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RĪGAS STRADIŅA  
UNIVERSITĀTE

Elīna Škapare

**MOLECULAR MECHANISMS  
OF CARBOHYDRATE METABOLISM -  
RELATED DISORDERS:  
INVESTIGATION IN EXPERIMENTAL MODELS  
AND CLINICAL SAMPLES**

Summary of promotion thesis for obtaining  
degree of a Doctor of Pharmacy  
Speciality – Pharmaceutical Pharmacology

Rīga, 2013

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ĪEGULDĪJUMS TAVĀ NĀKOTNĒ



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# INTRODUCTION

## Relevance of the study

Modern therapy of diabetes mellitus (DM) aims both to control blood glucose levels and also to prevent late complications, thus improving the patients' quality of life. Consequently, the molecular mechanisms of late complications, including glyoxalase enzyme system, are intensively studied. Given the fact that the carbohydrate metabolism disorders are associated with impaired energy metabolism, the optimization of glucose and fatty acid metabolism could improve the treatment of the disease as well as associated complications. The aim of the thesis was to study novel molecular mechanisms and regulation possibilities of the pathways of energy metabolism and molecular markers in carbohydrate metabolism-related disorders.

This study is the first to associate a lower blood glyoxalase 1 (Glo1) activity with development of the painful peripheral diabetic neuropathy in type 1 and 2 DM patients thus providing the experimental evidence for the role of altered Glo1 activity in the development of painful diabetic neuropathy. Lower Glo1 activity in the neuronal tissues was associated with higher blood glucose and triglyceride concentrations, as well as impaired endothelium-dependent relaxation to acetylcholine in aortic rings in the experimental model of type 2 diabetes. Obtained results show that blood Glo1 activity could not be used as an early marker of the development of vascular complications, but nevertheless the measurements of blood Glo1 activity might be an useful approach to evaluate the late complications of DM in advanced stages.

The thesis display novel insights into molecular mechanism of mildronate action and confirms the hypothesis that the reduction of L-carnitine concentration improves the disturbed energy metabolism related to carbohydrate metabolism disorders. A decrease of L-carnitine level induced by long term mildronate administration activates the peroxisome proliferator-activated receptor (PPAR) $\alpha$ /PPAR $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ) signaling pathway and induces redirection of fatty acid metabolism thus protecting mitochondria against long-chain fatty acid overload. The results of the study provide evidence that type 1 and type 2 diabetic patients with low blood L-carnitine levels have decreased occurrence of neuropathy and hypertension, therefore the reduction of L-carnitine bioavailability could be useful approach to delay the development of late complications of DM.

## Aim of the study

The aim of the doctoral thesis was to study novel molecular mechanisms and regulation possibilities of the pathways of energy metabolism in carbohydrate metabolism-related disorders.

## **Objectives of the study**

1. To study the relationship of the blood Glo1 activity as a biochemical marker for painful diabetic neuropathy in patients with type 1 and type 2 diabetes mellitus.

2. To study the changes in Glo1 and Glo2 enzyme activities and the development of diabetic complications in experimental animal models.

3. To study the molecular mechanisms underlying the cardioprotective effect of mildronate, an energy metabolism modulator.

4. To evaluate the association of reduced blood L-carnitine levels with the development and severity of late complications of DM.

## **Hypotheses of the study**

1. The Glo1 enzyme activity could be used to detect early development of late complications of DM and to predict phenotype of the diabetic neuropathy.

2. Reduced bioavailability of L-carnitine levels has cardioprotective effect in carbohydrate metabolism-related disorders.

## **Novelty of the study**

Obtained results demonstrate for the first time that Glo1 activity is lower in patients with both types of DM who were diagnosed with painful diabetic neuropathy, thus confirming the hypothesis of Glo1 as a molecular marker to study the late complications of DM. Novel molecular mechanisms of mildronate action have been shown experimentally to support the hypothesis that a reduction of L-carnitine bioavailability may improve the disturbed energy metabolism in carbohydrate metabolism-related disorders. The decrease of L-carnitine induced by long term mildronate administration activates the PPAR $\alpha$ /PGC1 $\alpha$  pathway and induces redirection of fatty acids metabolism thus protecting mitochondria against long chain fatty acid overload in ischemia and carbohydrate metabolism-related disorders.

# **1. MATERIAL AND METHODS**

## **1.1. Subjects of clinical study**

The study included patients with type 1 (n=108) and type 2 (n=109) diabetes mellitus treated at the Riga East Clinical university hospital, Clinic "Gailezers", Riga, Latvia from 2008 - 2010. For a control group, 132 non-diabetic individuals were recruited. The study was carried out in accordance

with the Declaration of Helsinki after approval by the Central Medical Ethics Committee of Latvia (Resolution A-11, 27th June 2008). A written informed consent was obtained from all subjects. In the case-control study, the concentrations of free L-carnitine in blood samples were determined in type 1 (n=93) and type 2 (n=87) diabetic patients with and without complications and in control subjects (n=122). Exclusion criteria for the participating in the study were vegetarian diet, mildronate, and L-carnitine intake. Selection of study subjects, evaluation of late complications of DM and blood sample collection was carried out in cooperation with Dr. med. I. Konrāde.

## 1.2. Animals and experimental design

Male Wistar (Laboratory of Experimental Animals, Riga Stradins University, Latvia), Zucker fa/fa, and Zucker lean (Charles River Laboratories, France), Goto-Kakizaki and Wistar-Kyoto rats (Taconic Farms, USA) were used for the experiments. All animal care and experimental procedures were performed in accordance with the guidelines of the European Community (86/609/EEC), local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia.

To evaluate the thermal pain sensitivity the tail-flick test was performed as described previously by Liepinsh et al. (Liepinsh, 2009a). The paw pressure was determined using an analgesy meter (Ugo Basile, Italy) (Skapare, 2012). Working memory performance of Zucker, Wistar and Goto-Kakizaki rats was assessed by recording spontaneous alternation behaviour in the Y-maze test as described previously (Yamada, 1999). Endothelial function was assessed in aortic rings of Zucker fa/fa, Zucker lean, Wistar and Goto-Kakizaki rats as described previously (Bartus, 2008).

The isolated rat heart infarction study was performed as described previously (Liepinsh, 2006). The infarct size was calculated as a percentage of the risk area. The control values were normalized to 100 %, and all of the results are expressed as values compared to the controls. Samples from the non-risk area (viable tissue) and the area at risk (necrotic tissue) of heart tissue were isolated for fatty acid, RT-PCR and Western blot analysis after 30 min ischemia and 60 min reperfusion.

Oxidation of radiolabeled palmitate was measured as described previously (Lopaschuk, 1997) with the exception that 9,10- $^3\text{H}$ palmitate was used for the assay (specific activity 60 Ci/mmol). To determine the CPT I-independent metabolism of long-chain fatty acids, 36  $\mu\text{M}$  palmitoylcarnitine was used for the respiration measurements according to previously described method (Kuka, 2012). To assess mitochondrial function after left anterior descending artery occlusion for 30 min, left ventricular cardiac fibers from the area at risk and the non-risk area were isolated and prepared as described

previously (Kuka, 2012). The fatty acid profile was determined by gas chromatography-mass spectrometry in cooperation with Dr. chem. O. Pugovičs and Dr. chem. S. Grinberga (Latvian Institute of Organic Synthesis).

Expression of PPAR $\alpha$ , PPAR $\gamma$  and other factors related to regulation of fatty acid metabolism in heart and liver tissue was determined by Western blot analysis as described by Liepinsh et al. (Liepinsh, 2011). Quantitative RT-PCR analysis was performed with Applied Biosystems 7500 Real Time PCR System according to the manufacturer's protocol and instructions.

### **1.3. Biochemical measurements**

Free fatty acids, triglycerides, glycogen, glucose, insulin and lactate concentrations in experimental samples were determined using kits from Wako, Instrumentation Laboratory, Millipore and Roche according to manufacturers' instructions.

Plasma glucose, HbA<sub>1c</sub> and hs-CRP concentrations were measured in venous blood samples of clinical study participants with commercially available kits in an accredited diagnostic laboratory of the hospital.

### **1.4. Assay of Glo1 and Glo2 activity**

A Glo1 activity in blood and tissue samples was measured by monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione from hemithioacetal for 5 min in a 96-well UV-transparent plate. The hemithioacetal was prepared fresh for each measurement by pre-incubating 2 mM methylglyoxal and 2 mM reduced glutathione in 50 mmol/L sodium phosphate buffer, pH 6.6, for 10 min at +37°C immediately before the use. Glo2 activity in blood and tissue samples was assayed by monitoring the decrease in absorbance at 240 nm due to the hydrolysis of S-D-lactoylglutathione for 5 min (Phillips, 1993). The enzyme activity in blood lysates was calculated based on standard curve of S-D-lactoylglutathione and defined as the formation or hydrolysis of  $\mu\text{mol}$  S-D-lactoylglutathione per min per g of haemoglobin (U/g Hb). Glyoxalase enzyme activity in tissue homogenates was defined as the formation or hydrolysis of  $\mu\text{mol}$  S-D-lactoylglutathione per min per g of protein g (U/g protein).

### **1.5. Data analysis**

Results are expressed as a mean  $\pm$  SEM, median (interquartile ranges) for quantitative variables or count (%) for qualitative variables. Differences in the continuous variables between the studied groups were tested using a Student's t or a Mann Whitney U-test, analysis of variance followed by Dunnett or Bonferroni test according to the specific indications, whereas a  $\chi^2$  or Fisher



test was used to compare categorical data. The differences were considered to be significant when  $p < 0.05$ . Spearman's or Pearson correlation analysis was used to examine the relationship of Glo1 activity with the measured variables from all groups. A multivariate regression analysis was used to determine independent association of Glo1 activity with painful DN after an adjustment for variables associated with painful DN in a univariate analysis, such as age, the duration of diabetes and BMI. The data were analyzed using Microsoft Excel 2003, the GraphPad Prism 3.0 (GraphPad Software Inc., USA) and SPSS 19.0 (SPSS, Chicago, IL, USA) statistical software.

## 2. RESULTS

### 2.1. Association of Glo1 activity and painful peripheral diabetic neuropathy

The demographic and clinical characteristics of the participants are presented in Table 2.1. As seen in the table, patients with type 2 diabetes mellitus were older and had significantly higher BMIs compared to patients with type 1 diabetes mellitus (Table 2.1).

