Rīgas Stradiņa universitāte

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PROTECTIVE EFFECTS AND MECHANISMS OF ACTION OF METHYL-GBB IN THE PRECLINICAL MODELS OF DIABETES AND ITS COMPLICATIONS

Summary of the Doctoral Thesis
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The Doctoral Thesis is available in the RSU library and at RSU webpage: www.rsu.lv.

Secretary of the Doctoral Council:

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ABBREVIATIONS

[^{3}H]-DOG – 2-[1,2-^{3}H]-deoxy-D-glucose

ADP – adenosine diphosphate

AMP – adenosine monophosphate

ATP – adenosine triphosphate

CoA – coenzyme A

COX-2 – Cyclooxygenase 2

CPT1 – carnitine palmitoyltransferase 1

FA – fatty acid

GB – guanabenz

GBB – γ-butyrobetaine

HDL – high-density lipoproteins

HFD – high-fat diet

IC_{50} – half maximal inhibitory concentration

LDL – low-density lipoproteins

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

Methyl-GBB – 4-[ethyl(dimethyl)ammonio]butanoate

Omy A – oligomycin A

OXPHOS – oxidative phosphorylation

OXPHOS state – ADP-stimulated mitochondrial respiration

P-Akt – phosphorylated protein kinase B

PC – palmitoylcarnitine

TNFα – tumor necrosis factor α.

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

INTRODUCTION

The global prevalence of diabetes has been continuously increasing for the past three decades, rising in the adult population from 4.7 % to 8.5 % (NCD Risk Factor Collaboration (NCD-RisC), 2016). The primary pathophysiology of type 2 diabetes mellitus (T2DM) is associated with the insufficient action of insulin (Kerner et al., 2014). T2DM is a cause for numerous cardiovascular complications, such as atherosclerosis and ischaemic heart disease (Defronzo, 2009; The Emerging Risk Factors Collaboration et al., 2010). Current treatments for T2DM are mainly based on several approaches intended to reduce the hyperglycaemia; however, these therapies possess limited efficacy and tolerability and significant mechanism-based side effects (Moller, 2001). Therefore, novel pharmacological targets and treatment strategies are needed to improve the clinical outcomes of patients with diabetes.

It has been suggested that insulin resistance and disturbances in glucose metabolism are induced by excessive fatty acid (FA) flux which results in incomplete FA oxidation and the accumulation of various lipid metabolites (Martins et al., 2012; Schooneman et al., 2013; van de Weijer et al., 2013). Among FA intermediates linked to insulin resistance are long-chain acylcarnitines (McCoin et al., 2015; Schooneman et al., 2013). Acylcarnitines are formed from activated FAs and L-carnitine to ensure transportation of long-chain FA into the mitochondrial matrix for further β-oxidation. The concentrations of long-chain acylcarnitines in the plasma and skeletal muscle are modestly increased among individuals with insulin-resistance and T2DM (Adams et al., 2009; Mihalik et al., 2010). In addition, the accumulation of long-chain acylcarnitines has been observed in experimental models of atherosclerosis (Gillies and Bell, 1976). Several previous studies have also noted accumulation of FA metabolites in cases of heart ischaemia/reperfusion (Corr et al., 1984; Ford et al., 1996; Idell-Wenger et al., 1978; Whitmer et al.,

1978). In a recent study it has been shown that decreasing long-chain acylcarnitine levels protects the heart against ischaemia/reperfusion-induced injury (Liepinsh et al., 2015). Thus, it has been hypothesized that the pharmacological reduction of acylcarnitine levels could be beneficial in patients with diabetes and cardiovascular complications of diabetes. Considering that long-chain acylcarnitines are produced from long-chain FAs and L-carnitine, decreasing the pools of L-carnitine and its derivatives might present a way to attenuate the development of insulin resistance and diabetes-related cardiovascular diseases. Recently, a series of compounds that inhibit the biosynthesis and transport of L-carnitine were synthesised and characterised (Liepinsh et al., 2014a; Tars et al., 2014). The best cardioprotective effect in the rat experimental heart infarction model was observed after treatment with 4-[ethyl(dimethyl)ammonio]butanoate (methyl-GBB), a methyl-derivative of γ-butyrobetaine (GBB) that effectively inhibits GBB dioxygenase (IC_{50} 2.8 μM) and organic cation transporter 2 (IC_{50} 3.0 μM) (Liepinsh et al., 2015). It is not yet known whether methyl-GBB treatment attenuates the development of insulin resistance and is beneficial for the treatment of diabetes.

Overall, further studies are necessary to clarify the role of acylcarnitine accumulation in the development of insulin resistance and cardiovascular complications of diabetes, and whether treatment with methyl-GBB serves as an effective strategy for the treatment of diabetes and its complications.

Aim of the study

To investigate the pharmacological mechanisms of action of an acylcarnitine concentration lowering drug methyl-GBB in experimental animal models of diabetes, cardiac ischaemia/reperfusion injury and atherosclerosis.

Objectives of the study

1. To study the molecular mechanisms of excessive accumulation of long-chain acylcarnitines in the accelerated development of insulin resistance.
2. To determine whether decreasing long-chain acylcarnitine content with methyl-GBB alone or in combination with exercise intervention induces antidiabetic effects in experimental mice models of insulin resistance.
3. To study the pathological mechanism and damaging effects of an increased long-chain acylcarnitine content on cardiac mitochondria during acute ischaemia/reperfusion injury.
4. To evaluate the impact of decrease in long-chain acylcarnitine content by methyl-GBB treatment on the development of atherosclerosis.

Hypothesis of the study

Pharmacologically induced decrease in the content of long-chain acylcarnitines by methyl-GBB facilitates glucose metabolism, improves insulin sensitivity, protects the heart mitochondria against ischaemia/reperfusion injury, attenuates the development of atherosclerosis and therefore represents an effective strategy for the treatment of diabetes and its complications.

Scientific novelty of the study

Within the framework of the research, the role of long-chain acylcarnitines in the regulation of energy metabolism was evaluated and protective effects of methyl-GBB treatment in experimental models of diabetes and its cardiovascular complications were studied. The current study resulted in following novel findings:

1. In the presence of glucose long-chain acylcarnitines facilitate insulin release to stimulate the transition from the fasted to fed state. The accumulation of long-chain acylcarnitines during the fed state limits

metabolic flexibility and accelerates hyperglycaemia and hyperinsulinemia.

2. The protective effects of methyl-GBB treatment in experimental models of diabetes are described for the first time. Methyl-GBB administration-induced decrease in acylcarnitine content improves insulin sensitivity and significantly reduces blood glucose and insulin levels in mice with insulin resistance and diabetes.
3. Long-chain acylcarnitines are the main FA intermediates that induce ischaemia/reperfusion-related damage. Acylcarnitine accumulation during ischaemia leads to inhibited oxidative phosphorylation, thus inducing mitochondrial membrane hyperpolarization and stimulating the production of reactive oxygen species in cardiac mitochondria.
4. For the first time, the protective effects of methyl-GBB treatment on the development of atherosclerosis in *apoE*$^{-/-}$ mice have been demonstrated. The anti-atherosclerotic mechanism of methyl-GBB treatment is mediated by decreased amounts of long-chain acylcarnitines and decreased infiltration of macrophages and monocytes into the aortic lesions of the aortic root.

1 MATERIALS AND METHODS

1.1 Animals and treatment

Male CD-1 (8–12 weeks old, Envigo Netherlands (former Harlan Laboratories BV), Netherlands), C57BL/6 male mice (8–12 weeks old, Envigo Netherlands), male *db/db* mice (10 weeks old, Envigo Netherlands), non-diabetic db/Lean male mice (Envigo Netherlands), male Wistar rats (10–16 weeks, Laboratory of Experimental Animals, Riga Stradins University, Latvia), female *apoE*$^{-/-}$ mice (7 weeks old, Taconic, Denmark) were used for 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. All animals were adapted to local conditions for two weeks before the start of experiments. The experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010).

To study the effects of long-chain acylcarnitines on glucose homeostasis, CD-1 mice were administered with palmitoylcarnitine intraperitoneally at a dose of 50 mg/kg. To ensure a continuous dosing of palmitoylcarnitine, osmotic minipumps (ALZET®, USA) filled with palmitoylcarnitine (10 mg/kg/day or 50 mg/kg/day) were implanted subcutaneously in the mice for 24 h (acute effect study) or 28 days (long-term effect study). In control animals osmotic minipumps loaded with saline (vehicle) were implanted. To inhibit endogenous insulin release, guanabenz (i.p. 1 mg/kg), an α_2-adrenoreceptor agonist (Saha et al., 2005), was used after the single-dose palmitoylcarnitine (50 mg/kg) administration. To determine the insulin-dependent effects of palmitoylcarnitine, insulin (0.3 IU/kg) was

administered subcutaneously 1 h after the intraperitoneal injection of guanabenz or guanabenz + palmitoylcarnitine.

To study the antidiabetic effects of pharmacological reduction of long-chain acylcarnitine levels and physical intervention, C57BL/6 mice were divided into 3 groups and *db/db* mice were divided into 4 groups as shown in Table 1.1. C57BL/6 mice were treated with normal chow or a high fat diet (HFD, Special Diets Services, UK) for 8 weeks to induce insulin resistance. Methyl-GBB phosphate (equivalent to 5 mg/kg of methyl-GBB) was administered with drinking water for 8 weeks. During the same time period, forced exercise was performed for *db/db* mice. For the exercise experiment, a 21-wheels forced exercise/walking wheel apparatus (PsymCon Model 35500, Lafayette Instrument, Lafayette, USA) was used.

Table 1.1

The design of the experiments with C57BL/6, db/Lean and *db/db* mice

No	Group	Mice with impaired insulin sensitivity	Mice with diabetes	Treatment
1	Non-diabetic control	Normal chow	db/Lean	water
2	Control with diabetes	HFD	*db/db*	water
3	Methyl-GBB	HFD	*db/db*	Methyl-GBB[1]
4	Exercise	–	*db/db*	Ex[2]
5	Methyl-GBB + Exercise	–	*db/db*	Methyl-GBB[1] + Ex[2]

[1] methyl-GBB once a day, p.o. 5 mg/kg; [2] Ex - forced walking five days a week, 60 min/day at a speed of 5 m/min.

To study the role of acylcarnitines in ischaemia/reperfusion damage, for the isolated heart experiments, Wistar rats were anaesthetized using sodium pentobarbital (60 mg/kg intraperitoneal injection) with the concomitant administration of heparin (1000 units/kg).

To evaluate the link between increased pools of plasma and tissue acylcarnitines and accelerated development of atherosclerosis and to study molecular anti-atherosclerotic mechanisms of methyl-GBB, *apoE*$^{-/-}$ mice were treated with methyl-GBB phosphate (equivalent to 10 mg/kg of methyl-GBB) dissolved in the drinking water. For the progression of atherosclerosis, all the experimental animals at the age of 8 weeks were switched to a Western RD (P) diet that contained 21 % fat and 0.15 % cholesterol from Special Diets Services (Essex, Great Britain) for 4 months.

1.2 Methods

1.2.1 *In vitro* methods

1.2.1.1 Western blot analysis of tissue lysates

The polyacrylamide gel electrophoresis and western blot analysis of tissue lysates was performed as described previously (Liepinsh et al., 2014b). To detect the phosphorylation level of Akt at Ser473, membranes were incubated with anti-P-Akt (#sc-7985-R; Santa Cruz Biotechnology, CA, USA or #4060S; Cell Signaling Technology, Danvers, MA, USA) specific antibodies and the obtained data were normalized against total Akt (#sc-8312; Santa Cruz Biotechnology) protein expression.

