were administrated *per os* at a dose of 20 mg/kg. To study the cardioprotective effects of the leading compound, Methyl-GBB at doses 1, 5, 10, 20 mg/kg was administrated *per os*. Meldonium at dose 100 mg/kg *per os* was used as a control for the comparison.

1.2. Methods

1.2.1. *In vitro* methods

1.2.1.1. Biochemical parameters

The concentrations of free fatty acids and triglycerides were measured using commercially available enzymatic kits from Wako (Neuss, Germany) and Instrumentation Laboratory (Lexington, Massachusetts, USA). The plasma

glucose and insulin concentration were determined using kit from Instrumentation Laboratory and Sensitive Rat Insulin RIA kit (Millipore, Billerica, USA), respectively. Lactate level was measured in samples using enzymatic kit from Roche Diagnostics (Mannheim, Germany). The FA profile was determined by gas chromatography/mass spectrometry of FA methyl esters as previously described (*Liepinsh*, 2013a). Acyl-carnitine and acylCoA levels in heart tissue homogenates and plasma samples were determined simultaneously in one run using a UPLC MS/MS method. The sample extraction was performed as previously described (*Blachnio-Zabielska*, 2011).

1.2.1.2. Oxidation of energy substrates in the isolated cardiac mitochondria and tissue homogenates

Mitochondria were isolated from cardiac tissues as previously described (*Kuka*, 2012). The oxidation rates of mitochondrial substrates were determined using respective radiolabelled substrate as described previously (*Campbell*, 2004; *Yoshida*, 2007). The mitochondrial and peroxisomal rates of palmitate oxidation were determined in cardiac tissue homogenate using [1-^{14}C]palmitate as described previously (*Degrace*, 2004), with the exception that specified L-carnitine concentration was used in the assay.

1.2.1.3. Respiration measurements in the isolated mitochondria and permeabilized cardiac fibers

Mitochondria were isolated from cardiac tissues as previously described (*Kuka*, 2012). The mitochondrial respiration was measured using a Clark-type electrode. To determine the CPT I-dependent oxidation of FAs, palmitoylCoA and specified L-carnitine concentrations (found in the cardiac tissue after the treatment) were used for the respiration measurements. Palmitoyl-carnitine was

used as a substrate to determine CPT I-independent FA oxidation. Pyruvate + malate were used as substrates to measure pyruvate metabolism. In addition, the CPT I and CrAT activities were measured in isolated cardiac mitochondria as described previously (*Kuka,* 2012; *Jaudzems,* 2009).

To assess mitochondrial function after ischemia-reperfusion injury, isolated rat hearts were subjected to 20 min of no-flow ischemia following 120 min reperfusion, and then cardiac fibers were prepared as described previously (*Kuka*, 2012). Respiration rates of cardiac fibers were measured at 37 °C with Clark-type electrode using pyruvate + malate (6 mM + 6 mM) as substrates. ADP-stimulated respiration (OXPHOS state) was achieved by adding 0.2 mM ADP. To determine the uncoupling of oxidative phosphorylation, LEAK state respiration was measured after the addition of 5 μM carboxyatractyloside.

1.2.1.4. BBOX activity and OCTN2-mediated transport of L-carnitine

The BBOX activity was assayed as described previously (*Tars*, 2010). The human recombinant BBOX or rat liver homogenate (1:10 (w/v) in PBS) were used as enzyme sources. The reaction was initiated by adding GBB, and to ensure the linear rate range the mixture was incubated at 37 ºC for 30 min (human BBOX) or 120 min (rat liver BBOX).

The transport of L-carnitine was measured as L-[N-methyl-^{3}H]-carnitine hydrochloride uptake by human embryonic kidney cells (HEK293). The assay was performed in 24-well plates in DMEM/F-12 medium. The cells were pre-incubated with the tested compounds 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, lysed and radioactivity in cell lysate was measured.

1.2.1.5. L-carnitine and administrated compound concentrations in cardiac tissues and plasma.

Determination of L-carnitine, Chloro-GBB, Dimethyl-GBB, Et-Me-meldonium, GBB-phosphinate and Methyl-GBB concentrations in cardiac tissues and plasma were performed by UPLC/MS/MS using the positive ion electrospray mode as described previously (*Dambrova*, 2008).

1.2.2. *Ex vivo* and *in vivo* methods

1.2.2.1. Oxidation of energy substrates in the isolated rat heart

The rates of radiolabelled glucose, lactate and palmitate oxidation were measured in different sets of Wistar rat hearts as previously described (*Lopaschuk*, 1997) with modifications. Briefly, the rat hearts were retrogradely perfused with the respective oxygenated Krebs-Henseleit (KH) buffer solution 'fed'('high insulin') supplemented with 10 mM glucose, 0.3 mM sodium palmitate bound to 2% BSA, 2 mM lactate, 0.2 mM pyruvate and 3 ng/ml insulin, or with KH buffer solution 'fasted' ('low insulin') supplemented with 5 mM glucose, 1.2 mM sodium palmitate bound to 2% BSA, 1 mM lactate, 0.1 mM pyruvate and 0.3 ng/ml insulin. Glucose and lactate oxidation rates were determined by measuring the $^{14}CO_2$ released from the metabolism of [U-^{14}C]glucose or [1-^{14}C]lactate, respectively. Palmitate oxidation was determined by measuring $^{3}H_2O$ released from [9,10-^{3}H]palmitate. Substrate uptake in the heart was calculated from the amount of radiolabelled substrate oxidized during the perfusion and the amount found in the cardiac tissue at the end of the perfusion.

1.2.2.2. *Ex vivo* and *in vivo* rat heart infarction study

The isolated heart infarction study was performed according to the Langendorff technique as described previously (*Kuka*, 2012).

In vivo infarction study was done as follows: after onset of anaesthesia, animals were intubated and ventilated, the chest was opened, and a polypropylene thread was placed around the LAD. The animals were allowed to adapt for 10 minutes. The coronary artery was occluded for 30 min. Successful occlusion was confirmed by ischemia-induced alterations in the ECG. Afterwards, the chest and skin were closed. After 24 h following the induction of reperfusion, the experimental animals was anaesthesid, the heart was excised and stained as described previously (*Liepinsh*, 2013b).

1.2.3. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). For statistical analysis, the Student's t-test, Mann-Whitney U-test, Chi-Square test or a one-way ANOVA with Tukey`s test were used. Spearman's and Pearson's correlation analysis were used to examine the relationship between the compound inhibition potency on BBOX and OCTN2 and OCTN2 inhibitor IC_{50} value and the L-carnitine content in heart tissues. P values less than 0.05 were considered to be statistically significant. Logistic regression analysis was used to determine IC_{50} values of the tested compounds. Statistical calculations were performed using Prism 5.03 software (GraphPad, San Diego, California).

2. RESULTS

2.1. The effects of energy metabolism pattern on myocardial infarction

2.1.1. The effects of fasted and fed states on myocardial infarction

The effects of fasted and fed states on infarct size were studied in an isolated rat heart infarction model. To mimic both states *ex vivo* in the isolated heart model, hearts from fasted and fed rats were perfused with 2 different solutions consisting of plasma components in concentrations found in the fed and fasted states (Table 2.1.) 'Fed' buffer solution contained higher concentrations of glucose, lactate and insulin, but lower concentration of palmitate, compared to 'fasted' buffer solution.

Table 2.1.

The concentrations of biochemical components in plasma of fed and fasted rats

	Fasted	Fed
Glucose, mM	5.0 ± 0.4	8.5 ± 0.2*
Fatty acids, mM	0.85 ± 0.09	0.40 ± 0.03*
Lactate, mM	1.2 ± 0.1	1.8 ± 0.1*
Insulin, ng/ml	0.22 ± 0.05	2.96 ± 0.48*

The presented results are mean ± SEM of at least 8 rats. *Significantly different from the fasted group (Student's t-test, $P < 0.05$).

The infarct size in fed Wistar rat hearts was almost 2-fold (46%) smaller than that observed in fasted rat hearts (Figure 2.1.). A similar effect was achieved when hearts from fasted rats were perfused with 'fed' buffer containing higher concentrations of glucose, insulin and lactate (Fasted+'fed' buffer group)).

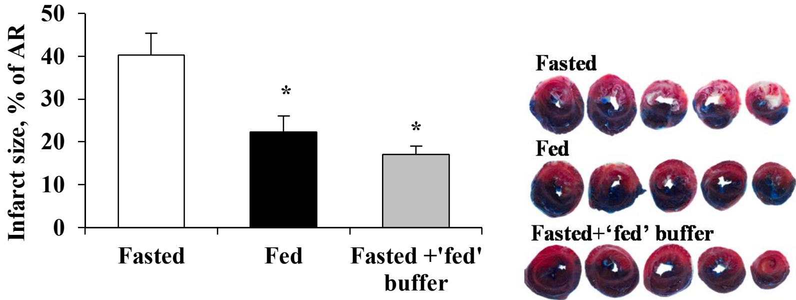

Figure 2.1. **Differences in the myocardial infarct size in hearts from fasted and fed rats**
The presented results are mean ± SEM of 8 rats. *Significantly different from the fasted group (Tukey's test, $P < 0.05$)

The effects of fasted and fed states on infarct size were studied also in the model of type 2 diabetes, Goto-Kakizaki rats. Similar to Wistar rats, there was almost a 2-fold difference in the infarct size in Goto-Kakizaki rats between hearts from fed and fasted animals. As a result of insulin resistance, the infarct size in hearts from fed and fasted Goto-Kakizaki rats was 20-50% larger than in Wistar rat hearts (Figure 2.2.).