Table 2.1

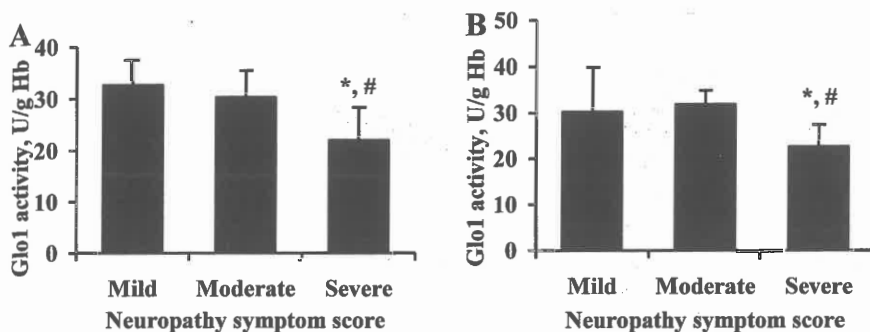
#### The demographic and clinical characteristics of the control and groups of patients with diabetes mellitus

	Control	Type 1 DM	Type 2 DM
n	132	108	109
Age, years	42.0 (33.0-52.8)	34.5 (27.0-50.0)	58.0 (51.5-64.0)
Male sex, n (%)	50 (38)	52 (48)	44 (40)
Duration of diabetes, years	-	11.0 (6.0-21.0)	7.0 (4.0-13.8)
Fasting plasma glucose, mM	5.0 (4.7-5.4)	8.6 (6.6-11.6)	7.8 (6.9-9.6)
HbA <sub>1c</sub> , %	5.1 (4.9-5.3)	8.6 (7.7-10.3)	7.9 (7.2-9.9)
BMI, kg/m <sup>2</sup>	24.9 (23.1-28.7)	24.7 (22.4-27.0)	31.6 (28.4-36.1)
hs-CRP, mg/l	0.9 (0.5-1.7)	2.2 (1.2-3.3)	2.0 (1.1-3.7)
Blood Glo1 activity, U/g Hb	28.5 (25.7-32.8)	31.2 (26.6-36.9)	31.9 (26.0-38.4)
Blood Glo2 activity, U/g Hb <sup>a</sup>	22.0 (18.7-24.2)	21.5 (19.0-23.7)	22.0 (18.0-27.0)

Values are presented as medians (interquartile ranges (IQR)) or n (%). <sup>a</sup>Glo2 activity was determined in blood samples of 18 control individuals, 36 - type 1 DM and 37 - type 2 DM patients.

The average Glo1 activity in patients with type 1 and type 2 diabetes mellitus was 31.2 (IQR 26.6-36.9) and 31.9 (IQR 26.0-38.4) U/g Hb, respectively. The Glo1 activity in the blood of non-diabetic individuals was 28.5 (IQR 25.7-32.8) U/g Hb and was significantly different from diabetic patients. The average blood Glo2 activity in patients with type 1 and type 2

diabetes mellitus was not different from control group. Glo1 activity was significantly reduced in patients with severe painful neuropathy symptoms compared to mild or moderate neuropathy symptom score (NSS) in both DM groups (Figure 2.1).



**Fig. 2.1 Association of haemolysate's Glo1 activity with severity of neuropathy symptom score (A) in type 1 (n = 88) and (B) type 2 (n = 74) DM patients.** Data are presented as medians  $\pm$  interquartile ranges. \*Significantly different from mild NSS symptoms (a one way ANOVA with Dunn's Multiple Comparison post-hoc test,  $p < 0.05$ ). #Significantly different from moderate NSS symptoms (a one way ANOVA with Dunn's Multiple Comparison post-hoc test,  $p < 0.05$ ).

Univariate correlation analysis of the study data showed that the haemolysate's Glo1 activity negatively correlated with the duration of diabetes ( $r = -0.261$ ,  $p = 0.007$ ) in patients with type 1 diabetes mellitus. No correlations of Glo1 activity with age, gender, glucose levels, HbA<sub>1C</sub>, hs-CRP results, lipid levels, BMI, alcohol consumption, smoking or the use of medications in both types of diabetes mellitus were found. In the control group, there were no significant associations of Glo1 with the different clinical variables.

As Glo1 activity was significantly decreased in patients with severe painful neuropathy symptoms, we compared biochemical parameters in diabetic patients with painless or painful diabetic neuropathy. Patients with painless and painful neuropathy had similar degree of nerve dysfunction. In type 1 and type 2 diabetic patients with painful diabetic neuropathy compared to patients with painless diabetic neuropathy, the Glo1 activity was significantly reduced by 12 and 14%, respectively (Table 2.2).

As shown in Table 2.2, type 1 and type 2 diabetic patients with painful neuropathy were significantly older by 19 and 7%, respectively, and had about a 2-fold longer duration of diabetes. Patients with painful neuropathy had a slightly increased BMI (by 10%) in the type 1 diabetes mellitus group. There were no significant differences between diabetic patients with painless or painful DN for other parameters.

Table 2.2

**The clinical characteristics of diabetic patients with painless and painful diabetic neuropathy**

	Type 1 DM			Type 2 DM		
	Painless neuropathy	Painful neuropathy	<i>p</i> value	Painless neuropathy	Painful neuropathy	<i>p</i> value
n	43	51		45	48	
Age, years	32.0 (24.0-47.0)	45.0 (29.0-57.0)	0.03	56.0 (50.5-62.0)	59.0 (54.2-68.0)	0.03
Duration of diabetes, years	10.5 (6.0-16.5)	19.0 (7.0-26.0)	0.01	5.5 (3.0-11.0)	11.0 (7.0-17.7)	<0.001
Glo1 activity, U/g Hb	33.0 (29.0-39.0)	28.9 (23.0-35.0)	0.01	32.6 (26.0-40.0)	29.2 (24.0-35.0)	0.04
BMI, kg/m <sup>2</sup>	24.0 (21.3-25.2)	25.8 (22.6-28.4)	0.01	31.9 (28.3-37.4)	31.2 (28.1-34.0)	ns

Values are presented as medians (interquartile ranges).

The increase in Glo1 activity per one unit was significantly associated with reduced risk of painful neuropathy after adjusting for age, the duration of diabetes and BMI by multivariate regression analysis (Table 2.3).

Table 2.3

**A multivariate regression analysis of association between the risk of painful neuropathy and Glo1 activity**

	Type 1 DM	
	ExpB (95% CI)	<i>p</i> value
Haemolysate Glo1 activity, U/g Hb	0.93 (0.87-1.00)	0.04
Duration of diabetes, years	1.04 (0.99-1.10)	ns
Age, years	1.00 (0.97-1.04)	ns
BMI, kg/m <sup>2</sup>	1.11 (0.96-1.28)	ns
	Type 2 DM	
	ExpB (95% CI)	<i>p</i> value
Haemolysate Glo1 activity, U/g Hb	0.92 (0.86-0.98)	0.01
Duration of diabetes, years	1.12 (1.04-1.21)	0.003
Age, years	1.02 (0.97-1.07)	ns

Values are presented as odds ratio (95% confidence interval (CI)).

In type 2 diabetic patients, but not in type 1, the increase in duration of diabetes was significantly associated with increased probability of painful neuropathy. The probability of painful neuropathy was not associated with age in both diabetes mellitus groups.

## 2.2. Alterations of glyoxalase enzyme activities in experimental animal models of obesity and type 2 diabetes mellitus

Goto-Kakizaki rats developed mild hyperglycemia, and at the end of the experiment, the plasma glucose level was approximately 1.5-fold greater in the fasted and fed states compared to age-matched Wistar control rats, while Zucker fa/fa rats presented an increase in the plasma glucose concentration of 12% only in the fasted state. The fed-state plasma glucose levels in the Zucker fa/fa rats were similar to those of the Zucker lean rats (Table 2.4). The concentration of HbA<sub>1C</sub> in the Goto-Kakizaki and Zucker fa/fa rats was 8.4±0.1 and 6.7±0.2%, respectively, and significantly differed from the control animals (Table 2.4). The fasting plasma insulin concentration in the Goto-Kakizaki rats was 1.5-fold greater compared to control rats, but the fed plasma insulin concentrations were similar in the Goto-Kakizaki and control rats. The Zucker fa/fa rats had a marked 10- and 8-fold increase in insulin concentration in the fed and fasted states, respectively, compared to the lean rats (Table 2.4).

Table 2.4

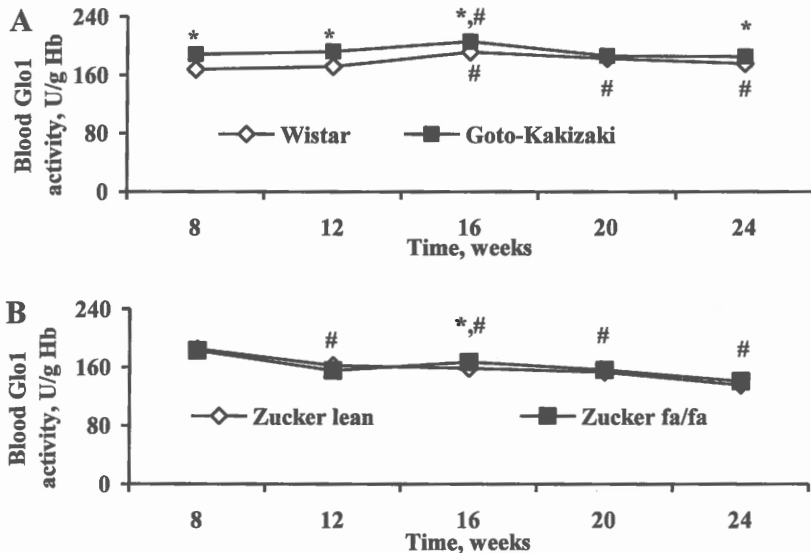
### Plasma biochemical parameters at the end of the study

	Wistar	Goto-Kakizaki	Zucker lean	Zucker fa/fa
	Fed state			
Plasma glucose, mM	7.65±0.15	12.11±0.78*	8.73±0.20	8.97±0.16
Triglycerides, mM	0.51±0.03	1.13±0.08*	0.70±0.10	6.37±0.78#
Insulin, ng/ml	2.15±0.43	3.67±0.86	1.44±0.18	11.39±1.31#
Free fatty acids, mM	0.37±0.03	1.05±0.13*	0.54±0.03	0.91±0.05#
HbA <sub>1C</sub> , %	7.59±0.33	8.39±0.14*	5.75±0.37	6.74±0.22#
	Fasted state			
Plasma glucose, mM	6.0±0.2	8.3±0.3*	7.4±0.2	8.3±0.2#
Triglycerides, mM	0.5±0.03	0.8±0.1*	0.8±0.2	7.9±1.0#
Insulin, ng/ml	0.31±0.03	0.48±0.07*	0.50±0.04	5.01±0.75#
Free fatty acids, mM	1.22±0.06	1.47±0.08*	1.23±0.07	1.28±0.07

The data are presented as the mean ± SEM of at least 11 animals. \*p<0.05 compared to the Wistar control (Student's *t* test). # p<0.05 compared to the Zucker lean control (Student's *t* test).