1.2.1.2 Determination of the acylcarnitines in the plasma and tissue

Determination of the acylcarnitine content in the samples was performed by UPLC/MS/MS (Makrecka et al., 2014). The sample extraction was performed as previously described (Blachnio-Zabielska et al., 2011).

1.2.1.3 Determination of methyl-GBB in the plasma

The determination of methyl-GBB concentrations in the plasma samples was performed using the UPLC/MS/MS method and a Quattro Micro triple-

quadrupole mass spectrometer (Micromass, Waters, Milford, MA, USA) using electrospray ionisation in the positive ion mode as previously described (Dambrova et al., 2008, 2013).

1.2.1.4 Determination of biochemical parameters in the plasma and blood

For biochemical measurements, blood samples were collected from the tail vein in heparin-containing tubes. To obtain plasma, the samples were centrifuged at 1000 g for 10 min at 4 °C.

Glucose concentrations in plasma samples were measured by Instrumentation Laboratory (Milan, Italy) and Roche Diagnostics (Mannheim, Germany) enzymatic kits. Blood glucose was measured using a MediSense Optium (Abbott Diabetes Care, Maidenhead, UK) blood glucose meter and strips. The concentration of free FAs was measured using enzymatic kits from Wako (Neuss, Germany). The plasma insulin concentrations were determined using a Sensitive Rat Insulin RIA kit and a Rat/Mouse Insulin ELISA kit (Millipore, Billerica, USA). Concentrations of triglycerides, HDL- and LDL-cholesterol were measured using kits from IL Laboratories (Lexington, USA). Concentration of tumour necrosis factor alpha (TNFα) in plasma was measured using TNFα ELISA Kit from Millipore.

1.2.1.5 Isolation of cardiac mitochondria

Mitochondria were isolated from the cardiac tissues as described previously (Kuka et al., 2012a). Isolated heart mitochondria were stored on ice until use or were frozen until analysis.

1.2.1.6 Determination of palmitoylcarnitine accumulation in mitochondrial fractions

Isolated rat cardiac mitochondria were incubated with 5.6 nM labelled [9,10-^{3}H]-palmitoylcarnitine (specific radioactivity 60 Ci/mmol; American Radiolabeled Chemicals) in the presence of 10 mM succinate, 1 μM rotenone and 1 mM ADP to ensure palmitoylcarnitine accumulation in mitochondria. The mitochondria were then washed, and mitochondrial fractions were prepared. The separation of the outer mitochondrial membrane and intermembrane space was performed as described previously (Ryu et al., 2011).

1.2.1.7 Mitochondrial respiration measurements

To determine the palmitoylcarnitine-induced mitochondrial damage, high-resolution respirometry with simultaneous fluorimetry was performed using an Oxygraph-2k (O2k, Oroboros Instruments) combined with the O2k-Fluorescence LED2-Module. Succinate (10 mM) with rotenone (1 μM) were used to determine complex II-linked respiration, and pyruvate and malate (5 mM and 0.5 mM respectively) with succinate (10 mM) were used to determine Complex I- and II-linked respiration. Measurements were performed in the OXPHOS state using 1 mM ADP. Oligomycin A (Omy A) at 10 μM was added to determine $LEAK_{Omy}$-linked respiration. In addition, simultaneously with respiratory measurements, tetramethylrhodamine methyl ester (TMRM, LST668, Life Technologies) was used to determine the mitochondrial membrane potential and H_2O_2 flux was measured using the H_2O_2-sensitive probe Amplex® Ultra Red (A36006, Life Technologies), as described previously (Makrecka-Kuka et al., 2015).

1.2.1.8 Quantification of the atherosclerotic lesions in the aortic sinus

Hearts containing the aortic sinus were processed for a quantitative atherosclerosis assay of the aortic sinus using a method described previously (Vilskersts et al., 2009). Images of the aorta were recorded using a Leica DFC490 digital camera (Wetzlar, Germany). The total area of the lesion was measured using Image-Pro Plus 6.3 software, as previously described (Liepinsh et al., 2013a).

1.2.1.9 Quantification of the infiltration of macrophages and monocytes in the atherosclerotic lesions

To determine the number of macrophages and monocytes in atherosclerotic lesions of the aortic sinus, 10 μm sections from aortic roots were prepared similarly as described previously (Vilskersts et al. 2009). Sections were mounted on poly-L-lysine coated slides, air-dried and fixed in cold acetone. Following fixation, the sections were stained with rat anti-MOMA-2 antibodies from Abcam (Cambridge, UK), as previously described (Hoyer et al., 2012).

1.2.1.10 Quantification of the atherosclerotic lesions in the aorta

The aortas, from arch to bifurcation, were fixed in 4 % formaldehyde. Following fixation, the whole aorta was longitudinally opened, pinned onto silicone plates and stained for lipids with Sudan IV. Images of the aorta were captured using a Sony α77 digital camera, and the total area of the lesion was calculated using the Image-Pro Plus 6.3 software. The extent of atherosclerosis was expressed as a percentage of the aortic surface covered by lesions compared to the total aortic surface.

1.2.2 *Ex vivo* and *in vivo* methods

1.2.2.1 Determination of glucose and insulin tolerance tests

To perform the glucose tolerance test, the mice were fasted overnight. Then, the glucose solution (0.5 or 1 g/kg of body weight) was administered intraperitoneally, and blood samples were drawn from the tail vein at 0 (fasting), 15, 30, 60, 120, 180 and 240 min. For the insulin tolerance test experiment, an insulin solution was administered intraperitoneally or subcutaneously (with the respective doses of 0.75 or 0.3–0.5 IU/kg) to the fed mice, and blood samples were drawn from the tail vein at 0 (fed), 30, 60, 120 and 240 min (additional blood sample at 24 hours was collected in the experiment with *db/db* mice). The blood glucose concentration was measured using a MediSense Optium Xceed blood glucose meter and strips.

1.2.2.2 Measurements of glucose uptake *in vivo*

To determine the glucose uptake *in vivo*, 1 μCi of 2-[1,2-^{3}H]-deoxy-D-glucose ([^{3}H]-DOG, specific activity, 60 Ci/mmol) were administered intravenously to the mice. After 10 min, the mice were sacrificed by cervical dislocation, and heart, skeletal muscle, liver and adipose tissue homogenates (1:5, w/v in MilliQ water) were prepared. The contents of [^{3}H]-DOG in the tissue samples were determined by a liquid scintillation method.

1.2.2.3 Isolated rat heart infarction study

Infarction was induced according to the Langendorff technique, as described previously (Kuka et al., 2012a). The infarct size was determined as described previously (Liepinsh et al., 2013a).

1.3 Statistical analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). Statistically significant differences in the mean values were evaluated using Student's t test, Chi-Square test or a one-way ANOVA with Tukey`s, two-tailed Student's t-test, Mann–Whitney U test or Dunnett's post-tests. The differences were considered significant when $P < 0.05$. The data were analysed using Graph Pad Prism 5.03 software (Graph Pad Inc., La Jolla, USA).

2 RESULTS

2.1 Effects of acute and long-term administration of palmitoylcarnitine on energy metabolism in mice

To study the role of acylcarnitines in the development of insulin resistance, acute and long-term palmitoylcarnitine administration was used to induce an increase in long-chain acylcarnitine concentrations in mice.

2.1.1 Effects of single-dose palmitoylcarnitine administration on glucose metabolism

Administration of palmitoylcarnitine at a dose of 50 mg/kg induced only a 3-fold increase in the intramuscular content of long-chain acylcarnitines (Figure 2.1). In comparison, after overnight fasting skeletal muscle long-chain acylcarnitine content was 5-fold higher than long-chain acylcarnitine content in fed state (Figure 2.1). Thus, palmitoylcarnitine treatment-induced effect on muscle long-chain acylcarnitine content is similar to short-term fasting.

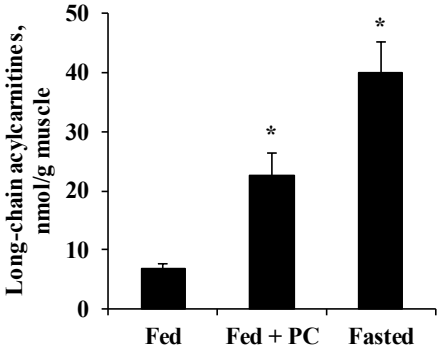

Figure 2.1 **The effect of acute administration of palmitoylcarnitine (PC, 50 mg/kg i.p. 1 h) on the concentrations of long-chain acylcarnitines in muscle tissues**
Each value represents the mean ± S.E.M. of 8 (Fed), 3 (Fed + PC) or 5 (fasted) animals. *Significantly different from the fed group (ANOVA following Tukey's test, $P < 0.05$).

The single-dose administration of palmitoylcarnitine induced a significant increase in the blood glucose concentrations of fasted (Figure 2.2 A) and fed (Figure 2.2 B) mice. The administration of insulin significantly reduced blood glucose with and without the co-administration of palmitoylcarnitine. Thus, additional increase in insulin concentration could decrease the palmitoylcarnitine-induced effect on blood glucose, suggesting that higher concentrations of insulin can overcome palmitoylcarnitine-induced effects on glucose metabolism.

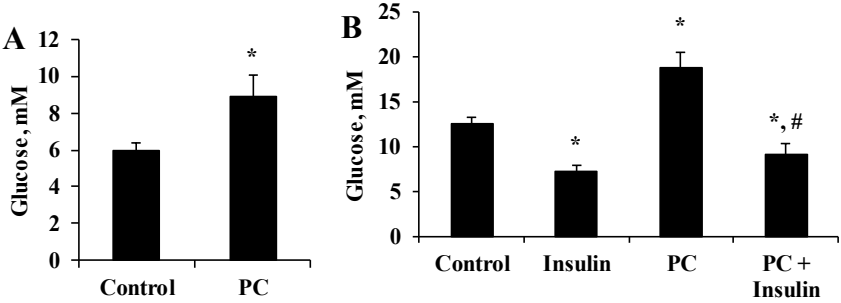

Figure 2.2 **The effects of acute administration of palmitoylcarnitine (PC, 50 mg/kg i.p. 1 h) on the concentrations of blood glucose in fasted (A) and fed (B) states**
Each value represents the mean ± S.E.M. of 5 animals. *Significantly different from the respective control group, # significantly different from the insulin control group (Student's t-test or ANOVA following Tukey's test, $P < 0.05$).

To test the 24-h effect of palmitoylcarnitine administration on glucose tolerance and energy metabolism balance in mice, palmitoylcarnitine (50 mg/kg/day) was administered by slow-release minipumps (ALZET®, USA, Figure 2.3). In the glucose tolerance test, a significantly higher increase was observed in blood glucose in the palmitoylcarnitine group mice (Figure 2.3). These results indicate that increased palmitoylcarnitine concentration delays the glucose metabolism transition from the fed to fasted state.

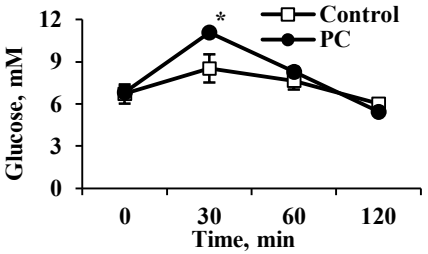

Figure 2.3 **The effects of the administration of palmitoylcarnitine (PC, 50 mg/kg/24h) with osmotic minipumps on glucose tolerance**
Each value represents the mean ± S.E.M. of 4 animals. *Significantly different from the respective control group (Student's t-test, $P < 0.05$).