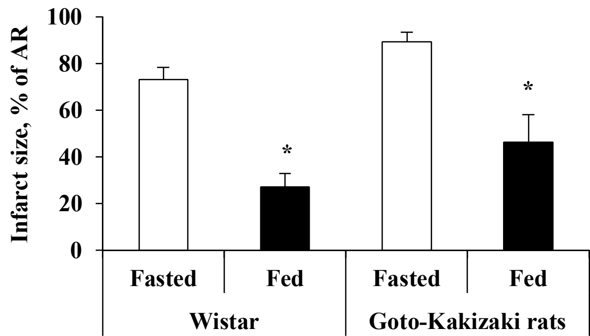

Figure 2.2. **Differences in myocardial infarct size in hearts from Wistar and diabetic Goto-Kakizaki rats**
The presented results are mean ± SEM of 5 rats. *Significantly different from the respective fasted group (Tukey's test, $P < 0.05$).

2.1.2. Differences in the energy metabolism pattern between fasted and fed states

In order to determine cardioprotective effect-underlying changes in substrate utilization pattern in the fed state, glucose, lactate and FA oxidation rates were measured in hearts isolated from fasted and fed rats.

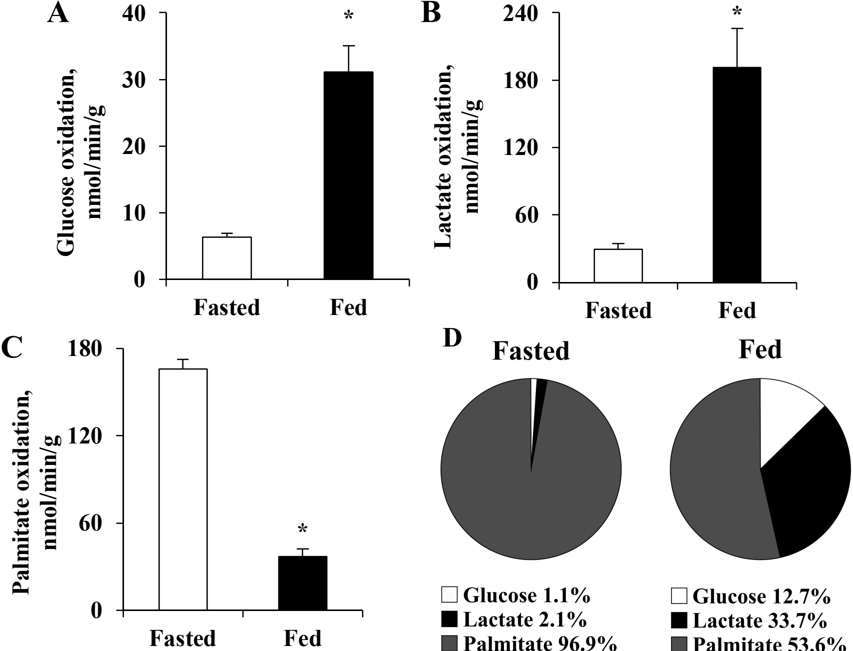

Figure 2.3. **Oxidation rates of energy substrates (glucose (A), lactate (B) and palmitate (C)) in isolated hearts from fasted and fed Wistar rats. Calculated energy substrate input in the energy production (D) in hearts from fasted and fed Wistar rats**

The presented results are mean ± SEM of at least 8 rats. *Significantly different from the fasted group (Student's t-test, $P < 0.05$).

In the fasted state, the oxidation of glucose and lactate was very limited (Figure 2.3.A and B). In contrast, glucose and lactate oxidation rates were increased by 5- and 4-fold (Figure 2.3.A and B), respectively, although the circulating concentrations of glucose and lactate were only 2-fold higher

(Table 2.1.) in the fed state relative to the fasted state. Thus, the increase in glucose, lactate and pyruvate oxidation rates directly depends on insulin signalling and indirectly on circulating glucose and lactate concentrations (Table 2.1.).

In contrast to glucose oxidation, FA oxidation depends on the levels of circulating FAs (Table 2.1.). The 3-fold reduction in the labelled palmitate oxidation rate was observed in the fed state compared to the fasted state (Figure 2.3.C). In the fasted state, FA oxidation overrides glucose and lactate oxidation and prevails as the predominant energy source (Figure 2.3.D). An increased circulating concentration of insulin and consequential stimulation of glucose metabolism together with lower FA load determine the protection against ischemia-reperfusion injury in the fed state.

2.2. The regulation of energy metabolism by long-chain acyl-carnitines

2.2.1. The effects of fasting on the concentrations of mitochondrial energy substrates in plasma and cardiac tissue

Despite a significant difference in the content of biochemical components in the plasma of fed and fasted animals (Table 2.1), the contents of lactate and long-chain FAs in cardiac tissues were similar in the fed and fasted states, at 15.5 ± 0.7 vs 14 ± 0.6 µmol/g and 450 ± 30 vs 520 ± 28 nmol/g, respectively. In the fasted state the contents of activated long-chain FAs in the form of acyl-carnitines and acylCoAs (73 ± 8 and 2.4 ± 0.1 nmol/g, respectively) were significantly 4.9- and 3.6-fold higher as compared to the fed state. The total amount of saturated long-chain acyl-carnitines was 23- and 31-fold higher than the total content of saturated acylCoAs in the fed and fasted states, respectively.

2.2.2. Substrate competition in isolated cardiac mitochondria

Measurements of pyruvate, lactate and palmitate oxidation in mitochondria showed that oxidation rates are highly dependent on the concentrations of these substrates (Figure 2.4. and 2.5.). Overall, the oxidation rate of pyruvate was 2-fold higher than the oxidation rate of lactate when similar substrate concentrations were used. An examination of the dependence of lactate and pyruvate metabolism on the palmitoyl-carnitine content demonstrated that palmitoyl-carnitine effectively reduced the rates of pyruvate and lactate oxidation in mitochondria in a dose-dependent manner (Figure 2.4.).

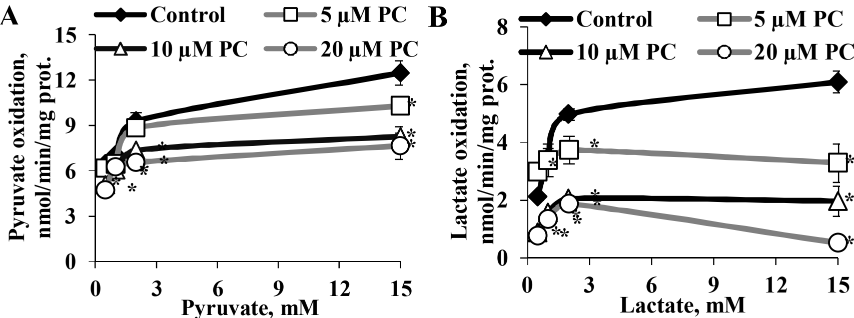

Figure 2.4. **The effects of palmitoyl-carnitine (PC) on pyruvate (A) and lactate (B) oxidation in isolated cardiac mitochondria**
The presented results are mean ± SEM of 5 rats. *Significantly different from the Control (Student's t-test, $P < 0.05$).

The inhibitory effect of palmitoyl-carnitine was more pronounced in the case of lactate metabolism (Figure 2.4.). Thus, 20 µM palmitoyl-carnitine reduced the oxidation rate of pyruvate in a similar manner as 5 µM palmitoyl-carnitine decreased the oxidation rate of lactate. In the presence of 10 or 20 µM palmitoyl-carnitine, the oxidation rate of lactate was severely depressed by more than 3-fold (Figure 2.4.B). Furthermore, pyruvate, but not lactate, was able to significantly reduce the palmitate oxidation rate in mitochondria

(Figure 2.5.). These results show that pyruvate and FAs can effectively compete for energy production pathways at the mitochondrial level; however, only the physiological content of acyl-carnitines has an impact on pyruvate and lactate oxidation in the mitochondria.

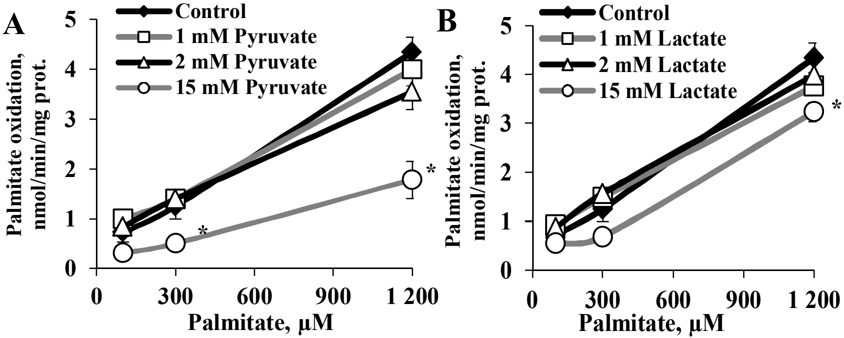

Figure 2.5. **The effects of pyruvate (A) and lactate (B) on FA oxidation in isolated cardiac mitochondria**

The presented results are mean ± SEM of 5 rats. *Significantly different from the Control (Student's t-test, $P < 0.05$).

2.2.3. The effects of long-chain acyl-carnitines on glucose metabolism in isolated rat heart model and *in vivo*

In order to determine the effects of long-chain acyl-carnitines on glucose utilization in the heart, we measured glucose uptake and oxidation rates in isolated rat hearts perfused[1] with or without 20 µM palmitoyl-carnitine. The increase in long-chain acyl-carnitine content induced by perfusion with palmitoyl-carnitine had no effect on glucose uptake rate in the isolated heart (Figure 2.6.A). However, the glucose oxidation rate was significantly decreased by 47% in palmitoyl-carnitine-treated hearts (Figure 2.6.B).

[1] KH buffer solution supplemented with 10 mM glucose, 0.3 mM sodium palmitate bound to 1% BSA, 2 mM lactate, 0.2 mM pyruvate and 3 ng/ml insulin.

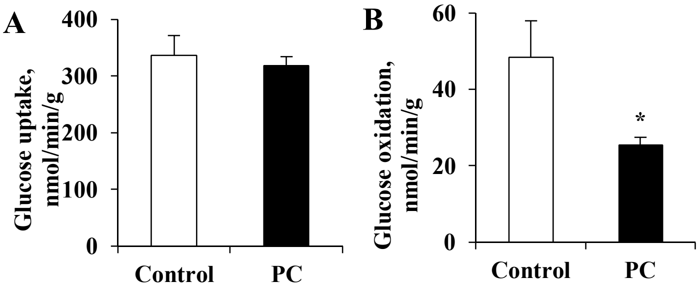

Figure 2.6. **The effects of palmitoyl-carnitine (PC) on glucose uptake (A) and oxidation (B) in isolated rat heart model**
The presented results are mean ± SEM of 8 rats. *Significantly different from the Control group (Student's t-test, P < 0.05).