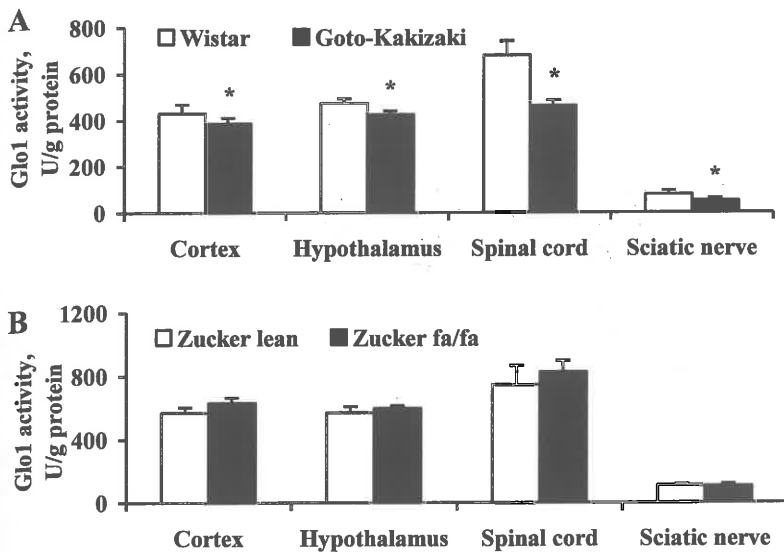
As expected, the Zucker fa/fa rats developed hyperlipidaemia, and the plasma triglyceride and free fatty acid levels in the fed state were 9- and 2-fold higher, respectively, compared to the lean control rats (Table 2.4). The Goto-Kakizaki rats had a significant 2- and 3-fold increase in plasma triglyceride and free fatty acid levels in the fed state and a 48 and 20% increase in the fasted state, respectively, compared to the Wistar rats (Table 2.4).

Blood Glo1 activity in the Goto-Kakizaki rats at the start of the experiment at 8 weeks of age was significantly increased by 12%, compared to the age-matched Wistar rats, but after 16 weeks, this difference decreased to 5% (Figure 2.2.A). Similar changes in blood Glo2 activity of Goto-Kakizaki rats were observed.



**Fig. 2.2** Changes in Glo1 activity in blood samples from Wistar and Goto-Kakizaki (A), and Zucker fa/fa and lean (B) rats. The data are presented as the mean  $\pm$  SEM of 11-12 animals. \* $p < 0.05$  compared to the control group (Student's *t* test), # $p < 0.05$  compared to 8 weeks in the respective group (Paired *t* test).

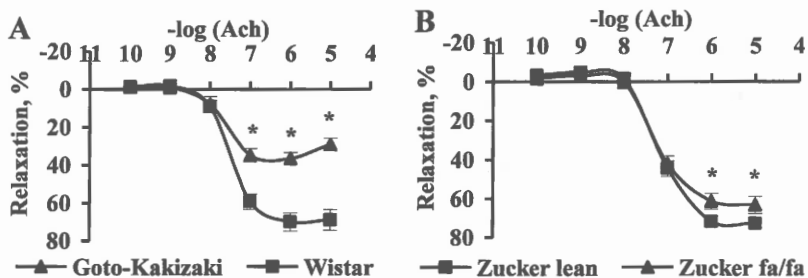
The Glo1 activity in the neuronal tissue (brain, spinal cord and sciatic nerve) of the Goto-Kakizaki rats was significantly reduced by 10, 32 and 36%, respectively (Fig. 2.3.A). Glo2 activity in the Goto-Kakizaki rats was significantly reduced only in the cortex and spinal cord tissue by 8 and 15%, respectively. GLO1 and GLO2 expression in the brain, spinal cord and sciatic nerve tissue of 24-week-old Goto-Kakizaki rats compared to the controls was not altered.



**Fig. 2.3** Glo1 activity in the cortex, hypothalamus, spinal cord and sciatic nerve tissues from Wistar and Goto-Kakizaki (A), and Zucker fa/fa and lean (B) rats at 24 weeks of age. The data are presented as the mean  $\pm$  SEM of at least 6 animals. \* $p < 0.05$  compared to the control group (Student's *t* test).

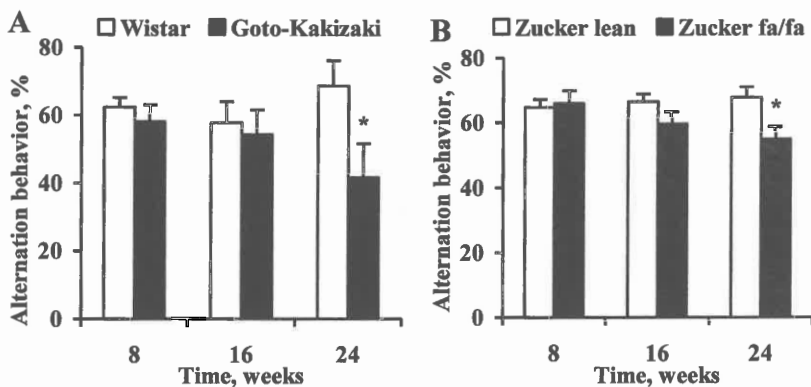
In the Zucker fa/fa rats, the blood and neuronal Glo1 activity was similar to that of the control animals (Figure 2.2.B and 2.3.B). Glo2 activity in the blood samples was increased on average from 5 till 20% during the 16-week period. Glo1 and Glo2 activities in the Zucker fa/fa rat tissue were unchanged or had a tendency to be increased compared to the lean Zucker rats.

Endothelium-dependent relaxation to acetylcholine in the aortic rings in the Goto-Kakizaki and Zucker fa/fa rats was significantly impaired compared to the controls (Figure 2.4). In addition, our results show that endothelial dysfunction was more pronounced in the Goto-Kakizaki rats compared to the Zucker fa/fa rats. The maximal aortic ring response to acetylcholine in the Goto-Kakizaki and Zucker fa/fa rats compared to the control animals were decreased by 48 and 13%, respectively (Figure 2.4).



**Fig. 2.4** Acetylcholine-induced endothelium-dependent relaxation of isolated aortic rings in Wistar and Goto-Kakizaki (A), Zucker lean and Zucker fa/fa (B) rats at 24 weeks of age. The data are presented as the mean % of relaxation  $\pm$  SEM of at least 11 animals. \* $p < 0.05$  compared to the control group (Student's *t* test).

The tail-flick, paw-pressure and Y-maze tests were performed at 8, 16, and 24 weeks. The results indicated no changes in peripheral thermal and mechanical pain perception until the 24th week in the Zucker fa/fa and Goto-Kakizaki rats compared to the control animals.



**Fig. 2.5** The spontaneous alternation behaviour in the Y-maze test in Wistar and Goto-Kakizaki (A), Zucker lean and fa/fa (B) rats at 8, 16, and 24 weeks of age. The data are presented as the mean % of alternation behaviour  $\pm$  SEM of at least 11 animals. \* $p < 0.05$  compared to the control group (Student's *t* test).

In the Y-maze test the spontaneous alternation behaviour in the Goto-Kakizaki and Zucker fa/fa rats compared to the control animals at 24 weeks of age were significantly reduced by 39 and 19%, respectively (Fig. 2.5). The working memory in the Goto-Kakizaki and Zucker fa/fa rats at 8 and 16 weeks of age was similar to that of the control animals (Fig. 2.5).

To detect if changes in Glo1 activity in the rat brain, spinal cord, and sciatic nerve tissue were associated with blood biochemical parameters or vascular complications, Spearman correlation analysis was performed. Correlation analysis of the study data showed that, in the Goto-Kakizaki rats, Glo1 activity in hypothalamus, spinal cord and sciatic nerve negatively correlated with the plasma glucose and triglyceride levels (Table 2.5).

Table 2.5

**Correlations of the biochemical parameters and vascular complications with Glo1 activity in various tissue samples of Wistar and Goto-Kakizaki rats**

	Hypothalamus Glo1 activity, U/g protein		Spinal cord Glo1 activity, U/g protein		Sciatic nerve Glo1 activity, U/g protein	
	<i>r</i>	<i>p</i> value	<i>r</i>	<i>p</i> value	<i>R</i>	<i>p</i> value
Plasma glucose, mM	-0.76	0.01	-0.78	0.01	-0.79	0.01
HbA <sub>1c</sub> , %	-0.67	0.02	-0.69	0.01	-0.52	ns
Triglycerides, mM	-0.63	0.03	-0.81	0.01	-0.81	0.01
Maximal endothelium- dependent relaxation, %	0.78	0.01	0.74	0.01	0.75	0.01
Alternation behaviour, %	0.10	ns	0.27	ns	0.33	ns

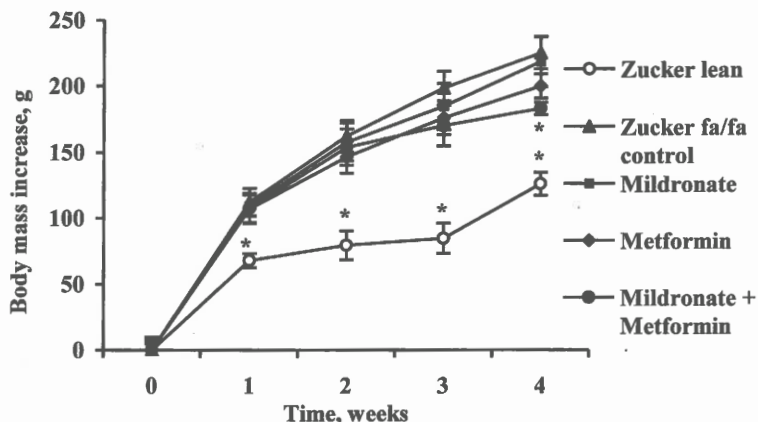
Values are presented as the Spearman correlation coefficients (*r*), ns – data are not statistically significant,  $p > 0.05$

A strong positive correlation with the maximal endothelial relaxation was found in all neuronal tissues from the Goto-Kakizaki rats (Table 2.5). There were no significant associations between blood and tissue Glo1 and Glo2 activities in Goto-Kakizaki rats, but a moderate positive correlation between blood Glo1 and Glo2 activity and Glo1 activity in the Zucker rat brain cortex was detected. There were no significant associations between the spontaneous alterations in Y maze test and Glo1 activities in neuronal tissues of Goto-Kakizaki and Zucker rats. No significant associations between tissue Glo1 activity and other measured parameters in the Zucker rats were found.