To determine the effect of long-chain acylcarnitines on insulin secretion, a bolus palmitoylcarnitine was administered to fasted mice. The administration of palmitoylcarnitine induced a significant 5-fold increase in insulin concentration (Figure 2.4 A). Up to a 2-fold increase in insulin concentration was also observed after palmitoylcarnitine administration in fed mice (Figure 2.4 B).

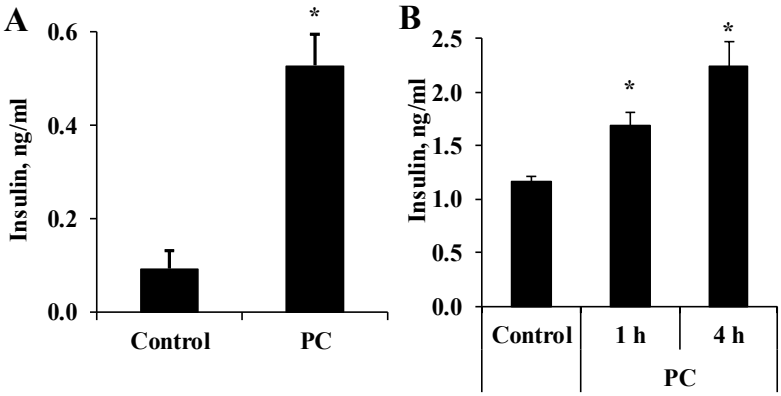

Figure 2.4 **The concentration of insulin in the plasma after the administration of palmitoylcarnitine (PC, 50 and 100 mg/kg) in fasted (A) and fed state (B)**
Each value represents the mean ± S.E.M. of 5 animals. *Significantly different from the respective control group (Student's t-test or ANOVA following Tukey's test, $P < 0.05$).

Overall, palmitoylcarnitine potentiates glucose-stimulated insulin release, thus suggesting that the long-chain acylcarnitine effect is important for the physiological transition from the fasted to fed state and that it induces hyperinsulinemia in the case of diabetes.

For more detailed analysis of the palmitoylcarnitine effect on insulin release, another experimental method suitable for the evaluation of both insulin-dependent and insulin-independent glucose homeostasis *in vivo* was used. To study the insulin-independent palmitoylcarnitine effects on plasma glucose, mice were administered the α_2-adrenoreceptor agonist guanabenz, which inhibits endogenous insulin release (Angel et al., 1988; Saha et al., 2005). For the evaluation of insulin-dependent effects, guanabenz administration was followed by insulin administration (Figure 2.5).

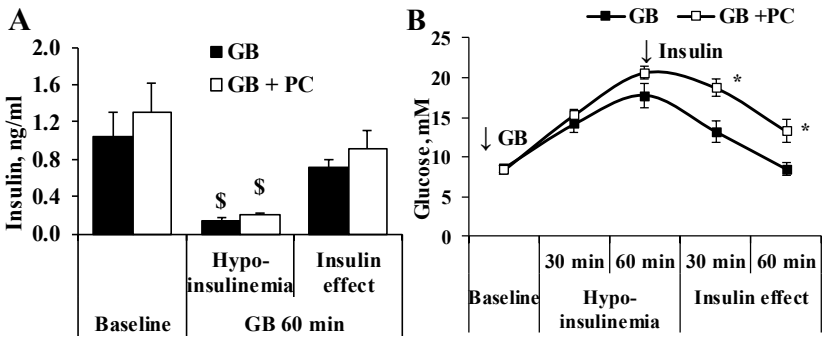

Figure 2.5 **Insulin-independent palmitoylcarnitine (PC, 50 mg/kg i.p.) effects on the levels of plasma insulin (A) and glucose (B)**
Each value represents the mean ± S.E.M. of 8 animals. *Significantly different from the respective control group, $ significantly different from the baseline (Student's t-test or ANOVA following Tukey's test, $P < 0.05$). GB – guanabenz.

In the fed control mice, administration of guanabenz caused hypoinsulinemia (Figure 2.5 A) and induced a marked increase in plasma glucose from 8.6 mM up to 17.7 mM, while the subsequent insulin administration increased insulin concentration (Figure 2.5 A) and significantly

decreased the glucose concentration back to the initial level (Figure 2.5 B). The administration of palmitoylcarnitine in combination with guanabenz induced an increase in the plasma glucose concentration to 3 mM higher concentration than compared to the guanabenz group. Palmitoylcarnitine also significantly diminished the insulin-induced blood glucose lowering effect (Figure 2.5 B). Thus, the blood glucose concentration in the palmitoylcarnitine group remained 5 mM higher than that in the control mice.

These effects could be explained by a palmitoylcarnitine-induced significant decrease in insulin-stimulated [^{3}H]-DOG uptake in skeletal muscles (Figure 2.6). Importantly, the palmitoylcarnitine effect on blood glucose is partially masked by the significantly stimulated [^{3}H]-DOG uptake in liver and adipose tissue (Figure 2.6).

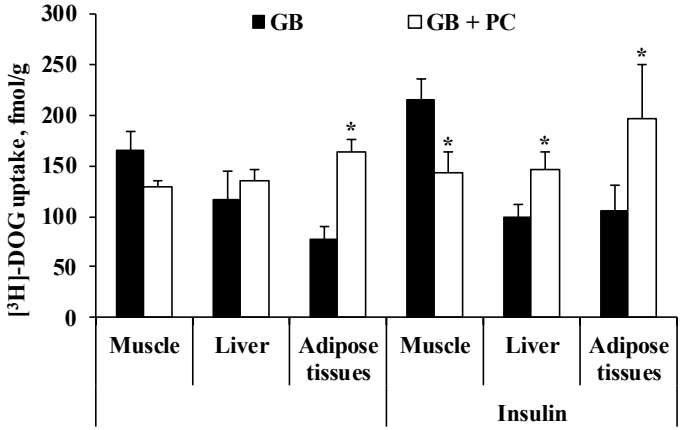

Figure 2.6 **The effects of acute administration of palmitoylcarnitine (PC, 50 mg/kg i.p. 1 h) with guanabenz (GB) and insulin on [^{3}H]deoxyglucose uptake**
Each value represents the mean ± S.E.M. of 8 animals. *Significantly different from the respective control group (ANOVA following Tukey's test, $P < 0.05$).

Overall, the palmitoylcarnitine administration limits insulin- related glucose uptake in muscles.

2.1.2 Mechanisms of palmitoylcarnitine action

To assess whether palmitoylcarnitine has an impact on insulin signalling pathway the phosphorylation of Akt Ser-473 was evaluated in mouse muscles after a single administration of palmitoylcarnitine (50 mg/kg, Figure 2.7). The single dose palmitoylcarnitine administration (50 mg/kg) decreased Akt phosphorylation (Figure 2.7). The increase in insulin concentration overcame the palmitoylcarnitine-induced inhibition of Akt phosphorylation (Figure 2.7). Thus, palmitoylcarnitine induces the inhibition of Akt phosphorylation, while increases in insulin concentration up to a certain level can overcome the palmitoylcarnitine-induced effects on insulin signalling.

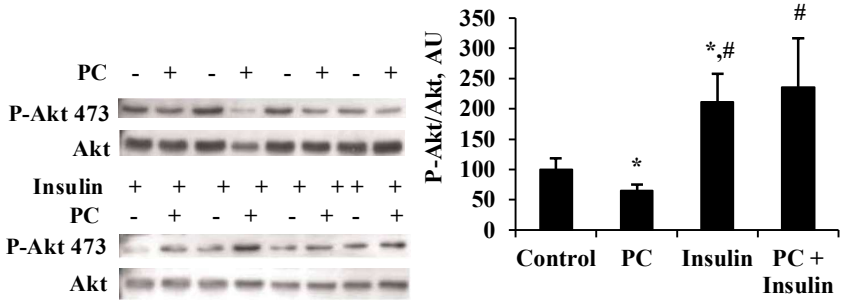

Figure 2.7 **The effect of palmitoylcarnitine (PC) with or without insulin on Akt (Ser-473) phosphorylation in fed mouse muscle *in vivo* (50 mg/kg PC i.p. 1 h)** Each value represents the mean ± S.E.M. of 5 animals. *Significantly different from the control group, #significantly different from the palmitoylcarnitine group (ANOVA following Tukey's test, $P < 0.05$).

Overall, the inhibitory effect on Akt phosphorylation and related insulin signalling is an important mechanism of palmitoylcarnitine action.

2.1.3 Effects induced by the long-term, slow-release administration of palmitoylcarnitine

To ensure the long-term administration of palmitoylcarnitine, we used slow-release osmotic minipumps (ALZET®, USA). This experimental setup

ensured a permanent increase in long-chain acylcarnitine content in muscles for 28 days. In the control mice minipumps loaded with saline (vehicle) were implanted.

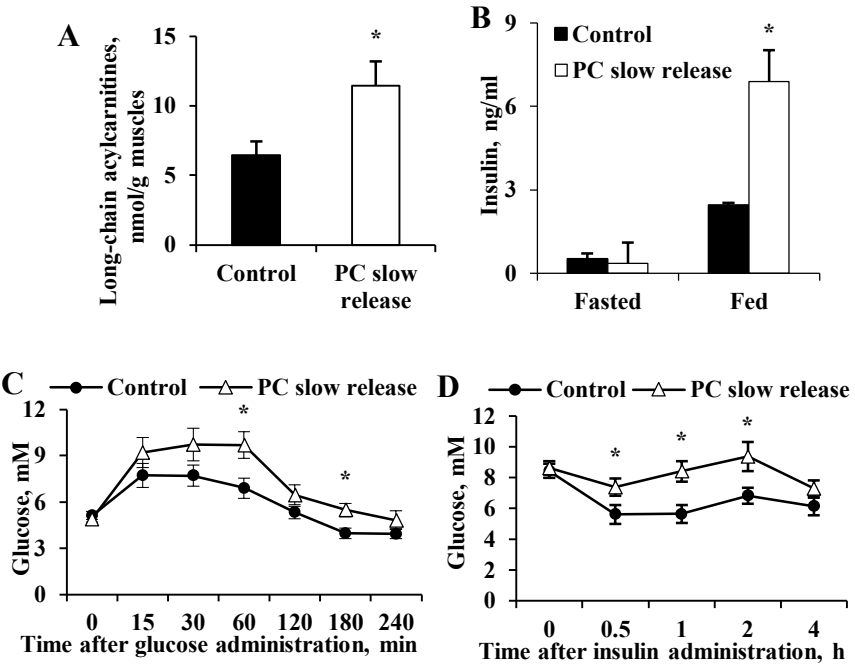

Figure 2.8 **Effects induced by the long-term administration of palmitoylcarnitine (PC, 10 mg/kg/day for 28 days) on the content of long-chain acylcarnitines in skeletal muscles in fed state (A), insulin concentration in plasma (B), glucose (C) and insulin (D) tolerance**

Each value represents the mean ± S.E.M. of 10 animals. *Significantly different from the respective control group, (Student's t-test, $P < 0.05$)

Palmitoylcarnitine administration at a dose of 10 mg/kg/day induced a substantial 2-fold increase in the content of total long-chain acylcarnitine content in muscles (Figure 2.8 A). A long-term increase in long-chain acylcarnitine content resulted in impaired glucose tolerance (Figure 2.8 C). The AUC calculated from the glucose tolerance test data in the palmitoylcarnitine

group was significantly increased by 30 %. Palmitoylcarnitine administration by minipumps blocked the insulin-induced blood glucose lowering effect in the insulin tolerance test (Figure 2.8 D). In the control animals, insulin administration induced an approximate 3 mM decrease in blood glucose concentration, while in the palmitoylcarnitine group blood glucose was not changed during the 4 h test period. In palmitoylcarnitine-treated mice, plasma glucose concentrations were similar to that of the control mice; however, it is likely that glucose concentrations were not affected due to the 2.8-fold increase in insulin concentration in the fed state (Figure 2.8 B). Overall, the long-term increase in long-chain acylcarnitine content induced insulin resistance and hyperinsulinemia.

