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

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ANNOTATION

Ischemic heart disease is a major cause of disability and death in millions of people annually. The important pathological consequences of ischemic heart disease arise from impaired cellular energy metabolism. Therefore, a pharmacological intervention that targets cardiac energy metabolism pathways is suggested for the development of novel treatment strategies to improve the clinical outcomes of patients with ischemic heart disease. L-carnitine, a cofactor of acyltransferases, participates in the regulation of mitochondrial energy metabolism. The aim of the thesis was to elucidate the roles of L-carnitine and its metabolites in the regulation of cardiac energy metabolism and to discover novel drug targets to achieve cardioprotection.

This thesis describes the importance of glucose and fatty acid energy metabolism pattern in the outcome of cardiac ischemia-reperfusion injury, the role of long-chain acyl-carnitines in the regulation of energy metabolism, and the decreasing of the L-carnitine concentration as a strategy to regulate acyl-carnitine availability. The results demonstrate that 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. A decrease in acyl-carnitine availability can be achieved by lowering the L-carnitine concentration. The inhibition of both L-carnitine transport via OCTN2 and its biosynthesis via γ-butyrobetaine dioxygenase represent potential stategies for decreasing L-carnitine. The results show that the selective inhibition of L-carnitine transport via OCTN2 compared with the selective inhibition of γ-butyrobetaine dioxygenase is a far more effective approach for decreasing the L-carnitine concentration and inducing cardioprotective effects. A novel cardioprotective agent, Methyl-GBB, a novel inhibitor of γ-butyrobetaine dioxygenase 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. Methyl-GBB decreases infarct size and improves survival after myocardial infarction *in vivo* in rats.

This thesis demonstrates that cardioprotection can be achieved by the inhibition of L-carnitine transport via OCTN2, thereby decreasing L-carnitine and acyl-carnitine availability and stimulating glucose metabolism.

DARBA ANOTĀCIJA

Sirds išēmiskā slimība ik gadu ir galvenais invaliditātes un nāves cēlonis miljoniem cilvēku. Sirds išēmiskās slimības patoģenēze saistīta ar traucētu šūnu enerģijas metabolismu. Tāpēc farmakoloģiska sirds enerģijas metabolisma signālceļu regulācija tiek piedāvāta kā jauna ārstēšanas stratēģija, lai uzlabotu klīnisko iznākumu pacientiem ar sirds išēmisko slimību. L-karnitīns ir aciltransferāžu kofaktors, kas piedalās enerģijas metabolisma regulācijā mitohondrijos. Promocijas darba mērķis bija noskaidrot L-karnitīna un tā atvasinājumu lomu sirds enerģijas metabolisma regulācijā un atklāt jaunus kardioprotektīvu zāļu mērķus L-karnitīna sistēmā.

Promocijas darbā aprakstīta glikozes un taukskābju enerģijas metabolisma izmaiņu ietekme uz sirds išēmijas-reperfūzijas bojājumu, garķēžu acil-karnitīnu loma enerģijas metabolisma regulācijā, kā arī L-karnitīna daudzuma samazināšana, lai regulētu acil-karnitīnu pieejamību. Iegūtie rezultāti parāda, ka garķēžu acil-karnitīnu koncentrācija nosaka enerģijas metabolisma norises sirdī. Turklāt, garķēžu acil-karnitīnu uzkrāšanās pasliktina glikozes metabolismu un palielina sirds išēmijas-reperfūzijas bojājumu. Garķēžu acil-karnitīnu daudzumu varētu samazināt, samazinot L-karnitīna koncentrāciju. Kā veidi L-karnitīna samazināšanai varētu izmantot OCTN2 nodrošinātā L-karnitīna transporta un enzīma γ-butirobetaīna dioksigenāzes veicināto L-karnitīna biosintēzes kavēšanu. Darbā parādīts, ka, salīdzinot ar selektīvu L-karnitīna biosintēzes kavēšana, inhibējot γ-butirobetaīna dioksigenāzi, selektīva OCTN2 nodrošinātā karnitīna transporta inhibēšana ir daudz efektīvāks veids, lai samazinātu L-karnitīna daudzumu un sasniegtu kardioprotektīvo efektu. Atrasts jauns kardioprotektīvs savienojums Metil-GBB, kas darbojas kā γ-butirobetaīna dioksigenāzes un OCTN2 inhibitors. Metil-GBB efektīvi samazina acil-karnitīnu daudzumu sirdī un mitohondrijos, kavē taukskābju oksidāciju un vienlaicīgi stimulē glikozes oksidāciju sirds audos. Metil-GBB samazina infarkta lielumu un uzlabo izdzīvošanu pēc miokarda infarkta *in vivo* žurku eksperimentālajā modelī.

Promocijas darbā parādīts, ka kardioptotektīvo efektu var sasniegt, kavējot OCTN2 nodrošināto L-karnitīna transportu, tādējādi samazinot L-karnitīna un garķēžu acil-karnitīnu daudzumu un stimulējot glikozes metabolismu.

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ABBREVIATIONS

ACBP – acylCoA binding proteins

ACC – acetylCoA carboxylase

ACS – long-chain acylCoA synthase

ADP – adenosine diphosphate

AMI – acute myocardial infarction

AMPK – adenosine monophosphate-activated protein kinase

AN – area of necrosis

AR – area at risk

ATP – adenosine triphosphate

BBOX – GBB dioxygenase

BSA – bovine serum albumin

CACT – carnitine translocase

cDNA – complementary deoxyribonucleic acid

Chloro-GBB – 4-((chloromethyl)dimethylammonio)butanoate

CoA – coenzyme A

CPT I – carnitine palmitoyltransferase I, CPT IA (liver type) and CPT IB (muscle type)

CPT II – carnitine palmitoyltransferase II

CrAT – carnitine acetyltransferase

Dimethyl-GBB – 4-[diethyl(methyl)ammonio]butanoate

DMEM – Dulbecco's Modified Eagle Medium

dP/dt max – contractility

dP/dt min – relaxation

ECG – electrocardiography

Et-Me-meldonium – 3-(1,1,2-trimethyl-1-propylhydrazin-1-ium-2-yl)propanoate

FA – fatty acids

FABP – fatty acid binding protein

$FADH_2$ – reduced flavin adenine dinucleotide

FAT/CD36 – FA translocase

FATP – FA transport protein

FBS – fetal bovine serum

GBB – γ-butyrobetaine

GBB-phoshinate – (3-(trimethylammonio)propyl)phosphinate

GIK – glucose-insulin-potassium

GK rats – Goto-Kakizaki rats

GLUT – glucose transporter

HEK293 – human embryonic kidney cell line

HK2 – hexokinase 2

HR – heart rate

HR*LVDP – cardiac workload

HSL – hormone-sensitive lipase

HTML – 3-hydroxy-TML

HTMLA – HTML aldolase

IC_{50} – half maximal inhibitory concentration

IS – infarct size

KH buffer – Krebs-Henseleit buffer

K_m – Michaelis constant

LAD – left anterior descending coronary artery

LDH – lactate dehydrogenase

LEAK state – respiration state induced by inhibition of adenine nucleotide translocator

LV – left ventricular

LVDP – left ventricular developed pressure

MCD – malonylCoA decarboxylase

Methyl-GBB – 4-(ethyldimethylammonio)butanoate

MPC – mitochondrial pyruvate carrier

mRNA – messenger ribonucleic acid

NAD –nicotinamide adenine dinucleotide

NADH – reduced nicotinamide adenine dinucleotide

OCTN – organic cation transporter

OXPHOS state – ADP-stimulated mitochondrial respiration

P-Akt – phosphorylated protein kinase B

PBS – phosphate buffer solution

PDH – pyruvate dehydrogenase

PDK – PDH kinase

PGC1-α – peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPAR-α – peroxisome proliferator-activated receptor alpha

RT-PCR – real-time polymerase chain reaction

TG – triglycerides

TMABA – 4-trimethylaminobutyraldehyde

TMABA-DH – TMABA dehydrogenase

TML – N^{ε}-trimethyllysine

TMLD – TML dioxygenase

UCP – uncoupling protein

UPLC MS/MS – ultra-performance liquid chromatography tandem mass-spectrometry

VLDL – very-low-density lipoproteins

WB – western blot

INTRODUCTION

Ischemic heart disease is a major health problem that causes 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. Therefore, pharmacological interventions that target cardiac energy metabolic pathways have been suggested as novel treatment strategies to improve the clinical outcomes of patients with ischemic heart disease (*Ardehali*, 2012; *Jaswal*, 2011; *Kloner*, 2013; *Lopaschuk*, 2010). In addition, the pharmacological manipulation of energy metabolism does not directly influence the hemodynamic parameters of the heart and may be combined with existing cardiovascular therapies (*Kalra*, 2012; *Palaniswamy*, 2011).

Heart function is highly dependent on energy derived from ATP generated during oxidative phosphorylation in mitochondria (*Dzeja*, 2000). The most important energy substrates in the heart are fatty acids (FAs) and glucose as well as their metabolic intermediates, such as lactate, pyruvate and ketone bodies (*Stanley*, 2005b). 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 for further β-oxidation. Another L-carnitine-dependent enzyme, carnitine acetyltransferase, regulates the acetylCoA/free CoA ratio, which is essential for pyruvate dehydrogenase activity. Because L-carnitine takes part in the regulation of both FA and glucose metabolism, changes in L-carnitine availability could optimize cardiac energy metabolism and represent a potential target for the treatment of ischemic heart disease. 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, 2011a, 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 cardiac tissues. 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. LITERATURE

1.1. Cardiac energy metabolism

An effective energy metabolism is essential for the maintenance of cardiac function. The energy requirements of the heart are very high (~30 μmol ATP/min/g wet weight at rest) and ATP storage capacity is very low (5 μmol ATP/g wet weight), thus the complete turnover of the cardiac ATP pool happens every 10 s (*Jaswal*, 2011; *Lopaschuk*, 2010; *Neely*, 1974). To sustain sufficient production of ATP heart acts as a metabolic omnivore and uses a variety of different substrates as energy sources. The most important energy substrates in the heart are fatty acids (FAs) and glucose as well as their intermediates, such as lactate, pyruvate and ketone bodies (*Stanley*, 2005b). The healthy normal heart possesses exceptional metabolic flexibility and is able to switch rapidly between the available energy substrates. The loss of this ability is associated with the development of cardiovascular disease (*Turer*, 2010). Therefore, clarification of factors that determine energy metabolism pattern in the heart can provide a novel drug targets for the treatment of cardiovascular diseases.

1.1.1. Fatty acid metabolism

The rate of FA metabolism in the heart is highly dependent on the concentration of non-esterified FAs in the plasma. FAs are transported in the plasma either bound to albumin or in form of lipoproteins (chylomicrons or very-low-density lipoproteins (VLDL) both containing triglycerides (TG)), and both forms significantly contribute to the overall FA supply to cardiac cells (*van der Vusse*, 2000). Normally the range of FA concentration in the plasma varies from 0.2 mM to 0.8 mM, however, under conditions of metabolic stress (e.g. starvation, ischemia, diabetes) the concentration of FAs can reach more than 1.0 mM (*Peterson*, 2012; *Roy*, 2013). This increase in circulating free FAs determines an increase in fatty acid uptake rate in the heart.

Fatty acids enter the cardiac cell by either passive diffusion or via protein carrier-mediated pathway (*Glatz*, 2010; *Lopaschuk*, 2010) (Figure 1.1.). Since passive diffusion is a relatively slow process and it is not sufficient to fulfil metabolic demands of the cardiac cell, the protein carrier-mediated FA transport is suggested as being predominant. There are three types of plasma membrane-associated proteins involved in the free FAs transport into the cell: plasma membrane-bound fatty acid binding protein (FABPpm), FA transport protein (FATP1 and heart specific FATP6) and FA translocase (FAT/CD36). Of these FA carriers, FAT/CD36 is considered to play a major role in the FA transport into the cardiac cell. The studies using

FAT/CD36 knockout mice demonstrated that 40-60% of FA oxidation by heart occurs via FAT/CD36-mediated transport (*Kuang*, 2004; *Pietka*, 2012). Moreover, patients with CD36 deficiency have reduced FA uptake in the heart, but not in the liver (*Hames*, 2014; *Tanaka*, 1997). Taken together, these data indicate a key role of FAT/CD36 in regulating the cardiac FA metabolism.

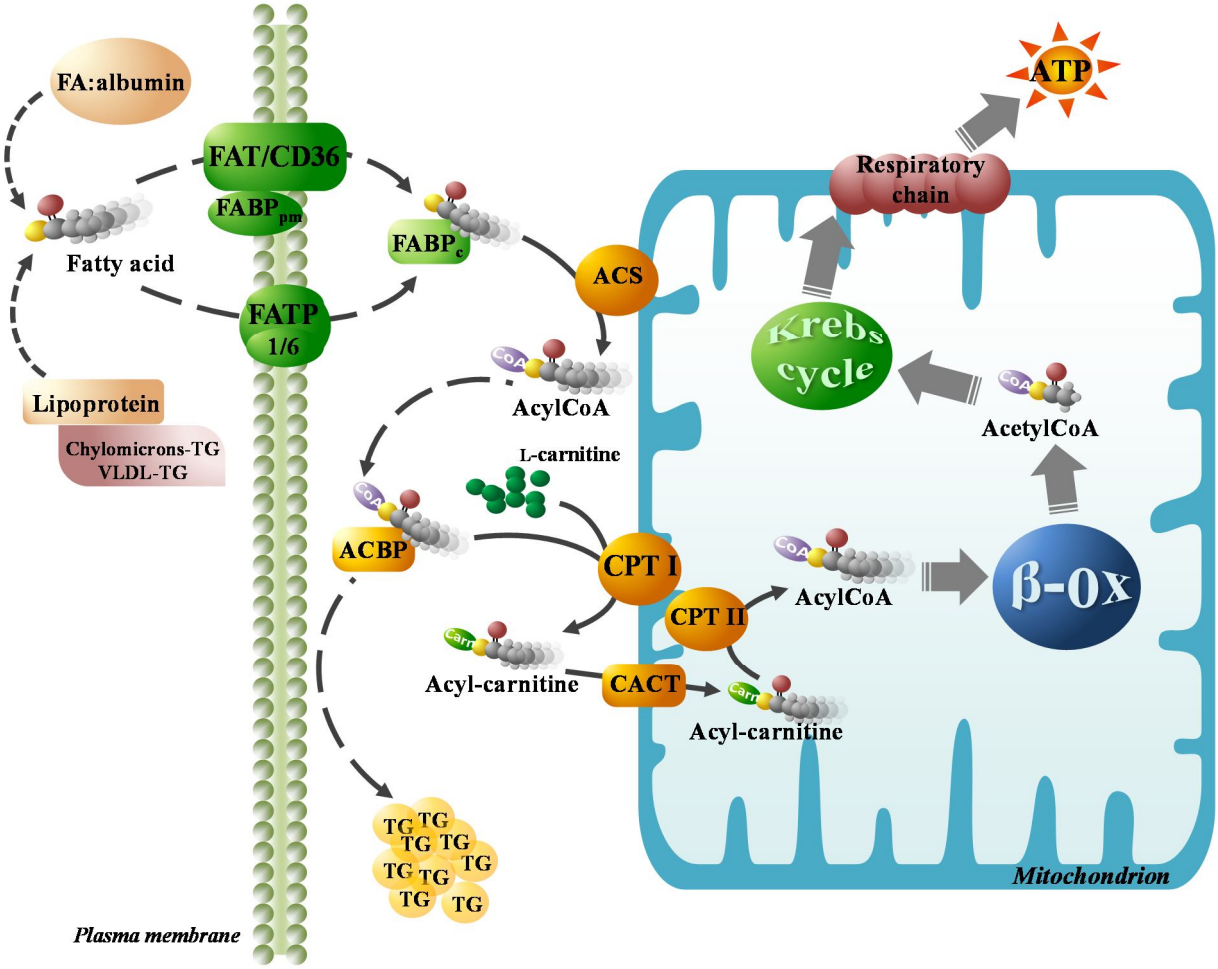


Figure 1.1. **Fatty acid metabolism in cardiac cell**
ACBP – acylCoA binding protein; ACS – long-chain acylCoA synthase; ATP – adenosine triphosphate; β-ox – β-oxidation; CACT – carnitine translocase; CPT I – carnitine palmitoyltransferase I; CPT II – carnitine palmitoyltransferase II; FA – fatty acid; FABP – fatty acid binding protein; FAT/CD36 – FA translocase; FATP – FA transport protein; TG – triglycerides; VLDL – very-low-density lipoproteins

Free FAs are highly hydrophobic molecules and can be harmful for cell; thus, intracellular FAs are associated with proteins or are covalently bound to coenzyme A (CoA) or L-carnitine. Therefore, immediately after being transported into the cell, FAs bind cytosolic FABP and are activated to acylCoAs via long-chain acylCoA synthase (ACS). The major cytosolic FABP in cardiac tissues is heart FABP (HFABP, FABP3) that appears to determine cellular FA traffic to mitochondria for further β-oxidation (*Storch*, 2010). It was suggested that the FAT/CD36 is physically coupled to FABP or ACS at the cytosolic side of the

membrane, thus supporting immediate FA activation (*Schaffer*, 2002). Nevertheless, a recent study demonstrated that, instead of physical, there is more likely to be a functional interaction between FAT/CD36 and ACS, increasing the overall efficiency of FA metabolism (*Schneider*, 2014). It was suggested that FAT/CD36 facilitates FA transport across the plasma membrane; subsequently FABP transports FAs between plasma membrane and intracellular membrane, where ACS acts as a metabolic trap of FAs (*Schneider*, 2014).

Since long-chain acylCoAs are water-soluble molecules and act as detergents, in order to protect membranes of cellular structures, once FAs are activated, the respective acylCoAs bind to acylCoA binding proteins (ACBP) which have similar properties as FABP (*Færgeman*, 2007; *Knudsen*, 2000). Depending on the cardiac metabolic demand, long-chain acylCoAs can be either delivered to mitochondria for subsequent β-oxidation or undergo synthesis of TG for temporary storage in cytosolic lipid droplets (Figure 1.1). The lipid droplets can be mobilized by lipid hydrolases in periods of energetic need; thus, the cardiac TG pool represents an important source of FAs (*Kienesberger*, 2013; *Murphy*, 2009). In healthy normal heart only 10-30% of FAs taken up by the heart enter cardiac TG pool, other 70-90% are oxidized in mitochondria (*Lopaschuk*, 2010; *Stanley*, 2005b).

Before entering the β-oxidation pathway, acylCoAs have to be transported into the mitochondria. In contrast to medium- and short- chain acylCoAs, long-chain acylCoAs cannot directly pass the inner mitochondrial membrane, and their mitochondrial uptake is mediated by the L-carnitine-dependent transport system. As the first step, acylCoAs are converted to respective acyl-carnitines by carnitine palmitoyltransferase I (CPT I). Formed acyl-carnitines are subsequently transported into the mitochondria by carnitine translocase (CACT) in exchange for free L-carnitine. Lastly, acyl-carnitines are converted back to the respective acylCoAs by carnitine palmitoyltransferase II (CPT II) in the mitochondrial matrix.

CPT I is a regulatory step in mitochondrial acylCoA uptake, which determines the acylCoA flux to β-oxidation. There are 2 enzyme isoforms in cardiac tissues: CPT IA (liver type) and CPT IB (muscle type) (*Weis*, 1994). Both isoforms have significantly different kinetic and regulatory properties (Table 1.1). For example, CPT IA demonstrates a higher affinity for L-carnitine than CPT IB. The activity of CPT I is regulated by changes in the concentration of malonylCoA. MalonylCoA is synthesized from the cytosolic acetylCoA by acetylCoA carboxylase and it acts as a potent allosteric inhibitor of CPT I (*Zammit*, 2008). CPT IA demonstrates lower affinity for malonylCoA than CPT IB. Moreover, in contrast to CPT IB, sensitivity of CPT IA to malonylCoA decreases during fasting. Although both CPT I isoforms are present in the cardiac tissue, no changes in IC_{50} value for malonylCoA were observed in the cardiac mitochondria during fasting, suggesting that in cardiac tissues changes

in malonylCoA concentration, rather than altered sensitivity, appear to be the main regulatory factor in FA transport into mitochondria (*Kerner*, 2000).

Table 1.1.

The characterisation of carnitine palmitoyltransferase I

	CPT IA	*CPT IB*	*Cardiac tissues (CPT IA + IB)*
K_m for L-carnitine, μM	30	500	200
K_m for palmitoylCoA, μM	30	60	50
MalonylCoA IC_{50}, μM	2.7	0.03	0.1
Modulation of sensitivity to malonylCoA by nutritional state	+	-	-

The data from *McGarry*, 1983; *Saggerson*, 1982.

Traditionally, the L-carnitine-dependent transport system was considered to be the only pathway of FA transport across the mitochondrial membrane. In 2004, the presence of FAT/CD36 in cardiac and muscle mitochondrial membrane was demonstrated (*Campbell*, 2004). Further studies in FAT/CD36 knock-out mice did not result in an unified conclusion about the role of FAT/CD36 in mitochondrial FA oxidation. It has been shown that FA oxidation is slightly reduced in mitochondria or permeabilized fibers isolated from skeletal muscle of FAT/CD36 knock-out mice (*Holloway*, 2009; *Smith*, 2011). However, in another study there was no difference between FAT/CD36 knock-out and control mice in FA oxidation rate by isolated mitochondria from cardiac tissues (*King*, 2007). Despite of controversial data, the general agreement is that FAT/CD36 is located on the outer mitochondrial membrane and it facilitates FA uptake into mitochondria via CPT I. This pathway via CPT I is supported by the fact that the removal of L-carnitine from the reagent mixture prevents palmitate oxidation by isolated mitochondria (*Campbell*, 2004). It is proposed that FAT/CD36 is not in direct contact with CPT I, but it acts as an acceptor which delivers FAs to ACS for further activation and transport into mitochondria via CPT I (Figure 1.2.) (*Lee*, 2011; *Smith*, 2011).

Once transported into the mitochondria, long-chain acylCoAs undergo β-oxidation, where they are repeatedly shortened by 2 carbon units producing acetylCoA, NADH and $FADH_2$. The β-oxidation pathway consists of four sequent steps regulated by respective enzymes: acylCoA dehydrogenase, enoylCoA hydratase, 3-hydroxyacylCoA dehydrogenase and 3-ketoacylCoA thiolase. Each of these enzymes exist in different isoforms, possess different chain length specificities and are sensitive to inhibition by the product of respective enzymatic reaction (*Lopaschuk*, 2010). Finally, acetylCoAs produced from β-oxidation are

further metabolized in the Krebs cycle generating more NADH and $FADH_2$, which act as electron donors for the mitochondrial respiratory chain and subsequent oxidative phosphorylation process.

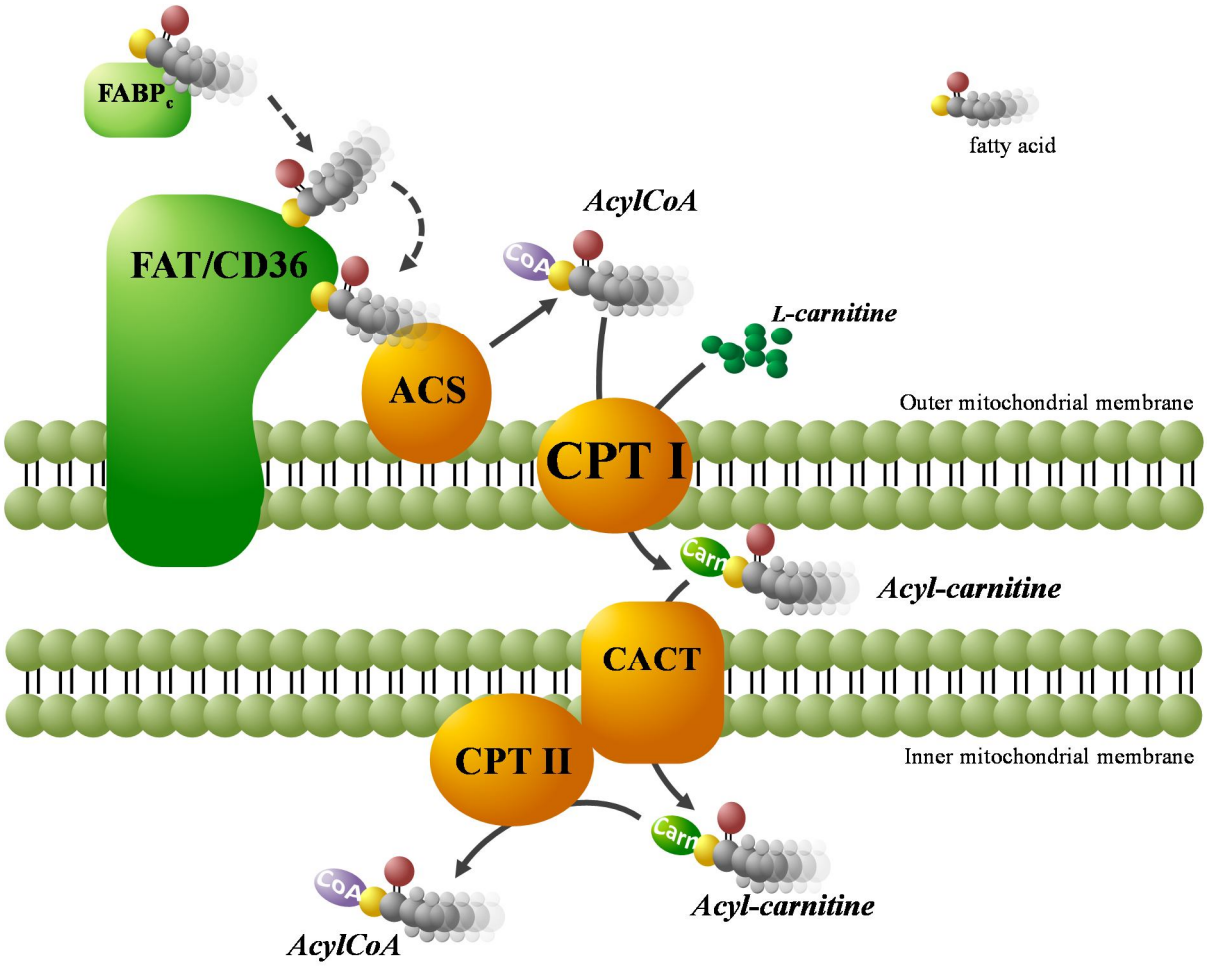


Figure 1.2. **Proposed model of fatty acid transport into the mitochondria**
Adapted from *Lee*, 2011; *Smith*, 2011.
ACS – long-chain acylCoA synthase; CACT – carnitine translocase; CPT I – carnitine palmitoyltransferase I; CPT II – carnitine palmitoyltransferase II; FABP – fatty acid binding protein; FAT/CD36 – FA translocase

1.1.2. Glucose metabolism

In normal healthy heart, glucose metabolism contributes to 10-40% of produced acetylCoAs for further energy production (Figure 1.3) (*Stanley*, 2005b). Glucose is supplied to the cell either by transport of circulating glucose or by release from intracellular glycogen.