### 2.3. Regulation of energy metabolism in experimental model of carbohydrate metabolism-related disorders

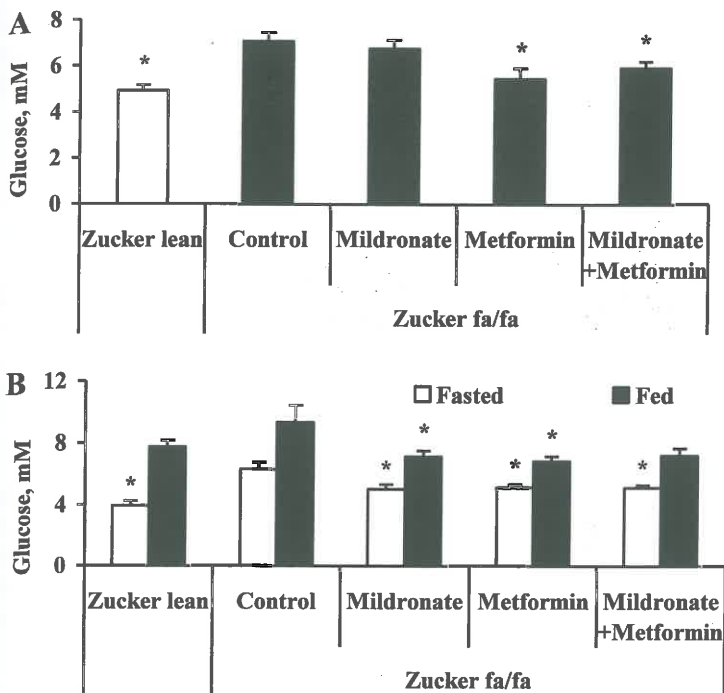
Before the start of experiment Zucker *fa/fa* rats were randomly divided into 4 groups of 10 rats in each group. Obese Zucker rats were p.o. treated daily with mildronate (200 mg/kg), metformin (300 mg/kg) and a combination of both compounds for 4 weeks. Zucker *fa/fa* control and Zucker lean rats received water. Before the experiment and during the treatment, Zucker rat body weights and food intake were monitored.



**Fig. 2.6** Effect of mildronate (200 mg/kg), metformin (300 mg/kg) and combination (200 mg/kg mildronate+300 mg/kg metformin) during 4 weeks of administration on body weight in Zucker *fa/fa* rats. Data are shown as the mean  $\pm$  SEM of at least 8 animals. \*Significantly different from Zucker *fa/fa* control group (Dunnett's test,  $p < 0.05$ ).

The weight gain of Zucker *fa/fa* rats was about two times higher than that of Zucker lean animals. Single drug treatment did not induce significant changes in weight gain, but the combination of both drugs significantly delayed body mass increase by 19% after 4 weeks of treatment, compared to Zucker *fa/fa* control group (Fig. 2.6).

At the beginning of treatment, blood glucose concentration in Zucker *fa/fa* rats was 1.5 times higher than in Zucker lean rats ( $7.1 \pm 0.4$  and  $4.9 \pm 0.2$  mM, respectively). Acute administration (1 h after drug administration) of metformin and the combination of both drugs significantly decreased blood glucose concentration by 22 and 17% (Fig. 2.7.A).

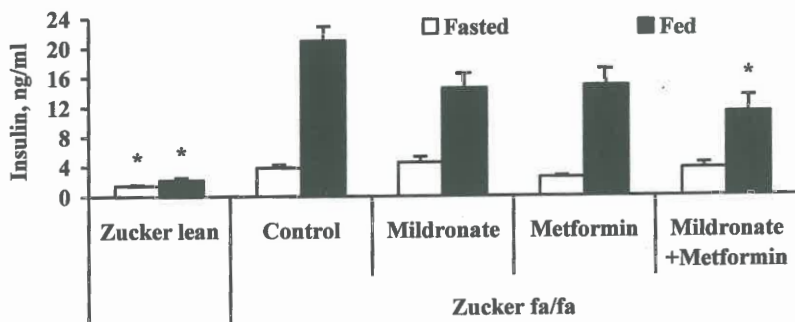


**Fig. 2.7 Acute (A) and long-term treatment (B) effect of mildronate, metformin and combination on blood glucose concentration in Zucker fa/fa rats.** Data are shown as the mean  $\pm$  SEM of at least 8 animals. \*Significantly different from Zucker fa/fa control group (Dunnett's test,  $p < 0.05$ ).

Chronic treatment with both drugs separately or in combination for 4 weeks prevented the progression of diabetes and induced a significant decrease of fasted and fed blood glucose concentration in Zucker fa/fa rats by 19 and 24%, respectively (Figure 2.7.B).

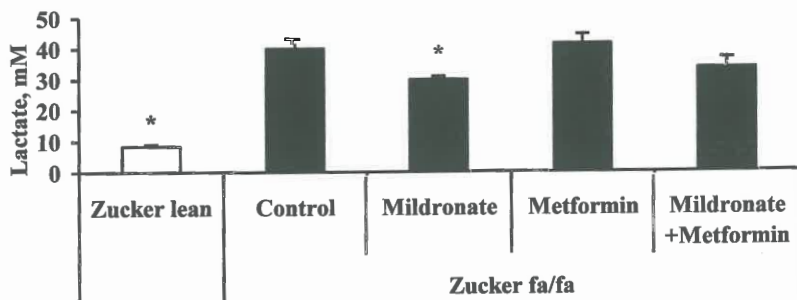
Insulin concentration in fasted Zucker fa/fa rat plasma was 2.6 times higher than in Zucker lean rats. No treatment produced any significant change in fasted insulin concentrations (Fig. 2.8). Treatment with mildronate or metformin resulted in a tendency to reduce elevated fed insulin concentrations (by 31 and 29%, respectively), but only the combination of both drugs significantly reduced fed insulin concentrations by about 47% (Fig. 2.8).





**Fig. 2.8 Effect of mildronate, metformin and combination on plasma insulin concentration in Zucker fa/fa rats.** Data are shown as the mean  $\pm$  SEM of at least 8 animals. \*Significantly different from Zucker fa/fa control group (Dunnett's test,  $p < 0.05$ ).

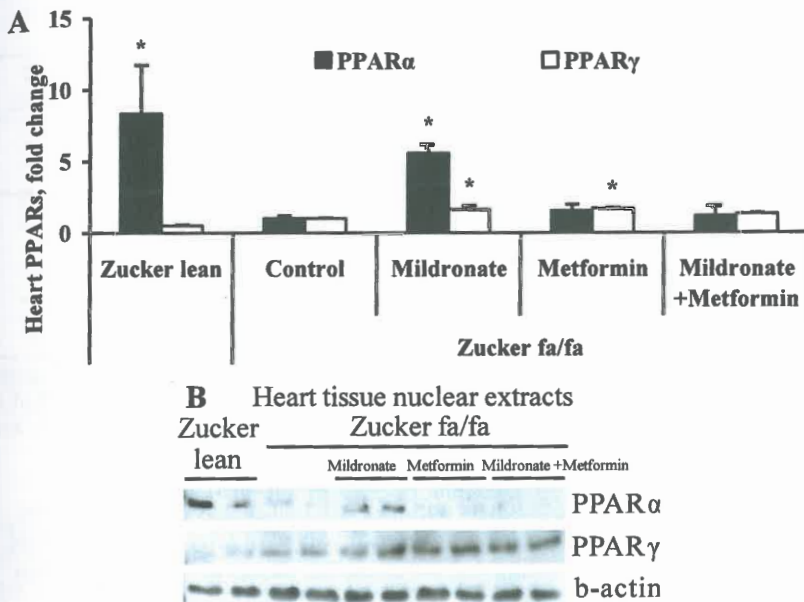
At the end of the experiment, a significant increase (5 times) in blood plasma lactate concentration was observed in Zucker fa/fa rats compared to Zucker lean rats (Fig. 2.9). Only mildronate alone significantly reduced lactate concentration for 25% in fed obese Zucker rat plasma (Fig. 2.9). Treatment with metformin or combination of both drugs did not significantly change plasma lactate concentration in Zucker fa/fa rats (Fig. 2.9).



**Fig. 2.9 Effect of mildronate, metformin and combination on plasma lactate concentration in Zucker fa/fa rats.** Data are shown as the mean  $\pm$  SEM of at least 8 animals. \*Significantly different from Zucker fa/fa control group (Dunnett's test,  $p < 0.05$ ).

Figure 2.10 displays the effects of drug treatment on PPAR $\alpha$  and PPAR $\gamma$  contents in the heart tissue. The PPAR $\alpha$  nuclear content was significantly decreased in the heart of Zucker fa/fa rats, compared to Zucker lean rats. In the heart, only mildronate treatment induced a 6-fold increase in

nuclear PPAR $\alpha$  content. Furthermore, PPAR $\gamma$  nuclear content was evaluated. In Zucker fa/fa rat heart nuclei, PPAR $\gamma$  content was 2 times higher than in Zucker lean rats. Mildronate and metformin treatments induced a similar statistically significant 1.7-fold increase in PPAR $\gamma$  heart nuclear (Fig. 2.10).



**Fig. 2.10** Effect of mildronate, metformin and combination on the PPAR $\alpha$  and PPAR $\gamma$  content in rat heart (A, B) tissue nuclear fractions. Data are shown as representative western blots and as calculated mean values  $\pm$ SEM of at least 4 animals. \*Significantly different from Zucker fa/fa control group (Dunnett's test,  $p < 0.05$ ).