2.2 Effects of methyl-GBB treatment combined with exercise on insulin sensitivity in experimental mice models of diabetes and insulin resistance

To study the potential protective effects of pharmacological acylcarnitine concentration decrease in diabetes, administration of methyl-GBB alone or combined with physical intervention was used to diminish levels of acylcarnitines in the plasma and muscles in the experimental insulin resistance and diabetes models of *db/db* mice and HFD fed C57BL/6 mice.

2.2.1 Content of acylcarnitines in *db/db* mice plasma and muscles

To evaluate whether acylcarnitines accumulate during diabetes, the acylcarnitine content was measured in the fed and fasted states (Figure 2.9).

In the fed db/Lean mice, the plasma concentrations of long-chain acylcarnitines were 13-fold lower than in the fasted state. However, in *db/db* mice the difference dropped to two-fold, suggesting it as a possible indicator of metabolic inflexibility. Thus, in the fasted state, the plasma content of long-chain acylcarnitines in *db/db* mice was 37 % lower than in db/Lean mice (Figure 2.9.A). However, in the fed state, plasma long-chain acylcarnitine

concentration was 3.5-times higher in *db/db* mice than in db/Lean mice (Figure 2.9.A), resulting in less difference between the content of long-chain acylcarnitines in the fed and fasted states in *db/db* control mice.

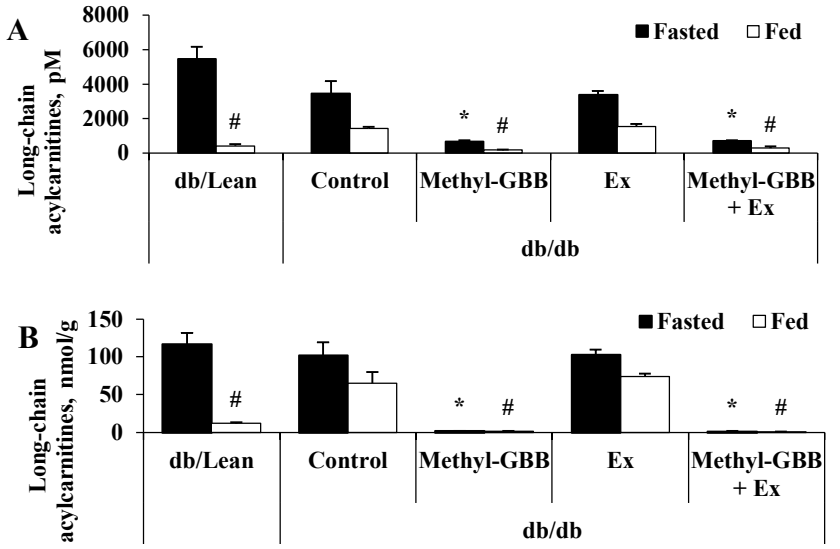

Figure 2.9 **The effect of methyl-GBB administration (5 mg/kg for 8 weeks) and exercise on long-chain acylcarnitine concentrations in *db/db* mouse plasma (A) and muscles (B)**

Each value represents the mean ± S.E.M. of 10 animals. *Significantly different from the fasted *db/db* control group, #significantly different from the fed *db/db* control group (ANOVA following Tukey's test $P < 0.05$).

The acylcarnitine content in the plasma fully reflects the acylcarnitine content in the muscles (Figure 2.9). In the muscles of fasted *db/db* and db/Lean mice, long-chain acylcarnitine contents were similar, while in fed *db/db* mice compared to db/Lean mice, a 5-fold higher long-chain acylcarnitine content was observed. These results demonstrate that accumulation of acylcarnitines in muscle tissue is related to the detrimental effects of acylcarnitines on glucose metabolism in the fed state. Exercise did not influence the concentrations of

short-, medium- and long-chain acylcarnitines in plasma and muscle (Figure 2.9). In contrast, methyl-GBB treatment induced a substantial decrease in all acylcarnitine plasma and muscle concentrations in both states as well as when it was combined with exercise (Figure 2.9 A). Thus, methyl-GBB induced up to a 60-fold decrease in the long-chain acylcarnitine concentration in muscles and up to a 7-fold decrease in the plasma (Figure 2.9). Results indicate that the postprandial plasma concentration of long-chain acylcarnitines could be a marker for insulin resistance. The methyl-GBB-induced decrease in acylcarnitine content was consistent in fed and fasted states and in combination with exercise.

2.2.2 Methyl-GBB and exercise induced effects on glucose and insulin tolerance

To test glucose metabolism disturbances in *db/db* mice and insulin resistant, HFD fed C57BL/6 mice, glucose and insulin tolerance tests were performed (Figure 2.10).

Results showed, that HFD-induced disturbances in glucose metabolism were prevented by methyl-GBB treatment, leading to significantly improved insulin sensitivity and glucose tolerance (Figure 2.10 AB). Also in *db/db* mice an insulin tolerance test showed serious disturbances in insulin sensitivity (Figure 2.10 C). Both exercise and methyl-GBB administration improved insulin sensitivity and a particularly pronounced effect on insulin sensitivity was induced by the combination of methyl-GBB treatment and exercise.

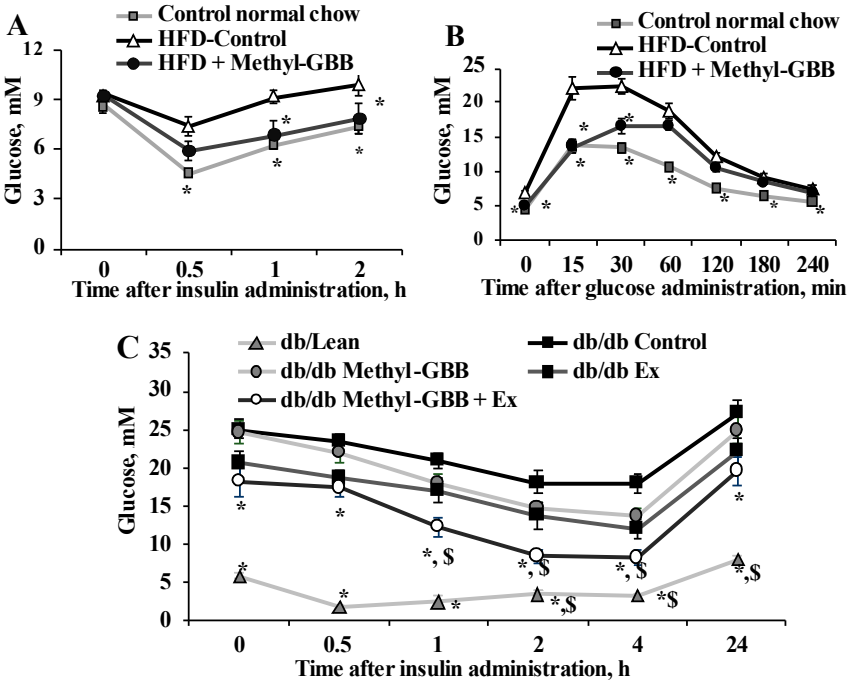

Figure 2.10 **Methyl-GBB administration (5 mg/kg for 8 weeks) and exercise induced effects on glucose concentration after insulin (A, C) and glucose (B) administration-induced changes in HFD (A, B) and *db/db* (C) mice**

Each value represents the mean ± S.E.M. of 10 animals. *Significantly different from the corresponding control group, $significantly different from the Ex group (ANOVA following Tukey's test $P < 0.05$).

2.2.3 Methyl-GBB and exercise induced effects on glucose and insulin concentrations

Compared to normal chow control group, in HFD fed C57BL/6 mice glucose concentration in blood was significantly increased. Similarly to the results observed in the tolerance tests, methyl-GBB significantly reduced glucose concentration to the normal chow control group level (Figure 2.11 A). In *db/db* mice, glucose was significantly more increased in both fed and fasted animals (Figure 2.10 C and Figure 2.11 B). This indicates that *db/db* mice are

characterized by severe hyperglycaemia and uncompensated type 2 diabetes. Exercise decreased postprandial glucose by 4 mM in *db/db* mice. An additional decrease of 6.5 mM in the fed state glucose concentration was induced by combined treatment (Figure 2.10 C). In addition, in fasted *db/db* mice, both methyl-GBB administration and exercise as well as the combination of both significantly decreased the plasma glucose concentration by 8.6 mM, 12.3 mM and 24.3 mM respectively (Figure 2.11 B).

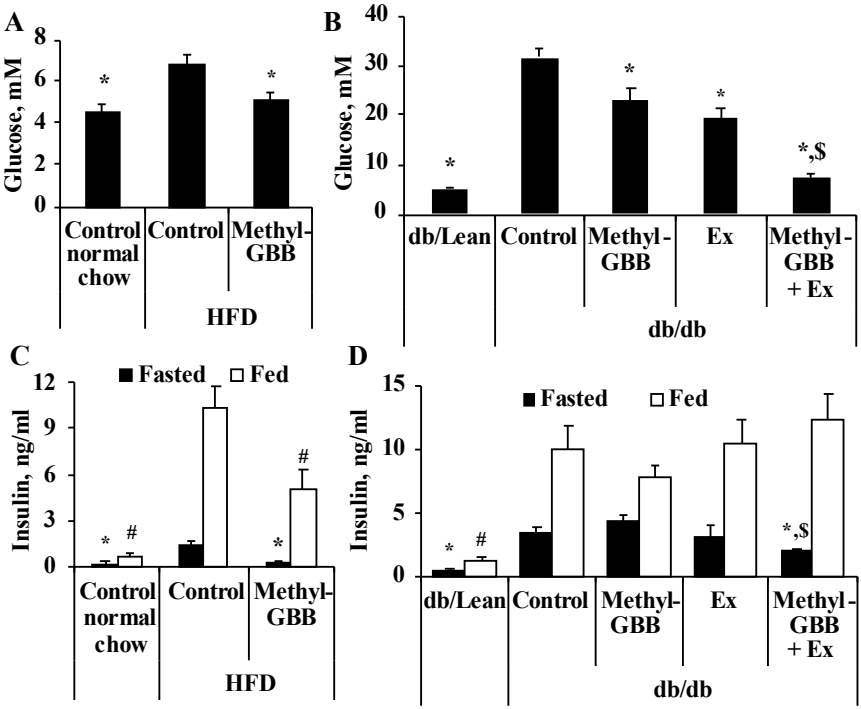

Figure 2.11 **Methyl-GBB administration (5 mg/kg for 8 weeks) and exercise induced effects on fasted plasma glucose, plasma insulin concentrations in HFD mice (A, C) and *db/db* mice (B, D)**

Each value represents the mean ± S.E.M. of 10 animals. *Significantly different from the corresponding fasted diabetes control group, #significantly different from the corresponding fed diabetes group, $significantly different from the Ex group (ANOVA following Tukey's test $P < 0.05$).

Thus, while accumulation of acylcarnitines was observed in the fed state, methyl-GBB- and combination-induced glucose-lowering effects were more pronounced in the fasting conditions. In HFD and *db/db* mice marked hyperinsulinemia was observed. In HFD mice methyl-GBB significantly decreased insulin concentration (Figure 2.11 C). In contrast, exercise and methyl-GBB administration did not significantly influence hyperinsulinemia in *db/db* mice (Figure 2.11 D).

These results suggest that the glucose lowering effect induced by treatments is related to increased insulin sensitivity.