One of the rate limiting steps in cardiac glucose metabolism is glucose uptake that is regulated by glucose transporters, predominantly, GLUT1 and GLUT4. Both transporters exist in the intracellular vesicles which circulate between the plasma membrane and the intracellular compartment. It has been demonstrated that under basal conditions 50-60% of GLUT1 is located in the plasma membrane, while more than 80% of GLUT4 is located

intracellularly (*Egert*, 1999; *Slot*, 1991). The GLUT4 translocates to the plasma membrane in the response to insulin stimulation, when glucose transport into the cardiac cell is significantly increased (*Egert*, 1997; *Fischer*, 1997). Study in cardiac specific GLUT4 knock-out mice demonstrated that the deletion of GLUT4 in the heart induces the upregulation of GLUT1 expression, thus, rate of glucose uptake is increased under basal conditions, however, no insulin stimulated action is observed (*Abel*, 1999). Moreover, in the heart GLUT1 expression is more highly regulated by metabolic alterations (for example, fasting and hyperinsulinemia) than is GLUT4 (*Abel*, 2002; *Montessuit*, 2013). Taken together, these data indicate that the rate of cardiac glucose uptake is regulated by both substrate and insulin availability.

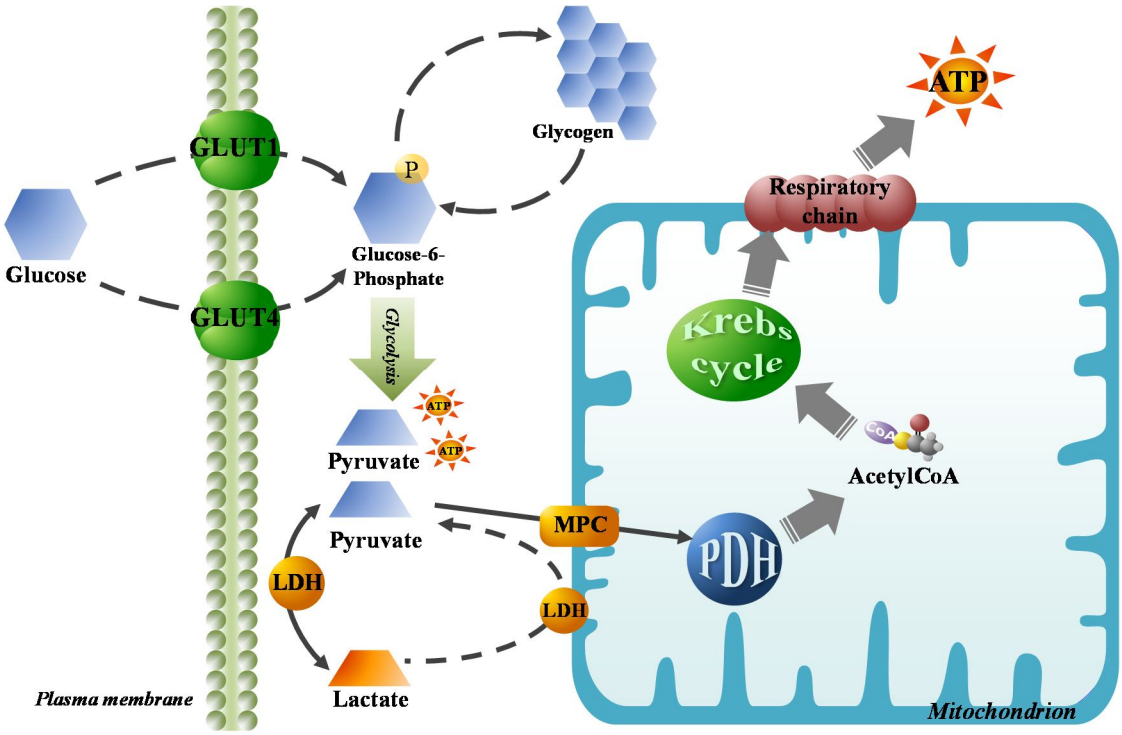


Figure 1.3. **Glucose metabolism in the cardiac cell**
ATP – adenosine triphosphate; GLUT – glucose transporter; LDH – lactate dehydrogenase; MPC – mitochondrial pyruvate carrier; PDH – pyruvate dehydrogenase

In the cell hexokinase phosphorylates glucose to yield glucose-6-phosphate which can act as a substrate for synthesis of glycogen or it can be metabolized in the glycolysis pathway. Traditionally, it has been considered that glycogen is just immediate source of glucose under metabolic stress conditions in the heart (*Depre*, 1999). However, it has been demonstrated that glycogen is used for energy production under physiological conditions and it appears to be preferentially oxidized compared to the exogenous glucose (*Goodwin*, 1998; *Henning*, 1996; *Karlstaedt*, 2012). These facts are also supported by the rapid turnover of glycogen and its relatively low concentration in the cardiac tissues (*Goodwin*, 1998; *Henning*, 1996; *Kokubun*, 2009; *Pederson*, 2004).

The next step in the glucose metabolism pathway is glycolysis, where 1 molecule of glucose-6-phosphate is converted to 2 molecules of pyruvate, also generating 2 molecules of ATP. Pyruvate produced in glycolysis process can be either decarboxylated to acetylCoA or converted to lactate by lactate dehydrogenase (LDH). Since mitochondria are not able to oxidize pyruvate as fast as it is produced, lactate as an inactive form of pyruvate is sometimes accumulating in the cell. Several studies have provided evidence that lactate can be directly taken up by the mitochondria and oxidized in the mitochondrial matrix, suggesting intracellular lactate shuttle hypothesis (*Brooks*, 2009; *Cruz*, 2012). However, recently it has been shown that conversion to pyruvate is required prior lactate can be oxidized in mitochondria (*Elustondo*, 2013; *Jacobs*, 2013). In addition, mitochondrial LDH location inside the outer mitochondrial membrane, but not in the matrix, has been demonstrated (*Elustondo*, 2013).

For further oxidation, pyruvate should be transported into the mitochondrial matrix. Pyruvate is a small molecule and it can freely diffuse from cytosol to the mitochondrial intermembrane space. However, due to the maintenance of the proton gradient by the inner mitochondrial membrane, such charged molecules like pyruvate cannot freely reach mitochondrial matrix (*Gray*, 2014). Recently mitochondrial pyruvate carrier (MPC) was identified (*Bricker*, 2012; *Herzig*, 2012). The MPC is not yet fully characterized and the mechanism of its regulation remains unknown. Meanwhile, MPC appears to be essential for cytosolic pyruvate transport to the mitochondrial matrix.

Despite that pyruvate has several potential metabolic paths in the mitochondrial matrix, the majority is decarboxylated to acetylCoA, which can be further oxidized in the Krebs cycle (*Depre*, 1999; *Gray*, 2014). Decarboxylation of pyruvate is catalysed by pyruvate dehydrogenase (PDH) complex. The PDH consists of 3 enzymes, pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3), which subsequently catalyzes the metabolism of pyruvate and formation of acetylCoA, CO_2 and NADH (*Patel*, 2014). Thus, PDH is a key step in glucose metabolism linking glycolytic pathway with Krebs cycle and oxidative phosphorylation. The activity of PDH is tightly regulated by phosphorylation and dephosphorylation processes activated by PDH substrate and products. PDH is inactivated by phosphorylation by a specific PDH kinase (PDK) and it is activated by dephosphorylation by a specific PDH phosphatase (*Holness*, 2003; *Kolobova*, 2001). Thus, the activity of PDH is determined by the balance between PDK and PDH phosphatase activities.

PDK appears to be the main regulator of PDH activity. The increase in acetylCoA/free CoA and NADH/NAD ratios activates PDK, leading to the reduction in PDH activity

(*Holness*, 2003; *Stanley*, 2005b). There are 3 out of 4 isoforms of PDK in the cardiac tissues: PDK1, PDK2 and PDK4 (*Bowker-Kinley*, 1998; *Wu*, 1998). Studies have demonstrated that in the heart PDK4 expression is highly regulated by such metabolic alterations as starvation and high fat diet (*Wu*, 1998; *Zhang*, 2012). Moreover, it has been demonstrated that the overexpression of PDK4 in the heart results in a decrease in glucose oxidation and a concomitant increase in FA oxidation (*Zhao*, 2008). Taken together, these data demonstrate that PDK4 plays an important role in the cardiac metabolic flexibility.

1.1.3. Energy metabolism in the ischemic heart

Normal healthy heart has sufficient oxygen delivery, thus, more than 90% of ATP are produced in mitochondrial oxidative phosphorylation process. However, during ischemia, the oxygen supply to the heart is insufficient and it results in dramatic decrease in ATP production due to the changes in cardiac energy metabolism. The lack of energy supply to myocardium can result in the depressed cardiac function.

In the glycolysis process, the ATP can be produced in the absence of oxygen and the amount produced can be sufficient to maintain ionic homeostasis in the cell during ischemia. However, due to the insufficient oxygen supply, the glycolytically-produced pyruvate cannot be subsequently oxidized and it is rapidly converted to lactate. The uncoupling of glycolysis from further glucose oxidation leads to increased production of lactate and protons. The accumulation of lactate and protons can induce intracellular acidosis, resulting in decreased cardiac efficiency and impaired cardiac function (*Dennis*, 1991; *Fillmore*, 2013; *Liu*, 2002).

The lack of oxygen induces decrease in FA oxidation. Although oxidation of FAs requires more O_2 per ATP produced compared to glucose, FA oxidation remains the predominant source of residual oxidative metabolism during ischemia (*Folmes*, 2009). As a result of insufficient rate of β-oxidation, the FAs and their active metabolites (acylCoAs and acyl-carnitines) can accumulate in both mitochondria and cytosol. The increased concentration of these metabolites may cause irreversible mitochondrial damage (*Korge*, 2003; *Tominaga*, 2008).

Overall, the main ischemia-induced disturbance in energy metabolism occurs at the mitochondrial level. Thus, increase in pyruvate oxidation and reduction of FA activation could be beneficial to improve the outcome of cardiac ischemia-reperfusion injury. Moreover, growing evidences suggest that pharmacological regulation of cardiac energy metabolism, by inhibition of FA oxidation and/or stimulation of glucose oxidation, can improve cardiac function of the ischemic or failing hearts (*Fillmore*, 2014; *Jaswal*, 2011; *Wang*, 2007). Thus,

better understanding of the regulation of cardiac energy metabolism pathways can provide novel strategies for the treatment of ischemic heart diseases.

1.1.4. The regulation of cardiac energy metabolism

Randle or glucose-fatty acid cycle describes the competition between FAs and glucose for metabolic pathways in the heart (*Randle*, 1963). The mechanism, how an increase in FA oxidation causes a subsequent decrease in glucose oxidation and vice versa, is controlled by many factors and it has been extensively studied over the past 50 years (*Hue*, 2009). Both glucose and FAs compete for further oxidative phosphorylation inside the mitochondria, which can play an essential role in the energy substrate selection (Figure 1.4).

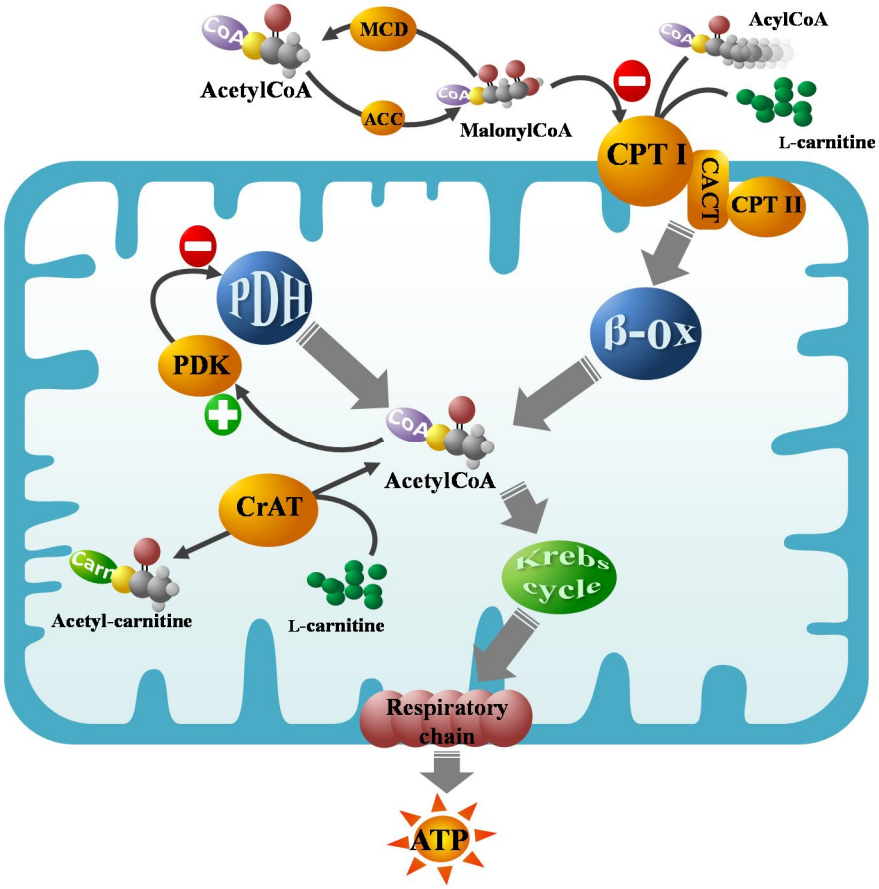


Figure 1.4. The regulation of mitochondrial energy metabolism
ACC – acetylCoA carboxylase; ATP – adenosine triphosphate; β-ox – β-oxidation; CACT – carnitine translocase; CPT I – carnitine palmitoyltransferase I; CPT II – carnitine palmitoyltransferase II; CrAT – carnitine acetyltransferase; MCD – malonylCoA decarboxylase; PDH – pyruvate dehydrogenase; PDK – PDH kinase

The competition between FAs and glucose for oxidation occurs at the level of PDH. High mitochondrial acetylCoA/free CoA and NADH/NAD ratios, which reflect high rate of β-oxidation, activate PDK and cause inhibition of PDH (*Zhang*, 2014a). Activation of PDH,

either directly by PDK inhibitor or indirectly by inhibition of β-oxidation, increases cardiac glucose oxidation and protects the heart against ischemia-reperfusion injury (*Roche*, 2007).

Another regulator of energy metabolism pattern in mitochondria is malonylCoA, the endogenous inhibitor of CPT I. The concentration of malonylCoA is determined by the balance between its synthesis by acetylCoA carboxylase (ACC) and degradation by malonylCoA decarboxylase (MCD). It has been demonstrated that the deletion of ACCβ (the isoform that predominates in the heart) expression induces a reduction of malonylCoA concentration that results in an increased cardiac FA oxidation rate (*Essop*, 2008; *Kolwicz*, 2012). Since no selective pharmacological inhibitors of ACC are available up to date, other approaches are used to inhibit ACC (*Ussher*, 2009). For example, the regulation of ACC activity by adenosine monophosphate-activated protein kinase (AMPK) appeared to be essential for FA oxidation in liver and skeletal muscles (*Fullerton*, 2013; *O'Neill*, 2014). However, in recent study using AMPK-ACC regulation resistant mice, despite 2-fold increase in malonylCoA cardiac content unchanged cardiac FA oxidation was observed, thus, demonstrating that AMPK-ACC signalling pathway control malonylCoA content, but they are not essential for the maintenance of cardiac FA oxidation suggesting the existence of other compensatory molecular pathways (*Zordoky*, 2014). The numerous studies have demonstrated that MCD is crucial in regulating malonylCoA content in the heart. The inhibition of MCD leads to an increase in malonylCoA content in cardiac tissues and it results in the reduction of FA oxidation rate and concomitant increase in glucose oxidation (*Dyck*, 2004; *Stanley*, 2005a). In addition, it has been demonstrated that inhibition or deletion of MCD results in improved outcome from cardiac ischemia-reperfusion injury (*Dyck*, 2006; *Stanley*, 2005a; *Ussher*, 2012). Overall, targeting malonylCoA availability to optimize cardiac metabolism has been proved beneficial (*Fillmore*, 2014; *Ussher*, 2009).

L-carnitine, as a cofactor of carnitine acyltransferases, participates in the regulation of mitochondrial energy metabolism pattern and represents potential target in the treatment of ischemic heart diseases. The most well-known function of L-carnitine is facilitating transport of long-chain FAs into mitochondria via CPT I for further β-oxidation. Another L-carnitine-dependent enzyme is carnitine acetyltransferase (CrAT) which is located in mitochondrial matrix where it participates in the regulation of glucose metabolism (*Stephens*, 2007). CrAT catalyses the formation of acetyl-carnitine from acetylCoA and L-carnitine and thus it regulates acetylCoA/free CoA ratio which is essential for PDH activity. Changes in L-carnitine availability may also influence acyl-carnitine availability, however, up to date the role of acyl-carnitine is limited to the transport of acyl moiety, and possible action of acyl-carnitine on cardiac energy metabolism regulation remains unclear.

1.2. L-carnitine system as a drug target

The homeostasis of L-carnitine is determined by its oral absorption, biosynthesis, transport into the tissues, and renal reabsorption (Figure 1.5). It is difficult to improve L-carnitine availability, since its homeostasis is tightly regulated by various enzymes and transporters.

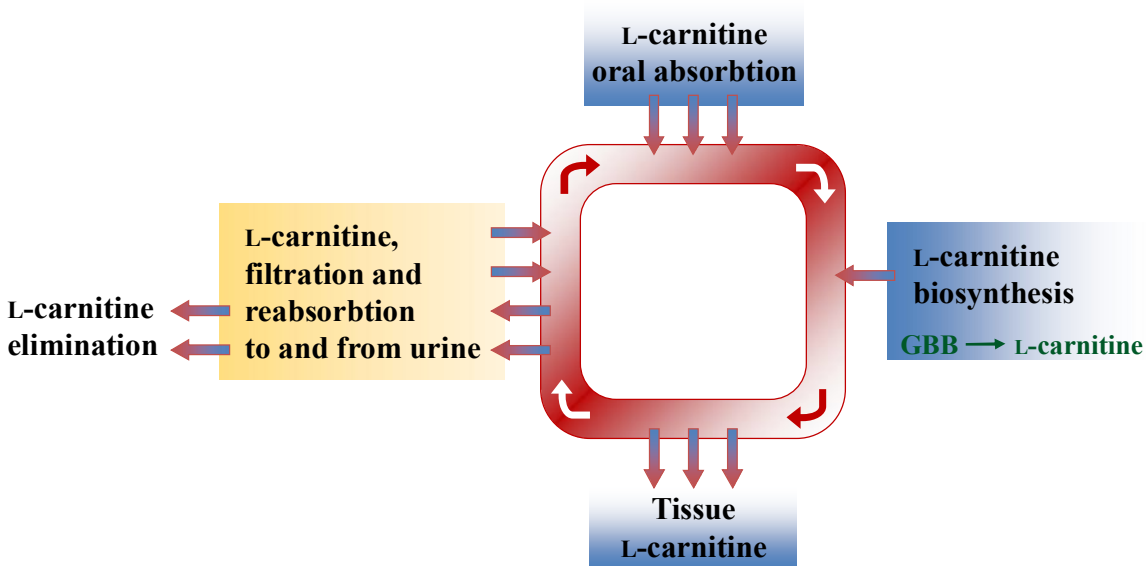


Figure 1.5. **Homeostasis of L-carnitine**

1.2.1. L-carnitine system

1.2.1.1. L-carnitine biosynthesis

The majority of L-carnitine is absorbed from the diet, particularly from meat and dairy products, however, at least 25% are produced endogenously. The L-carnitine is synthesized from N^{ε}-trimethyllysine (TML) in 4 subsequent enzymaticaly catalysed reactions (Figure 1.6) (*Reuter*, 2012; *Strijbis*, 2010; *Vaz*, 2002). TML is released after the degradation of such endogenous proteins like myosin, cytochrome C and histones (*Vaz*, 2002). Moreover, recent study demonstrated TML presence in vegetables, thus, together with previously published data, suggesting that mammals can uptake TML from diet (*Rebouche*, 1986; *Servillo*, 2014).

In the first L-carnitine biosynthesis reaction, TML is converted to 3-hydroxy-TML (HTML) by TML dioxygenase (TMLD). TMLD requires α-ketoglutarate, Fe^{2+} and molecular oxygen as cofactors (*Vaz*, 2001). In contrast to other L-carnitine biosynthesis enzymes, TMLD is localized in the mitochondrial matrix suggesting the existence of TML/HTML shuttle system across mitochondrial membranes (*Van Vlies*, 2007). In the second step, HTML aldolase (HTMLA) catalyzes aldolytic cleavage of HTML which results in 4-trimethylaminobutyraldehyde (TMABA) and glycine. Then TMABA is dehydrogenated to

γ-butyrobetaine (GBB) by TMABA dehydrogenase (TMABA-DH). In the final step, GBB is hydroxylated to L-carnitine by γ-butyrobetaine dioxygenase (BBOX). Similar to TMLD, BBOX requires α-ketoglutarate, Fe^{2+} and molecular oxygen as cofactors (*Wehbie*, 1988).

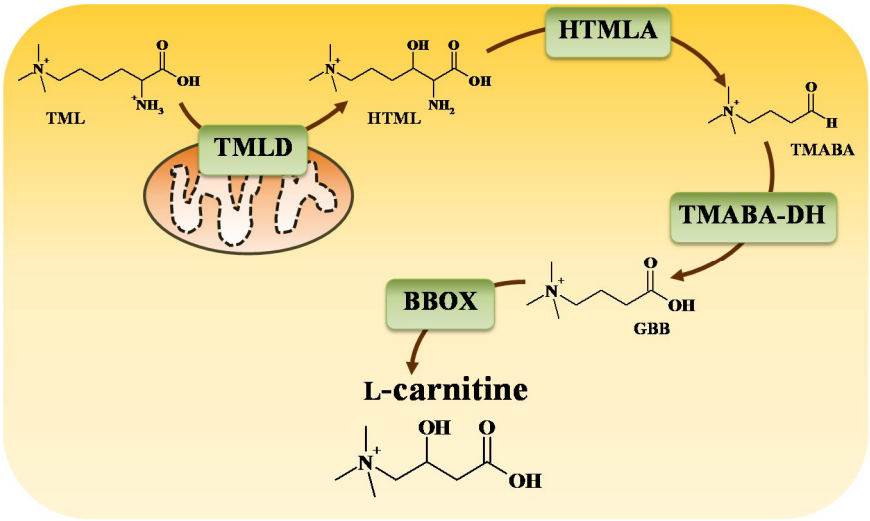


Figure 1.6. **L-carnitine biosynthesis**
BBOX – γ-butyrobetaine dioxygenase; GBB – γ-butyrobetaine; HTML – 3-hydroxy-TML; HTMLA – HTML aldolase; TMABA – 4-trimethylaminobutyraldehyde; TMABA-DH – TMABA dehydrogenase; TML – Nε-trimethyllysine; TMLD – TML dioxygenase

In contrast to other L-carnitine biosynthesis enzymes, BBOX has organ specific location and it has been found only in the kidneys, liver and brain (*Vaz*, 2002). In humans, the highest activity of BBOX was observed in kidneys, and it was 3.5 and 10 fold higher than in liver and brain, respectively (*Rebouche*, 1980). In contrast, BBOX activity is not present in kidneys and brain of rat, thus, L-carnitine is mainly synthesized in liver of this animal (*Cox*, 1974). Recently, the crystal structure of human BBOX was discovered and kinetic properties were characterized (*Leung*, 2010; *Tars*, 2010). Although BBOX is not the rate-limiting factor in L-carnitine biosynthesis (*Olson*, 1987), the treatment with BBOX inhibitor, meldonium, effectively decreases L-carnitine concentration in plasma and various tissues, including heart (*Dambrova*, 2008; *Kuka*, 2012; *Liepinsh*, 2006; *Svalbe*, 2011). Thus, BBOX can be used as a drug target to regulate L-carnitine availability.

1.2.1.2. L-carnitine transport

Cardiac tissues cannot synthesize L-carnitine, but require high concentrations of L-carnitine for FA oxidation. The L-carnitine concentration in the heart is > 20 times higher than in plasma (*Reuter*, 2012). High cardiac content of L-carnitine is ensured by the active uptake from the blood. The delivery of L-carnitine to the tissues is mediated by organic

cation/carnitine transporter family (OCTN1, OCTN2 and OCTN3), $ATB^{0,+}$ and OAT9 (Table 1.2.).