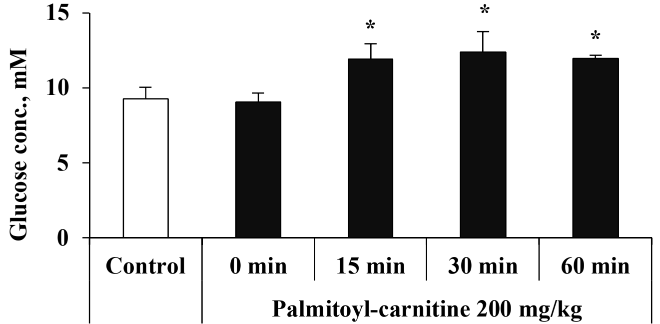

Figure 2.7. **The effects of acute palmitoyl-carnitine administration on glucose utilization *in vivo***
The presented results are mean ± SEM of 5 mice. *Significantly different from the Control group (Student's t-test, P < 0.05).

In addition, the single dose of palmitoyl-carnitine significantly increased the blood glucose concentration at all tested time points by 31-35% compared to control group (Figure 2.7.). Taken together, these results demonstrate that increased concentration of long-chain acyl-carnitines impairs glucose utilization.

2.3. The effects of sodium pivalate treatment on energy metabolism

In order to study the association of the decrease in L-carnitine content with cardioprotection, rats were treated with sodium pivalate (40 mM in drinking water for 14 days (500 mg/kg/day)), and the effects of the treatment on cardiac functionality and mitochondrial energy metabolism were studied.

2.3.1. The effects of sodium pivalate treatment on ischemia-reperfusion injury

Although the treatment with sodium pivalate significantly decreased L-carnitine concentration in the cardiac tissues by 37%, the infarct size after treatment with sodium pivalate was 64 ± 2.7% and did not differ from the control group (Figure 2.8.). In addition, the treatment with sodium pivalate had no effect on cardiac functional parameters (data not shown).

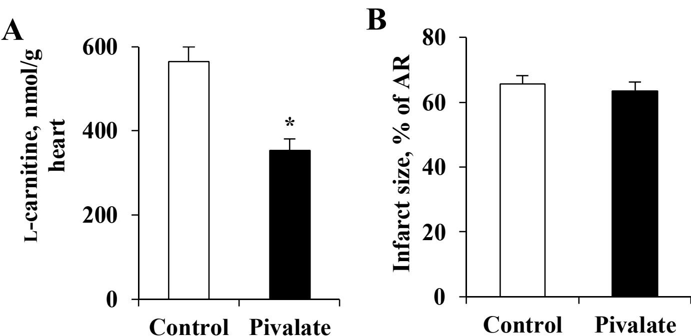

Figure 2.8. **The effects of sodium pivalate treatment on L-carnitine content in cardiac tissues (A) and infarct size (B)**

The presented results are mean ± SEM of 10 rats. *Significantly different from the Control group (Student's t-test, $P < 0.05$).

2.3.2. The effects of sodium pivalate treatment on L-carnitine-dependent enzymes and mitochondrial energy metabolism

To study the possible mechanisms behind the lack of cardioprotection after sodium pivalate treatment, L-carnitine dependent enzyme activities were measured in isolated cardiac mitochondria. CPT I activity was significantly decreased by 30% in the pivalate group compared to the control group (Figure 2.9.A). Similarly, CrAT activity was significantly decreased by 34% in the pivalate group compared to the control group (Figure 2.9.A).

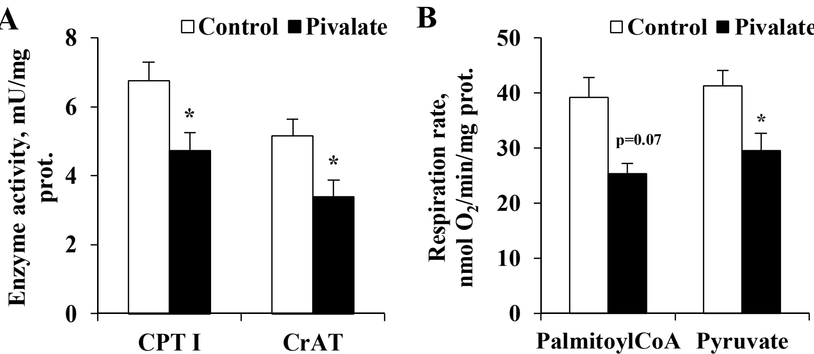

Figure 2.9. **The effects of sodium pivalate treatment on L-carnitine-dependent enzyme activities (A) and energy metabolism (B) in isolated cardiac mitochondria**

The presented results are mean ± SEM of 3-6 rats. *Significantly different from the Control group (Student's t-test or Mann-Whitney U-test, $P < 0.05$).

After the 14-day treatment with sodium pivalate, the CPT I-dependent mitochondrial respiration on palmitoylCoA was decreased by 35% ($p = 0.07$) (Figure 2.9.B). The respiration on pyruvate/malate after treatment with sodium pivalate was significantly decreased by 28% (Figure 2.9.B). Taken together, obtained results demonstrate that sodium pivalate treatment inhibits both fatty acid and pyruvate metabolism, thus it prevents any metabolic shift and lacks cardioprotective effect.

2.4. The effects of OCTN2 and BBOX inhibitors on L-carnitine availability

2.4.1. Screening on BBOX and OCTN2

In order to identify an effective target to decrease L-carnitine availability, 50 compounds (analogues of meldonium and GBB) were synthesized (*Tars*, 2014) and screened for the ability to inhibit L-carnitine biosynthesis (BBOX enzyme) and transport (OCTN2-mediated).The inhibition potency of meldonium analogues on BBOX activity and OCTN2-mediated L-carnitine transport is represented in Table 2.2. The inhibition potency of GBB analogues on BBOX activity and OCTN2-mediated L-carnitine transport is represented in Table 2.3.

Table 2.2.

Inhibition potency of meldonium analogues on BBOX activity and OCTN2-mediated L-carnitine transport

No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
	Meldonium	26 ± 2	62 (*Tars*, 2010)	62 ± 5
Modifications of the nitrogen at position 4				
10		0.17 ± 0.12	0.09 ± 0.05	54 ± 9
11		10.0 ± 2.0	7.2 ± 2.7	12.4 ± 2.5
12	Et-Me-meldonium	6.7 ± 4.4	3.1 ± 1.8	97 ± 5

The IC_{50} values are presented as mean ± SD of 3 independent experiments.

Table 2.3.

Inhibition potency of GBB analogues on BBOX activity and OCTN2-mediated L-carnitine transport

No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
	GBB	-	-	3.9 ± 0.1
Modifications of the trimethylammonium group				
16	Methyl-GBB	2.8 ± 0.6	3.3 ± 1.8	3 ± 0.3
19	Dimethyl-GBB	> 1000	100 ± 36	14.6 ± 3.0
20		34 ± 6	5.7 ± 1.6	4.8 ± 1.4
21		49 ± 8	127 ± 32	1.9 ± 0.5
22		30 ± 12	2.7 ± 0.8	12.7 ± 1.1
24		3.1 ± 0.6	2.1 ± 0.7	2.4 ± 0.5
30	Cl⁻ OH	1.40 ± 0.03	0.49 ± 0.24	4.4 ± 1.0
31		168 ± 18	> 1000	36 ± 6
33	Chloro-GBB	95 ± 3	268 ± 54	4.2 ± 2.5
36	F Cl⁻ OH	0.78 ± 0.36	0.61 ± 0.22	7 ± 1.8
37	Cl Cl⁻ OH	1.0 ± 0.2	0.26 ± 0.13	33 ± 6
Modifications of the core region				
41		452 ± 13	343 ± 117	26 ± 3

Table 2.3. (continued)

No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
43		> 1000	> 1000	7.7 ± 3.0
Modifications of the carboxylic acid				
46		10.2 ± 5.8	0.10 ± 0.03	> 100
47	GBB-Phosphinate	0.8 ± 0.1	0.52 ± 0.35	850 ± 122

The IC_{50} values are presented as mean ± SD of 3 independent experiments.

23 compounds displayed superior human BBOX inhibition relative to the meldonium. The most active compounds (No. **10** and **46**) were more than 600 times better BBOX inhibitors than meldonium. 35 compounds had the same potency or were more potent OCTN2 inhibitors than meldonium. The most active compounds (No. **21** and **24**) had IC_{50} values approximately 30 times better than the IC_{50} value of meldonium. The very strong correlation is present between compound inhibitory potency on rat liver and human recombinant enzyme ($r = 0.876$, $p < 0.0001$), indicating that synthesized compounds have very similar effectiveness in both species. No relationship between compound inhibitory potency of BBOX and OCTN2 was observed (rat liver BBOX $r = 0.172$, $p = 0.23$; human BBOX $r = 0.033$, $p = 0.82$).

2.4.2. The selection of BBOX and OCTN2 inhibitors for further experiments

To compare L-carnitine biosynthesis and transport as targets to reduce L-carnitine availability *in vivo*, for further experiments four compounds with a very different IC_{50} values on BBOX or OCTN2 were selected (Figure 2.10.). Chloro-GBB and Dimethyl-GBB were relatively potent inhibitors of OCTN2

and weak inhibitors of BBOX (human and rat liver) with IC_{50} values for OCTN2 of 4.2 μM and 14.6 μM, respectively. In contrast, Et-Me-meldonium and GBB-phosphinate were relatively potent inhibitors of BBOX and weak inhibitors of OCTN2 with IC_{50} values for BBOX (rat liver) of 6.7 μM and 0.8 μM, respectively and IC_{50} values for OCTN2 of 97 μM and 850 μM, respectively.