2.3 Role of the long-chain acylcarnitines in the development of ischaemia/reperfusion-induced damage in the heart mitochondria

Isolated rat heart infarction experiments and *in vitro* experiments with isolated cardiac mitochondria were used to study the mechanisms of the long-chain acylcarnitine accumulation-induced damage in the heart mitochondria during the ischaemia/reperfusion injury. *Wistar* rats were treated with methyl-GBB to test possible benefits of a decrease in acylcarnitine content.

2.3.1 Acylcarnitine contents in the heart and in cardiac mitochondria

The contents of acylcarnitines were measured both in heart tissues and in mitochondria in the area at risk and the non-risk area after 30 min of ischaemia (Figure 2.12). In infarcted hearts, in the area at risk the medium-chain acylcarnitine content was two times higher and the long-chain acylcarnitine content was three times higher compared with those in the non-risk area (Figure 2.12 A). In ischaemic mitochondria, accumulation of long-chain acylcarnitines was observed (Figure 2.12 B).

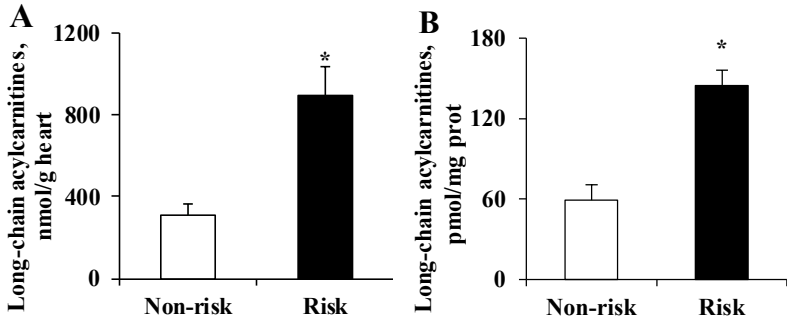

Figure 2.12 **The accumulation of long-chain acylcarnitines in heart tissues (A) and in the mitochondria (B) isolated from the at-risk and the non-risk areas**
Each value represents the mean ± S.E.M. for six hearts. *Significantly different from the corresponding non-risk group (Student's t test, $P < 0.05$).

To study long-chain acylcarnitine accumulation in mitochondrial fractions, we measured labelled [^{3}H]-palmitoylcarnitine content in mitochondria, and specifically in the inner and outer membranes, intermembrane space and mitochondrial matrix (Figure 2.13).

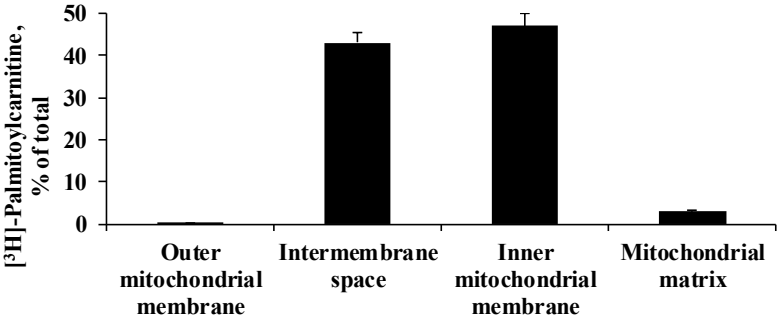

Figure 2.13 **The accumulation of palmitoylcarnitine in subfractions of mitochondria**
Each value represents the mean ± S.E.M. for four experiments. *Significantly different from the corresponding non-risk group (Student's t test, $P < 0.05$).

In the mitochondria, 43 % of [^{3}H]-palmitoylcarnitine was detected in the intermembrane space, and 47 % of [^{3}H]-palmitoylcarnitine was attached to the

inner mitochondrial membrane. In the mitochondrial matrix, only a negligible amount of [^{3}H]-palmitoylcarnitine accumulated. Therefore, the main locations of palmitoylcarnitine accumulation are the inner mitochondrial membrane and the intermembrane space.

2.3.2 Palmitoylcarnitine-induced mitochondrial damage

To determine the mechanisms responsible for mitochondrial damage induced by long-chain acylcarnitines, the effects of palmitoylcarnitine on mitochondrial respiration, H_2O_2 production and mitochondrial membrane potential were investigated using simultaneous high-resolution respirometry and fluorimetry. Palmitoylcarnitine decreased OXPHOS-dependent mitochondrial respiration with complex II and complex I and II substrates in a dose-dependent manner (Figure 2.14 A). The addition of 15 μM palmitoylcarnitine to the cardiac mitochondria did not induce the uncoupling of OXPHOS because no increase in mitochondrial respiration in the OXPHOS or $LEAK_{Omy}$ state was observed (Figure 2.14 B).

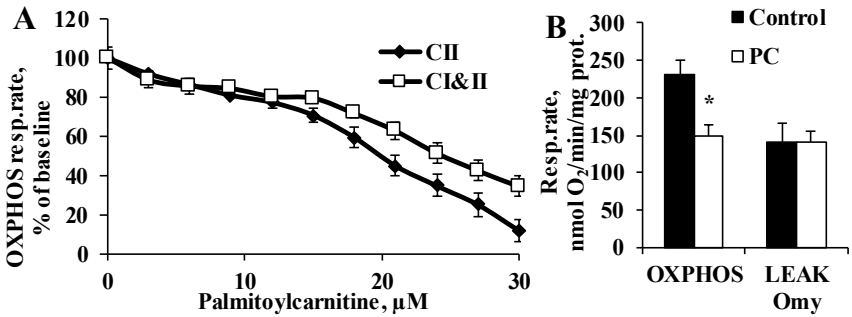

Figure 2.14 **The effects of palmitoylcarnitine (PC) on OXPHOS-dependent mitochondrial respiration**

Each value represents the mean ± S.E.M. for four or five experiments. *Significantly different from the control group (Student's t test, $P < 0.05$).

However, similarly to 10 μM Omy A addition, the presence of 15 μM palmitoylcarnitine induced a 2-fold increase in the mitochondrial membrane potential (Figure 2.15).

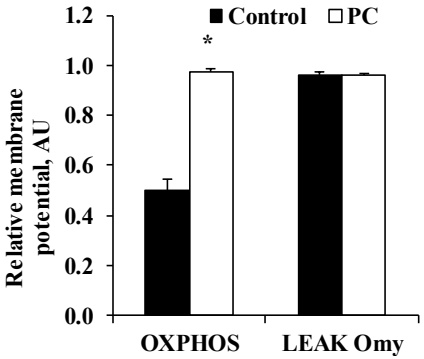

Figure 2.15 **The effect of palmitoylcarnitine (PC) on the mitochondrial membrane potential in the complex II-linked OXPHOS and LEAK states using TMRM**
TMRM - tetramethylrhodamine methyl ester. Each value represents the mean ± S.E.M. for four or five experiments. *Significantly different from the control group (Student's t test, $P < 0.05$).

The addition of palmitoylcarnitine increased the H_2O_2 production rate and the H_2O_2/O_2 ratio in mitochondria in a dose-dependent manner (Figure 2.16).

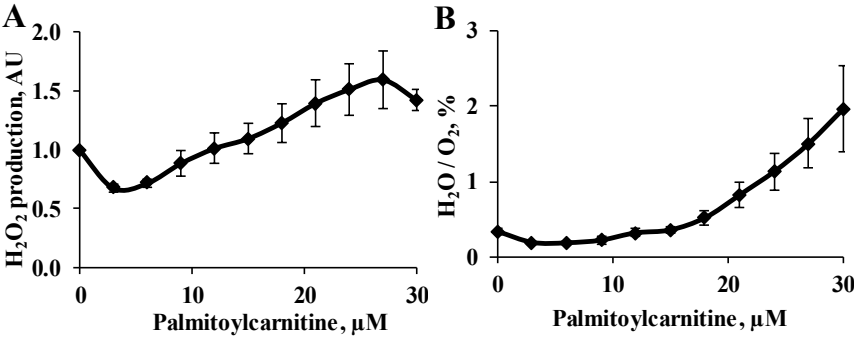

Figure 2.16 **The effect of palmitoylcarnitine on H_2O_2 production under complex II-linked OXPHOS state (A and B)**
Each value represents the mean ± S.E.M. for four or five experiments. *Significantly different from the control group (Student's t test, $P < 0.05$).

Taken together, these results demonstrate that long-chain acylcarnitines inhibit OXPHOS in cardiac mitochondria, thus inducing mitochondrial membrane hyperpolarization and stimulating the production of reactive oxygen species, which can lead to cell death.

2.3.3 Effects of increased and decreased acylcarnitine content on myocardial infarction

The harmful effects of palmitoylcarnitine were investigated in an isolated rat heart ischaemia/reperfusion model using palmitoylcarnitine at a concentration of 2 µM (Figure 2.17).

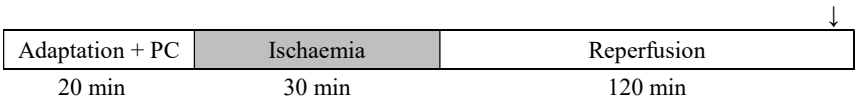

Figure 2.17 **The myocardial infarction experimental setup with palmitoylcarnitine (PC)**
PC was added to Krebs–Henseleit buffer at a concentration of 2 µM for 20 min before ischaemia.

In spontaneously beating isolated rat hearts, the addition of 2 μM palmitoylcarnitine to the perfusion buffer increased the palmitoylcarnitine concentration in the heart 4-fold (Figure 2.18 A). As shown in Figure 2.18 B, palmitoylcarnitine addition significantly increased the infarct size by 33 % relative to the control group's infarct size. Overall, palmitoylcarnitine seems to induce significant disturbances in heart function and an increase in the infarct size.

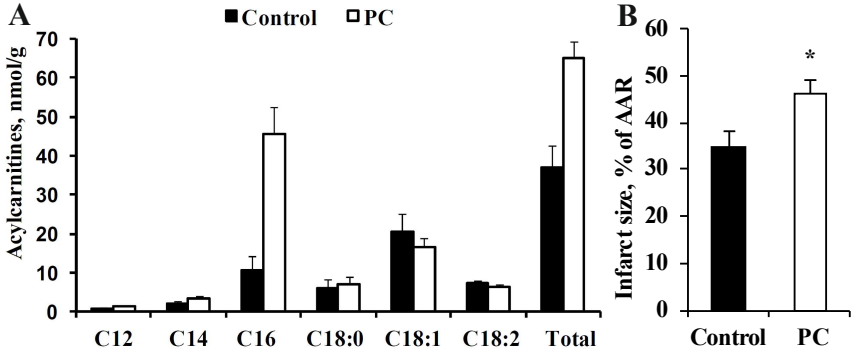

Figure 2.18 **The accumulation of acylcarnitines in heart tissues (A) and the infarct size (B) after reperfusion in the control and palmitoylcarnitine (PC) groups**
Each value represents the mean ± S.E.M. for five to ten animals. *Significantly different from the corresponding control group (Student's t test, $P < 0.05$). AAR – area at risk.

To test the effects of decreased acylcarnitine content, pre-treatment with methyl-GBB for 14 days before ischaemia/reperfusion injury was used. Results showed that methyl-GBB pre-treatment completely prevented acylcarnitine accumulation. Thus, methyl-GBB induced up to a 60-fold decrease in short-, medium- and long-chain acylcarnitine content in heart mitochondria isolated from the at-risk area (Figure 2.19 A). As a result, the infarct size decreased by 44 % (Figure 2.19 B). Thus, the methyl-GBB-induced decrease in the acylcarnitine content is protective against acute ischaemia/reperfusion-induced damage.