The first identified L-carnitine transporter was OCTN1 (*Tamai*, 1997). It mediates L-carnitine transport in Na^+-dependent manner; however, it has a relatively low affinity and specificity for L-carnitine. In human heart OCTN1 is localized in endothelial cells of microvessels (*Iwata*, 2008). In addition, it has been suggested that OCTN1 is also localized in mitochondria (*Lamhonwah*, 2006; *Shitara*, 2013). OCTN3 is a highly specific Na^+-independent transporter of L-carnitine; however, its distribution in tissues is limited (expressed mainly in testis) (*Alnouti*, 2006; *Durán*, 2005). $ATB^{0,+}$ has low affinity and specificity for L-carnitine in comparison with OCTN transporter family (Nakanishi 2001). In 2010, organic anion transporter (OAT9) has been identified as a novel L-carnitine transporter in mice kidney and liver; however, involvement of OAT9 in L-carnitine distribution in tissues needs to be investigated further (*Tsuchida*, 2010).

Table 1.2.

The characterisation of L-carnitine transporters

	Predominant substrates	*Affinity for L-carnitine K_m*	*Tissue distribution*	*Functional properties*	*References*
OCTN1	Zwitterions, organic cations	100 µM	Kidney (renal epithelium), intestine, spleen, heart, skeletal muscle, brain, testis	Na^+-dependent pH-dependent	*Pochini*, 2013; *Reuter*, 2012; *Tamai*, 2013, 1998
OCTN2	Zwitterions (L-carnitine), organic cations	3-10 µM	Skeletal muscle, heart, kidney (proximal tubules), placenta, prostate, thyroid	Na^+-dependent (L-carnitine transport) Na^+-independent	*Koepsell*, 2013; *Pochini*, 2013; *Reuter*, 2012; *Tamai*, 2013, 1998
OCTN3/ OCT6	L-carnitine	2-20 µM	Highly expressed in testis, weakly in liver peroxisomes, brain	Na^+-independent	*Koepsell*, 2013; *Pochini*, 2013; *Reuter*, 2012; *Tamai*, 2013
$ATB^{0,+}$	L-carnitine, amino acids	0.8-2 mM	Primary in intestinal tract, lung and mammary glands	Na^+, Cl^--dependent	*Reuter*, 2012; *Tamai*, 2013
OAT9 (short isoform)	Zwitterions (L-carnitine), organic cations and anions	3 µM	Mice liver, kidney	unknown	*Reuter*, 2012; *Tsuchida*, 2010

OCTN2 has the highest affinity for L-carnitine and therefore it plays the major role in L-carnitine uptake into the cardiac and skeletal muscle tissues (*Tamai*, 2013). OCTN2 is a sodium-dependent carnitine transporter, and it also determines L-carnitine absorption in small intestine and reabsorption of L-carnitine in the kidney (*Tamai*, 1998, 2001). The mutations in OCTN2 have been associated with systemic L-carnitine depletion, which in turn causes cardiomyopathy and skeletal muscle weakness (*Nezu*, 1999; *Shibbani*, 2014). Some therapeutic agents and short-chain acyl-carnitines are also transported by OCTN2 (Table 1.3.). Therefore, OCTN2 can be a drug target for the regulation of L-carnitine availability, and it also can play a role in the drug uptake into the cardiac cells.

Table 1.3.

The characterisation of OCTN2-dependent drug transport

Compound	*Therapeutic use*	*OCTN2 affinity, μM*	*References*
Acetyl-carnitine	Neurotropic	8.5	*Ohashi*, 1999
Verapamil	Anti-anginal, anti-arrhythmic	25	*Grube*, 2006
Meldonium	Anti-ischemic, cardioprotective	21	*Grigat*, 2009; *Rigault*, 2008
Spironolactone	Diuretic/antihypertensive	26	*Grube*, 2006

1.2.1.3. The role of L-carnitine and its metabolites in the cardiac energy metabolism and cardiovascular diseases

Numerous studies have addressed the question regarding the therapeutic potential of L-carnitine. For patients with genetically impaired OCTN2 function, L-carnitine treatment is life-saving (*Longo*, 2006). Systemic L-carnitine depletion is also observed in hemodialysis patients due to insufficient biosynthesis and excessive loss of L-carnitine through dialysis membrane (*Matera*, 2003). It has been demonstrated that L-carnitine administration reduces cardiac complications in dialysis patients (*Romagnoli*, 2002; *Sakurabayashi*, 2008; *Sgambat*, 2012). Meanwhile, data about benefits of L-carnitine treatment in ischemic heart diseases remain controversial. Moreover, the mechanisms underlying the effects of L-carnitine on cardiac energy metabolism and the outcome of ischemic injury are not well clarified.

Despite that the majority of experimental studies have demonstrated that L-carnitine, particularly when perfused at high dose, improves the recovery of cardiac function after ischemia-reperfusion injury, the lack of cardioprotective effect and, moreover, worsened ischemic injury and arrhytmogenic activity of L-carnitine treatment were also observed

(Table 1.4). Although it could be assumed that L-carnitine therapy primarily targets the metabolism of FAs, the experimental data show that protective effects of L-carnitine against ischemic injury are mainly due to stimulated glucose metabolism (*Broderick*, 1992, 1993, 1995b). Furthermore, it was shown that under conditions where L-carnitine does not improve glucose metabolism, the L-carnitine treatment can worsen injury (*Díaz*, 2008). The translational potential of observed L-carnitine cardioprotective effects should be evaluated with caution. The concentrations of L-carnitine used for *ex vivo* experiments (5 and 10 mM) cannot be easily achieved in plasma. Because of strict regulation of L-carnitine homeostasis high doses of L-carnitine supplementation may not provide beneficial effects due to low bioavailability (*Rebouche*, 2004).

Table 1.4.

The effects of L-carnitine on cardiac energy metabolism and ischemia-reperfusion injury in the experimental models

Model	*Dose of L-carnitine*	*Perfusion solution*	*Effects on cardiac energy metabolism*					*Effects on ischemia-reperfusion injury*	*References*
			L-carnitine pool	*Long-chain acylCoA*	*Glucose metabolism*	*FA oxidation*	*ATP content*		
Isolated rat heart	10 mM	11 mM glucose	-	-	Glycolysis = Oxidation =	-	-	-	*Broderick*, 1992
		11 mM glucose + 1.2 mM palmitate	Free ↑; SC ↑; LC ↑	↑	Glycolysis ↑ Oxidation ↑	↓	↓		
Isolated rat heart	10 mM	11 mM glucose + 1.2 mM palmitate + 100 μU/ml insulin	Normoxia: Free ↑; SC ↑; LC = After I/R: Free ↑; SC ↑; LC =	Normoxia: ↑ After I/R: ↓	Normoxia: = Glycolysis ↑ Oxidation After I/R: = Glycolysis ↑ Oxidation	-	Normoxia: = After I/R: =	Improved recovery of cardiac function	*Broderick*, 1993
Isolated rat heart	10 mM	11 mM glucose + 1.2 mM palmitate	After I/R: Free ↑; SC ↑; LC =	After I/R: =	Normoxia: = Glycolysis ↑ Oxidation After I/R: = Glycolysis ↑ Oxidation	-	After I/R: ↑	Improved recovery of cardiac function	*Broderick*, 1995
Isolated rat heart	5 mM	10 mM glucose	-	-	-	-	-	Improved recovery of cardiac function Reduced infarct size	*Cui*, 2003

Table 1.4. (continued)

Model	*Dose of L-carnitine*	*Perfusion solution*	*Effects on cardiac energy metabolism*					*Effects on ischemia-reperfusion injury*	*References*
			L-carnitine pool	*Long-chain acylCoA*	*Glucose metabolism*	*FA oxidation*	*ATP content*		
Isolated rat heart	5 mM	11 mM glucose + 0.4 or 1.2 mM palmitate	Free ↑	-	-	-	After I/R: ↑	Improved cardiac function in reperfusion	*Löster*, 2005
Isolated rat heart	1 mM	5 mM glucose	-	-	Lactate production ↓	-	-	Increased release of CK Delayed recovery after ischemia	*Díaz*, 2008
Isolated rat heart	5 mM 10 min before to 10 min after ischemia	12 mM glucose	-	-	-	-	-	Arrhythmogenic activity	*Najafi*, 2008
In vivo	200 mg/kg 9 days	-	Normoxia: Free =	-	-	-	After ligation: =	Reduced infarct size, better preserved cardiac function	*Mouhieddine*, 1993
In vivo	300 mg/kg 4 weeks	-	-	-	-	-	-	No effect on the infarct size	*Briet*, 2008

I/R - ischemia-reperfusion; SC – short-chain acyl-carnitine; LC – long-chain acyl-carnitines; ↑ - increase; ↓- decrease; = - no changes.

Clinical studies about the L-carnitine therapy in patients with acute myocardial infarction (AMI) also did not provide solid evidence of L-carnitine benefits. The first large randomized, multicentre trial CEDIM 1 (involved 472 patients) demonstrated that L-carnitine administration attenuates LV remodeling in patients with anterior AMI throughout the first year after the acute event, but it did not decrease all-cause mortality risk (*Iliceto*, 1995). Next CEDIM 2 trial (2329 patients) showed that L-carnitine administration reduces mortality within the first 5 days in AMI patients, however, it did not affect cumulative 6-month mortality (*Tarantini*, 2006). In addition, recent systematic review and meta-analysis of 13 controlled clinical trials concluded that a prospective large randomized controlled trial to assess the effects of L-carnitine is needed (*DiNicolantonio*, 2013). Several limitations of the available clinical studies have been discussed by Shang and colleagues (*Shang*, 2014). First, the dose of L-carnitine in most studies varies, thus making it difficult to compare and find the most effective dose of L-carnitine and duration of therapy. Second, the standard therapy for AMI patients has been changed since all included studies were performed (prior to 2007), thus, the benefits of L-carnitine supplementation should be re-evaluated in the context of current treatments (for example, earlier and more intensive statin use (*Steg*, 2012; *Van de Werf*, 2003, 2008)). Moreover, other factors like dietary pattern and L-carnitine bioavailability should be taken in to account to make conclusions about beneficial effects of L-carnitine treatment.

The correlation between the availability of L-carnitine or its metabolites and cardiovascular disease risk has been assessed in studies related to metabolic disorders and cardiovascular diseases. Despite the numerous small studies demonstrating the beneficial effects of L-carnitine supplementation in case of diabetes (*Ringseis*, 2012), the largest study conducted up to date demonstrated that there is no association of low L-carnitine concentration with the prevalence of late diabetic complications (*Liepinsh*, 2012). In addition, the diabetic patients with higher L-carnitine concentrations (> 30 μM) had higher prevalence of hypertension. Another study demonstrated that increased levels of long-chain acyl-carnitines in serum are associated not only with type 2 diabetes, but also with prediabetic states (*Mai*, 2013; *Zhang*, 2014b). Moreover, concentration of long-chain acyl-carnitines negatively correlates with glucose oxidation in fed and fasted states (*Ramos-Roman*, 2012).

The increased L-carnitine and acyl-carnitine levels were observed in plasma of patients with myocardial infarction (both ST-elevated, and non-ST-elevated) (*Khan*,

2013, 2014). Recent study in heart failure patients demonstrated that higher levels of plasma acyl-carnitines, and in particular palmitoyl-carnitine, are associated with the degree of heart failure (NYHA functional class) and poor prognosis (increased all-cause mortality and heart transplantation) (*Ueland*, 2013). In addition, recently, the role of L-carnitine metabolism by microbiota in the development of atherosclerosis has been demonstrated (*Koeth*, 2013), thus the safety of L-carnitine supplementation remains questionable. Taken together, these data provide some evidence that not only L-carnitine, but also acyl-carnitine can play a role in energy metabolism regulation, and the decrease in L-carnitine and/or acyl-carnitine could improve outcome from ischemia-reperfusion injury.

1.2.2. The effects of pharmacologically reduced L-carnitine availability on the cardiac energy metabolism

Some drugs have demonstrated the ability to decrease L-carnitine concentration; these drugs are suitable to study the role of L-carnitine in the regulation of energy metabolism pattern. The effects of drug-induced lowered L-carnitine state on cardiac energy metabolism and outcome of ischemia-reperfusion injury are discussed below.

1.2.2.1. The effects of pivalate on cardiac energy metabolism

The decrease in L-carnitine concentration in plasma was observed after treatment with pivalate-containing prodrugs such as pivampicillin, pivmecillinam, cefditoren pivoxil and tenofovir dipivoxil (*Brass*, 2002; *Jia*, 2013). Pivalic acid, which liberates from pro-drugs, is activated to form pivaloylCoA. Since pivaloylCoA cannot be oxidized to CO_2, it forms ester with L-carnitine, which is eliminated from the organism via urinary excretion (*Brass*, 2002; *Broderick*, 2006). Since the formation and excretion of pivaloyl-carnitine is the major route of pivalate elimination, it results in the excessive loss of L-carnitine. The decrease in L-carnitine availability depends on the duration of treatment (*Broderick*, 2006). Thus, it has been shown that after 2 weeks of treatment with 20 mM sodium pivalate the cardiac content of L-carnitine was decreased only by 24%. In addition, the depressed cardiac function was observed, but only during reperfusion, and not under normoxic conditions (*Broderick*, 2001). On the other hand, sodium pivalate treatment for up to 28 weeks resulted in 60% decrease in the cardiac

L-carnitine content and caused a severe depression in cardiac function even under normoxic conditions (*Broderick*, 1995a). In addition, it has been shown that treatment with pivalate for 11-12 weeks (decrease in cardiac L-carnitine content by 60%) shifts cardiac substrate utilization toward glucose metabolism without depressing cardiac functionality (*Morris*, 1995). Together, these data indicate that although the reduction of L-carnitine availability might result in impaired cardiac function, this effect is not unequivocal. Because the metabolic shift from fatty acid metabolism toward glucose metabolism can be cardioprotective (*Fillmore*, 2014; *Jaswal*, 2011; *Wang*, 2007), the detrimental effects of pivalate treatment on cardiac function may only occur when energy metabolism cannot adapt to decreased fatty acid metabolism. Thus, further studies are necessary to clarify the effects of pivalate treatment on cardiac energy metabolism, in particular, at mitochondrial level, where FAs and glucose compete for oxidation in metabolic pathways.

1.2.2.2. The effects of meldonium, inhibitor of L-carnitine biosynthesis and transport, on cardiac energy metabolism

Another approach to decrease L-carnitine availability is to inhibit its biosynthesis and/or transport in tissues. Thus far the only known synthetic inhibitor of L-carnitine biosynthesis was meldonium (3-(2,2,2-trimethylhydrazinium) propionate dihydrate; mildronate; THP; MET-88) (*Simkhovich*, 1988). Meldonium inhibits in a competitive manner the last enzyme in the L-carnitine biosynthesis, BBOX, (*Spaniol*, 2001). Moreover, it has been demonstrated that meldonium reduces L-carnitine transport into tissues and reabsorption in kidney by inhibiting OCTN2 (*Kuwajima*, 1999; *Spaniol*, 2001). Thus, in the experimental models, meldonium induces a pronounced decrease in L-carnitine concentration in plasma and various tissues, including heart, reaching plateau after 14 days of treatment (*Dambrova*, 2008; *Kuka*, 2012; *Liepinsh*, 2006; *Svalbe*, 2011). In addition, it has been demonstrated that long-term treatment with meldonium reduces L-carnitine concentration in plasma of non-vegetarian, healthy volunteers by 20% (*Liepinsh*, 2011b). Treatment with meldonium improved recovery after stroke in experimental model (*Svalbe*, 2011) and in clinical trials (*Vinichuk*, 1991; *Zhu*, 2013). Furthermore, meldonium treatment attenuated the development of atherosclerosis and prevented diabetic complications in experimental animal models (*Liepinsh*, 2009; *Vilskersts*, 2009a). The cardioprotective effects of meldonium have

been studied in different models of myocardial infarction and heart failure (*Hayashi*, 2000; *Liepinsh*, 2006, 2009; *Nakano*, 1999; *Sesti*, 2006; *Vilskersts*, 2009b). The 10-14 day-treatment with meldonium significantly reduces infarct size in the experimental cardiac ischemia-reperfusion injury models both *in vitro* and *in vivo* (*Liepinsh*, 2006; *Sesti*, 2006). The protective effect of meldonium against cardiac ischemia-reperfusion injury has been also demonstrated in Goto-Kakizaki rats, an experimental model of type 2 diabetes mellitus (*Liepinsh*, 2009). The reduction of infarct size after treatment with meldonium was not associated with significant changes in cardiac workload either before or during ischemia-reperfusion (*Kuka*, 2012; *Sesti*, 2006), indicating that mechanism of action is probably based on the optimization of cardiac energy metabolism (*Dambrova*, 2002). Recent study demonstrated that cardioprotective effect of meldonium is diminished after co-treatment with L-carnitine (*Kuka*, 2012). Taken together, these data indicate that the cardioprotective effect of meldonium is based on the regulation of cardiac energy metabolism via decreased L-carnitine availability.

The effects of meldonium treatment on the cardiac energy metabolism have been widely studied in the past 20 years. First, it has been suggested that meldonium-induced reduction of L-carnitine content inhibits FA oxidation in the heart (*Dambrova*, 2002; *Simkhovich*, 1988). However, in further studies, no changes in total cardiac FA oxidation were found after treatment with meldonium (*Degrace*, 2004; *Liepinsh*, 2013a). It has been demonstrated that meldonium treatment results in the significantly decreased CPT I activity and CPT I-dependent β-oxidation in cardiac mitochondria (*Kuka*, 2012). A further study demonstrated stimulated CPT I-independent β-oxidation after treatment with meldonium (*Liepinsh*, 2013a). In addition, the compensatory activation of PPAR-α/PGC1-α signalling pathway was observed (*Liepinsh*, 2011a, 2013a). The increase in nuclear content of PPARα and PGC1α resulted in the increased expression of genes related to FA oxidation, activated proliferation of peroxisomes, and subsequently stimulated peroxisomal FA oxidation (*Degrace*, 2004; *Liepinsh*, 2013a). The meldonium-induced reduction of cardiac L-carnitine content redirects FA oxidation from mitochondria to peroxisomes and, thus, diminishes FA accumulation in the mitochondria (*Liepinsh*, 2013a); this results in the maintenance of mitochondrial membrane integrity and bioenergetics function in ischemia-related conditions (*Kuka*, 2012; *Liepinsh*, 2013a).

The effects of the decreased L-carnitine availability induced by meldonium on the glucose metabolism regulation have been also studied. It has been shown that 20-day treatment with meldonium induces an increase in gene and protein expression related to glucose metabolism (*Liepinsh*, 2008). The increase in GLUT4 and insulin receptor protein expressions was followed by a significant increase in insulin-stimulated glucose uptake in the isolated mouse heart (*Liepinsh*, 2008). The upregulation of the expression of PDH complex genes in meldonium-treated animal hearts together with the observed decrease in lactate concentration in ischemic hearts indicate that meldonium treatment may also stimulate aerobic oxidation of glucose (*Asaka*, 1998; *Liepinsh*, 2008). In addition, the decrease in blood glucose concentration after treatment with meldonium was observed in the experimental models of diabetes and obesity (*Liepinsh*, 2009, 2011a; *Sokolovska*, 2011). Thus, decrease in L-carnitine content induced by meldonium not only modulates FA metabolism, but also stimulates glucose utilization. However, the mechanisms, how decrease in L-carnitine availability stimulates glucose metabolism, remain unclear.

Despite that the data mentioned indicate that the decrease in L-carnitine availability seems to be beneficial in the optimization of cardiac energy metabolism, the the mechanisms behind the effects of decreased L-carnitine content in cardiac tissues during ischemia-reperfusion injury are not fully understood. Moreover, the possible role of reduced acyl-carnitine content, secondary to the decrease in L-carnitine availability, has never been evaluated. Further studies are necessary to clarify the role of L-carnitine and its metabolites in the regulation of cardiac energy metabolism, and to elucidate if L-carnitine system can be considered as a drug target for the treatment of cardiovascular diseases.

2. MATERIALS AND METHODS

2.1. Animals and treatment

Male *Wistar* rats (Laboratory of Experimental Animals, Riga Stradins University, Latvia, weighing 200-250 g), *Goto-Kakizaki* and *Wistar-Kyoto* rats (Taconic Farms, USA, weighing 250 g) and *ICR* mice (Harlan, Netherlands, weighing 25-28 g) 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. All animals were adapted for two weeks prior to the experiments. Animals were housed under standard conditions (21-23 °C, 12 h light-dark cycle) with unlimited access to food (R70 diet, Lantmännen Lantbruk, Sweden) and water.

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. Fasting was started at the end of the light phase.

Wistar rats received a 40 mM sodium pivalate pH 7.0 (corresponds to approx. 500 mg/kg) as their drinking water for 14 days 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. Single administration and long-term administration (14 days) of compounds were performed.

To study the cardioprotective effects of the leading compound, Methyl-GBB at doses 1, 5, 10, 20 mg/kg was administrated *per os* daily for 3-14 days. Meldonium treatment (100 mg/kg for 14 days) was used as a control for the comparison.

2.2. Materials

Potassium dihydrogenphosphate, sodium chloride, sodium hydrogenphosphate, calcium chloride dihydrate, potassium chloride, sodium bicarbonate, sodium carbonate, sodium palmitate, taurine, saponin, imidazole, 2-[N-morpholino]ethanesulfonic acid

(MES), L-carnitine, sodium acetate and magnesium chloride hexahydrate were from Acros Organics (Geel, Belgium). Fatty acid free bovine serum albumin was from Europa Bioproducts Ltd (UK). Insulin was from Novo Nordisk (Denmark). Glucose was from Fresenius Kabi (Poland). Lactate dehydrogenase, collagenase, phosphocreatine, antimycin A, TMPD, rotenone, ethylenediamine-tetraacetate sodium salt (EDTA), leupeptin, pepstatin, aprotinin, phenylmethanesulfonyl fluoride (PMSF), NAD, ATP, ADP, sodium succinate, sodium lactate, dithiothreitol, triphenyl-tetrazolium chloride, 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pyruvic acid, malic acid, hyamine, Igepal CA 630 and coenzyme A were from Sigma-Aldrich (Schnelldorf, Germany). PalmitoylCoA and palmitoyl-carnitine were from Larodan (Malmö, Sweden). Methanol and acetonitrile were from Merck (Darmstadt, Germany). Sodium pentobarbital (Dorminal) solution was from Alfasan International BV (Holland). Heparin sodium was from Panpharma (Fougeres, France). Ketamine was from Vetoquinol Biowet (Poland) and xylasine was from Laboratorios Calier (Spain). Atropine sulfate was from Sopharma (Bulgaria). Tramadol was from KRKA (Slovenia). Sodium benzilpenicilinum was from Sandoz (Germany). [U-^{14}C]glucose, [1-^{14}C]lactate, [9,10-^{3}H]palmitate, [1-^{14}C]palmitate and L-[N-methyl-^{3}H] carnitine hydrochloride were from BIOTREND Chemikalien GmbH (Köln, Germany). Sodium pivalate was obtained from Alfa Aesar (Karlsruhe, Germany). Meldonium (3-(2,2,2-trimethylhydrazinium) propionate) was obtained from JSC Grindeks (Riga, Latvia). Synthesis of potential BBOX and OCTN2 inhibitors was performed according to methods described previously (*Tars*, 2014).

2.3. Methods

2.3.1. *In vitro* methods

2.3.1.1. Profile of FAs, acyl-carnitine and acylCoA in plasma and tissues

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 concentrations of acyl-carnitine and acylCoA were measured against a seven point standard curve for each analyte ranging from 10 ng/ml to 1000 ng/ml. The sample extraction was performed as previously described (*Blachnio-Zabielska*, 2011) with modifications. Briefly, 0.5 ml of

freshly prepared 100 mM KH_2PO_4, pH 4.9 and 1 ml of acetonitrile/2-propanol/methanol 3:1:1 (v/v) was added to 100 µl of plasma or 100 mg of tissues. The sample was sonicated for 30 s and centrifuged at 16000 g for 10 min. The supernatant was evaporated and lyophilised. The dry extract was re-suspended in 0.2 ml of acetonitrile/water 3:2 (v/v), vortexed and centrifuged at 13000 g for 10 min. The obtained supernatant was used for UPLC MS/MS analysis.

2.3.1.2. Biochemical parameters in plasma and tissues

The blood samples were collected from tail vein in heparin-containing tubes. To obtain plasma, samples were centrifuged at 1000 g for 10 min at 4 °C. The heart tissues were homogenized with a Cole Parmer 130-Watt ultrasonic processor set at 40 kHz for 25 s in an ice-cold phosphate buffer solution (PBS, 2.6 mM KCl, 136 mM NaCl, 10 mM Na_2HPO_4 and 1.75 mM KH_2PO_4) at w/v ratio of 1:10. The homogenate was centrifuged at 6000 g for 10 min at 4 °C. The supernatant was then decanted and used for biochemical analysis. All samples were stored at −80 °C until analysis.

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).

2.3.1.3. Mitochondrial and peroxisomal fatty acid oxidation in homogenates

The mitochondrial and peroxisomal rates of palmitate oxidation were determined as described previously (*Degrace*, 2004), with some modifications. The reaction mixture contained 1 mM NAD, 5 mM ATP, 100 µM CoA, 0.5 mM malate and specified concentration of L-carnitine in a Krebs-Henseleit buffer solution (KH buffer, 118 mM NaCl, 2.52 mM $CaCl_2$, 1.64 mM $MgCl_2$, 24.88 mM $NaHCO_3$, 1.18 mM KH_2PO_4 and 0.05 mM EDTA, pH 7.4 at 37 ºC). The reaction was started by the addition of 100 µM palmitate (supplemented with 1 µCi [1-^{14}C]palmitate, radiotracer specific activity 60 Ci/mmol) bound to fatty-acid-free BSA. After 60 min incubation at 37 °C, the

samples were treated with 10% $HClO_4$. $^{14}CO_2$ that was produced and trapped in hyamine-saturated filter paper placed in a tube stopper. After 1 h, the filter paper was removed, and $^{14}CO_2$ was counted using a scintillation cocktail. To measure peroxisomal palmitate oxidation, the mitochondrial β-oxidation activity was inhibited by pre-incubating the samples with 75 μM antimycin A, 10 μM rotenone and 250 μM KCN for 10 min. The rate of peroxisomal palmitate oxidation was calculated according to the radioactivity of the acid-soluble products and expressed as pmol of palmitate per min per mg protein. The rate of mitochondrial palmitate oxidation was expressed as the difference between total palmitate oxidation (without using inhibitors) and peroxisomal oxidation rate.