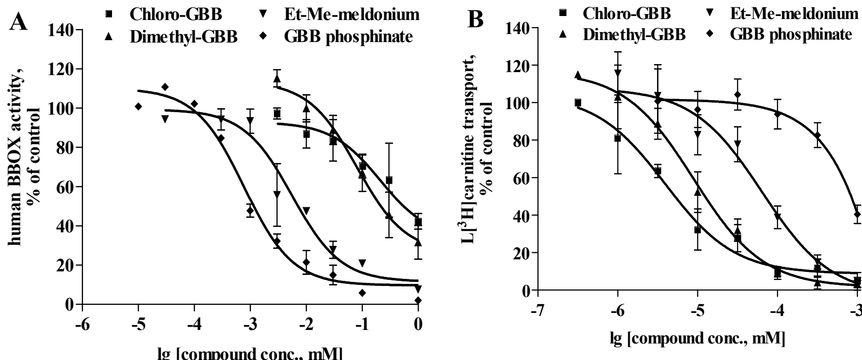

Figure 2.10. **The effect of selected compounds on BBOX activity (A) and OCTN2-mediated L-carnitine transport in HEK293 cells (B)**
The presented results are mean ± SD of at least 3 independent experiments.

2.4.3. The influence of long-term administration of OCTN2 and BBOX inhibitors on L-carnitine content in the heart tissues

The average content of L-carnitine in the rat heart tissues was 715 ± 29 nmol/g (Figure 2.11.). The long-term inhibition (14 days) of OCTN2 more effectively reduced the L-carnitine content in the heart compared to the inhibition of BBOX. Thus, the most pronounced decrease in L-carnitine content was induced by the most potent of the tested OCTN2 inhibitors, Chloro-GBB, which decreased L-carnitine content by 90% (Figure 2.11.). Dimethyl-GBB, which has a 3-fold lower potency relative to Chloro-GBB, decreased L-carnitine content by 35%. In comparison, administration of a very weak

OCTN2 inhibitor, Et-Me-meldonium, decreased L-carnitine content in the heart tissues only by 20%. GBB-phosphinate did not inhibit OCTN2 and therefore did not induce any changes in L-carnitine content in the heart tissues although it is the most potent inhibitor of BBOX used in this study (Figure 2.10.).

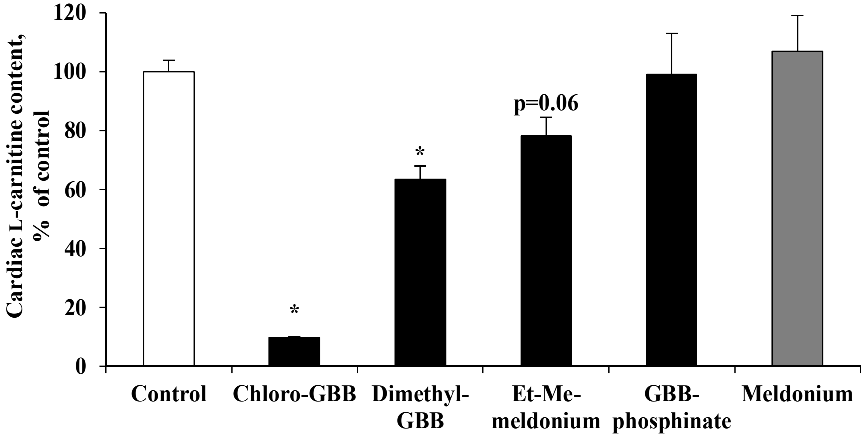

Figure 2.11. **The effect of selected compounds and meldonium (all at a dose of 20 mg/kg) on the L-carnitine content in the heart after 14 days of treatment**

The presented results are mean ± SEM of 8-10 rats. *Significantly different from control group (Tukey's test $P < 0.05$).

Along with the four selected compounds (Chloro-GBB, Dimethyl-GBB, Et-Me-meldonium, GBB-phosphinate), we tested also the long-term administration of 12 additional compounds (**24**, **16**, **30**, **20**, **36**, **43**, **11**, **22**, **41**, **37**, **10**, meldonium listed in Tables 2.2. and 2.3.) for the effects on L-carnitine content in the heart tissues. The reference compound meldonium as a relatively weak OCTN2 inhibitor did not induce any decrease of L-carnitine content in the heart tissues at the dose 20 mg/kg (Figure 2.11.). In addition, a significant and strong correlation between the IC_{50} values for OCTN2 of these compounds and the decrease of L-carnitine content in the heart tissues was found ($r = 0.854$; $p < 0.0001$). In comparison, IC_{50} values of tested compounds for BBOX did not correlate with the decrease of L-carnitine content in the heart tissues ($r=0.0603$).

2.4.4. The anti-infarction activity of OCTN2 and BBOX inhibitors after long-term administration

After a 14-day treatment with Chloro-GBB, the infarct size in the rat hearts was significantly decreased by 35% (Figure 2.12.). Another OCTN2 inhibitor, Dimethyl-GBB, decreased the infarct size by only 17%, and this weaker effect might be related to the insufficient decrease in L-carnitine content. BBOX inhibitors did not influence L-carnitine content and therefore at the tested dose did not decrease the infarct size in the rat hearts (Figure 2.12.). Similarly, the reference compound meldonium at a dose of 20 mg/kg did not induce a decrease in the infarct size even after long-term treatment (Figure 2.12.).

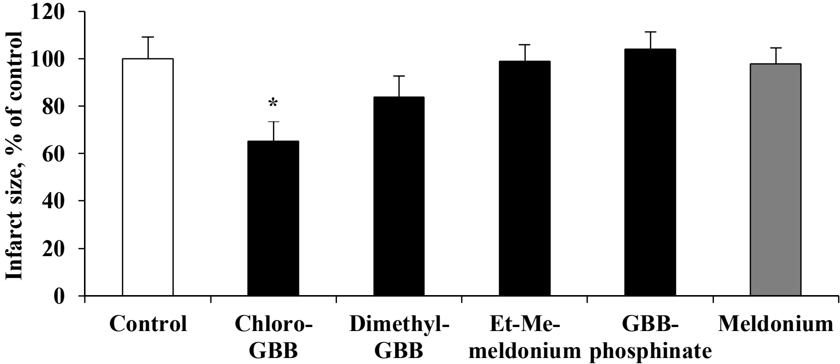

Figure 2.12. **The effect of selected compounds and meldonium on the infarct size after 14 days of treatment**

The presented results are mean ± SEM of 8-10 rats. *Significantly different from control group (Tukey's test $P < 0.05$).

2.5. The effects of long-term treatment of OCTN2 and BBOX inhibitor, Methyl-GBB, on energy metabolism and ischemia-reperfusion injury

2.5.1. The effects of Methyl-GBB treatment on L-carnitine availability

The effects of Methyl-GBB on the L-carnitine system can be explained by the inhibition of both BBOX and OCTN2 (Table 2.3.). In comparison to meldonium, Methyl-GBB is 8 times more potent at inhibiting L-carnitine synthesis by BBOX (IC_{50} 3 μM vs 26 μM) and 20 times more potent at inhibiting L-carnitine transport by OCTN2 (IC_{50} 3 μM vs 62 μM). Methyl-GBB not only inhibits L-carnitine transport by OCTN2, but itself is transported by OCTN2. Therefore a high affinity of Methyl-GBB to OCTN2 ensures that Methyl-GBB is easily transported into the tissues. After the 14 day treatment at doses of 1, 5 and 20 mg/kg Methyl-GBB concentrations in the heart were 47, 185 and 221 nmol/g heart, respectively (Figure 2.13.A). Concentration of Methyl-GBB in the heart increased in a time dependent manner (Figure 2.13.B) reaching a maximum after 14 days of treatment. As shown in Figure 2.13.A, treatment with meldonium for 2 weeks at a dose of 100 mg/kg induced a significant decrease in the L-carnitine heart content by 75%. In comparison, Methyl-GBB treatment at doses of 1, 5 and 20 mg/kg decreased the L-carnitine content in the heart by 42%, 84% and 95%, respectively.

To study the time-dependent effects of Methyl-GBB on L-carnitine content, we measured the concentration of L-carnitine cardiac tissues after 3, 7 and 14-day administrations of Methyl-GBB. As shown in Figure 2.13.B, Methyl-GBB decreased L-carnitine content in the heart in a time-dependent manner.

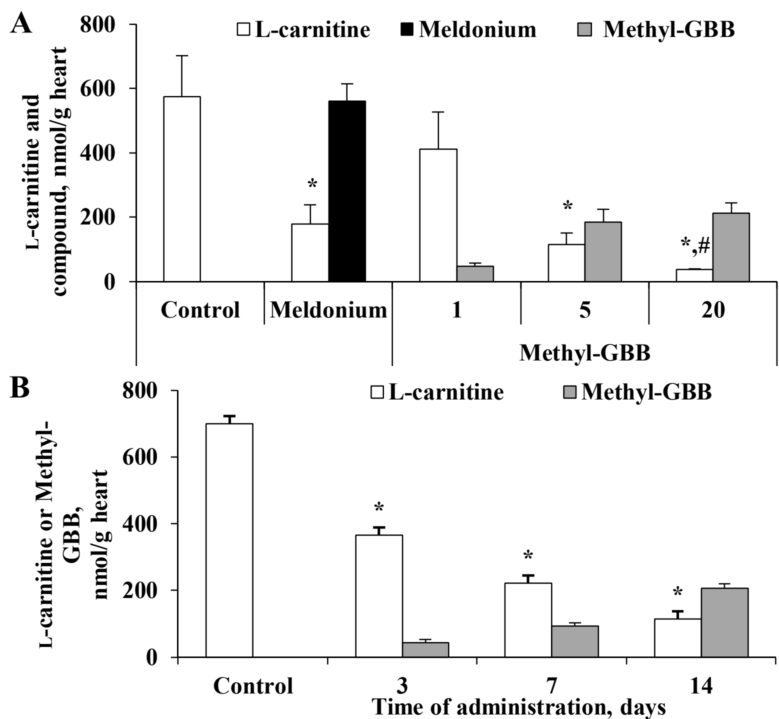

Figure 2.13. **Contents of L-carnitine and compound in cardiac tissues after 14 days of treatment by meldonium (100 mg/kg) or Methyl-GBB (1, 5 and 20 mg/kg) (A) and after 3, 7, 14 days of treatment by Methyl-GBB at a dose of 5 mg/kg (B)**

The presented results are mean ± SEM of 5 rats. *Significantly different from Control group (Tukey's test $P < 0.05$), #Significantly different from meldonium group (Tukey's test $P < 0.05$).