After a 4-week treatment, the mRNA expression of genes that are involved in glucose and fatty acid metabolism was measured in the heart and liver tissues by quantitative RT-PCR. As shown in Table 2.6, the mRNA expression of GLUT4 in the heart tissue of Zucker fa/fa rats was significantly reduced by 1.8-fold. Treatment with mildronate and a combination of both drugs induced a 1.4- and 4-fold increase in Glut-4 mRNA expression, respectively. The expression of genes related to fatty acid metabolism (LPL, mCPT I, ACOX, ACSL and PEX13) was reduced in the heart tissue of Zucker fa/fa rats in comparison to tissue from Zucker lean rats.

**Drug treatment-induced effects on heart gene expression in Zucker fa/fa rats**

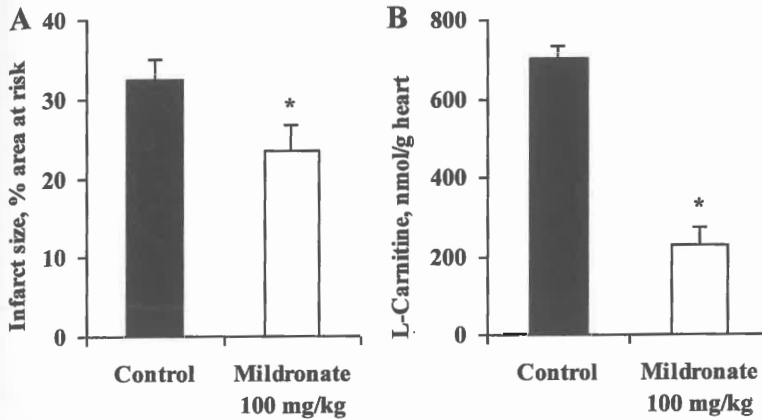
	<i>Zucker lean</i>	<i>Zucker fa/fa</i>			
		Control	Mildronate	Metformin	Mildronate +Metformin
GLUT4	1.8±0.5*	1.0±0.1	1.4±0.4	0.8±0.2	3.9±2.1*
LPL	1.6±0.1*	1.0±0.1	1.5±0.2*	1.0±0.1	1.8±0.3*
mCPT I	1.3±0.1	1.0±0.1	3.1±2.0	0.9±0.2	1.4±0.2*
LOX1	0.9±0.2	1.0±0.2	1.4±0.3	1.0±0.2	2.1±0.6*
ACOX	1.3±0.2*	1.0±0.1	1.6±0.3*	1.0±0.1	2.2±0.6*
ACSL	1.9±0.2*	1.0±0.1	1.6±0.4	1.0±0.1	2.1±0.5*
PEX13	1.4±0.1*	1.0±0.1	1.8±0.4*	1.1±0.1	2.1±0.5*

Effect of mildronate, metformin and combination on heart mRNA levels of genes related to glucose and fatty acid metabolism. Data are shown as the mean ± SEM of at least 4 animals. \*Significantly different from Zucker fa/fa control group (Dunnett's test,  $p < 0.05$ ).

Treatment with mildronate and, in particular, the combination of the two drugs increased the expression of genes involved in fatty acid metabolism. In contrast, metformin treatment did not affect PPAR $\alpha$  target gene expression in the heart tissue (Table 2.6).

#### 2.4. The molecular mechanisms of cardioprotective effect of mildronate

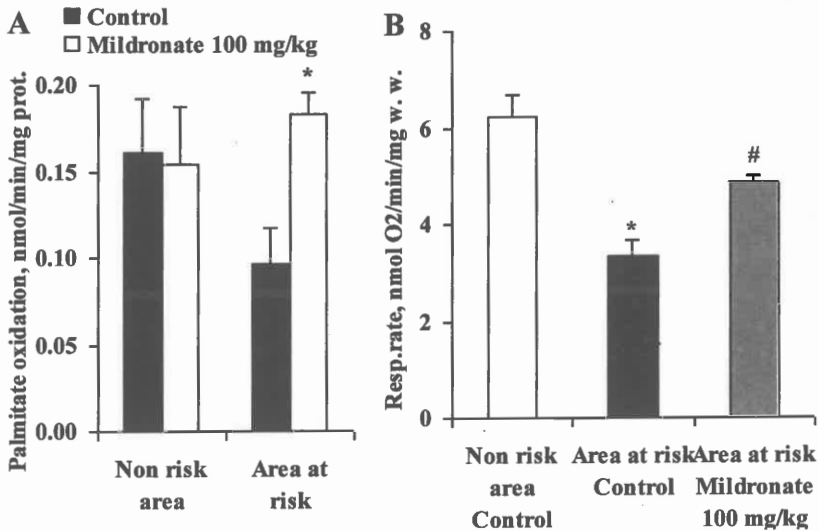
As the cardioprotective and energy metabolism regulatory effects of mildronate are based on the decreased L-carnitine concentration (Kuka, 2012), we tested whether the reduction of L-carnitine levels induced by treatment with mildronate results in a decreased infarct size and preserved peroxisomal and mitochondrial function in a myocardial infarction model. In isovolumetrically contracting hearts, there were no differences in the control and treated rat heart rates, peak left ventricular (LV) pressure, LV +dp/dt or LV -dp/dt. There were no significant differences between both experimental groups in hemodynamic parameters, which indicate that the observed effects on infarct size are not related to changes in the cardiac workload. Values for the area at risk were also similar in the hearts of both experimental groups. The infarct size in control rat hearts was 32.5% of the area at risk, while the treatment with mildronate, along with the reduction in L-carnitine content, significantly decreased the infarct size by 28% when compared to the control group (Fig. 2.11.A).



**Fig 2.11 Effect of a 2 week treatment with mildronate on infarct size (A) and L-carnitine concentration (B) in Wistar rat heart tissue.** Data are shown as the mean  $\pm$  SEM of 9-10 animals. \*Significantly different from the control group (Student's *t* test,  $p < 0.05$ ).

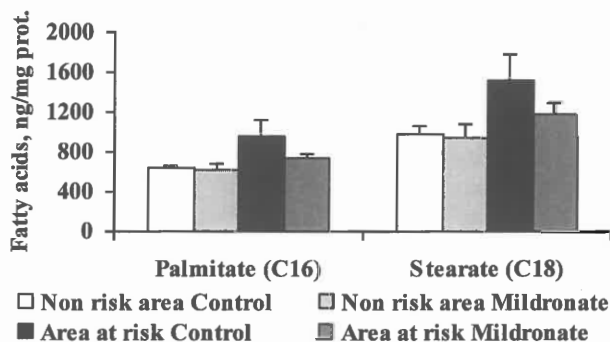
We previously found that to induce significant changes in energy metabolism, the L-carnitine content in the heart tissue should be decreased by at least 60%. As shown in Figure 2.11.B, the L-carnitine content in the control rat hearts was  $705 \pm 39$  nmol/g tissues. Mildronate treatment at a dose of 100 mg/kg for 2 weeks induced a significant decrease in the L-carnitine heart content of 67% (three fold).

In peroxisomes isolated from the non-risk area, the fatty acid oxidation (FAO) rate was similar in the control and treatment groups. In contrast, in area at risk, peroxisomal functioning and the FAO rate was diminished by ischemia, while mildronate treatment preserved the peroxisomal FAO rate (Fig. 2.12.A). Similarly, significant reduction in the ADP-stimulated respiration rates on pyruvate/malate were observed in fibers isolated from the area at risk. Decreased L-carnitine content preserved mitochondrial function after ischemia (Fig. 2.12.B). Altogether, these results suggest that a decrease in L-carnitine level induces compensatory changes in FAO pathways which preserve the mitochondrial and peroxisomal functions in the ischemic myocardium.



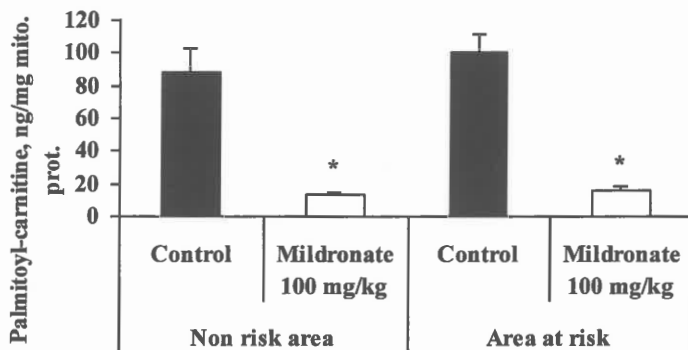
**Fig 2.12 Effect of treatment with mildronate on (A) palmitate oxidation in isolated peroxisomes and (B) ADP-stimulated mitochondrial respiration on pyruvate/malate and succinate in cardiac fibers.** Data are shown as the mean  $\pm$  SEM of 4-6 animals. \*Significantly different from the control group. #Significantly different from the respective ischemic control group (Student's t-test or a one-way ANOVA with Kruskal-Wallis post-test,  $p < 0.05$ ).

Because of the mildronate-induced decrease in long-chain FAO in mitochondria that was previously observed, an increase in the long chain fatty acid content in hearts was expected. However, despite the decreased L-carnitine content, we did not observe an increase in the contents of either palmitic (C16) or stearic (C18) acid in the heart tissues of non-risk area (Fig. 2.13). After 30 minutes of ischemia, accumulation of long chain fatty acid in the area at risk was observed and mildronate treatment did not affect the concentration of long chain fatty acids during ischemia. After 60 minutes of reperfusion, a considerable accumulation of long chain fatty acids was observed in the area at risk in the control hearts (Fig. 2.13). In contrast, a decrease in the content of long chain fatty acids was observed in the area at risk in mildronate treated hearts.



**Fig. 2.13** Effect of treatment with mildronate on palmitate (C16) and stearate (C18) content in the non-risk area and the area at risk of rat heart tissue after 60 min reperfusion. Data are shown as the mean  $\pm$  SEM of at least 4 animals.