2.3.1.4. The isolation of cardiac mitochondria and preparation of permeabilized cardiac fibers

Mitochondria were isolated from cardiac tissues as previously described (*Kuka*, 2012). Cardiac tissues were homogenized on ice in 1:10 w/v of the medium containing 180 mM KCl, 10 mM Tris-HCl, and 1 mM EGTA (pH 7.7 at 4 °C) with Teflon glass homogenizer. The homogenate was centrifuged at 750 g for 5 min at 4 °C and then the supernatant was centrifuged at 6800 g for 10 min at 4 °C. The obtained mitochondrial pellet was washed once (10 min at 6800 g) and resuspended in the buffer containing 180 mM KCl, 20 mM Tris-HCl, pH 7.2 at 4 ºC. The mitochondrial protein concentration was determined by Lowry method using BSA as a standard.

The permeabilized cardiac fibers were prepared as described previously (*Kuka*, 2012; *Toleikis*, 1996) with some modifications. Briefly, the bundles of fibers were permeabilized using 50 μg/ml saponin and 1 mg/ml collagenase at 4 ºC in a buffer A (20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 7.1 mM $MgCl_2$, 50 mM MES, 5 mM ATP, 15 mM phosphocreatine, 2.6 mM CaK_2EGTA, 7.4 mM K_2EGTA, pH 7.0 at 0 ºC). After 30 min incubation, the fibers were washed twice for 10 min in buffer B (20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 2.9 mM CaK_2EGTA, 7 mM K_2EGTA, pH 7.1 at 37 ºC).

2.3.1.5. Oxidation of energy substrates in the isolated cardiac mitochondria

To determine the oxidation rates of mitochondrial substrates, the reaction mixture containing 1 mM NAD, 5 mM ATP, 100 μM CoA, 0.5 mM malate and various concentrations of lactate or palmitate was supplemented with the respective radiolabelled substrate in KH buffer solution (*Campbell*, 2004; *Yoshida*, 2007).

To determine the rate of lactate oxidation in mitochondria, a reaction mixture containing lactate (0.5, 1, 2 or 15 mM non-labelled and 0.1 μCi/ml of [1-^{14}C]lactate radiotracer with a specific activity of 55 mCi/mmol) in the presence or absence of palmitoyl-carnitine (5, 10 or 20 μM) was used. The reaction was initiated by the addition of isolated cardiac mitochondria (approx. 3 mg protein). During the incubation at 37 °C, $^{14}CO_2$ was produced and trapped in hyamine-saturated filter paper placed in a tube stopper. The reaction was terminated by the addition of 25% $HClO_4$. After 1 h, the filter paper was removed, and $^{14}CO_2$ was counted using a scintillation cocktail.

The mitochondrial pyruvate oxidation rate was determined by a method similar to that used for lactate, except that the reaction mixture was pre-incubated for 15 min with lactate dehydrogenase (2.5 U) to convert the added lactate to pyruvate.

The mitochondrial palmitate oxidation rates were measured using 100, 300 or 1200 μM palmitate (supplemented with 1 μCi/ml of [9,10-^{3}H]palmitate radiotracer with a specific activity of 60 Ci/mmol) pre-bound to fatty acid-free BSA and 700 μM L-carnitine in the presence or absence of 1, 2 or 15 mM lactate or pyruvate. After a 10 min incubation at 37 °C, the samples were subsequently treated with a chloroform:methanol (1:2 v/v) solution, chloroform and a KCl/HCl (2/0.4 M) solution. Samples were then centrifuged at 1150 g for 15 min to separate the polar and non-polar phases, and a 50 μl aliquot from the polar phase (acid soluble FA metabolism products) was collected and counted for ^{3}H using a scintillation cocktail.

To determine the accumulation of fatty acids and their metabolites, after the perfusion with radiolabeled palmitate mitochondria were isolated and sonicated twice for 10 s with a Cole Parmer 130-Watt ultrasonic processor set at 20 kHz. To obtain fraction of acid-soluble metabolites, samples were treated identically as described above. The polar phase was taken and counted for acid-soluble metabolites, while non-polar phase was used to determine unmetabolized FAs.

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

The mitochondrial respiration was measured using a Clark-type electrode (Microelectrodes Inc., Bedford, USA) in the buffer containing 150 mM KCl, 2.25 mM $MgCl_2$, 10 mM Tris HCl, 5 mM KH_2PO_4 (pH 7.2. at 37 ºC). To determine the CPT I-dependent oxidation of FAs, palmitoylCoA (10 or 50 μM) and specified L-carnitine concentrations (found in the cardiac tissue after the treatment) were used for the respiration measurements. Palmitoyl-carnitine (10 or 36 μM) was used as a substrate to determine CPT I-independent FA oxidation. Pyruvate + malate (5 mM + 5 mM) were used as substrates to measure pyruvate metabolism.

The cardiac fiber respiration measurements were performed at 37 ºC with Clark-type electrode in a buffer B containing respiratory substrates (6 mM pyruvate + 6 mM malate or 10 μM palmitoylCoA + 700 μM L-carnitine + 2 mM malate).

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. 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.

2.3.1.7. CPT I and CrAT acitivities

The CPT I activity was measured as described previously (Kuka, 2012) in the presence of specified L-carnitine concentrations that were found in the heart tissue after the treatment. Enzyme activity was calculated per mg of protein. CrAT activity was measured as previously described (Jaudzems, 2009) with the exception that L-carnitine was not added for activity measurements so that the activity of the CrAT enzyme could be measured when only mitochondrial L-carnitine fraction is present.

2.3.1.8. mRNA isolation and quantitative RT-PCR

Total RNA from heart tissue was isolated using the TRI Reagent (Sigma, St. Louis, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, USA) following the manufacturer's instructions. Quantitative RT-PCR analysis for genes was performed by mixing synthesized cDNA, appropriate primers, and SYBR® Green Master Mix (Applied Biosystems™, Foster City, USA) and run in the Applied Biosystems Prism 7500 according to the manufacturer's protocol. The transcript levels for the constitutive housekeeping gene product β-actin were quantitatively measured for each sample, and PCR data were reported as the number of transcripts per number of β-actin mRNA molecules.

2.3.1.9. Western blot of cytosolic and nuclear extracts

Heart tissues were homogenized by an Ultra-Turrax® homogenizer (IKA, Germany) at a ratio of 1:10 (w/v) at 4 ºC in a buffer containing 100 mM Tris HCl, pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM glycerol 3-phosphate, 1 mM NaF and protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, and 100 μM PMSF). The nuclear extracts for western blot (WB) analysis were isolated and purified as described previously (*Liepinsh*, 2011a). PAGE and WB analysis of tissue lysates and nuclear extracts were performed as described by Liepinsh et al. (*Liepinsh*, 2011a). The blots were developed using chemiluminescence reagents (Millipore). Western blot images were scanned and then analyzed using Gel-Pro Analyzer 6.0 software.

2.3.1.10. BBOX activity

The BBOX activity was assayed by measuring the formation of L-carnitine from GBB and was carried out according to a slightly modified version of the method reported by Lindstedt and Lindstedt (*Lindstedt*, 1970). The human recombinant BBOX or rat liver homogenate (1:10 (w/v) in PBS) were used as enzyme sources. For the initial screening, the complete reaction mixture (final volume of 0.2 ml) contained: 20 mM KH_2PO_4, pH 7.0; 20 mM KCl; 3 mM 2-oxoglutarate; 0.25 mM $(NH_4)_2Fe(SO_4)_2$; 10 mM sodium ascorbate; 0.16 mg of catalase; 200 μM GBB; and the

appropriate amount of enzyme. The mixture was pre-incubated for 15 min in the absence (Control) or presence of the tested compound (100 or 1000 μM). 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 reaction was stopped with 0.8 ml of ice-cold acetonitrile:methanol (1:3 v/v). Then, mixture was spun at 20000 g for 10 minutes at 4 ºC. The supernatant was decanted and used for L-carnitine measurements. Compounds that decreased BBOX activity by at least 50% compared to the control were further tested to determine the IC_{50} value. The reaction mixture and the treatment were as previously described, and 6-8 different concentrations (concentrations were chosen depending on the inhibitory effect observed during initial screening) of the tested compounds were used to obtain a dose-activity curve.

2.3.1.11. OCTN2-mediated transport of L-carnitine

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

The measurements were performed in triplicate for each concentration of the tested compounds.

2.3.1.12. L-carnitine and administrated compound concentrations in cardiac tissues, plasma and urine

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

2.3.2. *Ex vivo* and *in vivo* methods

2.3.2.1. 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. The energy metabolism measurements were performed according to the Langendorff constant flow non-recirculation technique. Briefly, rats were anesthetised using sodium pentobarbital (60 mg/kg i.p.) with the concomitant administration of heparin (1000 U/kg). Hearts were excised and retrogradely perfused (perfusion pressure 70 mmHg) with the respective non-labelled oxygenated (95% O_2, 5% CO_2) 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. After 10 min, the perfusate was switched to the respective oxygenated radiolabelled KH buffer solution for 10 min at a constant flow of 10 ml/min. Then, the hearts were switched back to the respective non-labeled KH perfusion solution and perfused for 10 min.

Glucose and lactate oxidation rates were determined by measuring the $^{14}CO_2$ released from the metabolism of [U-^{14}C]glucose (specific activity, 300 mCi/mmol) or [1-^{14}C]lactate (specific activity, 55 mCi/mmol), respectively. Palmitate oxidation was determined by measuring $^{3}H_2O$ released from [9,10-^{3}H]palmitate (specific activity,

60 Ci/mmol). The perfusates were treated in the same manner as in the isolated mitochondria experiment with the exception that 9 N H_2SO_4 was used instead of $HClO_4$ to release $^{14}CO_2$. 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.

2.3.2.2. Palmitate uptake and oxidation *in vivo*

To determine the palmitate uptake and oxidation *in vivo*, 1 µCi of [9,10-^{3}H]palmitate (specific activity, 60 Ci/mmol) per 25 g of body weight was administered intravenously to the mice. After 10 min, the mice were sacrificed by cervical dislocation, and heart and muscle tissue homogenates (1:5, w/v in MilliQ water) were prepared. Samples were treated in the same manner as in the isolated mitochondria experiment.

2.3.2.3. Intraperitoneal glucose tolerance test

To perform the glucose tolerance test, the diabetic and control rats were fasted overnight. Then, the glucose solution (1 g/kg) was administered *i.p.*, and blood samples were then drawn from the tail vein at 0 (fasting), 5, 15, 30, 45, 60, 120 and 240 min. Blood glucose concentration was measured using a MediSense Optium Xceed blood glucose meter and strips.

2.3.2.4. Isolated rat heart infarction study and hemodynamic parameters

The infarction study was performed according to the Langendorff technique as described previously (*Kuka*, 2012), with some modifications. For the infarction studies, the hearts were perfused with specified oxygenated (95% O_2 - 5% CO_2) KH buffer solution supplemented with 10 mM glucose (if not indicated different) at a constant perfusion pressure of 60 (if not indicated different) mmHg. A water-ethanol mixture (1:1)-filled balloon connected to a physiological pressure transducer (ADInstruments, Chalgrove, UK) was inserted into the left ventricle, and the baseline end-diastolic pressure was set at 5-10 mmHg. The heart rate, left-ventricle developed pressure, contractility and relaxation were continuously recorded using a PowerLab 8/30 system

from ADInstruments. The coronary flow was measured using an ultrasound flow detection system connected to the PowerLab 8/30 instrument. The isolated rat hearts were adapted for 20 min, and the left anterior descending coronary artery (LAD) was subsequently occluded for 30 or 40 min followed by 120 min of reperfusion. Occlusion was confirmed by the 40% drop in coronary flow. The infarct size was determined as described previously (*Kuka*, 2012; *Liepinsh*, 2013b). Briefly, at the end of the reperfusion, the LAD was re-occluded, and the heart was perfused with 0.1% methylene blue dissolved in KH buffer solution. Afterwards, hearts were sectioned transversely from the apex to the base in 6 slices of 2 mm thickness and incubated in 1% triphenyl-tetrazolium chloride in phosphate buffer (pH 7.4, 37 ºC) for 10 min to stain viable tissue red and necrotic tissue white. The planemetric analysis of left-ventricle cross-sectional images was performed using Image-Pro Plus v6.3 software to determine the area at risk (AR) and area of necrosis (AN), each 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×100%.

2.3.2.5. Heart infarction *in vivo*

Before the induction of anaesthesia, experimental animals received subcutaneous injections of atropine sulfate at a dose of 50 µg/kg, tramadol at a dose of 20 mg/kg and sodium benzilpenicilinum at a dose of 150 mg/kg.

Anaesthesia was induced by *i.p.* injection of a mixture of ketamine and xylazine at doses of 100 mg/kg and 10 mg/kg, respectively. After the loss of nociceptive reflexes, the experimental animals were intubated using 16G intravenous catheters (Venflon, Sweden), ventilated with room air by a UB 7025 rodent ventilator (Hugo Sachs Elektronik - Harvard Apparatus GmbH, Germany) at a tidal volume of 1.5 ml/100 g animal and a rate of 55 strokes per minute. The chest was opened between ribs 5 and 6, a 5-0 polypropylene thread (SURGIPRO™ II, Covidien, Ireland) was placed around the left coronary artery at the level of the left atrium, and a polypropylene ligature was passed through a piece of plastic tubing. The ECG was recorded from a standard lead (II) using ADInstruments PowerLab system. After surgery, the experimental animals were allowed to adapt for 10 minutes. The coronary artery was occluded by applying tension to the plastic tube-polypropylene string arrangement. Successful occlusion was confirmed by ischemia-induced alterations in the ECG. After 30 minutes of occlusion,

reperfusion was initiated by removing the clamp from the plastic tube. Afterwards, the chest was closed using 3-0 silk threads (SOFSILK II, Covidien, Ireland), and the skin was closed using 4-0 nylon threads (MONOSOF, Covidien, Ireland). After the recovery of self-respiration, extubation was performed.

After 24 h following the induction of reperfusion, the experimental animals received an intraperitoneal injection of sodium pentobarbital at a dose of 60 mg/kg and heparin at a dose of 1000 U/kg. After the onset of anaesthesia, the heart was excised, connected via the aorta to the Langendorff apparatus, and washed with KH buffer. The coronary artery was re-occluded; staining and quantification of necrotic tissues were performed as in an isolated heart infarction setup.

2.3.3. 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).

3. RESULTS

3.1. The effects of energy metabolism pattern on myocardial infarction

3.1.1. 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 3.1.) 'Fed' buffer solution contained higher concentrations of glucose, lactate and insulin, but lower concentration of palmitate, compared to 'fasted' buffer solution.

Table 3.1.

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, $P < 0.05$).

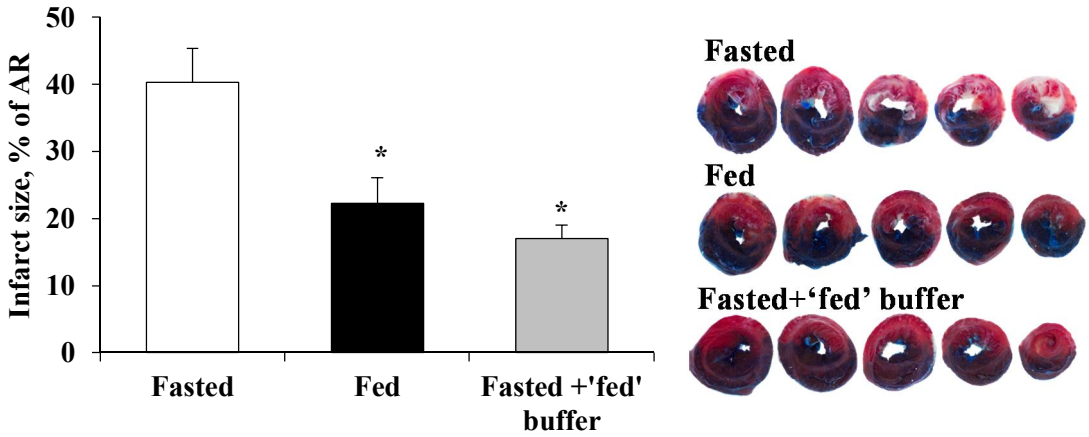


Figure 3.1. **Differences in the myocardial infarct size in hearts from fasted and fed rats**
The presented results are mean ± SEM of 8 rats. *Significantly different from the fasted group (Tukey's test, $P < 0.05$)

The infarct size in fed Wistar rat hearts was almost 2-fold (46%) smaller than that observed in fasted rat hearts (Figure 3.1.). 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)).

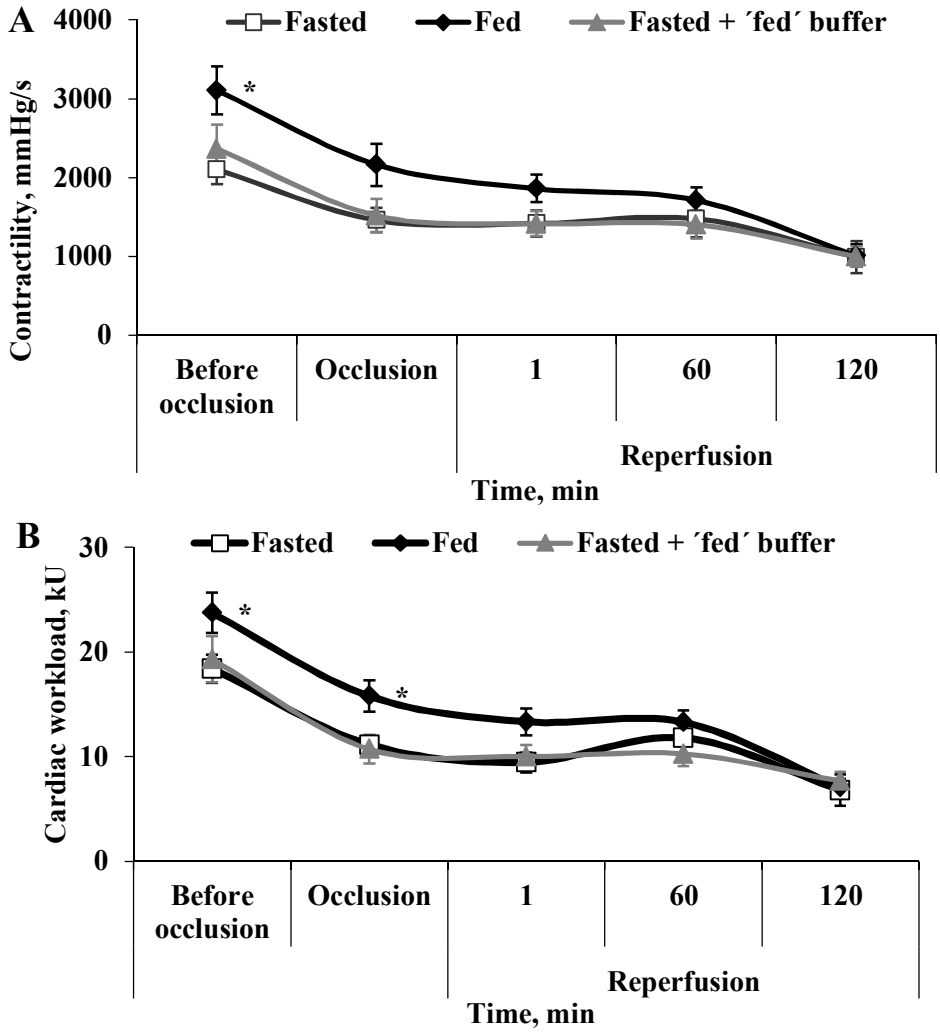


Figure 3.2. **Differences in the contractility (A) and cardiac workload (B) in hearts from fasted and fed rats**
The presented results are mean ± SEM of 8 rats. *Significantly different from the fasted group (Tukey's test, $P < 0.05$).

The basal contractility was significantly lower in fasted rat hearts compared to the fed group (Figure 3.2.A) although no significant differences were observed regarding the basal contractility and cardiac workload between the Fasted and Fasted+'fed' buffer groups. During occlusion the cardiac workload (HR*LVDP) was significantly lower in fasted rat hearts compared to fed rat hearts (Figure 3.2.B). To characterize ischemia-reperfusion tolerance changes in hemodynamic parameters were

normalized to the baseline function. All the parameters were found to be similar in fed and fasted states. In summary, although the changes in hemodynamic parameters relative to the baseline values were similar, the cardiac function in total was worse and the infarct size was significantly larger in hearts from fasted rats compared to those from fed rats. In addition, perfusion with 'fed' buffer was able to protect fasted hearts against infarction-induced damage but could not improve cardiac function.

The effects of fasted and fed states on infarct size were studied also in the model of type 2 diabetes, Goto-Kakizaki rats. The Goto-Kakizaki rats had markedly reduced insulin sensitivity and impaired glucose tolerance (Table 3.2.).

Table 3.2.

The concentrations of biochemical components in plasma and glucose tolerance test of Wistar and diabetic Goto-Kakizaki rats

	Wistar		*Goto-Kakizaki*	
	Fasted	Fed	Fasted	Fed
Glucose, mM	3.8 ± 0.2	5.2 ± 0.2*	6.1 ± 0.2$^{\#}$	8.0 ± 0.3*,$^{\#}$
Insulin, ng/ml	0.46 ± 0.05	0.99 ± 0.17*	0.77 ± 0.16	1.18 ± 0.60
Fatty acids, mM	0.92 ± 0.03	0.29 ± 0.02*	0.90 ± 0.07	0.46 ± 0.06*,$^{\#}$
Glucose tolerance test, AUC	850 ± 93		2345 ± 285$^{\#}$	

The presented results are mean ± SEM of 5 rats. *Significantly different from the respective fasted group (Tukey's test, $P < 0.05$). $^{\#}$ Significantly different from Wistar rats group (Student's t-test or Tukey's test, $P < 0.05$).

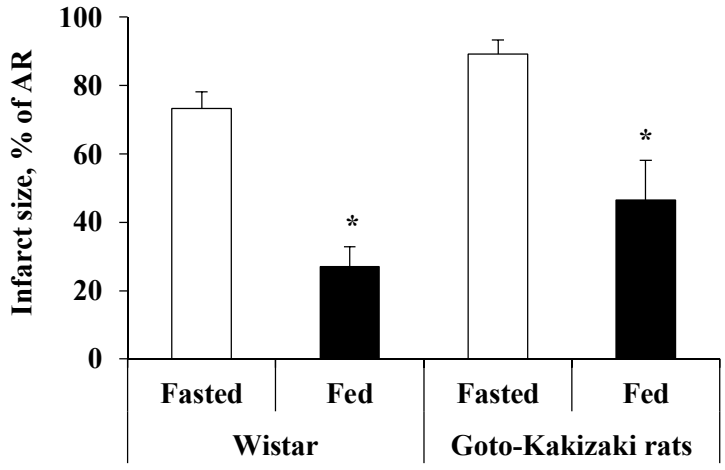


Figure 3.3. **Differences in myocardial infarct size in hearts from Wistar and diabetic Goto-Kakizaki rats**
The presented results are mean ± SEM of 5 rats. *Significantly different from the respective fasted group (Tukey's test, $P < 0.05$).

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 3.3.).

Similar to Wistar rats, in Goto-Kakizaki rats the basal contractility was significantly lower (Figure 3.4.A) in hearts from fasted animals, although no significant difference was observed in cardiac workload (Figure 3.4.B).

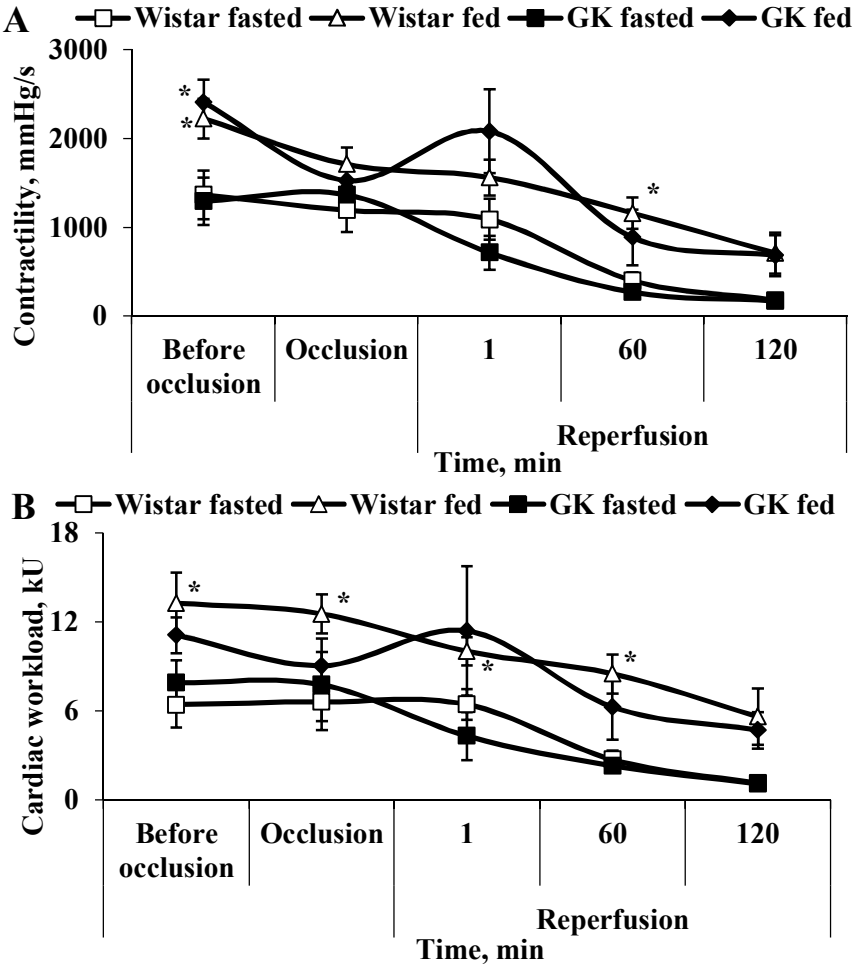


Figure 3.4. **Differences in contractility (A) and cardiac workload (B) in hearts from Wistar and diabetic Goto-Kakizaki (GK) rats**
The presented results are mean ± SEM of 5 rats. *Significantly different from the respective fasted group (Tukey's test, $P < 0.05$).