2.5.2. The effects of Methyl-GBB treatment on the cardiac acyl-carnitine content

In Methyl-GBB treated hearts the acyl-carnitine content was significantly decreased both in heart and mitochondria (Figure 2.14.A and B). Methyl-GBB treatment induced significant up to 50-fold reduction in the content of long-chain acyl-carnitines. Thus, the link between decrease in the

content of L-carnitine and preserved mitochondrial function during reperfusion is related to the decreased content of long-chain acyl-carnitines.

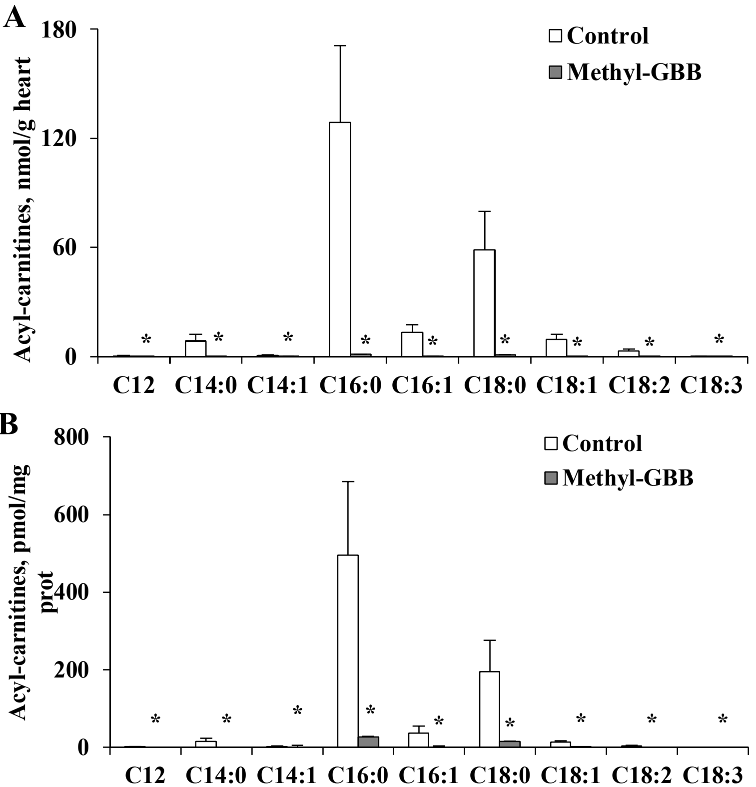

Figure 2.14. **The effects of a long-term Methyl-GBB (10 mg/kg) treatment on cardiac (A) and mitochondrial (B) content of acyl-carnitine**

The presented results are mean ± SEM of 5-8 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

2.5.3. The effects of Methyl-GBB treatment on the FA and glucose oxidation in the heart

Initially, the effects of Methyl-GBB on the metabolism of FAs were determined in mitochondria and peroxisomes. The treatment with Methyl-GBB significantly reduced both mitochondrial and peroxisomal palmitate oxidation

rates by 44% and 53%, respectively (Figure 2.15.A). After the 14-day Methyl-GBB treatment, the L-carnitine-dependent mitochondrial respiration rate with palmitoylCoA was significantly decreased by 27% (Figure 2.15.B), but the treatment had no effect on the L-carnitine-independent mitochondrial respiration rate with palmitoyl-carnitine (Figure 2.15.C).

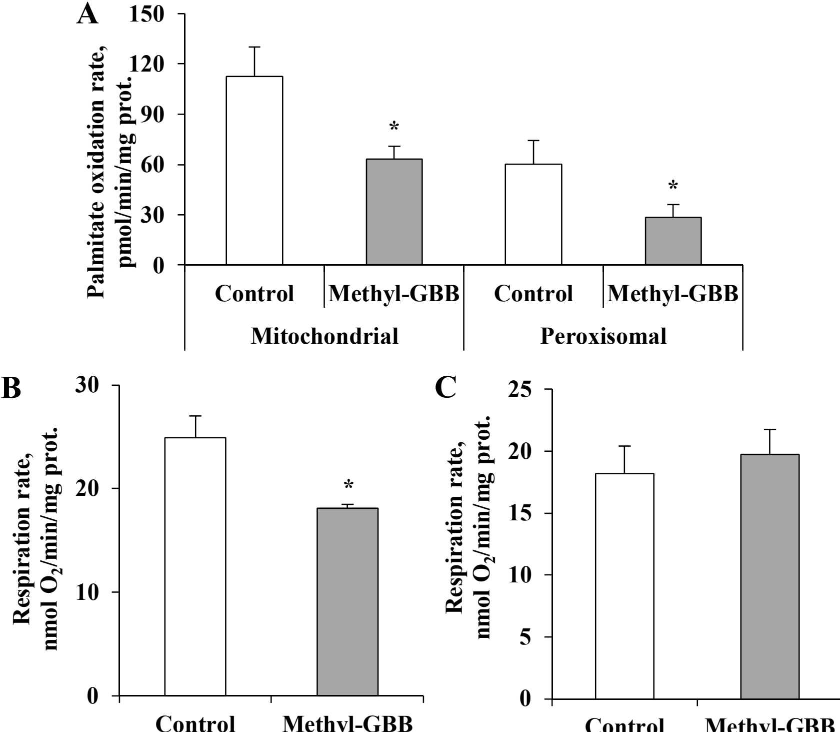

Figure 2.15. **The effects of a long-term Methyl-GBB (10 mg/kg) treatment on mitochondrial and peroxisomal [^{14}C]palmitate oxidation rates (A) and mitochondrial L-carnitine-dependent respiration with 10 µM palmitoylCoA (B) and L-carnitine-independent oxidation with 10 µM palmitoyl-carnitine (C)**

The presented results are mean ± SEM of 5-6 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

Then the effects of Methyl-GBB on the metabolism of FA were determined in an isolated heart. In contrast to the previously observed effects of meldonium (*Liepinsh*, 2013a), the oxidation rate of labelled palmitate in the isolated heart was significantly decreased by 40% (Figure 2.16.A). An increase in FA oxidation was observed after reperfusion in Methyl-GBB treated group (Figure 2.16.A). In addition, the Methyl-GBB treatment reduced the labelled palmitate concentration in the mitochondria and, thus, also reduced the risk of FA accumulation (Figure 2.16.B).

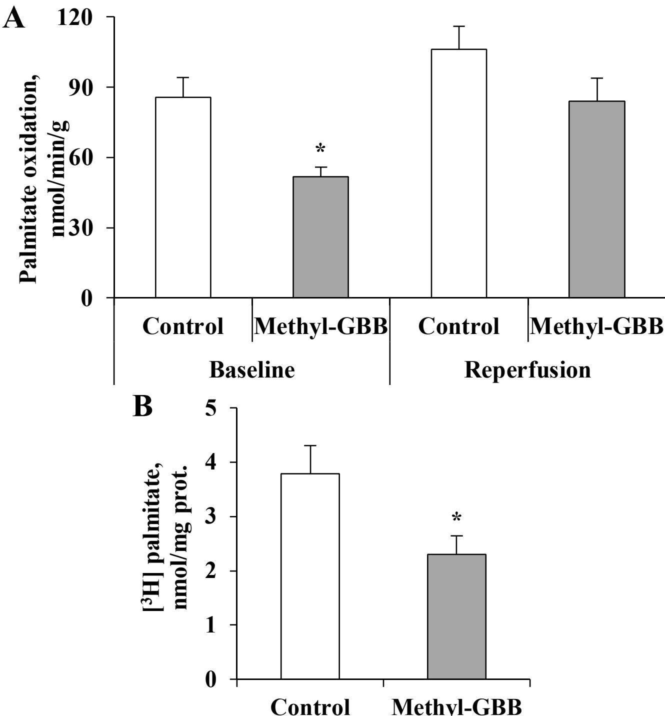

Figure 2.16. **The effects of long-term Methyl-GBB (10 mg/kg) treatment on [^{3}H]palmitate oxidation in isolated heart (A) and mitochondrial [^{3}H]palmitate content (B)**

The presented results are mean ± SEM of 5-6 animals. *Significantly different from Control group (Student's t-test $P < 0.05$).

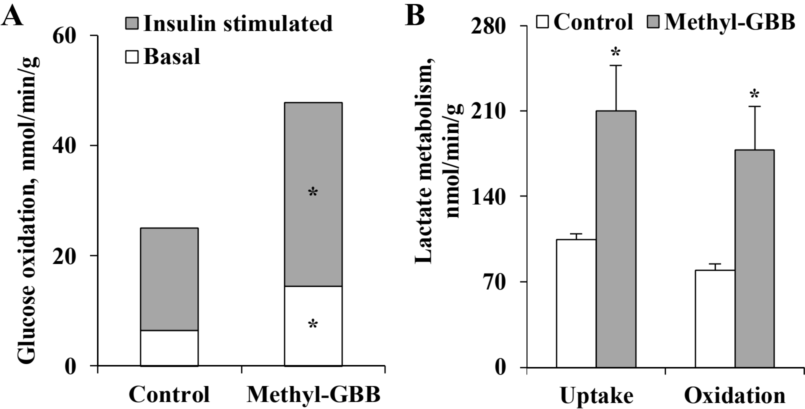

Figure 2.17. **The effects of long-term Methyl-GBB (10 mg/kg) treatment on basal and insulin stimulated [^{14}C]glucose oxidation (A), [^{14}C]lactate uptake and oxidation (B) in the isolated rat heart**

The presented results are mean ± SEM of 5-6 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

Treatment with Methyl-GBB induced a reduction in FA oxidation and in turn stimulated glucose oxidation (Figure 2.17.A). We observed a significant 2-fold increase in glucose oxidation in the Methyl-GBB-treated isolated rat hearts perfused with low and high concentrations of insulin. Similarly, Methyl-GBB treatment induced an increase in lactate uptake and oxidation rates in isolated hearts (Figure 2.17.B). Thus, Methyl-GBB treatment induces a partial switch of energy production from long-chain FAs to glucose oxidation.