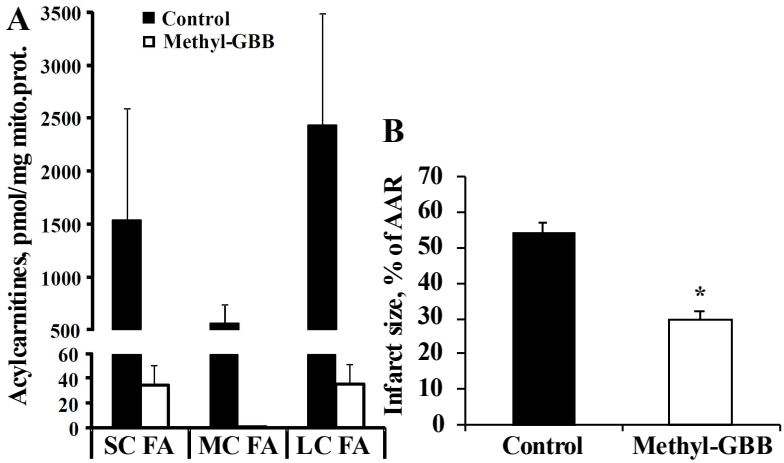

Figure 2.19 **The effects of long-term methyl-GBB (20 mg/kg) treatment on the acylcarnitine content (A) and the infarct size (B).**
Each value represents the mean ± S.E.M. for five to ten animals. *Significantly different from the corresponding control group (Student's t test, $P < 0.05$). AAR – area at risk, SC – short chain, MC – medium chain, LC – long chain.

2.4 Effects of methyl-GBB treatment on the development of atherosclerosis

In this study *apoE*$^{-/-}$ mice were treated with methyl-GBB to test the effects of the treatment on the development of atherosclerosis. CD-1 outbred mice received methyl-GBB and molecular anti-atherosclerotic mechanisms of the drug were evaluated.

2.4.1 Effects of the treatment on the acylcarnitine profile

Effects of the treatment with methyl-GBB on the acylcarnitine profile in the aortic tissues were assayed in the control and methyl-GBB receiving CD-1 outbred mice. As it can be seen in Figure 2.20, methyl-GBB administration to the CD-1 outbred mice at the dose of 16.8 mg/kg markedly decreased the amount of acylcarnitines in the aortic tissues.

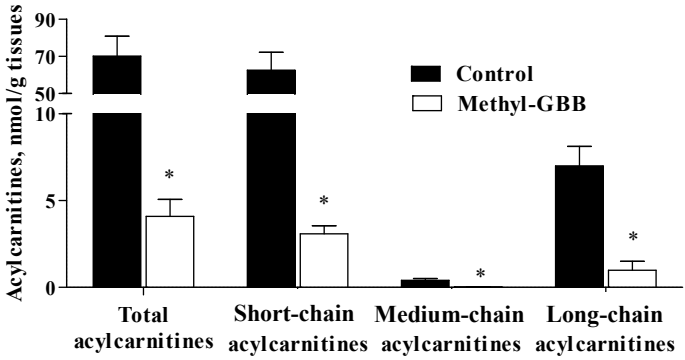

Figure 2.20 **The effects of methyl-GBB treatment on the acylcarnitine profile in the aortic tissues**

Each value represents the mean ± S.E.M. for ten animals. *Significantly different from the control group (two-tailed Student's t-test, $P < 0.05$).

Methyl-GBB treatment decreased the total and the short-chain acylcarnitine level by nearly seventeen-fold and the medium- and long-chain acylcarnitine level by seven-fold.

2.4.2 Effect of administration of methyl-GBB on the biochemical profile of plasma

Treatment of *apoE*$^{-/-}$ mice with methyl-GBB at the dose of 10 mg/kg for four months statistically significantly decreased the concentration of triglycerides, HDL- and LDL-cholesterol for 44 %, 29 % and 24 %, respectively (Table 2.1).

Table 2.1

Biochemical profile of plasma after treatment with methyl-GBB

Group	Triglycerides, mM	HDL, mg/dl	LDL, mg/dl
Control	1.8 ± 0.2	212 ± 22	1014 ± 53
Methyl-GBB	1.0 ± 0.1*	150 ± 18*	767 ± 47*

Each value represents the mean ± S.E.M. for ten animals. *Significantly different from the control group (two-tailed Student's t-test, $P < 0.05$).

2.4.3 Effect of administration of methyl-GBB on the TNFα concentration in plasma

The average concentration of TNFα in plasma of control group animals was 5.8 ± 1.5 pg/ml (Figure 2.21).

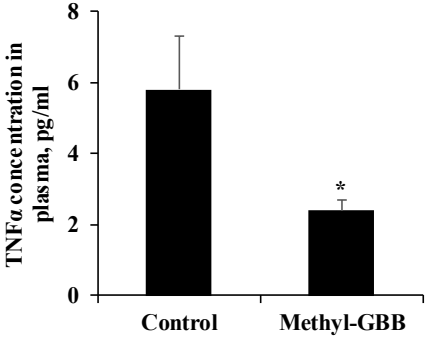

Figure 2.21 **The effects of methyl-GBB treatment on the TNFα concentration in plasma**
Each value represents the mean ± S.E.M. for ten animals. *Significantly different from the control group (two-tailed Student's t-test, $P < 0.05$).

Treatment with methyl-GBB significantly decreased the concentration of TNFα to 2.4 ± 0.3 pg/ml.

2.4.4 Effects of the treatment on the progression of atherosclerotic lesions in the whole aorta and aortic root

Following four months of treatment, atherosclerotic lesions were observed in the aortas of the *apoE*$^{-/-}$ mice both groups. In the control group, the plaques covered 16.0 ± 1.7 % of the whole aorta. Analysis of the Sudan stained aortas showed that the administration of methyl-GBB decreased the area covered by the plaques in the whole aorta to 10.3 ± 1.7 %, thereby significantly attenuating the development of atherosclerotic lesions in the whole aorta by 36 % (Figure 2.22 A). Similarly, methyl-GBB treatment attenuated also the development of atherosclerotic lesions in the aortic sinus for ~35 % (Figure 2.22 B).

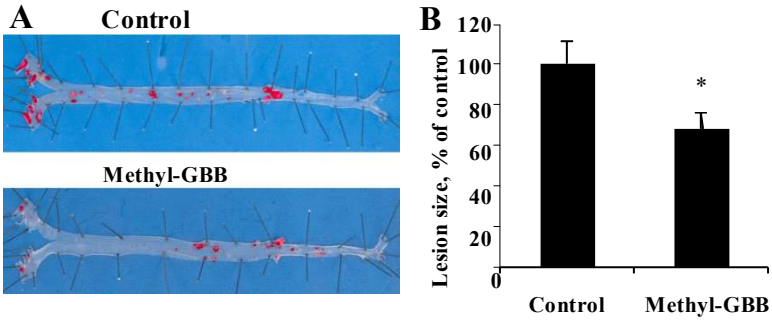

Figure 2.22 **The effects methyl-GBB administration on the development of atherosclerotic lesions in the aortas (A) and aortic roots (B) of *apoE*$^{-/-}$mice**
In Figure A are shown the representative digital pictures of the Sudan IV stained aortas from both experimental groups. Each value represents the mean ± S.E.M. for ten animals. *Significantly different from the control group (two-tailed Student's t-test or one-way ANOVA followed by Tukey's multiple comparison test, $P < 0.05$).

2.4.5 Effects of the treatment on the macrophage and monocyte counts in the atherosclerotic lesions

To assess the anti-atherosclerotic mechanism of methyl-GBB, the number of macrophages and monocytes was determined in the control and methyl-GBB groups (Figure 2.23).

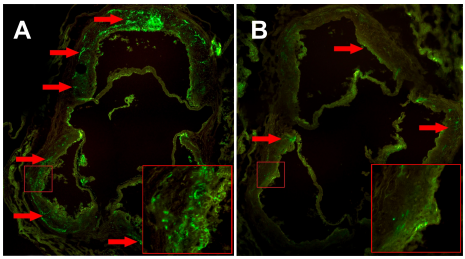

Figure 2.23 **The effect of methyl-GBB administration on the accumulation of macrophages and monocytes in the aortic lesions in the aortic roots of control (A) and methyl-GBB (B) treated *apoE*$^{-/-}$ mice**
The slides were stained with MOMA2 antibodies. Red arrows indicate the green fluorescent regions where the macrophages and monocytes are located. The red square illustrates the presence of stained cells in the atherosclerotic lesion.

Methyl-GBB treatment decreased the infiltration of macrophages and monocytes into the aortic lesions of the aortic root.

3 DISCUSSION

The present thesis describes the link between acylcarnitine accumulation and the pathological processes associated with cardiometabolic diseases: development of insulin resistance, atherosclerosis and heart ischaemia/reperfusion injury. The aim of the study was to determine, whether pharmacological decrease in the acylcarnitine concentration by methyl-GBB is beneficial for the treatment of the mentioned diseases and to investigate the pharmacological mechanisms of action of methyl-GBB. Initially, molecular mechanisms of excessive accumulation of long-chain acylcarnitines in the model of accelerated development of insulin resistance were investigated. Then, the antidiabetic effects of methyl-GBB treatment alone or in combination with physical intervention were studied. Further, the damage in the heart mitochondria induced by long-chain acylcarnitine accumulation in the ischaemia/reperfusion injury was described and methyl-GBB cardioprotective effects were assessed. Finally, the effects of methyl-GBB treatment on the development of atherosclerosis were evaluated.

3.1 Role of long-chain acylcarnitine accumulation in the development of insulin resistance

We have shown that long-chain acylcarnitines influence glucose metabolism *in vivo* and therefore are important players in the physiological regulation of energy metabolism. Moreover, the accumulation of long-chain acylcarnitines during the fed state can accelerate diabetes progression by the simultaneous induction of insulin resistance and insulin release *in vivo*. A single-dose administration of palmitoylcarnitine in mice inhibits Akt phosphorylation in muscles and the downstream signalling pathways involved in glucose uptake. The long-term administration of palmitoylcarnitine induces insulin resistance, hyperinsulinemia and disturbances in glucose tolerance.

In previous studies, we hypothesized that long-chain acylcarnitines are not only markers for incomplete FA oxidation but also active FA metabolites involved in the regulation of energy metabolism (Makrecka et al., 2014). Our present data confirm this hypothesis through the results indicating that the acute and chronic administration of palmitoylcarnitine *in vivo* limits insulin signalling-induced effects and insulin-related glucose uptake in muscles. According to our results, the mechanism behind long-chain acylcarnitine action in muscles *in vivo* is the inhibition of Akt phosphorylation and the subsequent inhibition of downstream signalling. This is in line with previous studies in C2C12 myotubes *in vitro* (Aguer et al., 2015; Koves et al., 2008). Interestingly, an increased concentration of insulin can overcome the long-chain acylcarnitine-induced effects and stimulate Akt phosphorylation to the appropriate level. This explains previous results showing that the increased concentrations of insulin in response to glucose stimulation in the fed state can overcome the inhibitory effects of high long-chain acylcarnitine content (Consitt et al., 2016; Soeters et al., 2009). In addition, to support the transition from the fasted to fed state and overcome transient intramuscular insulin insensitivity, long-chain acylcarnitines facilitate insulin release.