3.1.2. 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. In the fasted state, the oxidation of glucose and lactate was very limited (Figure 3.5.A and B). In contrast, glucose and lactate oxidation rates were increased by 5- and 4-fold (Figure 3.5.A and B), respectively, although the circulating concentrations of glucose and lactate were only 2-fold higher (Table 3.1.) 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 3.1.).

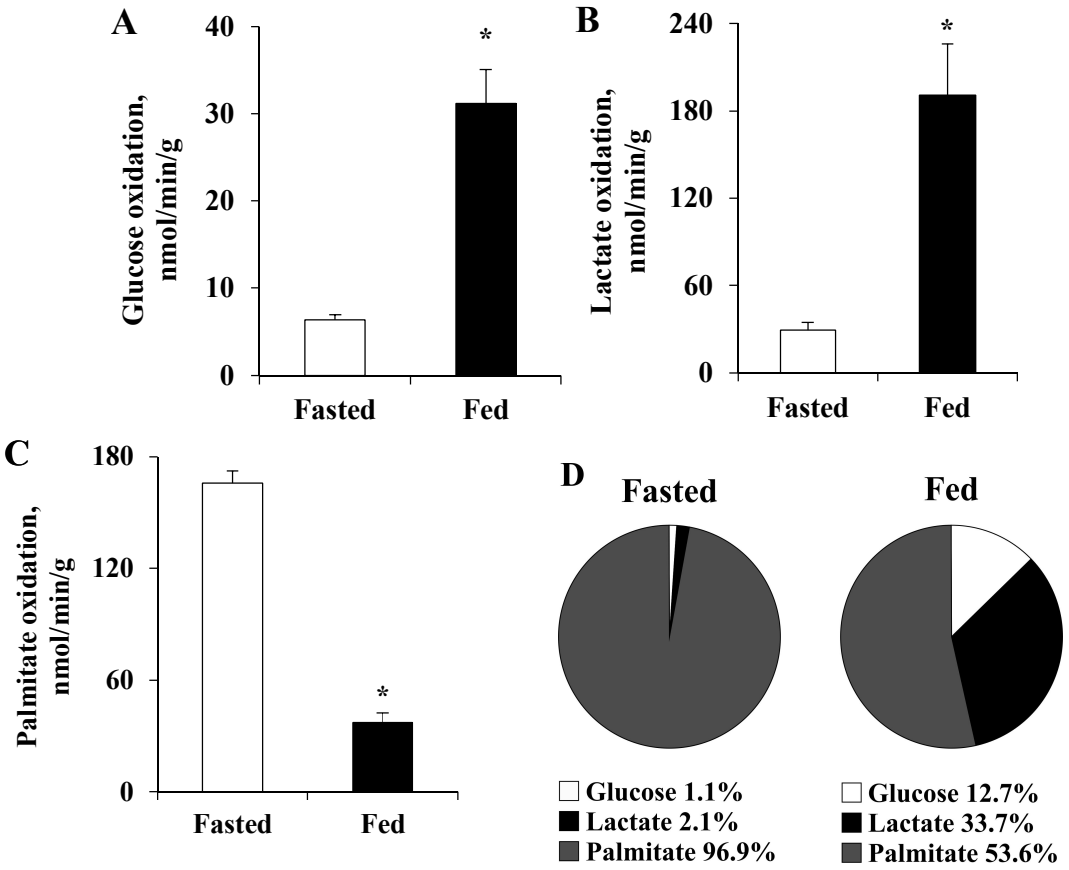


Figure 3.5. **Oxidation rates of energy substrates (glucose (A), lactate (B) and palmitate (C)) in isolated hearts from fasted and fed Wistar rats. Calculated energy substrate input in the energy production (D) in hearts from fasted and fed Wistar rats**

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 contrast to glucose oxidation, FA oxidation depends on the levels of circulating FAs (Table 3.1.). The 3-fold reduction in the labelled palmitate oxidation rate was observed in the fed state compared to the fasted state (Figure 3.5.C). In the fasted state, FA oxidation overrides glucose and lactate oxidation and prevails as the predominant energy source (Figure 3.5.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.

3.1.3. The effects of fasted and fed states on insulin and PPAR-α/PGC-1α mediated changes in the gene expression

In the fed state, the higher concentration of circulating insulin (Table 3.1.) together with activated insulin pathway (Figure 3.6.A) induced a significant 2-fold increase in the expression of genes involved in glucose metabolism (uptake (GLUT1, GLUT4), phosphorylation (HK2) and pyruvate decarboxylation (PDHx)) (Figure 3.7.). Changes in gene expression in the fed state are associated with the increased oxidation of glucose and lactate in the whole heart (Figure 3.5.A and B). Moreover, the respiration of cardiac fibers with pyruvate and malate was increased by 20% (Table 3.3.).

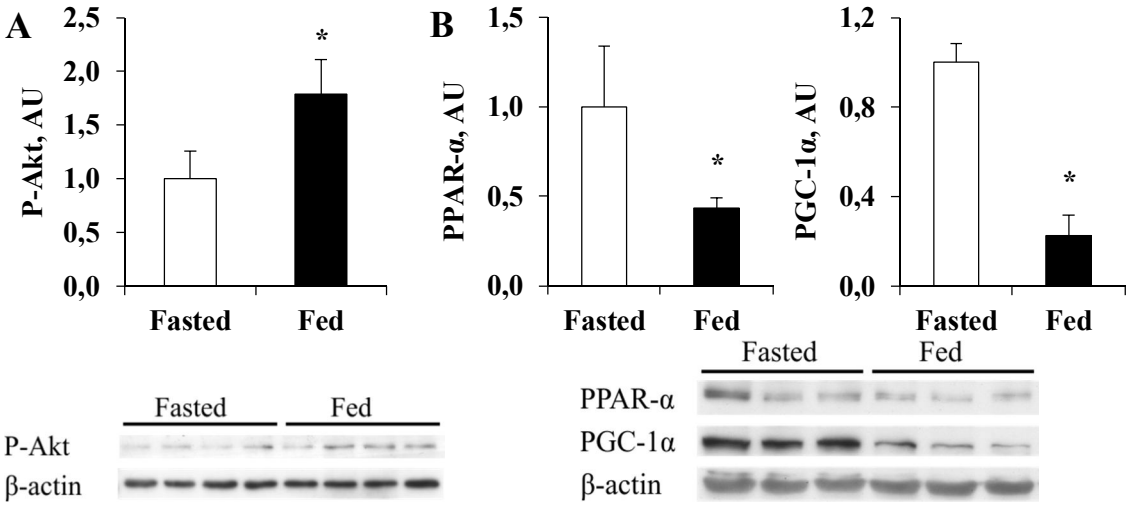


Figure 3.6. **Nuclear contents of phosphorylated Akt (P-Akt) (A), PPAR-α and PGC-1α (B) in the hearts from fed and fasted Wistar rats**

The presented results are mean ± SEM of 4 rats. *Significantly different from the fasted group (Mann–Whitney test, $P < 0.05$).

In contrast, in the fasted state the PPAR-α/PGC-1α pathway and genes involved in lypolysis (HSL) and FA transport (FATP1, CPT1A) were upregulated (Figure 3.6.B). These gene expression results are consistent with the differences in FA metabolism between the fasted and fed states (Figure 3.5.C) in the heart. The expression of PDK4 (an inhibitor of pyruvate decarboxylation) was 10-fold higher in the fasted state compared to the fed state (Figure 3.7.). Thus, an increased expression of PDK4 provides a mechanism for the arrest of lactate oxidation in the fasted state (Figure 3.5.B) as indicated by respiration of cardiac fibers using pyruvate and malate as substrates (Table 3.3.).

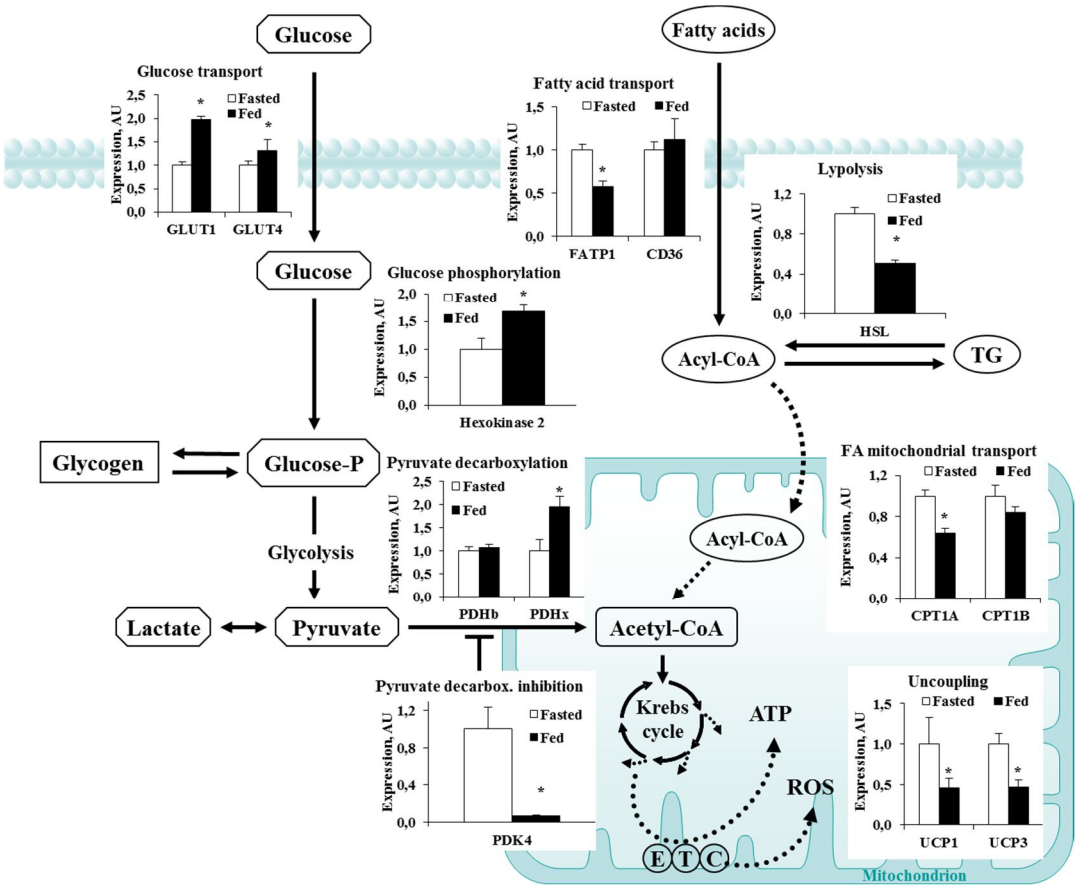


Figure 3.7. **Differences in the gene expression profile in the hearts from fasted and fed Wistar rats**

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

Meanwhile no significant difference was observed in mitochondrial FA oxidation rate using palmitoylCoA as a respiratory substrate (Table 3.3.). Although changes in gene expression induced by nutritional state are associated with energy metabolism pattern in the whole heart (Figure 3.5.), present results indicate that these changes do not regulate energy metabolism in mitochondria.

Table 3.3.

Mitochondrial respiration rate measurements using different substrates in cardiac fibers under fasted and fed states

Substrate[1]	*Fasted*	*Fed*
	Respiration rate, nmol O_2/min/mg w.w.	
Pyruvate	7.0 ± 0.4	8.6 ± 0.6*
PalmitoylCoA	2.9 ± 0.1	2.7 ± 0.4

The presented results are mean ± SEM of 5 rats. *Significantly different from the fasted group (Mann–Whitney test, $P < 0.05$).

[1] Pyruvate – 6 mM pyruvate + 6 mM malate;
PalmitoylCoA – 10 µM palmitoylCoA + 700 µM L-carnitine + 2 mM malate.

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

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

The concentrations of biochemical components in cardiac tissue were measured to determine the availability of energy substrates in tissues under fed and fasted states. Despite a significant difference in the content of biochemical components in the plasma of fed and fasted animals (Table 3.1), 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 (Figure 3.8.A and B). 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.

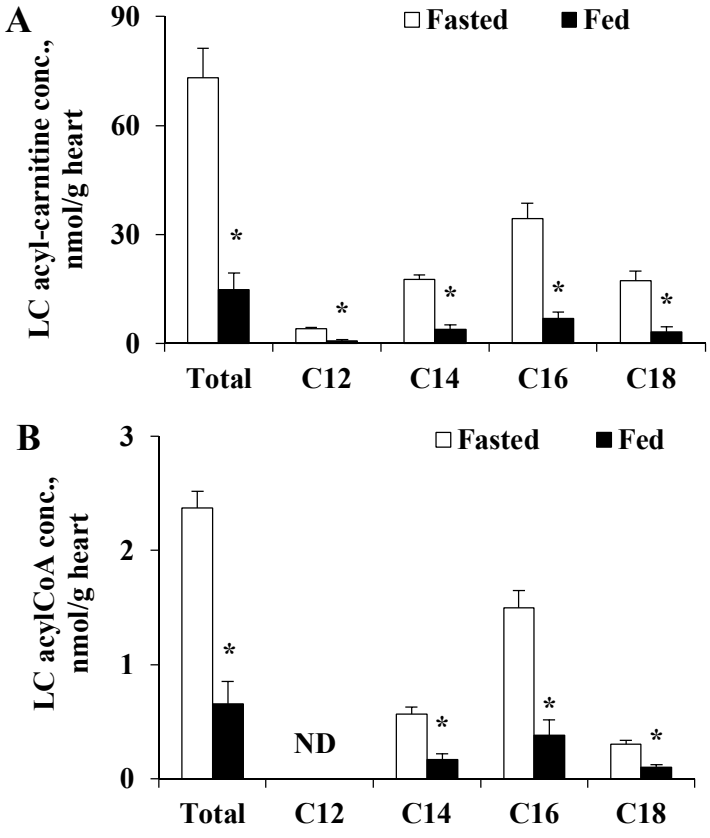


Figure 3.8. **The contents of long-chain (LC) acyl-carnitines (A) and acylCoAs (B) in the hearts of fasted and fed Wistar rats**

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

3.2.2. Substrate competition in isolated cardiac mitochondria

To determine the effects of substrate availability and competition for energy production at the mitochondrial level, substrate oxidation experiments were performed in isolated cardiac mitochondria using a wide range of substrate concentrations. Measurements of pyruvate, lactate and palmitate oxidation in mitochondria showed that oxidation rates are highly dependent on the concentrations of these substrates (Figure 3.9. and 3.11.). 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 3.9.).

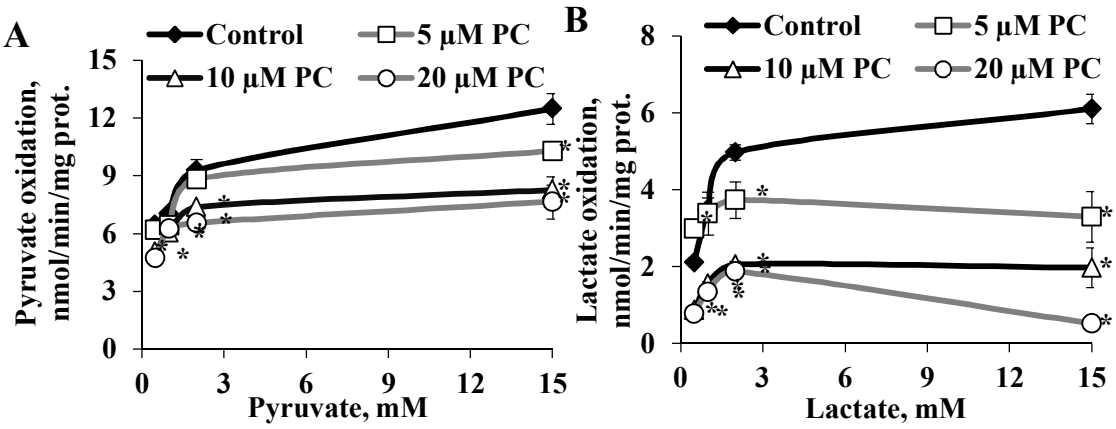


Figure 3.9. **The effects of palmitoyl-carnitine (PC) on pyruvate (A) and lactate (B) oxidation in isolated cardiac mitochondria**
The presented results are mean ± SEM of 5 rats. *Significantly different from the Control (Student's t-test, $P < 0.05$).

The inhibitory effect of palmitoyl-carnitine was more pronounced in the case of lactate metabolism. 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. In contrast to palmitoyl-carnitine, palmitoylCoA had no impact on pyruvate oxidation (Figure 3.10.).

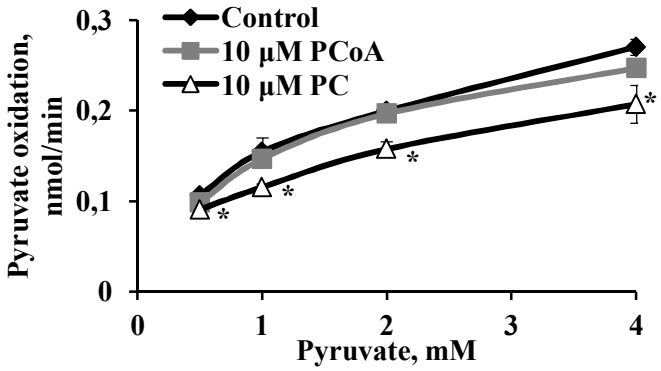


Figure 3.10. **The effects of palmitoyl-carnitine (PC) or palmitoylCoA (PCoA) on pyruvate oxidation in isolated cardiac mitochondria**

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

Furthermore, pyruvate, but not lactate, was able to significantly reduce the palmitate oxidation rate in mitochondria (Figure 3.11.). 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.

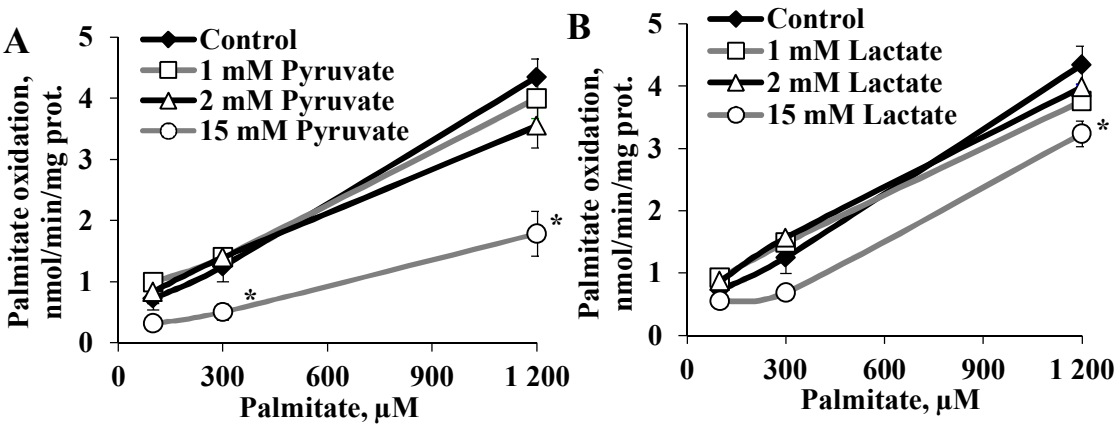


Figure 3.11. **The effects of pyruvate (A) and lactate (B) on FA oxidation in isolated cardiac mitochondria**

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

3.2.3. 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[2] with or without 20 μM palmitoyl-carnitine.

Table 3.4.

The acyl-carnitine content in cardiac tissues after perfusion with buffer supplemented with 20 μM palmitoyl-carnitine

	Control	*Palmitoyl-carnitine*
	Acyl-carnitines, nmol/g heart	
Short-chain (C2-C4)	128 ± 14	156 ± 13
Medium-chain (C6-C10)	0.5 ± 0.1	0.8 ± 0.1
Long-chain (C12-C20)	318 ± 54	536 ± 70*

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

The increase in long-chain acyl-carnitine content (Table 3.4.) induced by perfusion with palmitoyl-carnitine had no effect on glucose uptake rate in the isolated heart (Figure 3.12.A). However, the glucose oxidation rate was significantly decreased by 47% in palmitoyl-carnitine-treated hearts (Figure 3.12.B).

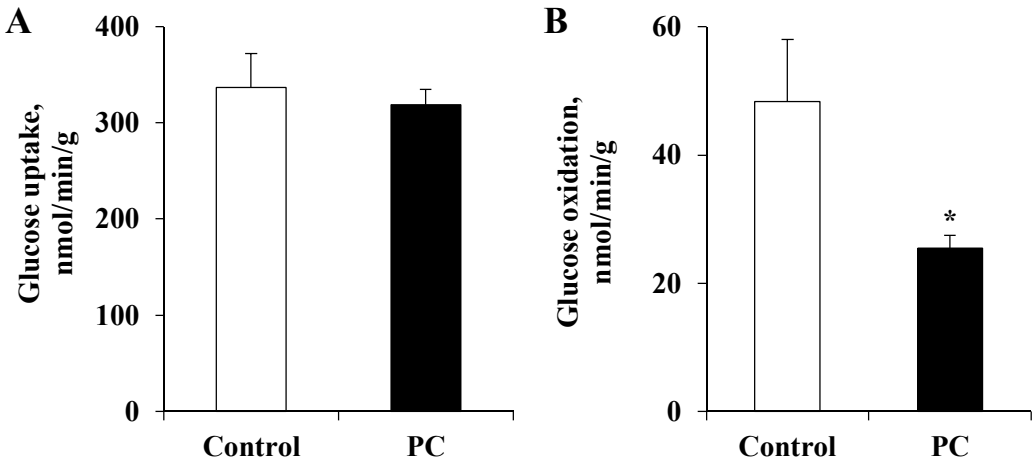


Figure 3.12. **The effects of palmitoyl-carnitine (PC) on glucose uptake (A) and oxidation (B) in isolated rat heart model**
The presented results are mean ± SEM of 8 rats. *Significantly different from the Control group (Student's t-test, $P < 0.05$).

[2] 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.

To determine the effects of long-chain acyl-carnitine on glucose utilization *in vivo*, blood concentration of glucose was measured before and 15, 30 and 60 min after the acute administration of palmitoyl-carnitine that significantly increased long-chain acyl-carnitine concentration in plasma and cardiac and muscle tissues (Table 3.5.).

Table 3.5.

The effect of acute administration of palmitoyl-carnitine on the concentrations of long-chain acyl-carnitines in plasma and cardiac and muscle tissues

	Control	*Palmitoyl-carnitine 200 mg/kg i.p.60 min*
	Long-chain acyl-carnitines	
Plasma, μM	1.0 ± 0.2	164 ± 13*
Cardiac tissues, nmol/g	28 ± 8	135 ± 11*
Muscle tissues, nmol/g	14 ± 3	101 ± 17*

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

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 3.13.) indicating the overall decrease in glucose utilization. Taken together, these results demonstrate that increased concentration of long-chain acyl-carnitines impairs glucose utilization.

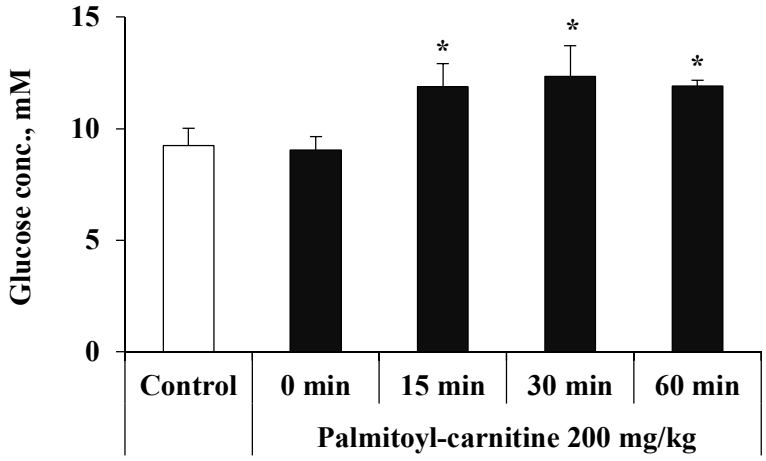


Figure 3.13. **The effects of acute palmitoyl-carnitine administration on glucose utilization *in vivo***

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

3.3. 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 (approx. 500 mg/kg/day)), and the effects of the treatment on cardiac functionality and mitochondrial energy metabolism were studied.

3.3.1. 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 3.14.).

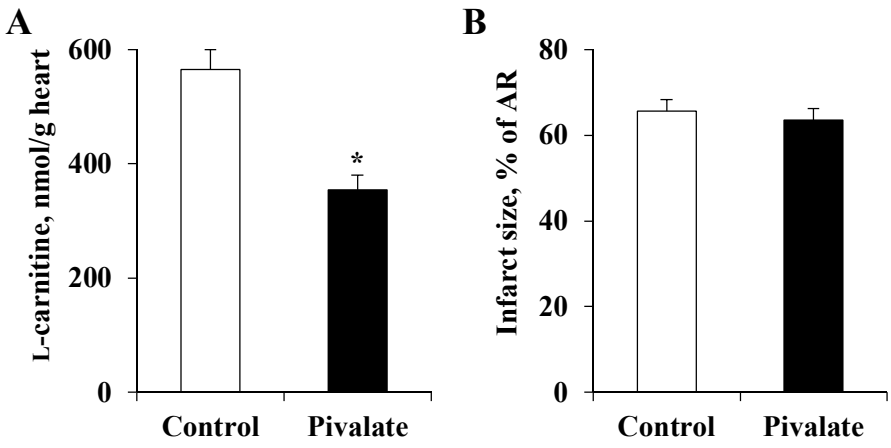


Figure 3.14. **The effects of sodium pivalate treatment on L-carnitine content in cardiac tissues (A) and infarct size (B)**
The presented results are mean ± SEM of 10 rats. *Significantly different from the Control group (Student's t-test, $P < 0.05$).