2.5.4. The effects of Methyl-GBB treatment on the cardiac ischemia-reperfusion injury

The anti-infarction effect of the meldonium and Methyl-GBB was investigated in both an isolated rat heart *in vitro* ischemia-reperfusion model and *in vivo*. As observed in Figure 2.18.A, meldonium treatment at a dose of 100 mg/kg decreased the infarct size by 25% compared to the Control group infarct size. Methyl-GBB at doses of 5 and 20 mg/kg had significantly better

effects and decreased the infarct size by 45-48%. Thus, Methyl-GBB with a dose 20 times lower is almost 2 times more effective than meldonium.

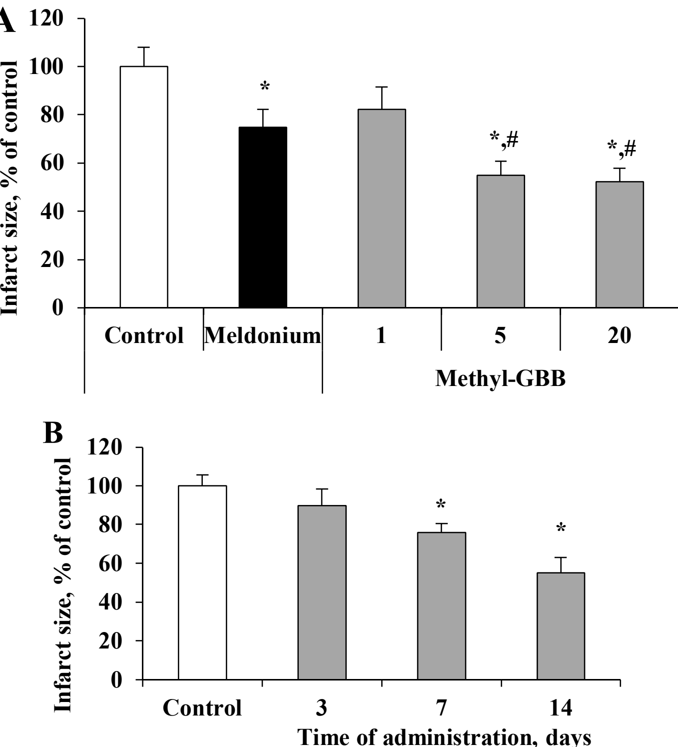

Figure 2.18. **The effects of meldonium (100 mg/kg) and Methyl-GBB (1, 5 and 20 mg/kg) on the infarct size after 14 days of treatment (A). The effects of Methyl-GBB (5 mg/kg) on the infarct size after 3, 7, 14 days of treatment (B)**

The presented results are mean ± SEM of 8-10 rats. *Significantly different from Control group (Tukey's test $P < 0.05$), #Significantly different from meldonium group (Tukey's test $P < 0.05$).

It was shown previously that meldonium exhibits significant cardioprotective effects only after 14 days of treatment (*Liepinsh*, 2006); therefore, the time-dependent effects of Methyl-GBB administration were tested. After 7 days of treatment, Methyl-GBB significantly decreased the

infarct size by 24% (Figure 2.18.B). Overall, similar to meldonium, long-term treatment with Methyl-GBB is required to provide maximal cardioprotection.

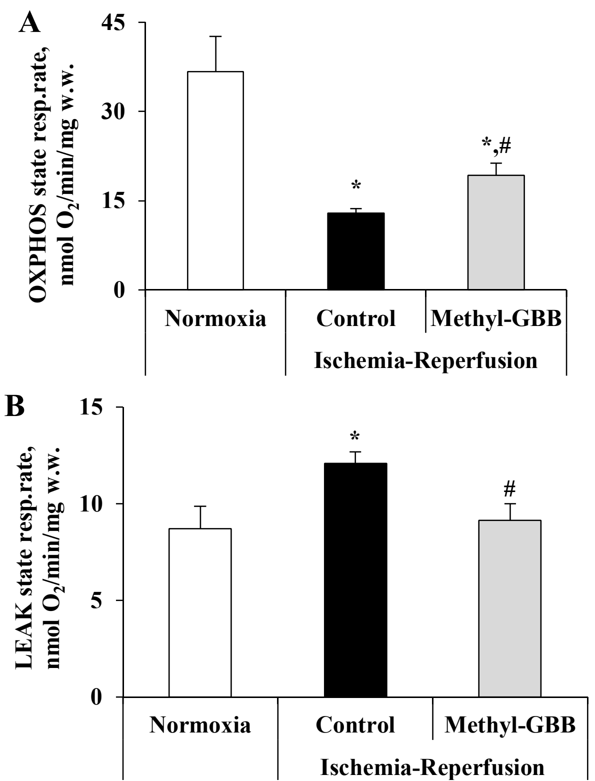

Figure 2.19. **The effects of Methyl-GBB (20 mg/kg) treatment on ADP-stimulated mitochondrial respiration (OXPHOS state) (A) and uncoupling of oxidative phosphorylation (LEAK state) (B) in cardiac fibers isolated after ischemia-reperfusion**

The presented results are mean ± SEM of 5 rats. *Significantly different from normoxia (Tukey's test $P < 0.05$), #Significantly different from ischemic control group (Tukey's test $P < 0.05$).

The ischemia-reperfusion induced a 2.8-fold decrease in OXPHOS state respiration and a 40% increase in LEAK state respiration (Figure 2.19.). The Methyl-GBB treatment significantly protected against the ischemia-

reperfusion-induced mitochondrial dysfunction. Mitochondria respiration rate at OXPHOS state were increased by 50% compared to ischemic control (Figure 2.19.A). According to LEAK state respiration measurement, Methyl-GBB completely protected against ischemia-reperfusion induced mitochondrial uncoupling (Figure 2.19.B).

In the *in vivo* myocardial infarction model, the effects of a 14-day pretreatment with Methyl-GBB at doses of 10 and 20 mg/kg were evaluated. Treatment with Methyl-GBB attenuated, in a dose-dependent manner, the infarct size by 18% to 45% in Wistar rats undergoing LAD occlusion and reperfusion *in vivo* (Figure 2.20.A). Moreover, Methyl-GBB treatment improved the 24-h survival of rats by 20-30% (Figure 2.20.B).

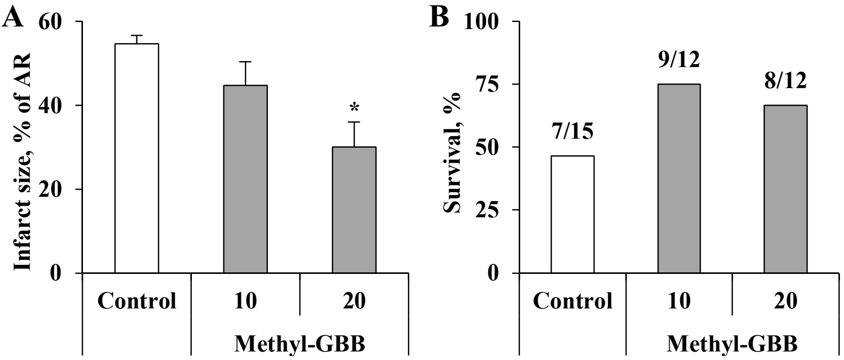

Figure 2.20. **The effects of Methyl-GBB (10 and 20 mg/kg) treatment on the infarct size (A) and rat 24 h survival (B)**

The presented results are mean ± SEM at least 7 rats. *Significantly different from control group (Tukey's test $P < 0.05$).

Thus, Methyl-GBB administration possesses cardioprotective effects in experimental models of ischemia-reperfusion injury *ex vivo* and *in vivo*.

3. DISCUSSION

The present thesis describes the association of cardiac energy metabolism pattern with the outcome of cardiac ischemia-reperfusion injury, the role of long-chain acyl-carnitines in the regulation of energy metabolism, and the reduction of L-carnitine availability as a strategy for the regulation of acyl-carnitine availability. First, the fed and fasted nutritional states were used to determine the cardiac energy metabolism pattern association with the outcome of cardiac ischemia-reperfusion injury. Second, the role of long-chain acyl-carnitines in the regulation of energy metabolism pattern in the heart was determined. Third, different potential strategies were compared to determine the most effective tool for reducing L-carnitine and acyl-carnitine availability. Fourth, the effects of a leading compound on cardiac energy metabolism and ischemia-reperfusion injury were studied.

3.1. The association of cardiac energy metabolism pattern with the outcome of ischemia-reperfusion injury

We have shown that in the healthy control and diabetic hearts, the infarct size is significantly smaller in the fed state compared to the fasted state. In the fed state, the heart tissues oxidise more glucose and less palmitate than in the fasted state as a result of activated insulin signalling pathway-induced expression of glucose metabolism genes and reduced inhibitory activity of activated FAs. FA overloading in the fasted state leads to an uncoupling of mitochondrial oxidative phosphorylation and energy dissipation. Our results demonstrate that ***cardiac recovery from ischemia-reperfusion injury is improved in the fed state*** due to enhanced glucose and lactate oxidation and lower load of FAs.