In the fasted state, the low level of insulin is unable to inhibit long-chain acylcarnitine production, and the high long-chain acylcarnitine content continues to inhibit the Akt pathway. As a result, glucose uptake and metabolism is limited, while long-chain acylcarnitine synthesis by CPT1 and subsequent FA oxidation increases. Therefore, in starved individuals, the intracellular content of long-chain acylcarnitines is higher than in the fasted state (Soeters et al., 2009). In our study, both a bolus and long-term administration of palmitoylcarnitine (C16) increased the content of C18 acylcarnitines, suggesting that a high long-chain acylcarnitine content blocks the insulin-induced CPT1 inhibition and stimulates an even higher increase in long-chain acylcarnitine content. Many studies indicate that acylcarnitine

accumulation is a result of incomplete FA oxidation (Aguer et al., 2015; McCoin et al., 2015; Samuel and Shulman, 2012; Schooneman et al., 2013). However, our results also indicate that in certain conditions such as the fasted state, the physiologically important long-chain acylcarnitine accumulation is a result of their CPT1-driven overproduction even if coupled to a high FA oxidation rate. Overall, long-chain acylcarnitines, as inhibitors of Akt phosphorylation, are active participants in an intercellular feedback mechanism of insulin signalling and are a substantial part of the energy metabolism regulation program.

In healthy subjects in the fed state, the increased concentration of insulin inhibits long-chain acylcarnitine production via the increased tissue content of malonyl-CoA (Schooneman et al., 2013; Soeters et al., 2009). That occurs due to the insulin-induced inhibition of AMP-activated protein kinase activity, which results in the stimulation of acetyl-CoA carboxylase (synthesis of malonyl-CoA) and inhibition of malonyl-CoA decarboxylase (degradation of malonyl-CoA, Ruderman et al., 1999; Valentine et al., 2014). The inability of insulin to inhibit long-chain acylcarnitine production in the fed state induces disturbances in glucose uptake and metabolism. In the early stage of insulin resistance, hyperinsulinemia can compensate for insulin resistance and also overcome the long-chain acylcarnitine-induced effects. In the later stages of the disease, insulin resistance leads to the inability to inhibit long-chain acylcarnitine production and is accompanied by increased concentrations of long-chain acylcarnitines, which continuously inhibit the Akt-mediated signalling pathway and further stimulate the progression of glucose intolerance. Thus, the accumulation of long-chain acylcarnitines can accelerate the progression of insulin resistance.

In conclusion, taking into account the importance of muscle metabolic flexibility and the ability to switch between fed and fasted states, long-chain acylcarnitines play a role in the regulation of energy metabolism. These results

link the development of skeletal muscle insulin resistance to the excessive accumulation of long-chain acylcarnitines and suggest that acylcarnitines could induce insulin resistance.

3.2 Reduction of long-chain acylcarnitine content: an effective pharmacologic strategy to prevent the development of diabetes

The present study demonstrates that long-term acylcarnitine accumulation in the fed state is a feature of type 2 diabetes. Therefore, decreased acylcarnitine content by methyl-GBB administration improved insulin sensitivity and significantly reduced blood glucose and insulin levels in mice with impaired insulin sensitivity and diabetes. Exercise and the combination of methyl-GBB treatment with exercise improved insulin sensitivity in *db/db* mice. Thus, decrease in acylcarnitine levels is sufficient to restore insulin sensitivity in the early stage of diabetes, while in case of severe diabetes an additive insulin sensitizing effect of forced physical activity and decreased acylcarnitine content by methyl-GBB would be necessary to achieve substantial antidiabetic effect.

Several previous studies have attempted to associate acylcarnitines with diabetes; however, the content of acylcarnitines in relation to the fed and fasted states and the efficiency of treatment were not monitored (Rodríguez-Gutiérrez et al., 2012; Su et al., 2005; Zhang et al., 2014). In this study, acylcarnitine content was measured in the plasma and muscles of fed and fasted animals, and the results clearly demonstrated that the main pathological role of acylcarnitine accumulation in type 2 diabetes is related to the postprandial or fed state. Acylcarnitine accumulation in the fed state is a consequence of insulin resistance or the inability of insulin to sufficiently decrease the production of acylcarnitines. In turn, acylcarnitines inhibit pyruvate and lactate oxidation

leading to an increase in circulating glucose and lactate concentrations and the induction of common hyperglycaemia-related complications (publication..).

Previously, inhibition of FA oxidation was suggested as a target for diabetes treatment. In this study, a 60-fold decrease in long-chain acylcarnitines did not result in a similar decrease in the FA metabolism rate in skeletal muscles. Also previously it has been indicated that the inhibition of CPT1 and decreased acylcarnitine content does not fully reflect the FA uptake or oxidation rate (Liepinsh et al., 2013b, 2015; Luiken et al., 2009). Methyl-GBB-induced improvements in insulin sensitivity are mainly due to the decreased content of acylcarnitines. In addition, this suggests that in the muscles, methyl-GBB decreases only acylcarnitine overproduction, thus balancing acylcarnitine synthesis and utilization. Moreover, the marked decrease in acylcarnitine content only partially restores insulin sensitivity, and additional improvements could be possible through other mechanisms such as the inhibition of FA oxidation.

There is very good clinical evidence that physical activity is important for prevention and effective therapy for diabetes (Balducci et al., 2014). However, implementation of this strategy in clinical practice has not always led to the expected outcomes (De Feo and Schwarz, 2013). In the present study, low intensity exercise was beneficial for *db/db* mice. Higher intensity exercise provides greater benefit in diabetes treatment; however, it also increases the risk of injury and cardiovascular complications (Balducci et al., 2014). Thus, low-risk moderate intensity exercise such as walking could provide the best overall health benefit for patients with diabetes. Additional improvements could be achieved by pharmacological interventions that facilitate physical activity and improve exercise-induced antidiabetic effects. Patients with diabetes walking an additional 1,000 steps per day could reduce postprandial blood glucose by 1.6 mM over a period of 2 years (Tudor-Locke and Bassett, 2004). In our experiment, improvements in insulin sensitivity and a decrease in

postprandial glucose of 4 mM in *db/db* mice were achieved by walking an additional 3,600 steps/day on working days. Meanwhile, an additional decrease in the fed state glucose concentration and a significant improvement in insulin sensitivity were achieved by combined intervention. In addition, the cardioprotective properties of methyl-GBB can reduce the possible ischaemia induced cardiovascular complications (Liepinsh et al., 2015). Thus, pharmacological decrease of acylcarnitine levels by methyl-GBB would be particularly beneficial for patients with diabetes along with physical activity.

3.3 Role of long-chain acylcarnitines in the development of cardiovascular complications of diabetes

The present study demonstrates that the accumulation of long-chain acylcarnitines in mitochondria determines the ischemia-reperfusion induced damage. Acylcarnitine accumulation during ischaemia leads to inhibited oxidative phosphorylation in cardiac mitochondria, thus inducing mitochondrial membrane hyperpolarization and stimulating the production of reactive oxygen species. A pharmacologically induced decrease in the mitochondrial acylcarnitine content by methyl-GBB treatment reduces the infarct size.

Acylcarnitine accumulation during ischaemia was discovered approximately three decades ago (Corr et al., 1984; Ford et al., 1996; Idell-Wenger et al., 1978; Whitmer et al., 1978), but many aspects remain unclear. In general, acylcarnitine content depends on both the production and the utilization rates of particular FA intermediates. Consequently, overproduction of FA intermediates in the FA metabolism pathway occurs during stimulated FA flux or due to insufficient utilization in mitochondria. Thus, in the fasted state, the acylcarnitine content in the heart is up to 5-fold higher than in the fed state (Makrecka et al., 2014). Taking into account that fasting is a physiological condition and does not induce damage to heart mitochondria, an average 1.5 to 3-fold increase in FA intermediates during ischaemia is harmful only to fasted

or starved animals. Similarly, in diabetes patients, the higher risk of heart disease (Peters et al., 2015) might be attributable to higher acylcarnitine content in the heart. Our previous study, which demonstrated that ischaemic damage to the heart is significantly lower in the fed state compared with the fasted state (Liepinsh et al., 2014b), also indicated that the accumulation of FA intermediates is at least partially responsible for the more pronounced heart damage in ischaemia.

Acyl-CoAs and acylcarnitines accumulate during ischaemia, but acyl-CoAs have been rated as the most prominent FA intermediates responsible for mitochondrial damage (Drosatos and Schulze, 2013; Li et al., 2010). However, in a previous study, we demonstrated that the physiological acylcarnitine content, but not the acyl-CoA content, is sufficient to inhibit pyruvate and lactate metabolism in mitochondria (Makrecka et al., 2014). In the present study, supplementation of the heart perfusion buffer with palmitoylcarnitine induced a 2-fold increase in the acylcarnitine content and significantly increased the infarct size. Thus, our results indicate that acylcarnitines are the main FA intermediates that induce ischaemia/reperfusion-related damage.

To date, only some studies have addressed the mechanisms of mitochondrial dysfunction induced by long-chain acylcarnitines (Korge et al., 2003; Siliprandi et al., 1992; Tominaga et al., 2008). Inhibition or uncoupling of oxidative phosphorylation, mitochondrial membrane depolarization, opening of the mitochondrial permeability transition pore and production of reactive oxygen species were proposed to be mechanisms by which FAs and their intermediates induce mitochondrial damage and subsequent cell damage. In a study using permeabilized isolated rat ventricular myocytes, it was demonstrated that the mechanism of palmitoylcarnitine-induced mitochondrial damage is different at low and high concentrations of acylcarnitines (Tominaga et al., 2008). The important finding of the present study is the demonstrated localization of palmitoylcarnitine accumulation in mitochondria, suggesting

that either CPT1-generated acylcarnitines or acylcarnitines transported from cytosol tend to accumulate between the mitochondrial membranes and on the inner mitochondrial membrane. To a certain extent, mitochondria are protected by binding proteins localized on mitochondrial membranes, but overproduction of acylcarnitines results in an increased unbound acylcarnitine concentration in the intermembrane space and corresponding damaging effects. Overall, long-chain acylcarnitines inhibit oxidative phosphorylation and thus induce accumulation of protons and mitochondrial membrane hyperpolarization, which stimulates reverse proton flux through the respiratory chain and subsequent production of reactive oxygen species.

The inhibition of acylcarnitine production has been suggested as a cardioprotective drug target (Keung et al., 2013; Liepinsh et al., 2013b, 2015). CPT1 is also considered to be an important FA mitochondrial transport-limiting enzyme and is often directly or indirectly targeted to achieve inhibition of FA metabolism and facilitation of glucose oxidation. It is apparent that any inhibition of the FA pathway upstream of CPT1 would also limit acylcarnitine production, and at least part of the protective mechanism of inhibited FA flux is related to decreased acylcarnitine content. Methyl-GBB treatment has been shown as a powerful tool for reducing the infarct size after ischaemia/reperfusion injury by decreasing the acylcarnitine content in the heart and mitochondria and limiting long-chain FA oxidation in favour of glucose oxidation (Liepinsh et al., 2015). Also in this study the administration of methyl-GBB completely prevented acylcarnitine accumulation and decreased the infarct size. Therefore, the main advantage of the pharmacological decrease of acylcarnitine content might be the reduction of direct damage by the long-chain acylcarnitines on mitochondria in an ischaemic heart.

3.4 Methyl-GBB attenuates the development of atherosclerosis by decreasing levels of long-chain acylcarnitines

In this study, the effects of lowering the long-chain acylcarnitine levels by methyl-GBB treatment were studied on the development of atherosclerosis in *apoE*$^{-/-}$ mice. We found that methyl-GBB administration significantly attenuated the development of atherosclerotic lesions in the whole aorta and markedly decreased the amount of acylcarnitines in the aortic tissues.