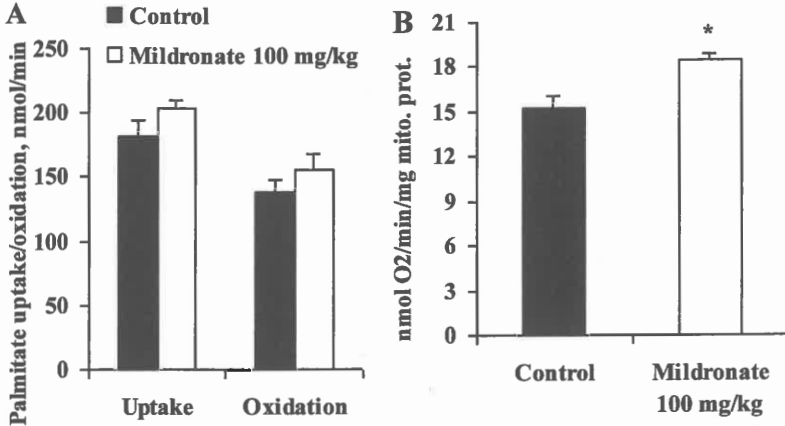
In addition, the palmitoyl-carnitine contents in mitochondria were significantly decreased both in non-risk area and area at risk (Fig. 2.14) suggesting redirection of long chain fatty acid metabolism from mitochondria to peroxisomes. Altogether, these results suggest that the decrease in the content of L-carnitine preserves mitochondrial function to restore the fatty acid metabolism during reperfusion.



**Fig. 2.14** Effect of treatment with mildronate on palmitoyl-carnitine mitochondrial content in the non-risk area and the area at risk after ischemia-reperfusion injury. Data are shown as the mean  $\pm$  SEM of 6-8 animals. \*Significantly different from the control group (Student's *t* test,  $p < 0.05$ )

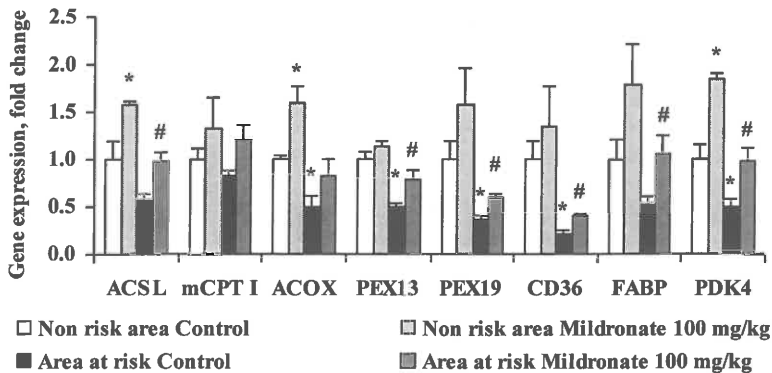


To test the changes in mitochondria induced by the mildronate treatment, we determined the FAO in the heart and its organelles. Despite the long-term 3-fold reduction in L-carnitine content, the uptake and oxidation rates of long chain fatty acids in the heart in normoxia were not significantly influenced (Fig. 2.15.A). In addition, we found that the mitochondrial respiration of palmitoyl-carnitine was significantly increased by 20% in the mildronate treated hearts (Fig. 2.15.B).



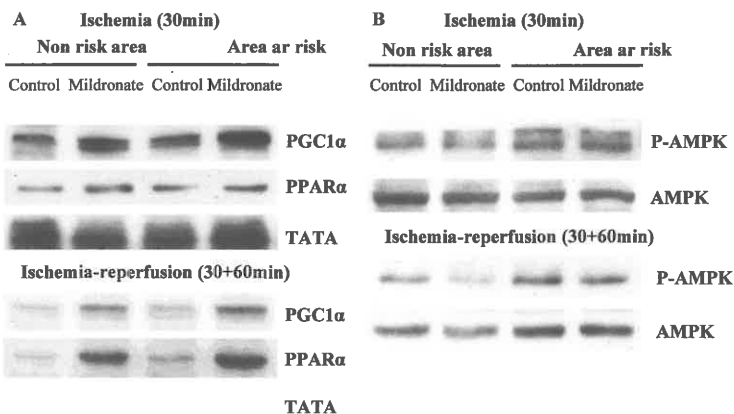
**Fig 2.15** Effect of treatment with mildronate on labeled palmitate uptake and oxidation in isolated heart (A) and mitochondrial respiration on 36  $\mu$ M palmitoyl-carnitine (B). Data are shown as the mean  $\pm$  SEM of 5-6 animals.\*Significantly different from the control group (Student's *t* test,  $p < 0.05$ ).

To detect changes in gene expression after 2 weeks of mildronate treatment, mRNA was separately isolated from the area at risk and the non-risk area of heart tissue after 30 min ischemia followed by 60 min reperfusion. The expression of acyl-CoA oxidase (ACOX), an enzyme involved in peroxisomal  $\beta$ -oxidation, was significantly increased in the non-risk area of the treated hearts (Fig. 2.16). The results of gene expression coincide with the stimulated FAO in peroxisomes. After 60 min of reperfusion, the expression of genes related to fatty acid metabolism in the area at risk was significantly diminished, while treatment significantly prevented this decrease in gene expression (Fig. 2.16). These results, along with the preserved function of mitochondria and peroxisomes in the area at risk, suggest that a decreased L-carnitine content preserves energy supply and cardiac cell functions during and after ischemia.



**Fig. 2.16** Effect of mildronate treatment on gene expression related to fatty acid and glucose metabolism in the non-risk area and the area at risk of rat heart tissue after 60 min reperfusion. Data are shown as the mean  $\pm$  SEM of 4 animals. \*Significantly different from the from the non-risk area of the control group. #Significantly different from the area at risk of the control group (Mann-Whitney U test  $p < 0.05$ ).

From the obtained gene expression data, it was necessary to clarify the nuclear factors involved in the regulation of fatty acid metabolism and signaling pathways that were stimulated by mildronate treatment-induced metabolic changes. Mildronate treatment stimulates the increase in PPAR $\alpha$  and also PGC1 $\alpha$  nuclear content in the non-risk area and the area at risk in Wistar rat hearts in particular after 60 min of reperfusion (Figure 2.17.A).



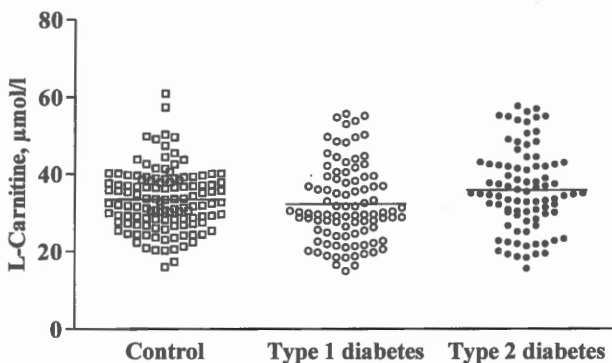
**Fig. 2.17** Effect of mildronate treatment on the nuclear contents of PPAR $\alpha$ , PGC1 $\alpha$  (A) and cytosolic content of P-AMPK (B) in the non-risk area or the area at risk after 30 min ischemia or following 60 min reperfusion.

To clarify the upstream signaling events of the PPAR/PGC1 $\alpha$  pathway, we tested whether the activation of AMPK via its phosphorylation could play a role in the activation of the PPAR $\alpha$ /PGC1 $\alpha$  pathway. We detected an increased amount of P-AMPK (4 times) in cytosolic extracts of the area at risk of hearts after 30 min of ischemia following 60 min of reperfusion (Fig. 2.17.B). In contrast, unchanged or decreased levels of phosphorylated AMPK in ischemic hearts were observed in mildronate treated group. Thus, the mildronate-induced activation of the PPAR $\alpha$ /PGC1 $\alpha$  pathway in hearts cannot be explained by changes in AMPK activity.

### 2.5. Association of blood L-carnitine levels with the development of late complications of DM

L-carnitine is involved in the regulation of both fatty acid and carbohydrate metabolism. To determine the relationship of blood L-carnitine levels with biochemical parameters and development of late complications of DM, free L-carnitine concentrations were measured in blood samples of healthy individuals and patients with type 1 and type 2 DM. The mean ages were 42 and 47 years in the control and diabetic patient groups, respectively, and on average, 43% of the study participants were male. Diabetic patients had significantly higher fasting plasma glucose and HbA1c concentrations, compared with the control group.

The average L-carnitine concentration in nondiabetic individuals was  $33 \pm 8 \mu\text{mol/l}$  ( $n=122$ ) and this concentration did not differ significantly in patients with DM (Fig. 2.18). The mean L-carnitine concentrations in patients with type 1 and type 2 diabetes were  $32 \pm 10$  and  $36 \pm 11 \mu\text{mol/l}$  ( $n=93$  and  $87$ ), respectively.



**Fig. 2.18 Concentrations of free L-carnitine in blood samples from the control group and type 1 and type 2 DM group**

Data are shown as the mean from individual measurements.

Low L-carnitine concentrations (<20  $\mu\text{mol/l}$ ) were found in 9% of diabetic patients. Only hospitalized diabetic patients were included in our study group; therefore, the prevalence of late complications was quite high. In this study late complications of diabetes were diagnosed in 88% (159/180) of patients with DM.

Table 2.7

**Average L-carnitine concentration in diabetic patients with and without neuropathy, retinopathy, nephropathy, or hypertension**

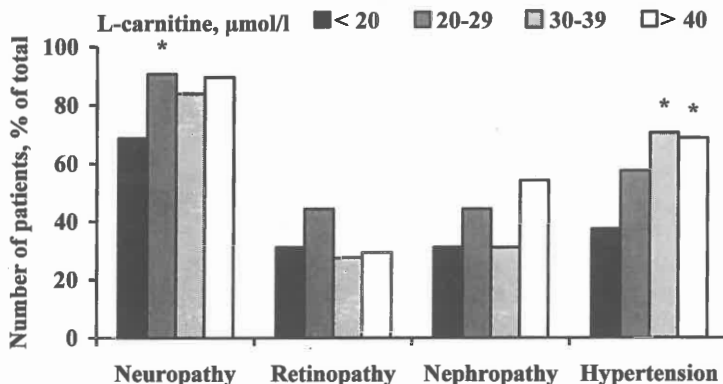
	L-carnitine, $\mu\text{mol/l}$
Neuropathy	
Without (n=25)	32 $\pm$ 11
Painless (n=71)	35 $\pm$ 10
Painful (n=84)	34 $\pm$ 11
Retinopathy	
Without (n=120)	34 $\pm$ 11
Nonproliferative (n=40)	31 $\pm$ 9
Preproliferative (n=16)	36 $\pm$ 11
Proliferative with macular edema (n=4)	42 $\pm$ 8
Nephropathy	
Without (n=105)	32 $\pm$ 10
Microalbuminuria (n=66)	36 $\pm$ 11
Macroalbuminuria (n=8)	33 $\pm$ 8
Hypertension	
Without (n=66)	32 $\pm$ 11
With (n=113)	35 $\pm$ 10*

Data are presented as means  $\pm$  SD. \*Significantly different from the patients without late complications (Student's *t* test,  $p < 0.05$ )

Neuropathy was diagnosed in 86% (155/180 patients), retinopathy – in 33% (60/180 patients), nephropathy – in 41% (74/180 patients) and hypertension - in 63% (113/180 patients) of the diabetic subjects (Table 2.7). The average L-carnitine concentrations in type 1 and type 2 diabetic patients with neuropathy, retinopathy and nephropathy were not different from those observed in patients without complications (Table 2.7). The average L-carnitine concentrations in diabetic patients with hypertension were significantly increased compared to diabetic patients without hypertension (Table 2.7).