The treatment with sodium pivalate had no effect on cardiac functional parameters, including heart rate (HR), left ventricular developed pressure (LVDP), coronary flow, contractility/relaxation (dP/dt max and min), or the cardiac workload (HR*LVDP), compared to the control group (Table 3.6.). During the occlusion of LAD, the coronary flow was decreased by an average of 47-48%, and the cardiac workload was decreased by an average of 21-26% compared to the values observed prior to occlusion; however, no significant changes were observed between both groups. No significant differences were observed between the groups during the reperfusion stage (Table 3.6.).

Table 3.6.

The effect of sodium pivalate administration on isolated rat heart hemodynamic

	Heart rate, BPM	Coronary flow, ml/min	LVDP, mmHg	dP/dt max, mmHg/sec.	dP/dt min, mmHg/sec.	Cardiac workload, kU
	Basal hemodynamic parameters					
Control	258±9	12.5±0.7	150±7	4631±227	-2771±165	38821±2685
Pivalate	246±21	11.3±0.5	146±8	4648±318	-2674±188	36423±4056
	Hemodynamic parameters during ischemia					
Control	256±11	6.7±0.4*	104±7*	2923±220*	-1868±127*	27004±2634*
Pivalate	221±19	6.0±0.4*	105±10*	2916±335*	-1829±163*	23735±3241*
	Hemodynamic parameters at the end of reperfusion					
Control	247±8	7.2±0.6*	85±10*,#	2862±329*	-1585±169*,#	21762±3229*,#
Pivalate	219±24	6.4±0.5*	81±8*,#	2681±356*	-1492±150*,#	18647±3363*,#

The presented results are mean ± SEM of 10 rats. *Significantly different from the same group before occlusion (Tukey's test, $P < 0.05$). #Significantly different from the same group during ischemia (Tukey's test, $P < 0.05$).

3.3.2. 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 (CPT I and CrAT) activities were measured in isolated cardiac mitochondria.

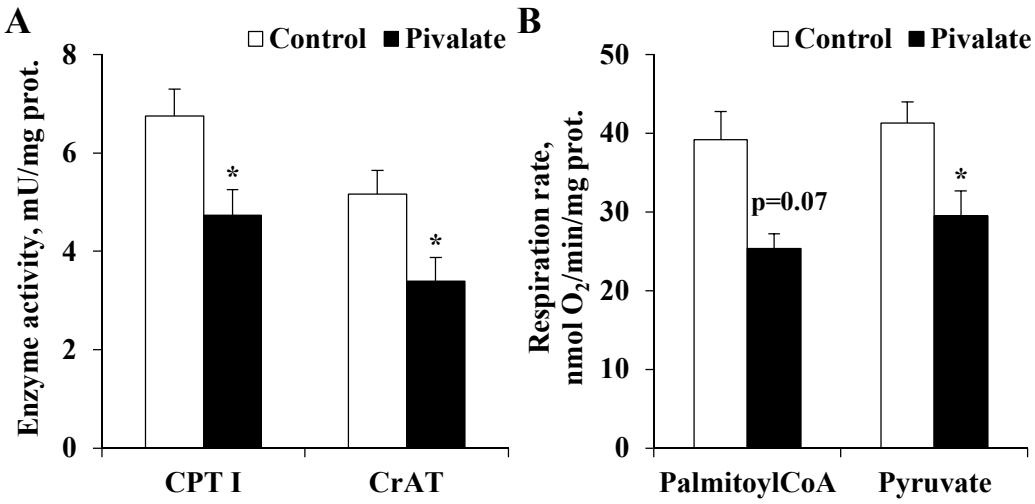


Figure 3.15. **The effects of sodium pivalate treatment on L-carnitine-dependent enzyme activities (A) and energy metabolism (B) in isolated cardiac mitochondria**
The presented results are mean ± SEM of 3-6 rats. *Significantly different from the Control group (Student's t-test or Mann-Whitney U-test, $P < 0.05$).

CPT I activity was significantly decreased by 30% in the pivalate group compared to the control group (Figure 3.15.A). Similarly, CrAT activity was significantly decreased by 34% in the pivalate group compared to the control group (Figure 3.15.A). Taken together, these results indicate that sodium pivalate treatment may impair energy metabolism in cardiac mitochondria.

To determine the effects of sodium pivalate treatment on mitochondrial energy metabolism, mitochondrial respiration measurements using different substrates in isolated cardiac mitochondria were performed. After the 14-day treatment with sodium pivalate, the CPT I-dependent mitochondrial respiration on palmitoylCoA was decreased by 35%; however, this effect was not statistically significant (Figure 3.15.B). The treatment with sodium pivalate had no significant impact on CPT I-independent mitochondrial respiration on palmitoyl-carnitine (16.2 ± 3.3 nmol O_2/min/mg protein compared to 16.4 ± 1.2 nmol O_2/min/mg protein for the control group). The respiration on pyruvate/malate after treatment with sodium pivalate was significantly decreased by 28% (Figure 3.15.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.

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

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).

3.4.1. Screening on BBOX and OCTN2

Fifty compounds were synthesized and characterized, among them 15 were analogues of meldonium and 35 were GBB analogues. The inhibition potency of meldonium analogues on BBOX activity and OCTN2-mediated L-carnitine transport is presented in Table 3.7. The inhibition potency of GBB analogues on BBOX activity and OCTN2-mediated L-carnitine transport is presented in Table 3.8.

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.

Table 3.7.

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

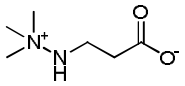
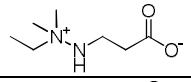
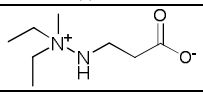
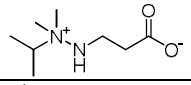
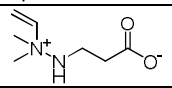
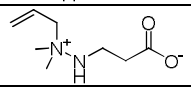
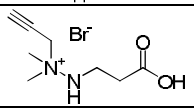
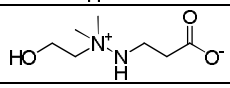
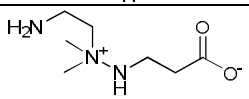
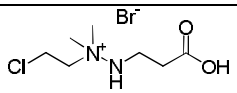
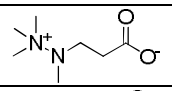
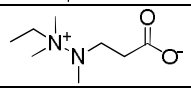
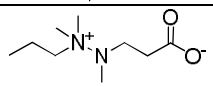
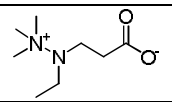
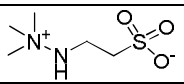
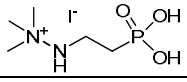
No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
	 Meldonium	26 ± 2	62 (*Tars*, 2010)	62 ± 5
Modifications of the trimethylammonium group				
1		> 1000	140 ± 52	21 ± 13
2		118 ± 65	> 1000	64 ± 20
3		378 ± 27	> 1000	10.6 ± 5.2
4		8 ± 6	6.8 ± 1.7	38 ± 8
5		> 1000	119 ± 43	47 ± 10
6		67 ± 2	24 ± 8	46 ± 1
7		> 1000	1000	73 ± 0.5
8		> 1000	> 1000	100
9		160 ± 26	10.2 ± 4.4	> 100
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
13		3.4 ± 0.9	2.8 ± 1.9	99 ± 16

Table 3.7. (continued)

No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
Modifications of the carboxylic acid				
14		91 ± 31	87 ± 33	> 100
15		449 ± 10	117 ± 21	> 100

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

Table 3.8.

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

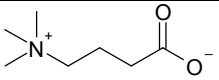
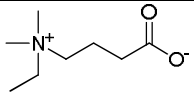
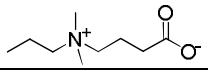
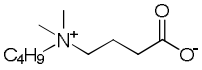
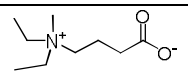
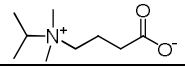
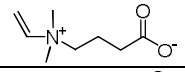
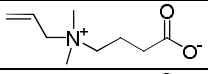
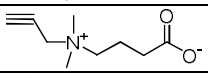
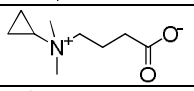
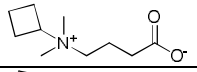
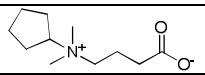
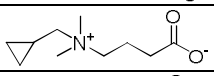
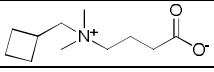
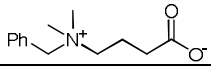
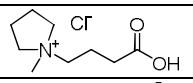
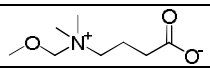
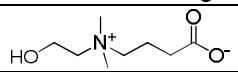
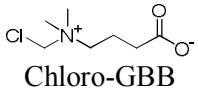
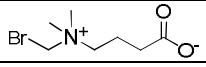
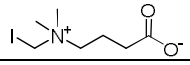
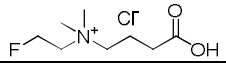
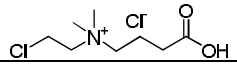
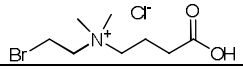
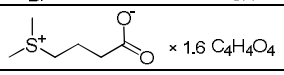
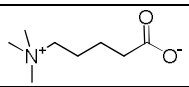
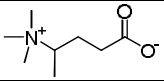
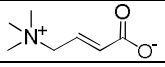
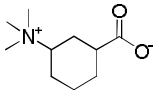
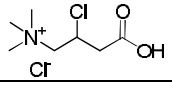
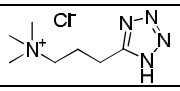
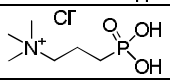
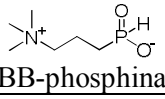
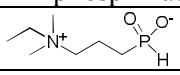
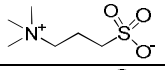
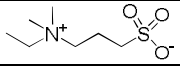
No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
	 GBB	-	-	3.9 ± 0.1
Modifications of the trimethylammonium group				
16	 Methyl-GBB	2.8 ± 0.6	3.3 ± 1.8	3 ± 0.3
17		> 1000	> 1000	59 ± 10
18		> 1000	> 1000	> 100
19	 Dimethyl-GBB	> 1000	100 ± 36	14.6 ± 3.0
20		34 ± 6	5.7 ± 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
23		10 ± 4	1.4 ± 0.6	19.3 ± 2.6
24		3.1 ± 0.6	2.1 ± 0.7	2.4 ± 0.5
25		2.1 ± 0.2	0.81 ± 0.37	4.4 ± 0.8
26		> 1000	> 1000	> 100
27		> 1000	> 1000	55 ± 1
28		> 1000	> 1000	40 ± 4
29		> 1000	> 1000	100

Table 3.8. (continued)

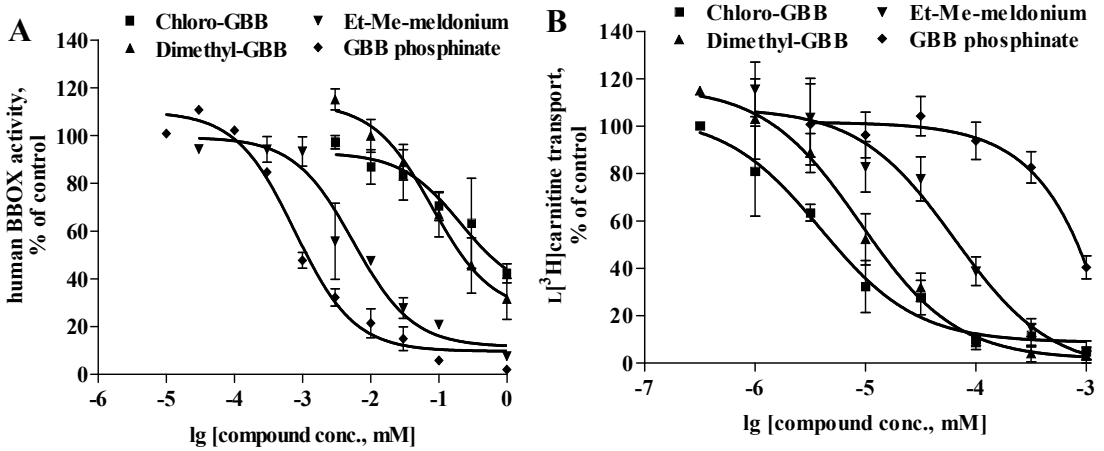
No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
30		1.40 ± 0.03	0.49 ± 0.24	4.4 ± 1.0
31		168 ± 18	> 1000	36 ± 6
32		> 1000	1000	49 ± 6.4
33	 Chloro-GBB	95 ± 3	268 ± 54	4.2 ± 2.5
34		674 ± 71	> 1000	7.9 ± 1.2
35		> 1000	1000	11.4 ± 7.3
36		0.78 ± 0.36	0.61 ± 0.22	7 ± 1.8
37		1.0 ± 0.2	0.26 ± 0.13	33 ± 6
38		18 ± 8	1.2 ± 0.3	65 ± 11
39		232 ± 36	388 ± 32	93 ± 13
Modifications of the core region				
40		> 1000	> 1000	67 ± 26
41		452 ± 13	343 ± 117	26 ± 3
42		10.3 ± 3.0	3.8 ± 1.8	50 ± 15
43		> 1000	> 1000	7.7 ± 3.0
44		1000	459 ± 66	24 ± 14
Modifications of the carboxylic acid				
45		> 1000	> 1000	6.5 ± 1.3
46		10.2 ± 5.8	0.10 ± 0.03	> 100
47	 GBB-phosphinate	0.8 ± 0.1	0.52 ± 0.35	850 ± 122
48		10.5 ± 2.2	0.53 ± 0.17	> 100
49		71 ± 18	1.7 ± 0.4	> 100
50		203 ± 28	6.7 ± 1.2	100

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

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 $r = 0.033$, $p = 0.82$).

3.4.2. 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 3.16.).



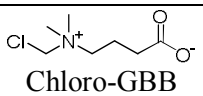
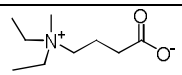
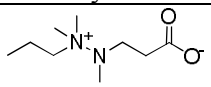
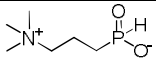
No.	Structure	Rat liver BBOX IC_{50}, μM	Human BBOX IC_{50}, μM	OCTN2 IC_{50}, μM
33	 Chloro-GBB	95 ± 3	268 ± 54	4.2 ± 2.5
19	 Dimethyl-GBB	> 1000	100 ± 36	14.6 ± 3.0
12	 Et-Me-meldonium	6.7 ± 4.4	3.1 ± 1.8	97 ± 5
47	 GBB-phosphinate	0.8 ± 0.1	0.52 ± 0.35	850 ± 122

Figure 3.16. **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.

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.

3.4.3. The influence of administration of a single dose of selected compounds on L-carnitine content in the heart tissues, plasma and urine

The average concentration of free L-carnitine in the control rat plasma was 35 ± 2 μM (Figure 3.17.). A single dose administration of BBOX inhibitors Et-Me-meldonium and GBB-phosphinate did not induce changes in the plasma L-carnitine concentration. In contrast, L-carnitine concentration in the rat plasma increased during the first 24 hours after the treatment with the OCTN2 inhibitors Chloro-GBB and Dimethyl-GBB (Figure 3.17.A). This could be explained by the inhibited OCTN2 mediated L-carnitine transport in tissues.

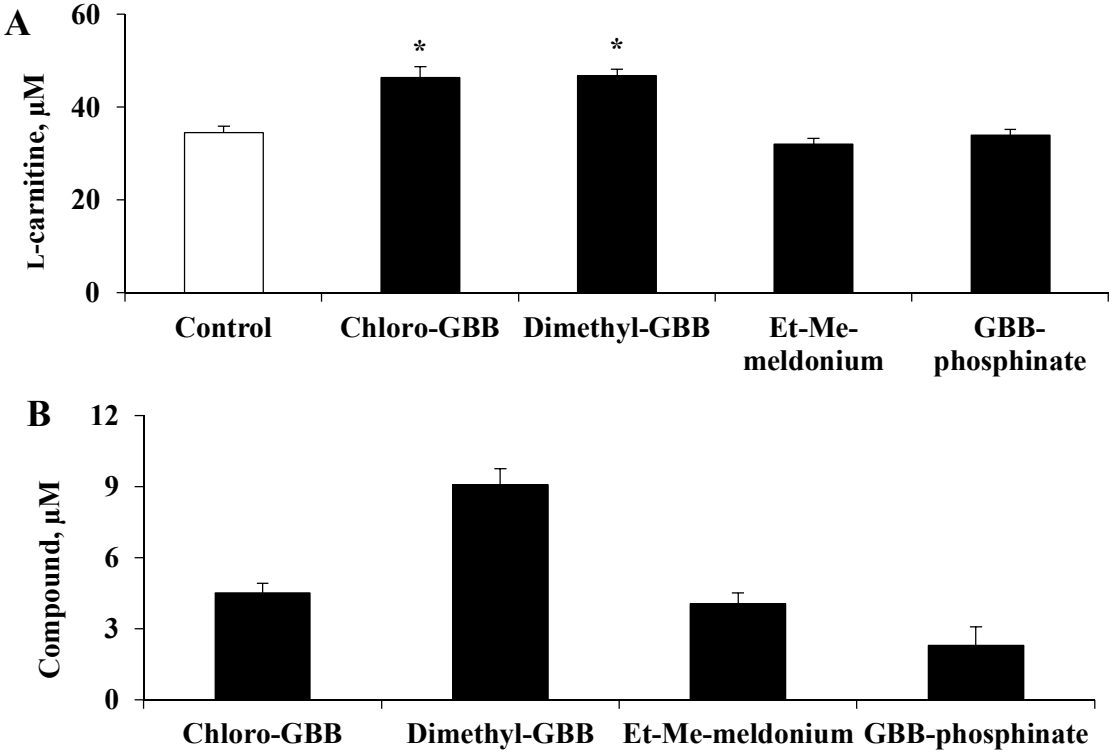


Figure 3.17. **L-carnitine (A) and compound (B) concentrations in the plasma 24 h after a single administration of BBOX and OCTN2 inhibitors**
The presented results are mean ± SEM of 5 rats. *Significantly different from the Control group (Tukey's test, $P < 0.05$).

The plasma concentrations of the compounds 24 h after the administration represent the sum of compound transport into tissues and elimination of the compound by the urine (Figure 3.17.B). Thus, GBB-phosphinate had the lowest concentration in the plasma due to the compound being eliminated most effectively through the urine.

None of the compounds had changed L-carnitine content in the heart tissues 24 h after a single administration (Figure 3.18.A). This finding was in line with the previous observations, which suggested that long-term treatment is necessary to induce a significant reduction in L-carnitine content.

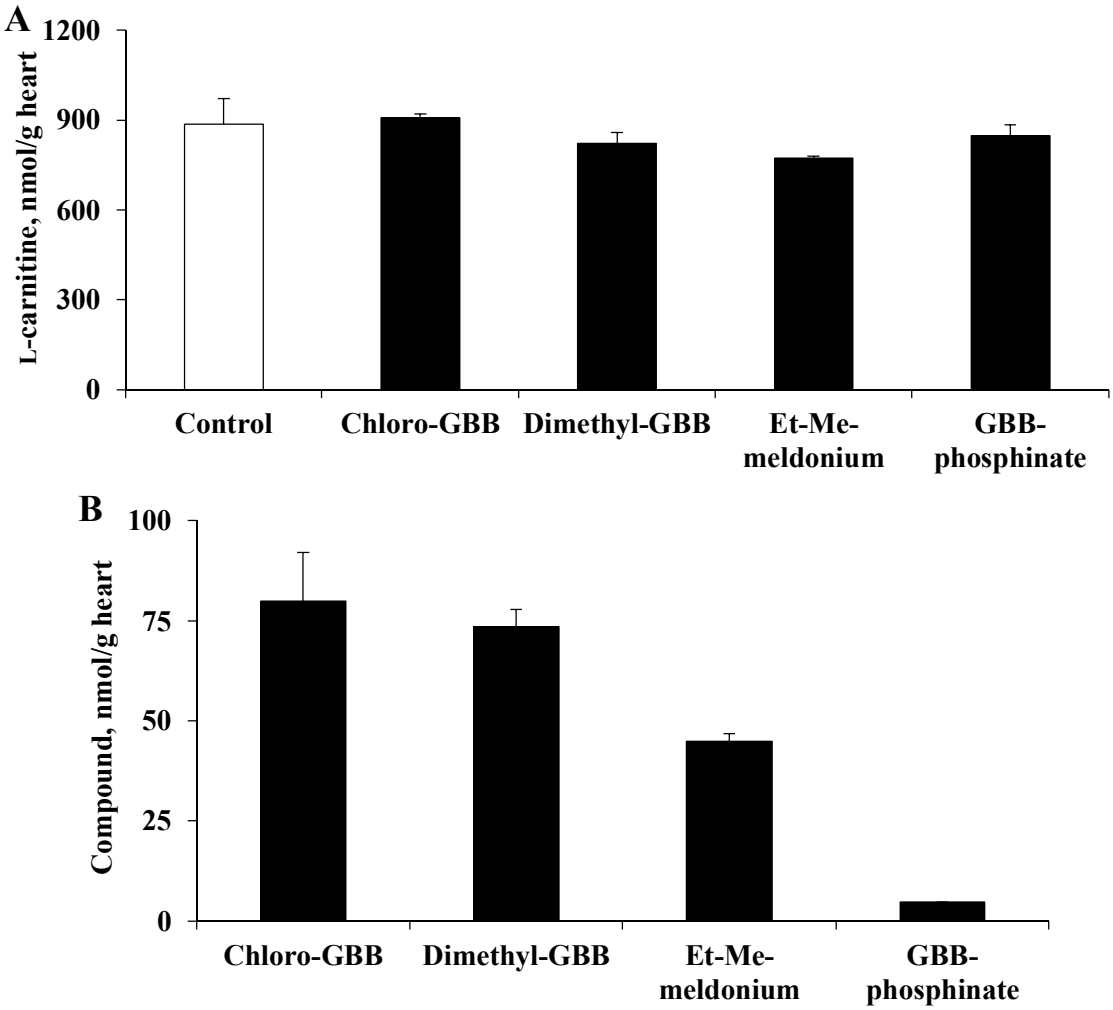


Figure 3.18. **L-carnitine (A) and compound (B) concentrations in the heart 24 h after a single administration of BBOX and OCTN2 inhibitors**
The presented results are mean ± SEM of 5 rats.

In contrast, the content of compounds in the heart tissues varied considerably. Twenty-four hours after a single administration, the concentrations of Chloro-GBB and Dimethyl-GBB reached approximately 80 nmol/g of heart tissue (Figure 3.18.B). In

comparison, the concentrations of Et-Me-meldonium and GBB-phosphinate only reached 45 and 5 nmol/g of heart tissue, respectively (Figure 3.18.B). Thus, the measured IC_{50} for OCTN2 not only characterizes the inhibition of OCTN2-mediated L-carnitine transport, but it is also a measure of the efficacy of OCTN2-mediated transport of the respective compound in tissues.

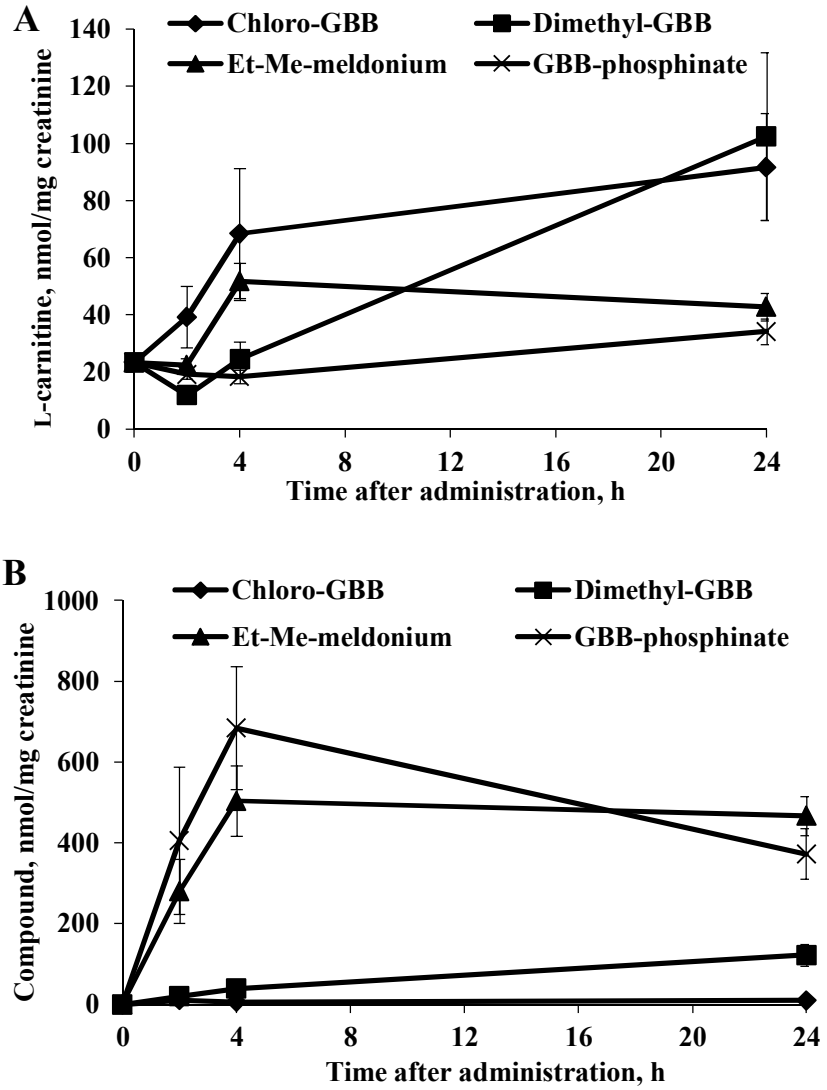


Figure 3.19. **L-carnitine (A) and compound (B) concentration in the urine up to 24 h after a single administration of BBOX and OCTN2 inhibitors**
The presented results are mean ± SEM of 5 rats.