There is abundant evidence that increased glucose oxidation is protective against myocardial infarction-induced cell damage (*Hafstad*, 2007;

Liu, 1996; *Ussher*, 2012). In the fasted state, the oxidation of glucose, lactate and pyruvate contributes to less than 5% of produced ATP, which leads to a marked heart tissue damage in cases of myocardial infarction. In the fed state, significantly increased glucose oxidation ensures better survival in ischemic conditions and improved recovery in reperfusion. The increases in glucose, lactate and pyruvate oxidation rates depend on insulin signalling rather than on circulating glucose and lactate concentrations. Insulin signalling influences glucose transport and metabolism by altering gene expression and enzyme activities. Similarly, FA oxidation is partially down-regulated in hearts isolated from fed rats. Insulin-activated pathways induce inhibition of FA metabolism, resulting in significantly lower concentrations of activated long-chain FAs in the heart and mitochondria. As a result, in the fed state, there is a lower risk of mitochondrial damage by FAs in cases of myocardial infarction.

In line with previous studies (*Mjos*, 1971; *Simonsen*, 1978), we also observed that high FA flux in the fasted state leads to reduced cardiac efficiency. Increased oxidation of FAs in the fasted state results in higher oxygen requirement, which increases the risk of heart damage under hypoxia-related conditions. Overall, these results provide additional evidence that FAs is a less-effective energy substrate and that high FA load increases the risk for permanent heart damage and the possibility of lethal cardiovascular events.

Although preoperative fasting is mandatory for adults to prevent perioperative complications, this fasting could also cause hypoglycaemia-related effects (*Awad*, 2012; *Ljungqvist*, 2009; *van Hoorn*, 2005). A growing body of data on the interplay between glucose and FA metabolism during acute myocardial infarction supports the need for heart-specific glucose metabolism-stimulating agents (*Diks*, 2005; *Luttikhold*, 2013; *van den Brom*, 2013; *van Hoorn*, 2005). GIK infusion has long been suggested for myocardial protection (*Sievers*, 1966), although the results of clinical trials are controversial (*Grossman*, 2013; *Zhao*, 2010). The present results suggest that, in subjects in

the postprandial state, concentrations of glucose and insulin are already relatively high, and the possibilities for inducing additional increases in glucose oxidation by insulin and glucose infusion are very limited. Thus, protective effects could be expected only if the GIK is administered in the fasted state. In addition, the improvement of the clinical outcome can be achieved when GIK administration is started within the first hours after the onset of symptoms of acute coronary syndrome (*Grossman*, 2013). Thus, in case of symptoms of cardiovascular event during fasting the intake of foods or beverages rich in carbohydrates could be potentially life-saving.

Obesity-induced insulin resistance is an important risk factor for cardiovascular diseases (*Bhat*, 2013; *Lavie*, 2009). Therefore, long-term low calorie diets are beneficial for preventing diabetes and cardiovascular complications. However, acute extreme fasting or starving could result in low plasma glucose concentrations, which can trigger cardiovascular events. Moreover, an increased FA availability in the fasted state enhances the severity of cardiovascular events. In low-carbohydrate diets, low plasma glucose levels or even hypoglycaemia are observed in the postprandial state. Our results demonstrate that it is important to suggest for high-risk patients to avoid very low-carbohydrate diets and prolonged fasting periods.

In conclusion, our results suggest that glucose and lactate oxidation is important for the survival of ischemic hearts, and even overnight fasting-induced hypoglycaemia and increased FA load could trigger cardiovascular events, such as angina pectoris and arrhythmias.

3.2. The role of long-chain acyl-carnitines in the regulation of cardiac energy metabolism

We examined whether the availability of activated FAs determines the energy metabolism pattern in cardiac mitochondria. Our results demonstrate

that FA and glucose intermediates compete for metabolic pathways not only in the heart (Randle cycle), but also in the cardiac mitochondria. In cardiac mitochondria, energy metabolism is mainly driven by the availability of substrates, particularly the long-chain acyl-carnitines. Moreover, the increase in long-chain acyl-carnitine concentration induces the reduction of glucose oxidation in the heart. Taken together, our results provide evidence that the rate of acyl-carnitine production determines the energy metabolism pattern in cardiac mitochondria and subsequently in the heart. Furthermore, the increase in long-chain acyl-carnitine concentration can contribute to the development of insulin resistance.

The energy metabolism pattern in the heart is mainly determined by the availability of energy substrates in plasma and insulin signalling (*Lopaschuk*, 2010; *Randle*, 1998). Nevertheless, according to the results of present study, the concentrations of the most prominent intracellular energy substrates, lactate and long-chain FAs, did not differ between the fed and fasted states in cardiac tissues. In contrast, the content of activated long-chain FAs was significantly increased in both plasma and cardiac tissues in the fasted state compared to fed state. In addition, the physiological content of long-chain acylCoAs in the heart is too low to have an impact on glucose metabolism. Meanwhile, the content of long-chain acyl-carnitines is 29 times higher than the content of long-chain acylCoAs and it is sufficient to determine the energy metabolism pattern in cardiac tissues.

Substrate competition experiments demonstrated that neither lactate nor pyruvate has a major impact on long-chain FA oxidation at physiological concentrations. In accordance with a previous study (*Yoshida*, 2007), we demonstrated that lactate fails to inhibit FA oxidation in isolated cardiac mitochondria at physiological concentrations. In addition, pyruvate could only significantly reduce FA oxidation at supraphysiological concentration (15 mM). Therefore, the physiological content of pyruvate found in cardiac cells (*Brooks*,

1999; *Zhao*, 2008) is too low to have any effect on FA metabolism. Meanwhile, the physiological content of long-chain acyl-carnitines in cardiac tissue is sufficient to effectively reduce the oxidation of pyruvate and lactate in cardiac mitochondria and subsequently glucose utilization in the heart. Taken together, the present results suggest that ***the content of long-chain acyl-carnitines determines the rates of pyruvate and lactate metabolism in mitochondria, but not vice versa.***

The development of insulin resistance has been linked to the incomplete FA oxidation and subsequent accumulation of FA metabolites (*Bell*, 2010; *Koves*, 2005, 2008; *Muoio*, 2012). In addition, it has been demonstrated that FA-induced impairment in glucose metabolism in L6 myotubes occurs only in the presence of carnitine (*Koves*, 2008). These data indicate that acyl-carnitines can contribute to the development of insulin resistance, however, thus far no direct evidence has been provided. In the present study, we have demonstrated that palmitoyl-carnitine effectively reduces glucose (pyruvate-lactate) utilization in mitochondria, isolated rat heart and *in vivo*. These results link the development of insulin resistance to the increased availability of long-chain acyl-carnitines. Moreover, it has been demonstrated that activated long-chain FAs induce mitochondrial dysfunction (*Koves*, 2008; *Tominaga*, 2008) and the reduction of long-chain acyl-carnitine content in mitochondria can be beneficial to protect against ischemia-reperfusion injury (*Liepinsh*, 2013a). The present findings indicate that targeting the availability of long-chain acyl-carnitines could serve as a new strategy for the treatment of insulin resistance.

Taken together our results provide evidence that long-chain acyl-carnitine content orchestrates the interplay between the metabolism of glucose (pyruvate-lactate) and long-chain FAs in cardiac mitochondria and heart. Furthermore, the mitochondrial accumulation of long-chain acyl-carnitines impairs glucose metabolism.

3.3. The reduction of L-carnitine availability as a cardioprotective drug target

3.3.1. The effects of pivalate treatment on cardiac energy metabolism and ischemia-reperfusion injury

The present study examined the effects of short-term, high-dose sodium pivalate treatment on cardiac energy metabolism. Our results demonstrate that in addition to decreased CPT I-dependent FA oxidation, treatment with sodium pivalate impairs pyruvate metabolism in cardiac mitochondria. As a result the overall energy metabolism in the heart can be disturbed.

The shift from long-chain fatty acid metabolism to glucose utilization is cardioprotective. Thus, a decrease in fatty acid metabolism through malonylCoA inhibition (*Ussher*, 2009) or the administration of meldonium, an inhibitor of L-carnitine biosynthesis and transport (*Kuka*, 2012; *Liepinsh*, 2006), has been proven to be cardioprotective against ischemia-reperfusion injury. Sodium pivalate does not induce a sufficient decrease in L-carnitine concentration to reduce CPT I activity in L-carnitine-dependent manner as the K_m value of heart CPT I for L-carnitine is significantly lower (*McGarry*, 1983) than the measured concentration of L-carnitine in the pivalate treated heart. Therefore, in contrast to meldonium, pivalate decreases CPT I-dependent long-chain FA metabolism independently of decreased L-carnitine concentration. Moreover, despite the reduction of free L-carnitine content in cardiac tissues and CPT I activity, no changes in cardiac long-chain acyl-carnitine content have been observed after treatment with sodium pivalate (*Broderick*, 1995).

Previously, it has been shown that pivalate treatment depletes the mitochondrial free CoA content in hepatocytes due to the formation of pivaloylCoA (*Ruff*, 1991). Moreover, the decrease in acetyl-carnitine content in various tissues, including heart was observed after the treatment with pivalate (*Broderick*, 1995; *Kato*, 2012; *Nakajima*, 1999). Our results obtained in cardiac

mitochondria are in agreement with the previously mentioned observations because the CrAT activity was significantly decreased. CrAT modulates the acetylCoA/free CoA ratio (*Zammit*, 2009), and it has been shown to regulate the myocardial substrate supply with respect to metabolic demand (*Schroeder*, 2012). The accumulation of acetylCoA and the decrease in free CoA content is known to decrease PDH complex activity (*Stanley*, 2005) and it can explain the observed decrease in mitochondrial respiration with pyruvate and malate. Thus, unlike meldonium, pivalate inhibits both FA and pyruvate metabolism and depletes CoA content, and thus it does not induce a metabolic shift and lacks any cardioprotective effect.

In conclusion, present findings indicate that pivalate induces changes in the L-carnitine homeostasis without affecting cardiac functionality, although pivalate or its metabolites directly impair mitochondrial energy metabolism. Thus, pivalate is not an appropriate tool to reduce the availability of L-carnitine and acyl-carnitines.