Atherosclerosis is characterised as an inflammation of blood vessels with the participation of immune cells (Libby, 2012). Moreover, it has been shown that acylcarnitines accumulate in the aortic tissues during the development of atherosclerotic lesions (Gillies and Bell, 1976). Previously, a study showed that acylcarnitines induce the pro-inflammatory activation of macrophages and promote the expression of COX-2 and the secretion of TNFα and other pro-inflammatory cytokines (Rutkowsky et al., 2014). In addition, palmitoylcarnitine has been shown to disrupt the function of the vascular endothelium and decrease the synthesis of nitric oxide (Inoue et al., 1994), pathways that could promote the progression of atherosclerosis. The analysis of the mice aortic tissue extracts revealed that methyl-GBB administration significantly decreased the amount of short-, medium- and long-chain acylcarnitines. Thus, the methyl-GBB anti-atherosclerotic mechanism could include the inhibition immune cell infiltration in atherosclerotic lesions. In support of previously mentioned presumption are the results obtained from the analysis of atherosclerotic lesions in the aortic root, which showed that methyl-GBB treatment decreases the accumulation of macrophages and monocytes in the atherosclerotic lesions. Moreover, administration of methyl-GBB significantly decreased the TNFα concentration in plasma that could be a result of decreased secretion of TNFα by activated macrophages. Thus, this study demonstrates that methyl-GBB attenuates the development of atherosclerosis

by inhibiting infiltration of macrophages and monocytes into the aortic lesions of the aortic root, decreasing their secretion of pro-inflammatory cytokines and possibly by reducing deleterious effects of acylcarnitines on the vascular endothelium.

Postprandial hypertriglyceridemia and elevated LDL levels are risk factors for the development of atherosclerosis (Fujioka and Ishikawa, 2009; Odden et al., 2014). Methyl-GBB treatment induced decrease in triglyceride concentration, thus, the decrease of triglyceride concentration could contribute the attenuation of development of atherosclerosis. The administration of methyl-GBB also decreased the concentration of LDL-cholesterol, but the effects of methyl-GBB on acylcarnitine and triglyceride levels were significantly more pronounced than that on plasma cholesterol concentrations.

Several studies have shown that treatment with substances that markedly decrease the pools of L-carnitine (pivalate moiety releasing molecules and D-carnitine) or genetic mutations of proteins that are involved in the homeostasis and synthesis of L-carnitine induce impairment of functioning of different organs (Broderick, 2006; Magoulas and El-Hattab, 2012). In our experimental setup, treatment of *apoE*$^{-/-}$ mice with methyl-GBB significantly decreased pools of L-carnitine but we did not observe any signs of muscle weakness or elevations of ALT and AST. Previously it has been shown, that 2-week treatment with methyl-GBB at the dose 20 mg/kg decreased L-carnitine pools in heart tissues by 95 %, but the systolic function and anatomical parameters of the left ventricle was unchanged (Liepinsh et al., 2015). Thus, we conclude that the decrease of L-carnitine pools may not be the main factor that determines the development of organ dysfunction and it could be a matter of pharmacological properties of the studied substance. In favour of that is a study with sodium pivalate which showed that treatment with it decreased amount of L-carnitine in heart tissues only by 37 % and simultaneously impaired metabolism of pyruvate and decreased metabolism of palmitoyl-CoA (Kuka et al., 2012b).

This could lead to a depletion of ATP levels and development of ventricular dysfunction, which was observed after prolonged administration of pivalate (Broderick, 2006). On the contrary, treatment with methyl-GBB not only limited FA metabolism, but also facilitated glucose metabolism in heart tissues (Liepinsh et al., 2015), thus, preserving ATP pools and heart function. Overall, since the *apoE*$^{-/-}$ mouse model closely resembles the development of vascular lesions in humans (Nakashima et al., 1994), we can hypothesise that the treatment with methyl-GBB could be a powerful approach to attenuate the development of atherosclerosis also in clinics.

Overall, we have shown that by inhibiting Akt phosphorylation and glucose metabolism the accumulation of long-chain acylcarnitines accelerates hyperglycaemia and hyperinsulinemia leading to metabolic inflexibility and insulin resistance. Long-chain acylcarnitine accumulation during ischaemia leads to mitochondrial damage in cardiac cells, indicating that long-chain acylcarnitines are the main FA intermediates that induce ischaemia/reperfusion-related injury. Long-chain acylcarnitines participate in various inflammatory processes, including the expression and secretion of TNFα and other pro-inflammatory cytokines, and their accumulation is observed in atherosclerotic lesions. Our data on the harmful effects of increased acylcarnitine content and the protective effects of decreased acylcarnitine content by methyl-GBB treatment suggest that inhibition of acylcarnitine production in mitochondria is a valuable cardiometabolic drug target.

In conclusion, pharmacologically induced decrease in the content of long-chain acylcarnitines by methyl-GBB facilitates glucose metabolism, improves insulin sensitivity, protects the heart mitochondria against ischaemia/reperfusion injury and attenuates the development of atherosclerosis and therefore represents an effective strategy for the treatment of diabetes and its complications.

4 CONCLUSIONS

1. The accumulation of long-chain acylcarnitines during the fed state limits metabolic flexibility, induces insulin resistance, hyperinsulinemia and disturbances in glucose tolerance. Inhibition of Akt phosphorylation and related insulin signalling is an important mechanism of long-chain acylcarnitine-induced detrimental effects on glucose metabolism.
2. Methyl-GBB administration-induced decrease in acylcarnitine content improves insulin sensitivity and significantly reduces blood glucose and insulin levels in mice with impaired insulin sensitivity and diabetes.
3. Long-chain acylcarnitines are the main FA intermediates that induce ischaemia/reperfusion-related damage in heart mitochondria. Acylcarnitine accumulation during ischaemia leads to inhibited oxidative phosphorylation in cardiac mitochondria, thus inducing mitochondrial membrane hyperpolarization and stimulating the production of reactive oxygen species. Treatment with methyl-GBB protects the cardiac mitochondria against ischaemia/reperfusion-induced accumulation of long-chain acylcarnitines.
4. Treatment with methyl-GBB attenuates the development of atherosclerosis in *apoE*$^{-/-}$ mice. The anti-atherosclerotic mechanism of methyl-GBB treatment is mediated by decreased amounts of long-chain acylcarnitines and decreased infiltration of macrophages and monocytes into the aortic lesions of the aortic root.

APPROBATION OF THE STUDY – PUBLICATIONS AND THESIS

Doctoral thesis is based on following SCI publications:

1. Liepinsh, E., Makrecka-Kuka, M., Makarova, E., Volska, K., Vilks, K., Sevostjanovs, E., et al. 2017. Acute and long-term administration of palmitoylcarnitine induces muscle-specific insulin resistance in mice. *BioFactors*. 43(5), 718–730.
2. Liepinsh, E., Makrecka-Kuka, M., Makarova, E., Volska, K., Svalbe, B., Sevostjanovs, E., et al. 2016. Decreased acylcarnitine content improves insulin sensitivity in experimental mice models of insulin resistance. *Pharmacol Res*. 113(Pt B), 788–795.
3. Liepinsh, E., Makrecka-Kuka, M., Volska, K., Kuka, J., Makarova, E., Antone, U., et al. 2016. Long-chain acylcarnitines determine ischaemia/reperfusion-induced damage in heart mitochondria. *Biochem J*. 473(9), 1191–1202.
4. Vilskersts, R., Kuka, J., Liepinsh, E., Makrecka-Kuka, M., Volska, K., Makarova, E., et al. 2015. Methyl-γ-butyrobetaine decreases levels of acylcarnitines and attenuates the development of atherosclerosis. *Vascul Pharmacol*. 72, 101–107.

Results are reported in the following international conferences:

1. Volska, K., Liepinsh, E., Makarova, E., Makrecka-Kuka, M., Kuka, J., Dambrova, M. 2018. The mechanisms of long-chain acylcarnitine accumulation during ischemia, 13th Conference on Mitochondrial Physiology: The role of mitochondria in health, disease and drug discovery, Jurmala, Latvia, 18–21 Sep 2018, Book of Abstracts, p. 22.
2. Volska, K., Makrecka-Kuka, M., Makarova, E., Kuka, J., Vilskersts, R., Liepinsh, E., Dambrova, M. 2017. Protective effects of pharmacologically decreased long-chain acylcarnitine contents in the preclinical models of

diabetes and its complications, 2nd International Conference in Pharmacology: From Cellular Processes to Drug Targets (ICP2017RIGA), Riga, Latvia, October 19–20, 2017. doi:10.25006/IA.5.S2-A2.28

3. Vilks, K., Volska, K., Makarova, E., Makrecka-Kuka, M., Dambrova, M., Liepinsh, E. 2017. Impact of long-chain acylcarnitines on muscle insulin sensitivity and interaction with Akt-related insulin signalling pathway, 2nd International Conference in Pharmacology: From Cellular Processes to Drug Targets (ICP2017RIGA), Riga, Latvia, October 19–20, 2017. doi:10.25006/IA.5.S2-A2.20
4. Vilks, K., Volska, K., Makarova, E., Makrecka-Kuka, M., Dambrova, M., Liepinsh, E. 2017. Elevated acylcarnitine levels induce muscle insulin insensitivity through the interaction with Akt-related insulin signalling pathway, 42nd FEBS Congress – From molecules to cells and back, Jerusalem, Israel, September 10, 2017, p.5.4-013.
5. Vilks, K., Volska, K., Makarova, E., Makrecka-Kuka, M., Dambrova, M., Liepinsh, E. 2017. Palmitoylcarnitine interacts with Akt-related insulin signalling pathway and induces muscle-specific insulin resistance. *Latvijas Universitātes 75. konference, Molekulārās bioloģijas sekcija*, Rīga, Latvija, 2017. gada 31. janvāris.
6. Makarova, E., Makrecka-Kuka, M., Volska, K., Vilks, K., Grinberga, S., Dambrova, M., Liepinsh, E. 2017. The impaired change in plasma long-chain acylcarnitine level as a marker of insulin resistance in *db/db* and high fat diet-fed mice, 50th Miami Winter Symposium, Diabetes: Today's Research – Tomorrow's Therapies, Miami, USA, January 22–25, 2017, p.028.
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8. Liepinsh, E., Makrecka-Kuka, M., Volska, K., Kuka, J., Dambrova, M. Long chain acylcarnitines: new target to prevent ischemia-reperfusion induced damage in heart mitochondria. 2016. *Mitochondrial Medicine: Developing New Treatments for Mitochondrial Disease*, Hinxton, Cambridge, UK, May 4–6, 2016, Book of Abstracts, p. 58.
9. Dambrova, M., Volska, K., Makrecka-Kuka, M., Makarova, E., Kuka, J., Vilskersts, R., Liepinsh, E. 2016. Cardioprotective effects of pharmacologically decreased long-chain acylcarnitine contents in experimental models of myocardial infarction, atherosclerosis, and diabetes, *7th European Congress of Pharmacology***,** Istanbul, Turkey, June 26–30, 2016, Book of Abstracts, p. 81–82.
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Results are reported in following local conferences:

1. Konrāde, I., Makarova, E., Tonne, I., Dambrova, G., Kalere, I., Vilks, K., Voļska, K., Dambrova, M. 2017. Acilkarnitīnu koncentrācijas mērījumi – jauna diagnostikas metode insulīna rezistences noteikšanai, *RSU 2017. gada zinātniskā konference*, Sekc. "Hroniskās slimības, terapiju algoritmi, jaunas ārstniecības metodes" [Rīga, Latvija, 6. un 7. aprīlis, 2017. gads]: Tēzes, 274. lpp.
2. Voļska, K., Kūka, J., Makrecka-Kūka, M., Alonso Garcia-Mauriño, M., Dambrova, M. 2016. Garķēžu acilkarnitīnu nozīme sirds išēmijas-reperfūzijas bojājuma attīstībā, *RSU 2016. gada zinātniskā konference* [Rīga, Latvija, 17.–18. marts, 2016]: Tēzes, 65. lpp.
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