The prevalence of neuropathy in patients with low L-carnitine concentrations (<20  $\mu\text{mol/l}$ ) was 69%. In contrast, in patients with higher L-carnitine concentrations, the prevalence of neuropathy was 88% (Figure 2.19). However, L-carnitine concentrations did not correlate with either the

neuropathy disability or symptom scores. The prevalence of retinopathy in patients with low L-carnitine concentrations was 31%, which was similar to the average prevalence of retinopathy in patients with higher concentrations of L-carnitine (Figure 2.19).



**Fig. 2.19** The prevalence of neuropathy, retinopathy, nephropathy, and hypertension among different L-carnitine concentration groups in patients with type 1 and type 2 diabetes. All patients were stratified into the following subgroups by L-carnitine concentrations: less than 20, 20 to 29, 30 to 39, greater than 40 μmol/l. The data are represented as a percentage of the total number of patients in each group.

\*Significantly different from the low L-carnitine (<20 μmol/l) group (Fisher exact test,  $p < 0.05$ ).

The prevalence of nephropathy in patients with low L-carnitine concentrations was 31%, which was lower than that seen in patients with higher L-carnitine concentrations (42%); however, this difference was not statistically significant (Figure 2.19). The prevalence of hypertension in patients with low L-carnitine concentrations was 38%, which was significantly lower than that seen in patients with higher L-carnitine concentrations (69%; Figure 2.19).

### 3. DISCUSSION

The promotion work describes new molecular mechanisms along with regulation possibilities of the pathways of energy metabolism in carbohydrate metabolism-related disorders. The aim of the study was to establish whether the glyoxalase enzyme activity could be used to detect early development of late complications of diabetes and to predict phenotype of the diabetic neuropathy, as well as to investigate the regulation possibilities of energy metabolism pathways in carbohydrate metabolism-related disorders.

### 3.1. Alterations of glyoxalase enzyme activities in diabetes mellitus

The present study was undertaken to investigate the Glo1 activity in control individuals without diabetes and diabetic patients with peripheral neuropathy by analyzing the largest amount of clinical material thus far. In the present study, while the haemolysate Glo1 activity in patients with DM was increased by 10% compared to healthy individuals, haemolysate Glo2 activity detected in a smaller subgroup of DM patients was not different from control group. The previous investigations of glyoxalase enzyme activity in diabetes have provided inconsistent results. After short-term incubation of human erythrocytes with high concentrations of glucose *in vitro*, the cell concentrations of the glyoxalase substrates, methylglyoxal and S-D-lactoylglutathione increased 2-3 times, but similarly to our study the activities of Glo1 and Glo2 were not significantly changed (Thornalley, 1988). Also, in one of the first clinical study of glyoxalase enzyme activity in diabetic patients, despite of higher glyoxalase substrate concentrations, Glo1 and Glo2 activities in red blood cells did not differ from the control group (Thornalley, 1989). In another clinical study with type 1 and type 2 diabetic patients concentrations of glyoxalase system metabolites – methylglyoxal, S-D-lactoylglutathione and D-lactate, and glyoxalase enzyme activities were increased (McLellan, 1994). Glo1 activity in erythrocytes of patients with both types of DM was increased by 30-40%, but Glo2 activity was increased by 11% only in patients with type 2 diabetes compared to healthy subjects (McLellan, 1994). The increase in metabolic flux through the glyoxalase system in diabetes suggests that the rate of methylglyoxal formation is enhanced during hyperglycaemia (Thornalley, 1988), which could contribute to activation of the defence system and increase the glyoxalase enzyme activity. However, regardless of changes in blood glyoxalase enzyme activity, glyoxalase enzyme system is unable to compensate for the increasing amount of methylglyoxal in cells (Thornalley, 1995).

Interestingly, studies that included diabetic patients with poor metabolic control found an association between a higher Glo1 activity and the development of microvascular complications (McLellan, 1994, Ratliff, 1996). There were no correlation between Glo1 activity in leukocytes and HbA<sub>1C</sub>, thus the authors of the study concluded that glucose control was not a factor that regulates Glo1 and Glo2 activity (Ratliff, 1996). This is supported by the results presented in our study, since the correlations of glucose, HbA<sub>1C</sub> and lipid levels with activity of Glo1 were not observed. There was no significant association of haemolysates Glo1 activity with other clinical variables in healthy controls or type 2 diabetic patients. Although age-dependent changes in Glo1 activity have been reported (Sharma-Luthra, 1994), our study did not find any significant correlation of Glo1 activity with age in the control or diabetes mellitus groups. Instead, our study provides evidence for correlation of decreased Glo1 activity with the duration of diabetes in type 1 diabetic, but not

in type 2 diabetic patients. Interestingly, in a previous clinical study association of increased methylglyoxal concentrations with the duration of diabetes was observed only in patients with type 1 DM (McLellan, 1994).

The primary finding of the experimental part of this study was that in 24-week-old Goto-Kakizaki rats, which exhibited mild hyperglycaemia and marked glucose intolerance, there were no considerable alterations in Glo1 and Glo2 activities in the blood samples, but the Glo1 and Glo2 activities in neuronal tissues was significantly reduced. Zucker fa/fa rats developed hypertriglyceridaemia, obesity and lipid overload-induced insulin resistance, but Glo1 activity in the blood samples and neuronal tissue of obese Zucker rats was similar to that of the control animals. The overall changes in Glo2 activity followed the pattern of changes in the activity of Glo1, which functions as the most important methylglyoxal-detoxifying enzyme. Additionally, our data showed that in Goto-Kakizaki and Zucker rats, Glo1 activity in blood and neuronal tissues was 3-4 fold higher compared to Glo2 activity. Our measurements of enzyme activities in Goto-Kakizaki rats are in line with previous studies, which have shown that Glo1 and Glo2 activities in erythrocytes of streptozotocin-induced diabetic rats were not altered (Nagaraj, 2002, Phillips, 1993). However the Glo1 activities in sciatic nerve and brain tissue of streptozotocin-induced diabetic rats were not different from control animals (Brouwers, 2011, Phillips, 1993), which were of younger age than those used in the present study. To our knowledge the only study thus far that investigated glyoxalase system in experimental model of obesity found that Glo2 activity was significantly increased in erythrocytes of obese (ob/ob) mice (Atkins, 1989) similarly as in obese Zucker rats in our study. However, while in our study Glo1 activity in blood of obese Zucker rats was not different compared to lean rats, the Glo1 activity was decreased in ob/ob mice at a similar age (Atkins, 1989). Thus, the observed changes in Glo1 and Glo2 activities in diabetic animals vary depending on experimental model, age and duration of diabetes. Correlation analysis of the study data showed that in Goto-Kakizaki rats, Glo1 activity in the hypothalamus, spinal cord and sciatic nerve negatively correlated with plasma glucose and triglyceride levels. The model of obesity and hypertriglyceridaemia did not result in any significant changes in blood or tissue Glo1 activity, but Glo2 activity was slightly increased in blood samples of Zucker fa/fa rats. Thus obtained results suggest that in Goto-Kakizaki rats, blood Glo1 activity was less sensitive to hyperglycaemia than neuronal Glo1.

In our study, before the appearance of functional impairments in the neuronal tissue of 24-week-old Goto-Kakizaki rats, reduced Glo1 activity was found in the brain, spinal cord and sciatic nerve. Although the RAGE-dependent sustained activation of the pro-inflammatory pathway could induce downregulation of GLO1 expression in tissue (Rabbani, 2011), in our study GLO1 expression at the gene level was not significantly altered. Thus, it could

be hypothesised that the changes in Glo1 activity are not due to any pro-inflammatory signalling at the expressional level but rather reflect glycototoxicity-induced changes in glyoxalase system which might be mediated by altered cellular concentration of reduced glutathione (Thornalley, 2003). It must be noted that the experimental Goto-Kakizaki model represents both lipoid and glycototoxicity because the respective biochemical measures are elevated compared to the control animals and changes in neuronal Glo1 activity correlate with elevated glucose and triglyceride concentrations. However, Glo1 activity is less sensitive to elevated lipid levels, as we did not find significant changes in Glo1 activity in Zucker fa/fa rats.

### **3.2. Association of Glo1 activity and development of late complications of DM**

Our results demonstrate that decreased Glo1 enzyme activity in haemolysates from both type 1 and type 2 diabetes mellitus patients is associated with painful neuropathy. A recent study showed that the expression of GLO1 varies in the peripheral nervous system from different inbred strains of mice that display significant differences in mechanical allodynia, suggesting that Glo1 directly contributes to the phenotype of neuropathy (Jack, 2011). Recently it was found that concentrations of plasma methylglyoxal above 600 nM discriminate between type 2 diabetes patients with pain and those without pain. In addition, methylglyoxal induced primary hyperalgesia in experimental mouse model of streptozotocin-induced diabetes and GLO +/- mouse model (Bierhaus, 2012). Although previous studies have shown that Glo1 activity is increased in diabetic patients with late complications (McLellan, 1994, Ratliff, 1996, Thornalley, 1989), thus far the relationship between Glo1 activity and phenotype of diabetic neuropathy in patients with DM was not studied.