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3.3.2. The effects of BBOX and OCTN2 inhibitors on L-carnitine availability and cardiac ischemia-reperfusion injury

Since BBOX and OCTN2 represent two approaches for reduction of L-carnitine availability, the effects of 50 compounds on L-carnitine formation by BBOX and OCTN2-mediated L-carnitine transport were tested, and the cardioprotective activity of selected compounds was evaluated. We found a number of compounds with inhibitory activity in the low µM range, and several of the tested compounds were found to be relatively selective inhibitors of either BBOX or OCTN2. Thus, these compounds could be used to compare BBOX and OCTN2 as molecular drug targets to reduce L-carnitine content and to protect the heart against ischemia-reperfusion induced myocardial infarction.

A small fraction of the total L-carnitine content circulates in the blood because the OCTN2-mediated reabsorption capacity of L-carnitine is limited to 50 μM (*Pochini*, 2013). In addition, OCTN2 is widely expressed in tissues and maintains a high tissue concentration of L-carnitine. Therefore, to induce significant changes in the L-carnitine content in the heart and other tissues, long-term simultaneous inhibition of OCTN2-dependent L-carnitine transport in tissues and reabsorption in the kidneys is necessary. After 14 days of treatment by OCTN2 inhibitors induced a significant reduction in the L-carnitine content in the cardiac tissues, and the inhibition of OCTN2 (IC_{50} values) strongly correlated with the reduction of L-carnitine content in the heart. In comparison, selective inhibitors of BBOX after long-term treatment at the same dose reduced L-carnitine in the cardiac tissues by only 20%. Overall, ***selective inhibitors of OCTN2 more effectively decrease the availability of L-carnitine in the cardiac tissues compared to the inhibitors of BBOX.***

The decrease in L-carnitine cardiac content protects cardiac mitochondria against an overload of long-chain acyl-carnitines (*Liepinsh*, 2013a), and in case of meldonium 14 days of treatment are required to induce the cardioprotective effect in the heart (*Liepinsh*, 2006). Previously, it has been demonstrated that it is necessary to reduce L-carnitine content in the heart by at least 60% to decrease L-carnitine-dependent FA metabolism and concomitantly stimulate glucose metabolism (*Kuka*, 2012; *Liepinsh*, 2008, 2009, 2013a). In the present study, only treatment with the most potent OCTN2 inhibitor Chloro-GBB induced a significant reduction in the myocardial infarct size in the rat heart. In comparison, the less potent inhibitor Dimethyl-GBB did not significantly reduce infarct size at the same dose. This can be explained by an insufficient reduction of L-carnitine content in the heart. Treatment by potent inhibitors of BBOX did not induce any significant changes in L-carnitine content in the cardiac tissues and therefore did not protect against the myocardial infarction. Thus***, the inhibition of OCTN2, compared to the***

inhibition of BBOX, is a far more effective approach to decrease L-carnitine content and to reduce infarct size in the heart.

3.4. The reduction of L-carnitine availability as a tool to prevent acyl-carnitine accumulation and achieve cardioprotection

This study demonstrates that Methyl-GBB, a novel inhibitor of BBOX and OCTN2, protects myocardium from ischemia and reperfusion induced damage in the isolated rat heart and *in vivo* setups. Methyl-GBB effectively reduces content of acyl-carnitines in heart and mitochondria, limits FA oxidation and in turn stimulates glucose oxidation in the cardiac tissues by lowering L-carnitine availability. Despite these significant changes in cardiac metabolism, no evidence of cardiac dysfunction was observed. Different approaches have been used to partially inhibit FA oxidation and switch energy metabolism from FA to glucose oxidation (*Dyck*, 2004; *Kantor*, 2000; *Lopaschuk*, 1989). CPT I is considered to be a rate-limiting enzyme for FA transport into mitochondria and is often targeted to achieve the inhibition of FA metabolism (*Bentebibel*, 2006; *Lopaschuk*, 1989; *Unger*, 2005). In addition to the direct inhibition of CPT I or to an increase in malonylCoA content, a decrease in L-carnitine concentration in cardiac tissues was found to be one of the most effective ways to decrease the activity of CPT I. For a long time, the only non-toxic and effective compound that decreased the concentration of L-carnitine and in turn had protective effects against cardiovascular diseases was meldonium (*Dambrova*, 2002; *Schürch*, 2010). In comparison, newly synthesized Methyl-GBB is a 10- to 20-times more potent inhibitor of BBOX and OCTN2, and it is more effective in reducing the L-carnitine concentration in tissues. Thus, at a dose 20 times lower than a dose of meldonium, Methyl-GBB induced a similar L-carnitine-lowering effect in the cardiac tissues.

As it has been demonstrated previously, the cardioprotective effects of meldonium depend on a decreased L-carnitine content in the heart tissues (*Kuka*, 2012). In the present study, the time-dependent reduction in L-carnitine content correlated with the cardioprotective effect induced by Methyl-GBB; nevertheless, we observed some significant differences between the actions of Methyl-GBB and meldonium. After only 7 days of treatment, Methyl-GBB reached the same maximal infarct size-limiting effect induced by meldonium, while after 14 days of treatment with Methyl-GBB, the size of the infarcted area in an isolated heart model was 2-times smaller compared to the meldonium treated hearts. The effect of Methyl-GBB on limiting the infarct size was also confirmed in an *in vivo* myocardial infarction model. Similar to the effects observed in isolated hearts, the effects of limiting the infarct size induced by Methyl-GBB in the *in vivo* model were almost 2-times better than the effects induced by meldonium (*Sesti*, 2006). In addition, Methyl-GBB treatment improved rat survival at 24 h after myocardial infarction *in vivo*. Overall, ***Methyl-GBB is more potent cardioprotective agent than meldonium.***

Treatment with Methyl-GBB induced a decrease in L-carnitine availability and production of acyl-carnitines and it reduced palmitate oxidation in isolated mitochondria and peroxisomes and in isolated hearts. The main advantage of this pharmacological approach is the reduction of direct damage by the long-chain acyl-carnitines on mitochondria in an ischemic heart. In contrast, the metabolism of FAs in Methyl-GBB-treated hearts was not decreased in reperfusion. Thus, while the protection against long-chain acyl-carnitine accumulation-induced damage is beneficial in ischemia when the oxygen supply is limited, the activation of FA metabolism in reperfusion would help to restore energy production in the heart.

Enhanced glucose oxidation is beneficial during ischemia because of the reduced proton production and less oxygen spent per ATP produced (*Ussher*, 2012). In this study we also observed that ***after Methyl-GBB treatment the***

decreased content of long-chain acyl-carnitines and inhibited FA oxidation in turn induced stimulation of glucose oxidation in the heart. These results suggest that reduced infarct size is at least partially associated with significantly stimulated glucose and lactate oxidation.

In conclusion, these results demonstrate that Methyl-GBB treatment leads to cardioprotection and improves animal survival by limiting FA oxidation and facilitating glucose metabolism.

4. CONCLUSIONS

1. Cardiac ischemia-reperfusion injury is less severe in the fed state due to enhanced glucose and lactate oxidation and a lower load of FAs.
2. The long-chain acyl-carnitine concentration orchestrates the interplay between the metabolism of glucose (pyruvate/lactate) and long-chain FAs in cardiac mitochondria and the heart. In addition, the accumulation of long-chain acyl-carnitines impairs glucose metabolism.
3. Sodium pivalate induces changes in L-carnitine homeostasis without affecting cardiac functionality, although pivalate or its metabolites can directly impair mitochondrial energy metabolism. Thus, pivalate is not an appropriate pharmacological tool to reduce the availabilities of L-carnitine and acyl-carnitines.
4. The selective inhibition of OCTN2 compared with that of BBOX is a far more effective approach for decreasing the L-carnitine concentration and inducing cardioprotective effects.
5. Methyl-GBB, a novel inhibitor of BBOX and OCTN2, effectively reduces the concentrations of acyl-carnitines in the heart and mitochondria and limits FA oxidation, thereby stimulating glucose oxidation in heart tissues by lowering L-carnitine availability. Methyl-GBB treatment leads to cardioprotection and improved survival after myocardial infarction.

5. APPROBATION OF THE STUDY – PUBLICATIONS AND THESIS

Doctoral thesis is based on following SCI publications:

1. Kuka J, Makrecka M, Grinberga S, Pugovics O, Liepinsh E, Dambrova M. A short-term high-dose administration of sodium pivalate impairs pyruvate metabolism without affecting cardiac function. *Cardiovascular Toxicology*, **2012**, 12(4):298–303.
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Results are reported in the following international conferences:

1. Dambrova M, Makrecka M, Kuka J, Vilskersts R, Liepinsh E. Discovery of a novel regulator of cardiac energy metabolism, cardioprotective drug methyl-GBB. *The 17th World Congress of Basic and Clinical Pharmacology (WCP2014)*, Cape Town, South Africa, July 13–18, **2014**. Basic & Clinical Pharmacology & Toxicology, 115 (Suppl. 1), p.19.
2. Liepinsh E, Makrecka M, Kuka J, Antone U, Dambrova M. The heart is better protected against myocardial infarction in the fed state compared to the fasted state: role of insulin, glucose and fatty acids. *The 17th World Congress of Basic and Clinical Pharmacology (WCP2014)*, Cape Town, South Africa, July 13–18, **2014**. Basic & Clinical Pharmacology & Toxicology, 115 (Suppl. 1), p.49.
3. Makrecka-Kūka M. Acyl-carnitines as a druggable target in mitochondria. *Balticum Organicum Syntheticum (BOS-2014) pre-conference round table "New Therapeutic Targets: Chemistry and Biology"*, Malpils, Latvia, July 3–4, **2014**.
4. Makrecka M, Kuka J, Volska K, Dambrova M, Liepinsh E. The accumulation of long-chain acyl-carnitines determines the severity of cardiac ischemia-reperfusion injury. *SHVM 2014 Twelfth annual scientific sessions - Strategies for recovering metabolic homeostasis and ventricular function in the diseased heart,* Tromso, Norway, June 24–27, **2014,** Book of Abstracts, p. 43.
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