OCTN2 is important for the reabsorbtion of L-carnitine and also the tested compounds from the urine. The average concentration of free L-carnitine in control rat urine was 23 ± 2 nmol/mg of creatinine. Chloro-GBB and Dimethyl-GBB induced a significant (up to 4-fold) increase in L-carnitine content in urine 2-24 h after the administration (Figure 3.19.A). In contrast, GBB-phosphinate did not facilitate

L-carnitine excretion via urine. Similarly to the transport into heart tissues, higher affinity towards OCTN2 was associated with significantly lower elimination of the compounds by the kidneys (Figure 3.19.B). Thus, the concentrations of Et-Me-meldonium and GBB-phosphinate in urine were 5-60 times higher than the concentrations of Chloro-GBB and Dimethyl-GBB, which suggests that there are lower reabsorbtion rates for the compounds with lower affinities towards OCTN2.

3.4.4. 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 3.20.A). 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 3.20.A). 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 3.16.).

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 3.7. and 3.8.) 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 3.20.A). 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 (Figure 3.20.B). In comparison, IC_{50} values of tested compounds for BBOX did not correlate with the decrease of L-carnitine content in the heart tissues ($r = 0.0603$).

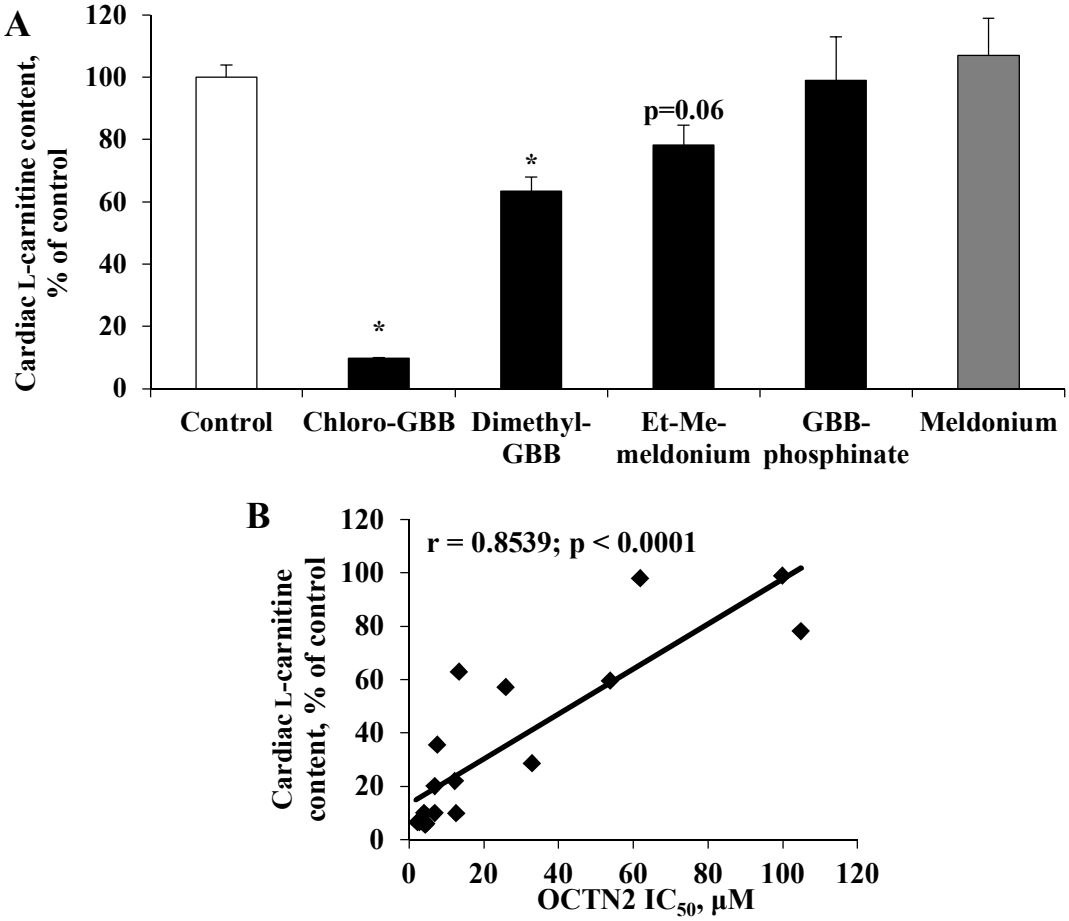


Figure 3.20. **The effect of selected compounds and meldonium (all at a dose of 20 mg/kg) on the L-carnitine content in the heart after 14 days of treatment (A). Correlation between OCTN2 IC_{50} and reduction of L-carnitine content after 14 days of treatment (B)**
The presented results are mean ± SEM of 8-10 rats. *Significantly different from control group (Tukey's test $P < 0.05$).

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

The cardioprotective effects of the compounds were evaluated after a 14-day treatment in an isolated rat heart experimental ischemia-reperfusion model. In the control group, the infarct size was approximately 45% of the area at risk. After a 14-day treatment with Chloro-GBB, the infarct size in the rat hearts was significantly decreased by 35% (Figure 3.21.). 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 3.21.). 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 3.21.).

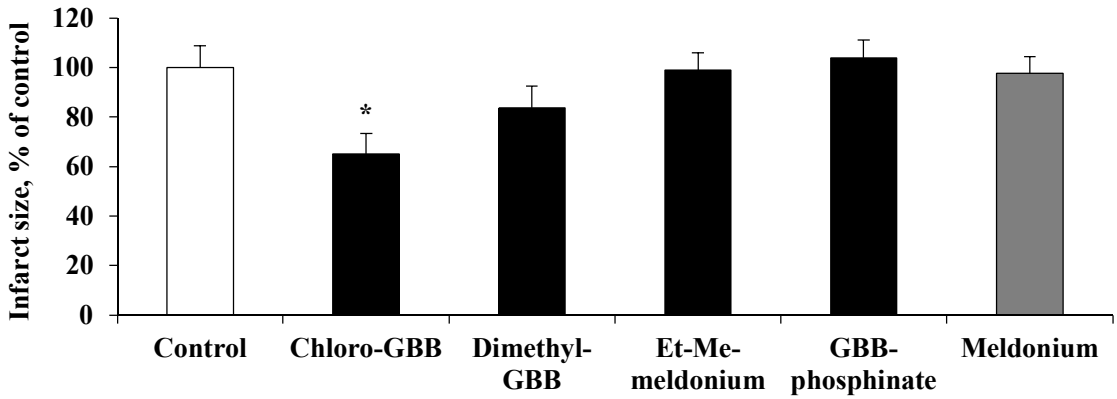


Figure 3.21. **The effect of selected compounds and meldonium on the infarct size after 14 days of treatment**
The presented results are mean ± SEM of 8-10 rats. *Significantly different from control group (Tukey's test $P < 0.05$).

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

3.5.1. 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 3.8.). 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). After 3 days of the treatment at doses of 5 and 20 mg/kg plasma concentration of Methyl-GBB exceeded 10 μM and according to measurements *in vitro* almost completely inhibited L-carnitine transport by OCTN2 (Table 3.9.).

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 3.22.A). Concentration of Methyl-GBB in the heart increased in a time dependent manner (Figure 3.22.B) reaching a maximum after 14 days of treatment. As shown in Figure 3.22.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.

Table 3.9.

The concentrations of L-carnitine and compounds in plasma after 3, 7, 14 days of treatment

		L-carnitine, μM			Compound, μM		
		3 days	7 days	14 days	3 days	7 days	14 days
Control		32 ± 2	33 ± 5	32 ± 3	-	-	-
Meldonium 100 mg/kg		24 ± 1*	19 ± 1*	11 ± 1*	104 ± 15	63 ± 10	19 ± 2
Methyl-GBB	1 mg/kg	38 ± 4$^{\#}$	39 ± 3$^{\#}$	41 ± 5$^{\#}$	1.6 ± 0.2	2.6 ± 0.3	3.4 ± 0.3
	5 mg/kg	47 ± 2*,$^{\#}$	29 ± 1$^{\#}$	14 ± 2*	11 ± 2	15 ± 1	17 ± 1
	20 mg/kg	23 ± 1*	8.7 ± 0.8*,$^{\#}$	5.5 ± 0.2*,$^{\#}$	37 ± 5	30 ± 1	25 ± 2

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 $P < 0.05$).

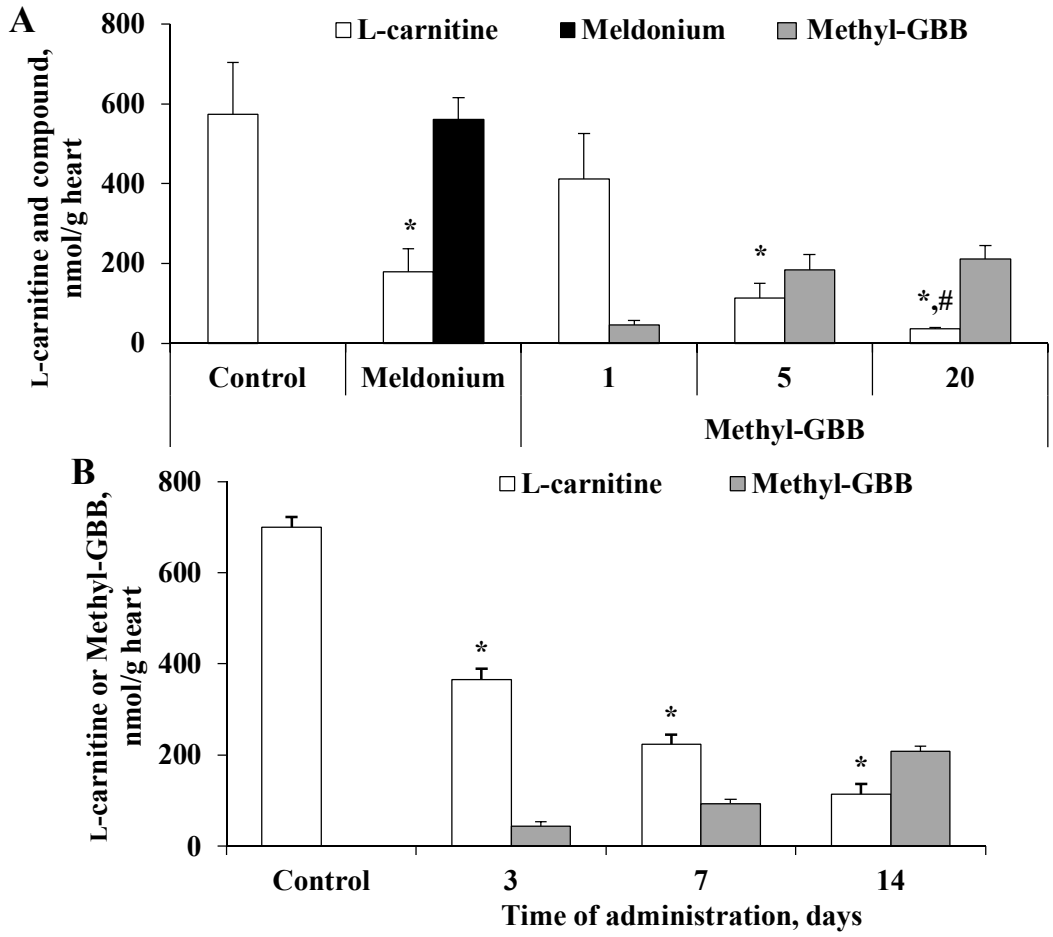


Figure 3.22. **Contents of L-carnitine and compound in cardiac tissues after 14 days of treatment by meldonium (100 mg/kg) or Methyl-GBB (1, 5 and 20 mg/kg) (A) and after 3, 7, 14 days of treatment by Methyl-GBB at a dose of 5 mg/kg (B)**

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 $P < 0.05$).

To study the time-dependent effects of Methyl-GBB on L-carnitine content, we measured the concentration of L-carnitine in plasma samples after 3, 7 and 14-day

administrations of Methyl-GBB. As shown in Figure 3.22.B, Methyl-GBB decreased L-carnitine content in the heart in a time-dependent manner. Meanwhile, after short-term (up to 7 days) treatment with Methyl-GBB, the plasma concentration of L-carnitine did not correlate with the changes in L-carnitine content in the heart (Table 3.9.). Thus, in contrast to the effects of Methyl-GBB on L-carnitine content in the heart, a maximal reduction of plasma L-carnitine concentration was observed only after 14 days of the treatment. Unexpectedly, Methyl-GBB at dose of 1 mg/kg did not decrease L-carnitine concentration in plasma even after 14 days of treatment, while in heart tissue, significant reduction in L-carnitine content was observed (Figure 3.22.A).

3.5.2. 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 3.23.A and B).

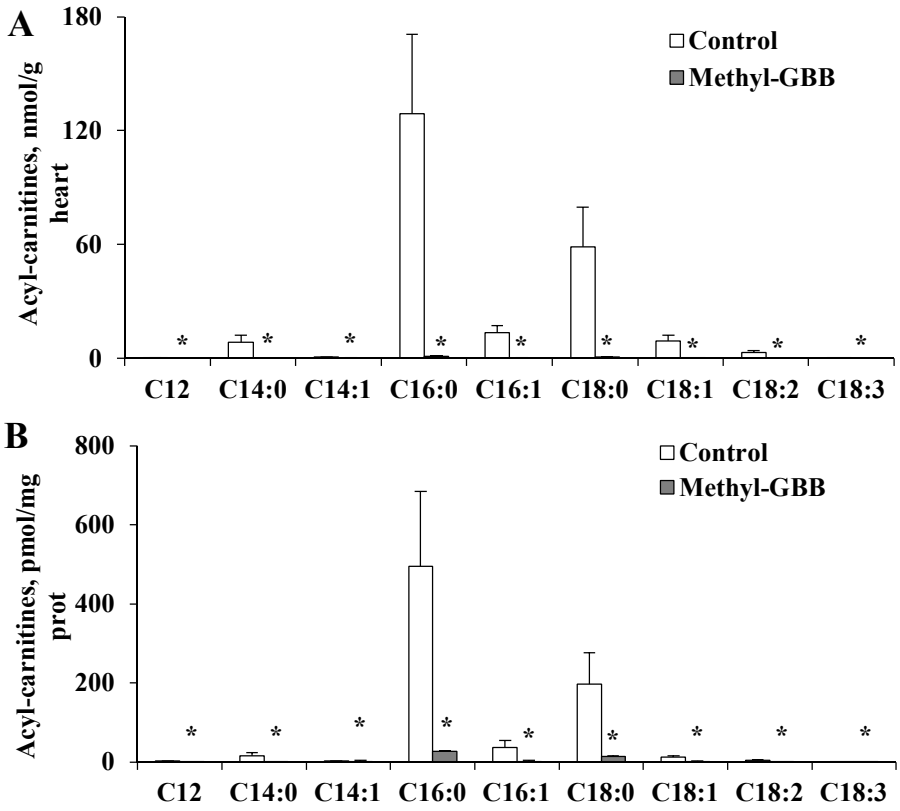


Figure 3.23. **The effects of a long-term Methyl-GBB (10 mg/kg) treatment on cardiac (A) and mitochondrial (B) content of acyl-carnitine**
The presented results are mean ± SEM of 5-8 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

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.

3.5.3. The effects of Methyl-GBB treatment on the FA and glucose oxidation

To test the changes in energy metabolism induced by Methyl-GBB treatment, the FA oxidation rates were measured in the isolated heart and its organelles. 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 3.24.A).

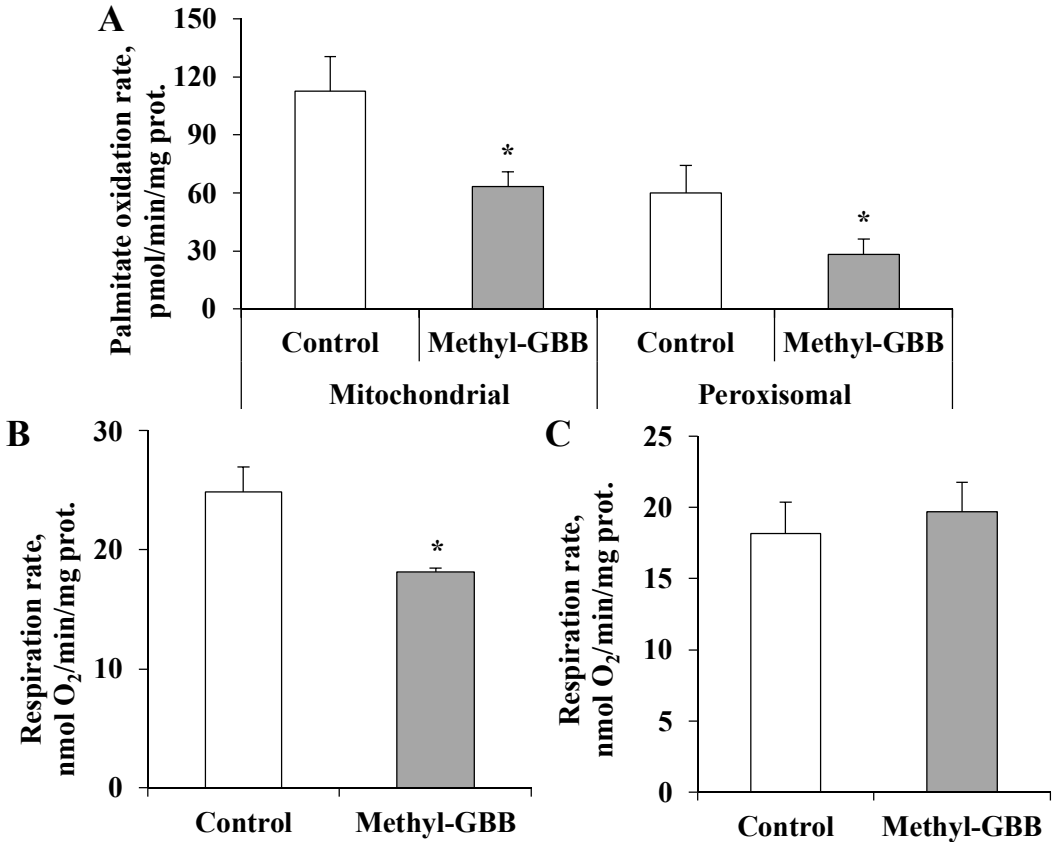


Figure 3.24. **The effects of a long-term Methyl-GBB (10 mg/kg) treatment on mitochondrial and peroxisomal [^{14}C]palmitate oxidation rates (A) and mitochondrial L-carnitine-dependent respiration with 10 µM palmitoylCoA (B) and L-carnitine-independent respiration with 10 µM palmitoyl-carnitine (C)**
The presented results are mean ± SEM of 5-6 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

To elucidate the role of decreased L-carnitine content in the regulation of FA oxidation, both the L-carnitine-dependent and -independent mitochondrial respiration rates were determined. After the 14-day Methyl-GBB treatment, the L-carnitine-dependent mitochondrial respiration rate with palmitoylCoA was significantly decreased by 27% (Figure 3.24.B), but the treatment had no effect on the L-carnitine-independent mitochondrial respiration rate with palmitoyl-carnitine (Figure 3.24.C).

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 3.25.A). An increase in FA oxidation was observed after reperfusion in Methyl-GBB treated group (Figure 3.25.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 3.25.B).

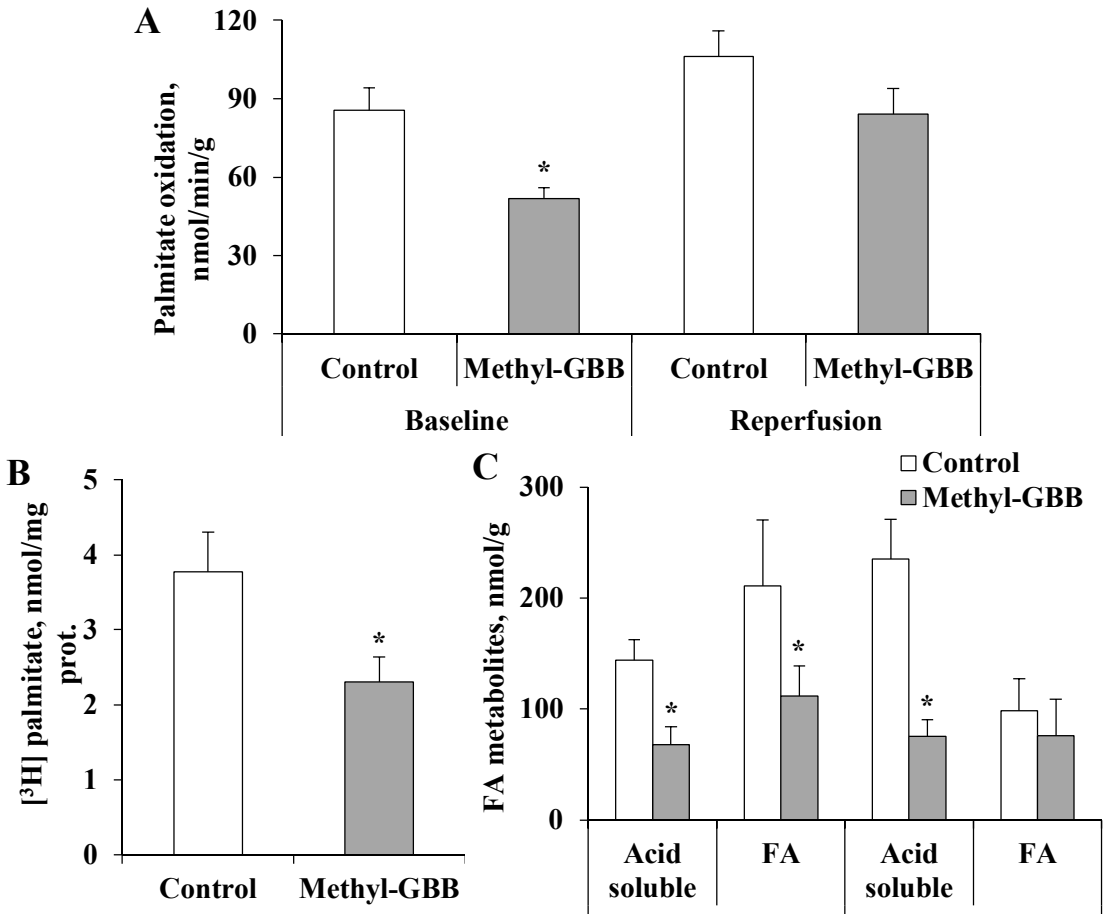


Figure 3.25. **The effects of long-term Methyl-GBB (10 mg/kg) treatment on [^{3}H]palmitate oxidation in isolated heart (A), mitochondrial [^{3}H]palmitate content (B) and [^{3}H]palmitate oxidation in heart and skeletal muscles *in vivo* (C)**
The presented results are mean ± SEM of 5-6 animals. *Significantly different from Control group (Student's t-test $P < 0.05$).

Additionally, we tested the labelled palmitate uptake and metabolism in mice *in vivo* (Figure 3.25.C). In Methyl-GBB-treated mice hearts and skeletal muscles we observed a significant 2-fold decrease in the labelled palmitate uptake and oxidation rate, which coincides with the results observed in the isolated heart and organelles.

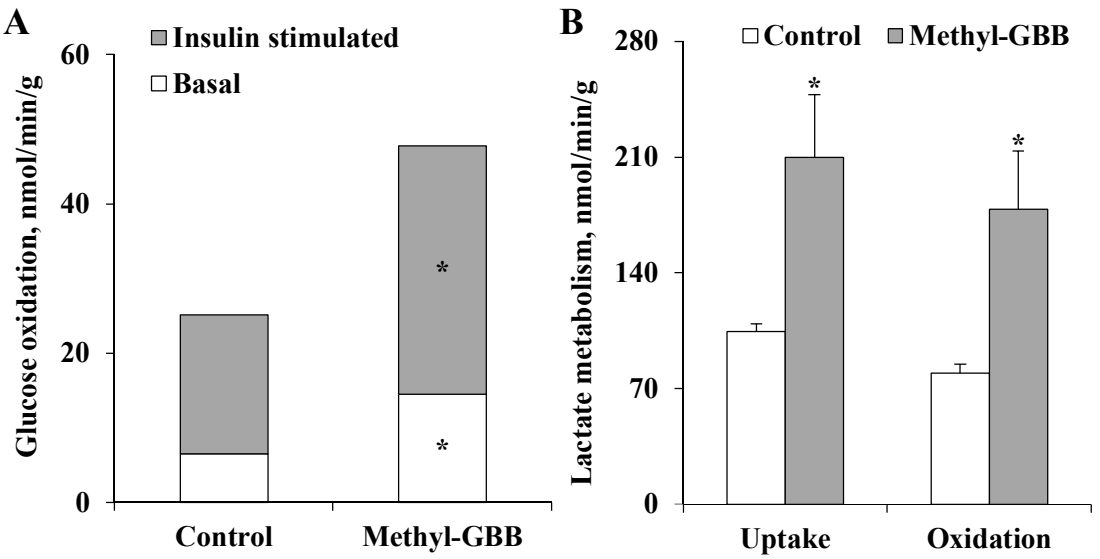


Figure 3.26. **The effects of long-term Methyl-GBB (10 mg/kg) treatment on basal and insulin stimulated [^{14}C]glucose oxidation (A), [^{14}C]lactate uptake and oxidation (B) in the isolated rat heart**
The presented results are mean ± SEM of 5-6 rats. *Significantly different from Control group (Student's t-test $P < 0.05$).

Treatment with Methyl-GBB induced a reduction in FA oxidation and in turn stimulated glucose oxidation (Figure 3.26.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 3.26.B). Thus, Methyl-GBB treatment induces a partial switch of energy production from long-chain FAs to glucose oxidation.

3.5.4. 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*. In isolated spontaneously beating rat hearts, the substances did not affect heart rate, LVDP,

coronary flow and contractility (dP/dt max) or relaxation (dP/dt min) either before (Table 3.10.), during or after ischemia compared to the Control group. During LAD occlusion, the coronary flow in all experimental groups was decreased by 39% to 44%. Moreover, the drop of LVDP by 29% to 36% was observed. No changes in the heart rate during the occlusion period were observed. In the reperfusion stage, coronary flow recovered up to 77% to 87% of the baseline values.

Table 3.10.

The effect of long-term Methyl-GBB treatment on isolated rat heart hemodynamic parameters

		Heart rate, BPM	Coronary flow, ml/min	LVDP, mmHg	dP/dt max, mmHg/sec.	dP/dt min, mmHg/sec.	Cardiac workload, kU
		Basal hemodynamic parameters					
Control		250±11	11.3±1.8	117±5	3616±167	-2244±131	28989±1537
Meldonium		233±13	9.7±0.4	115±5	3605±204	-2103±98	26296±1080
Methyl-GBB	1	238±14	12.1±1.5	118±4	3821±190	-2203±87	27801±1439
	5	246±12	11.2±0.8	122±5	3872±195	-2262±85	29637±1056
	20	231±12	11.9±0.7	125±7	3772±191	-2244±98	28472±1855
		Hemodynamic parameters during ischemia					
Control		249±10	6.9±1.3*	85±6*	2676±193*	-1686±138*	21374±1860*
Meldonium		226±9	5.6±0.3*	77±5*	2565±266*	-1489±138*	17620±1653*
Methyl-GBB	1	243±15	6.8 ±1.1*	84±6*	2772±236*	-1696±140*	20131±1563*
	5	228±13	6.7±0.6*	95±5*	3020±200*	-1815±110*	21570±1450*
	20	227±15	6.9±0.5*	88±7*	2742±197*	-1614±116*	19644±1874*
		Hemodynamic parameters at the end of reperfusion					
Control		237±23	8.5±1.4	75±3*	2535±222*	-1348±95*,#	17940±1753*
Meldonium		229±15	6.7±0.4*,#	64±5*	2377±202*	-1167±91*	14794±1573*
Methyl-GBB	1	245±18	8.5±1.1*	63±6*,#	2305±240*	-1196±109*,#	14837±1489*,#
	5	241±14	8.3±0.5*,#	78±3*,#	2808±180*	-1423±55*,#	18849±1305*,#
	20	226±19	8.8±0.5*,#	75±8*,#	2518±270*	-1284±123*	14983±2719*

The presented results are mean ± SEM of 10 rats. *Significantly different from the same group before occlusion (Tukey's test, $P < 0.05$). #Significantly different from the same group during ischemia (Tukey's test, $P < 0.05$).

The values for the AR were similar in the hearts of all the experimental groups, and the AR was approximately 45-50% of the area of the LV. As observed in Figure 3.27.A, meldonium treatment at a dose of 100 mg/kg decreased the infarct size by 25% ($p = 0.03$) 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.

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 3.27.B). Overall, similar to meldonium, long-term treatment with Methyl-GBB is required to provide maximal cardioprotection.

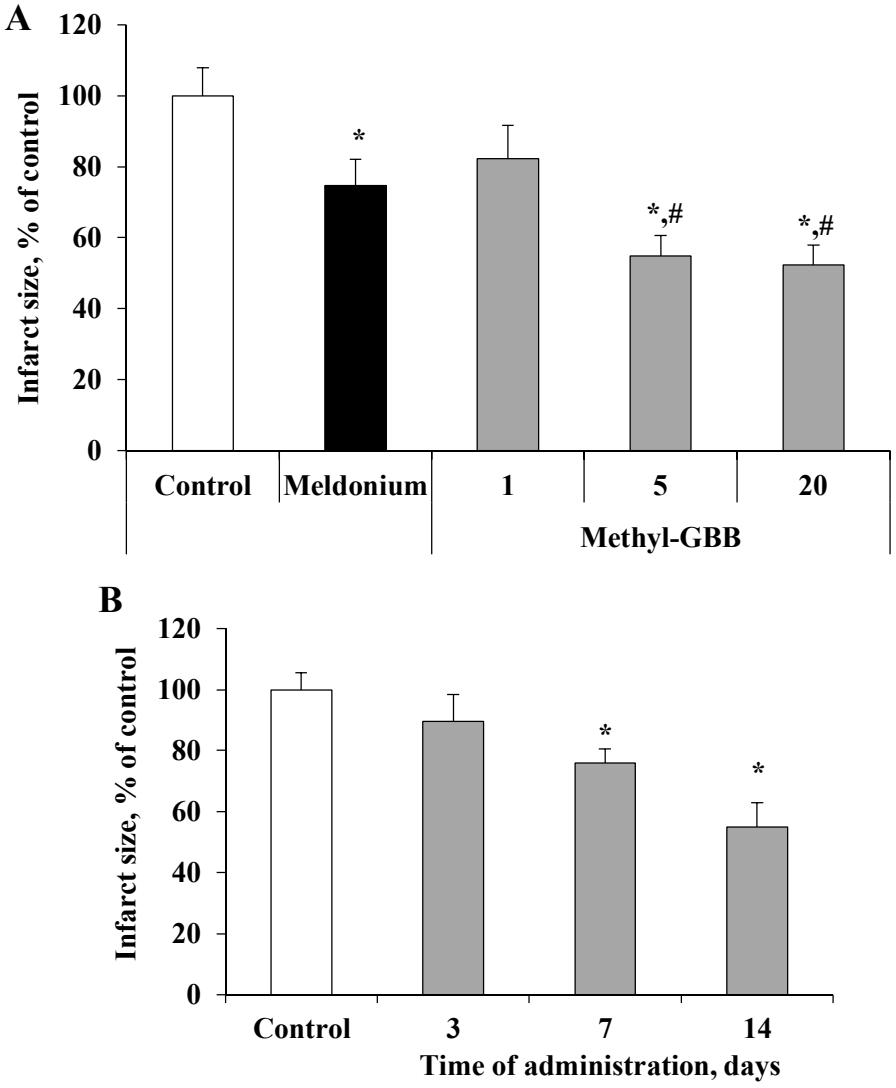


Figure 3.27. **The effects of meldonium (100 mg/kg) and Methyl-GBB (1, 5 and 20 mg/kg) on the infarct size after 14 days of treatment (A). The effects of Methyl-GBB (5 mg/kg) on the infarct size after 3, 7, 14 days of treatment (B)**

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 $P < 0.05$).

The ischemia-reperfusion induced a 2.8-fold decrease in OXPHOS state respiration and a 40% increase in LEAK state respiration (Figure 3.28.A and B). 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 3.28.A). According to LEAK state respiration measurement, Methyl-GBB completely protected against ischemia-reperfusion induced mitochondrial uncoupling (Figure 3.28.B).

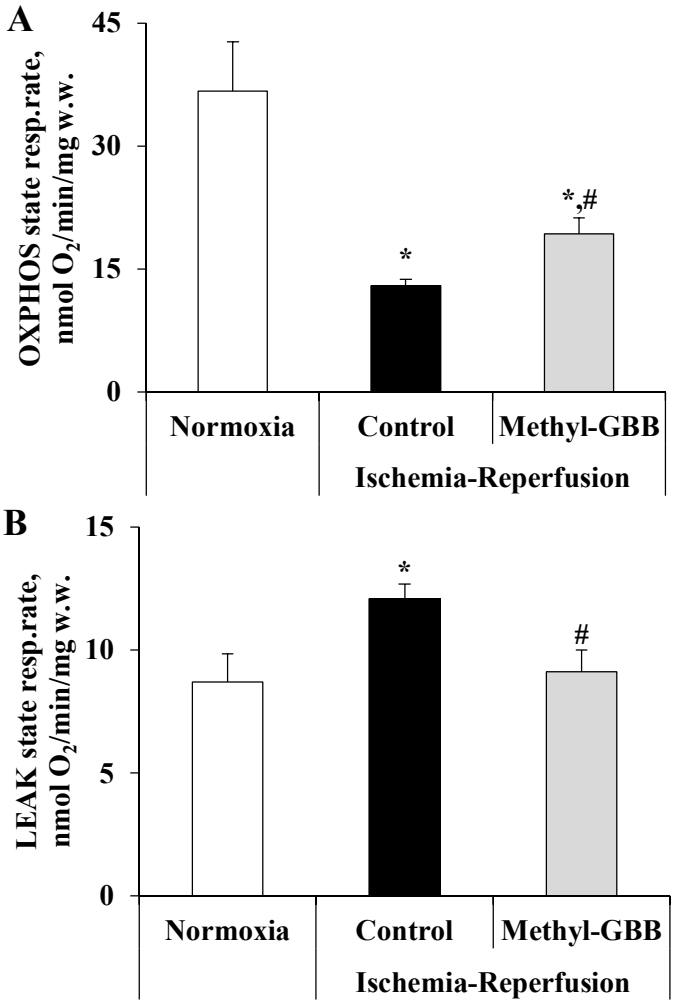


Figure 3.28. **The effects of Methyl-GBB (20 mg/kg) treatment on ADP-stimulated mitochondrial respiration (OXPHOS state) (A) and uncoupling of oxidative phosphorylation (LEAK state) (B) in cardiac fibers isolated after ischemia-reperfusion**
The presented results are mean ± SEM of 5 rats. *Significantly different from normoxia (Tukey's test $P < 0.05$), #Significantly different from ischemic control group (Tukey's test $P < 0.05$).

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%

(p = 0.001) in Wistar rats undergoing LAD occlusion and reperfusion *in vivo* (Figure 3.29.A). Moreover, Methyl-GBB treatment improved the 24-h survival of rats by 20-30% (Figure 3.29.B).

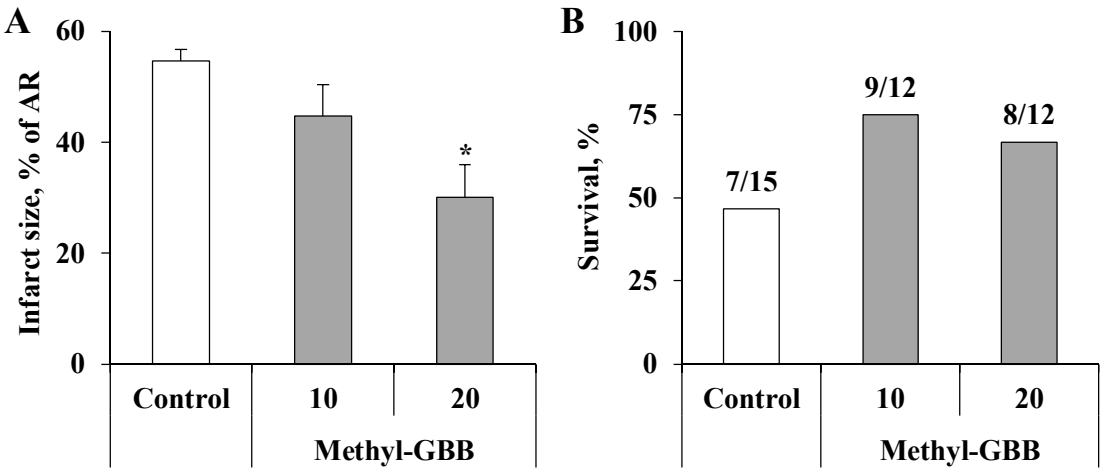


Figure 3.29. **The effects of Methyl-GBB (10 and 20 mg/kg) treatment on the infarct size (A) and rat 24 h survival (B)**
The presented results are mean ± SEM at least 7 rats. *Significantly different from control group (Tukey's test P < 0.05).

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

4. 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 targets were compared to determine the most effective strategy 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.

4.1. 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. Since FAs are intrinsic activators of the uncoupling protein (UCP), increase in cardiac UCP1 and UCP3 expression observed in this study explains the reduced efficiency of oxidative phosphorylation and reduced ATP yield (*Cole*, 2011; *Ray*, 2002; *Rider*, 2013), which leads to decreased cardiac function in the fasted state. 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.

Several previous studies have used *ex vivo* isolated perfused hearts to examine the differences between fed and fasted states in a global ischemia-reperfusion model (*Doenst*, 1996; *Montessuit*, 1996, 2000; *Schaefer*, 1997; *Schneider*, 1991). These studies concluded that fasted rat hearts are better protected from mechanical dysfunction induced by ischemia-reperfusion. However, all previous studies used only one buffer solution that does not mimic both states in the *ex vivo* isolated heart model. Two studies performed by Montessuit and colleagues used a buffer that contained glucose, palmitate and insulin in concentrations similar to fed conditions (*Montessuit*, 1996, 2000), while other studies used glucose as the only energy substrate (*Schaefer*, 1997; *Schneider*, 1991). Previous results indicate the beneficial effects of acutely stimulated glucose metabolism rather than the effects of changes in energy metabolism pattern. Meanwhile, present results highlight the importance of insulin-signalling and FA metabolism intensity to the outcome of ischemia-reperfusion injury.

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.

4.2. 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.***

Increased expression of pyruvate dehydrogenase kinase 4 (PDK4, an inhibitor of pyruvate decarboxylation) has been suggested as an effective mechanism to reduce pyruvate and lactate oxidation in the fasted state (*Sugden*, 2000; *Wu*, 1998), but in the present study, only a 20% decrease in the respiration rate of cardiac fibers was observed in the fasted state using pyruvate as a substrate. Moreover, previous studies have

demonstrated that palmitoyl-carnitine could inactivate pyruvate dehydrogenase in adipocytes, skeletal muscle and cardiac mitochondria (*Ashour*, 1983; *Hansford*, 1977; *Smith*, 1979). Present data indicate that the metabolism inside mitochondria in different nutritional states depends on alterations in availability and the rate of production of acyl-carnitines rather than on changes in gene expression induced by the nutritional state.

Metabolic disorders, like insulin resistance and metabolic syndrome, are accompanied by increased serum long-chain FA levels and are associated with mitochondrial dysfunction (*Bugger*, 2010; *Martins*, 2012). However, the concentration of long-chain free FAs in plasma depends on fat storage tissues (*Viscarra*, 2013) and does not reflect the content of FAs in the oxidizing tissues and thus cannot serve as a marker of FA flux in mitochondria. Meanwhile, the concentration of long-chain acyl-carnitines in plasma can predict the intracellular metabolism pattern and has been suggested to be a marker of metabolic dysfunction (*Ramos-Roman*, 2012; *Soeters*, 2009). The present results demonstrate that the changes in concentration of long-chain acyl-carnitines in plasma reflect changes in long-chain acyl-carnitine content in cardiac tissues, and that concentration of acyl-carnitines in plasma could serve as an indicator of the energy metabolism pattern in cardiac mitochondria. Thus, the concentration of plasma long-chain acyl-carnitines is suggested as a marker of metabolic dysfunction (*Koves*, 2008), but ***it is essential to measure long-chain acyl-carnitines in both nutritional states for diagnostic purposes due to the differences in their concentrations under fasted and fed states.***

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 L-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.

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

4.3.1. 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 FA metabolism to glucose utilization is cardioprotective. Thus, a decrease in FA 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*, 1995a).

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*, 1995a; *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*, 2005b) 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 (Table 4.1).

Together, the results show that a high-dose administration of sodium pivalate over a 2-week period does not affect cardiac functionality. However, the detrimental effects on overall mitochondrial and particularly pyruvate energy metabolism should be viewed with caution, especially when the mid- or long-term administration of high doses of pivalate moiety-liberating prodrugs is considered in the presence of common background diseases with affected mitochondrial energy metabolism like diabetic neuropathy (*Vincent*, 2011) and heart failure (*Ajith*, 2014; *Lemieux*, 2011). 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.

Table 4.1.

The comparison between meldonium and sodium pivalate

Parameters	Meldonium	Sodium pivalate
Dose and duration of the treatment	100 mg/kg p.os. 14 days	40 mM (approx. 500mg/kg) 14 days
L-carnitine content in cardiac tissues	↓	↓
Cardioprotection	+	-
Mitochondrial CPT I-depedent (L-carnitine- dependent) FA oxidation	↓	↓
Mitochondrial pyruvate oxidation	=	↓

The data for meldonium from *Kuka*, 2012.

4.3.2. 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. We observed that 24 h after a single dose administration of OCTN2 inhibitors the concentration of L-carnitine in the urine was significantly increased. However, during the first 24 h, only 6-7.5% of the whole L-carnitine pool were eliminated via the urine, and L-carnitine concentration in the plasma and cardiac tissues was not significantly decreased. Selective inhibitors of BBOX did not induce any changes in L-carnitine concentration in the urine, plasma or heart tissues after 24 h of treatment. Prolonged elimination of L-carnitine 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.***

OCTN2 is not a general drug transporter, but it was found to be a highly specific carrier for L-carnitine and closely related molecules (*Grigat*, 2009). Some studies recently have tested the potential of drug conjugates with L-carnitine as prodrugs, which could target OCTN2 to increase the tissue bioavailability of drugs (*Diao*, 2011). In the present study, we also observed that a higher affinity to OCTN2 is related to an increased transport efficiency of the tested compounds. For example, 24 h after a single administration, the contents of OCTN2 inhibitors Chloro-GBB and Dimethyl-GBB were 15 times higher than the content of BBOX inhibitor GBB-phosphinate in the cardiac tissues. In addition, the IC_{50} values for OCTN2 strongly correlate with the inhibitor content in the cardiac tissues. Furthermore, OCTN2 transport ensures compound reuptake in the kidneys. We observed efficient urinary excretion of both compounds that are weak OCTN2 inhibitors. Thus, comparable doses of these compounds are inefficient, and higher doses would be required to achieve similar pharmacological effects as for potent OCTN2 inhibitors. Taking into account that OCTN2 transporters are widely located in different organs, OCTN2 could potentially be an efficient tool to increase drug transport in tissues and to reduce drug elimination via urine. Overall, OCTN2 is a major determinant of the pharmacokinetic and efficacy profiles of novel cardioprotective drugs in this study.

In conclusion, the obtained results confirm that selective inhibition of OCTN2, compared to selective inhibition of BBOX, is a far more effective approach to decrease L-carnitine content and to induce cardioprotective effects. OCTN2-mediated transport

improves drug delivery in target tissues and therefore plays significant roles in both drug delivery and interaction with the L-carnitine system.

4.4. 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 plasma and 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. Previously, it was shown that long-term meldonium treatment decreases the plasma concentrations of L-carnitine through the inhibition of reabsorbtion in kidneys (*Kuwajima*, 1999; *Liepinsh*, 2011b). Similarly, Methyl-GBB increased L-carnitine content in the urine even after few hours of administration. This effect is attributed to a highly efficient inhibition of OCTN2 and it results in rapid L-carnitine excretion. Overall, the decreased L-carnitine content in tissues is the result of Methyl-GBB effects on L-carnitine transport into tissues and reabsorbtion in the kidneys.

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 (Table 4.2). 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.***

Table 4.2.

The comparison between meldonium and Methyl-GBB

Parameters	Meldonium	Methyl-GBB
Dose and duration of the treatment	100 mg/kg p.os. 14 days	5-20 mg/kg p.os. 14 days
L-carnitine content in cardiac tissues	↓	↓↓
Acyl-carnitine content in cardiac mitochondria	↓	↓
Cardioprotection	+	++
Energy metabolism under normoxic conditions		
Cardiac FA oxidation	=	↓
Peroxisomal FA oxidation	↑	↓
Mitochondrial FA oxidation	CPT I-dependent ↓ CPT I-independent ↑	CPT I-dependent ↓ CPT I-independent =
Cardiac glucose utilization	↑	↑

The data for meldonium from *Asaka*, 1998; *Kuka*, 2012; *Liepinsh*, 2008, 2013a.

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, both in isolated hearts and *in vivo*. 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.

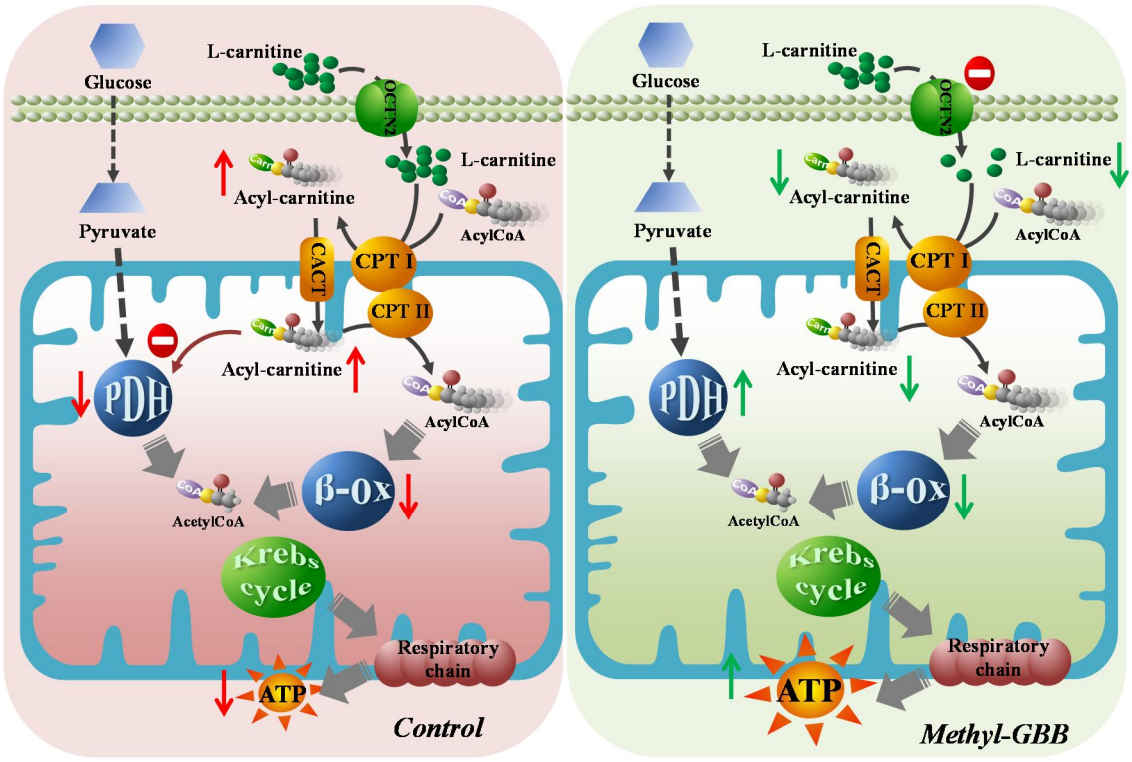


Figure 4.1. **The effects of Methyl-GBB treatment on cardiac energy metabolism during ischemia-reperfusion injury.**
ATP – adenosine triphosphate; β-ox – β-oxidation; CACT – carnitine translocase; CPT I – carnitine palmitoyltransferase I; CPT II – carnitine palmitoyltransferase II; OCTN2 – organic cation/carnitine transporter type 2; PDH – pyruvate dehydrogenase

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*** (Figure 4.1.). 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.

5. 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, sodium 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. OCTN2-mediated transport improves drug delivery to target tissues and therefore plays significant roles in both drug delivery and interaction with the L-carnitine system.
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.

6. 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.
2. Liepinsh E, Makrecka M, Kuka J, Makarova E, Vilskersts R, Cirule H, Sevostjanovs E, Grinberga S, Pugovics O, Dambrova M. The heart is better protected against myocardial infarction in the fed state compared to the fasted state. *Metabolism*, **2014**, 63(1):127–136.
3. Makrecka M, Kuka J, Volska K, Antone U, Sevostjanovs E, Cirule H, Grinberga S, Pugovics O, Dambrova M, Liepinsh E. Long-chain acylcarnitine content determines the pattern of energy metabolism in cardiac mitochondria. *Molecular and Cellular Biochemistry*, **2014**, 395(1-2):1–10.
4. Tars K, Leitans J, Kazaks A, Zelencova D, Liepinsh E, Kuka J, Makrecka M, Lola D, Andrianovs V, Gustina D, Grinberga S, Liepinsh E, Kalvinsh I, Dambrova M, Loza E, Pugovics O. Targeting Carnitine Biosynthesis: Discovery of New Inhibitors against γ-Butyrobetaine Hydroxylase. *Journal of Medicinal Chemistry*, **2014**, 57(6):2213–2236.
5. Liepinsh E, Makrecka M, Kuka J, Cirule H, Makarova E, Sevostjanovs E, Grinberga S, Vilskersts R, Lola D, Loza E, Stonans I, Pugovics O, Dambrova M. 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. *Pharmacological Research*, **2014**, 85:33–38.
6. Liepinsh E, Makrecka-Kuka M, Kuka J, Vilskersts R, Makarova E, Cirule H, Loza E, Lola D, Grinberga S, Pugovics O, Kalvinsh I, Dambrova M. Inhibition of L-carnitine biosynthesis and transport by methyl-γ-butyrobetaine decreases fatty acid oxidation and protects against myocardial infarction. *British Journal of Pharmacology,* **2015,** 172(5):1319–1332.

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.
5. Kuka J, Makrecka M, Dambrova M, Liepinsh E. Contents of long-chain acyl-carnitines determine the pattern of energy metabolism in the heart. *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. 37.
6. Liepinsh E, Makrecka M, Kuka J, Dambrova M. The heart is better protected against myocardial infarction in the fed state. *The 29th Congress of the Federation of the International Danube Symposia on Diabetes mellitus,* Jurmala, Latvia, June 24-28, **2014**. Book of Abstracts, p. 37.
7. Makrecka M, Volska K, Kuka J, Liepinsh E, Dambrova M. The accumulation of long-chain acyl-carnitines is a major cause of mitochondrial damage during ischaemia. *MiP 2013, 9th MiPconference - Comparative Mitochondrial Physiology*, Obergurgl, Austria, September 23-27, **2013**, Book of Abstracts, p.72.
8. Makrecka M, Kuka J, Sevostjanovs E, Liepinsh E, Dambrova M. Activated insulin signalling and reduction in fatty acid load determine the protection against

myocardial infarction in the fed state, *ESC Basic Science Summer School*, Sophia Antipolis, France, June 16-20, **2013,** Abstract Nr. P33.

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