In our study, we assessed both NSS and NDS as measures of subjective pain perception and objective neuropathic deficits according to previously described methods (Doupsis, 2009, McIntosh, 2003). Glo1 activity was significantly reduced in patients with severe painful neuropathy symptoms for both type 1 and type 2 diabetes mellitus patients, but was not different between different NDS groups. Previous studies in patients with painful neuropathy found elevated CRP values, which suggested that inflammatory processes and endothelial dysfunction are linked to the peripheral sensitization resulting in pain sensation and diabetic neuropathy (Doupsis, 2009, Herder, 2009). The results of the present study however did not confirm any correlations with CRP. This difference could be explained by the slightly elevated CRP values in both the painful and painless diabetic neuropathy groups due to poor metabolic control, which had been associated with an elevated CRP in both type 1 (Jenkins, 2008) and type 2 (Arnalich, 2000) DM. ADA guidelines recommend reaching HbA1c 7.0% and EASD guidelines



recommend less than 6.5% as a target value for DM patients. DM patient population in Latvia is overall at the level of poor compensation which is reflected by the median HbA1c values for patients included in our study: type 1 was characterized by 8.6% and type 2 was 7.8% which corresponds to poor metabolic compensation and do not reach the target values suggested by guidelines. Consequently, the elevated CRP concentrations were found in both patients with diabetic neuropathy without pain and with painful diabetic neuropathy. It should be pointed out that the pathogenesis of type 2 diabetes mellitus is considerably heterogeneous and both glycation stress and inflammation-related processes could be cooperatively driving forces in the development of late complications of DM.

The multivariate regression analysis, which was adjusted for confounders, showed an inverse significant association of Glo1 activity with probability of painful diabetic neuropathy, independent of age, diabetes duration and BMI. The increase in Glo1 activity per one unit was significantly associated with reduced risk of painful diabetic neuropathy by 10% after adjusting for age, the duration of diabetes and BMI by multivariate regression analysis. Although analysis showed that age and duration of diabetes do not associate with the probability of painful diabetic neuropathy in both types of DM, the observed differences in Glo1 activity might be additionally influenced by these factors. Another limitation is too small number for statistical analysis of the participants in diabetes patient subgroups that did not present neuropathy. Although our study shows that the decreased activity of Glo1 is related to an increased pain perception, the molecular mechanisms that are linked to the altered activity of Glo1 deserve further investigations. In summary, the study provides evidence that the activity of the Glo1 enzyme is lower in blood samples from both type 1 and type 2 diabetic patients diagnosed for painful diabetic neuropathy. In addition, in patients with type 1 diabetes, Glo1 activity negatively correlates with the duration of diabetes. These findings support the hypothesis that Glo1 activity modulates the phenotype of diabetic neuropathy.

No changes in peripheral thermal and mechanical pain perception were observed in the Goto-Kakizaki or obese Zucker fa/fa rats compared to the respective control animals, even though the Glo1 activity in the neuronal tissues was decreased by 10-36%. Taking into account the impaired vascular reactivity in both rat strains, this indicates an early stage of diabetes and obesity-related complications. It has been reported before that changes in vascular reactivity to acetylcholine in obese Zucker rats precedes impaired neural function (Oltman, 2005) and obese, normoglycaemic Zucker rats develop vascular and neural dysfunction at 32-40 weeks of age (Oltman, 2005, Oltman, 2008). Impaired endothelium-dependent relaxation in conductance and resistance blood vessels has already been reported before in Zucker fa/fa (Serpillon, 2009, Walker, 1999) and Goto-Kakizaki rats (Cheng, 2005, Gupte,

2010). It has been shown recently that hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation can be significantly improved by Glo1 overexpression indicating the role of methylglyoxal in the development of oxidative stress and vascular dysfunction in diabetes (Brouwers, 2010). Our results provide evidence for preserved endothelial function in diabetic Goto-Kakizaki rats with higher neuronal Glo1 activity. We found a positive correlation between Glo1 activity in neuronal tissues and endothelial function in the aortic rings from Goto-Kakizaki rats, but not with acetylcholine-induced maximal relaxation of the aortic rings from Zucker rats. Moreover, we did not find any relationship between blood Glo1 activity and endothelial function in both rat strains. Thus, it can be concluded that blood Glo1 activity does not reflect the functional status of vascular endothelium in experimental models of type 2 diabetes or metabolic syndrome. The measurement of blood Glo1 activity might be a useful approach to study late complications; however, our data from experimental models show that this activity does not reflect early disturbances in vascular reactivity.

It has been reported that type 2 diabetes mellitus is one of the risk factors for cognitive impairment (Reijmer, 2010). At the end of experiment we observed working memory deficits in spontaneous alternation in rat model of obesity and type 2 diabetes. The pathogenesis of cognition dysfunction is only partially understood. Although many studies suggest that changes in cerebral structure and function in diabetes are related to hyperglycemia-induced end organ damage (through the activation of polyol pathway and protein kinase C, as well as accumulation of advanced glycation end-product), also macrovascular disease and insulin resistance may play a role in pathogenesis of memory impairment in type 2 diabetes and obesity (de la Monte, 2010, Vijayakumar, 2012). Present study provides evidence for disturbed neuronal Glo1 activity under conditions of hyperglycaemia prior to measurable functional changes in pain perception but in the presence of impaired endothelium-dependent relaxation and cognitive function. In addition, blood Glo1 activity could not be used as an early marker of the development of diabetic complications in experimental models of type 2 diabetes or metabolic syndrome.

### **3.3. Regulation of energy metabolism in experimental models of carbohydrate metabolism-related disorders**

In experimental model of obesity and metabolic syndrome (Zucker fa/fa rats) we evaluated the effects of mildronate treatment in comparison with the drug of first choice for the treatment of type 2 DM – metformin, and tested possible beneficial outcomes of treatment with a combination of both drugs. Our results show that mildronate and metformin treatment similarly reduced blood glucose and insulin concentrations in Zucker rats. Moreover, treatment

with a combination of mildronate and metformin prevented weight gain and improved insulin sensitivity. A common feature of obesity and metabolic syndrome is insulin resistance, which is characterized by a diminished ability of insulin sensitive tissues to take up and metabolize glucose in response to insulin (Shulman, 2000). Insulin resistance contributes to the development of energy metabolism disturbances and leads to the inability of cells to appropriately switch between energy substrates (Larsen, 2008). Metabolic syndrome significantly increases the risk of development of both type 2 diabetes and cardiovascular disease. Furthermore metabolic disturbances and lipotoxicity are both associated with fatty acid accumulation in cellular compartments, in particular, in the mitochondria (Koves, 2008, Wang, 2007). It is therefore important to characterize new therapeutic strategies that could effectively reduce lipid overload-induced damage and improve insulin sensitivity in obese patients. Mildronate treatment, due to a decrease in L-carnitine availability, partially reduces CPT I-related fatty acid transport and, in turn, prevents the accumulation of fatty acid in the mitochondria (Asaka, 1998, Kirimoto, 1996, Spaniol, 2003). It has been shown previously that mildronate treatment reduces CPT I activity and CPT I-related long chain fatty acid  $\beta$  oxidation in mitochondria (Kuka, 2012, Simkhovich, 1988). Metformin has multiple anti-diabetic effects, such as inhibition of gluconeogenesis and delay of gastrointestinal absorption of glucose and it reduces food intake or prevents body weight gain in obese patients with type 2 diabetes and in animal models of obesity (Bailey, 1996, Wiernsperger, 1999). In contrast to metformin acute administration of mildronate did not decrease blood glucose concentration in fed Zucker fa/fa rats that suggests that mildronate does not directly influence the gastrointestinal absorption of glucose. Neither acute administration of mildronate in vivo, nor in vitro application with perfusion buffer in isolated heart experiments, induced any effects on glucose concentration in blood or uptake in the mouse hearts. Mildronate long-term treatment significantly increased the rate of insulin-stimulated glucose uptake and the expression of GLUT4, HK II, and insulin receptor proteins in mouse hearts (Liepinsh, 2008). Similarly, it was observed that mildronate treatment for 6 - 8 weeks decreased blood glucose levels and prevented development of late complications in experimental rat models of type 1 diabetes (Sokolovska, 2011a, Sokolovska, 2011b) and type 2 diabetes (Liepinsh, 2009b). In the present study, mildronate treatment and especially treatment with the combination of mildronate and metformin increased fatty acid metabolism-related gene expression in the heart tissue of experimental rat model of obesity and metabolic syndrome. Obtained results suggest, that long-term mildronate treatment is required to induce the changes in glucose and fatty acid metabolism-related gene and protein expression.

In our study, treatment with the combination of mildronate and metformin induced effects that were different than the effects produced by

monotherapy. While the mechanisms of action of mildronate and metformin are different, treatment with a combination of the two drugs did not induce a synergistic or additive glucose-lowering effect. In contrast, an additive effect on fed stage insulin concentration was observed along with an increase in the expression of Glut-4. Thus, increased Glut-4 gene expression and enhanced insulin sensitivity are benefits of the treatment with the drug combination and it might underlie more pronounced cardioprotective effect of combinatory therapy. One of the well-known side effects of metformin is related to an increased lactate level. Mildronate treatment separately or in combination with metformin stimulated the glucose metabolism and in this way reduced the lactate concentration in Zucker fa/fa rat plasma. In previous studies, a decrease in lactate concentration in ischemic hearts of mildronate-treated animals has been noted (Asaka, 1998, Hayashi, 2000). Obtained results suggest that treatment with mildronate stimulates both glucose uptake and glycolysis, as well as enhances anaerobic oxidation of glucose in tissues, thus mildronate could reduce the metformin-induced lactate accumulation.

In our study mildronate and metformin monotherapy did not significantly affect the weight of Zucker fa/fa rats. In previous studies, an effect of mildronate on rat weight was not observed (Liepinsh, 2009b), whereas data concerning the effects of metformin are controversial (Yasuda et al., 2004). However, clinical studies have shown that the treatment of patients with type 2 diabetes with sulfonylurea agents and insulin-sensitizing drugs results in undesirable weight gain (UKPDS, 1998, Khan, 2002). In contrast to monotherapy, treatment with the combination of mildronate and metformin significantly prevented weight gain. In addition, drug treatment did not influence food intake, but mildronate and metformin might influence the absorption of glucose after food intake. The prevention of weight gain may also be due to the activation of AMPK and increased energy expenditure. The obtained results provide evidence that mildronate administration improves the hyperglycemia- and hyperlipidaemia- disturbed cellular energy metabolism through modulation of glucose and fatty acid metabolism in cells and mitochondria.

Peroxisome proliferator-activated receptors (PPARs) are central regulators of energy metabolism and mediate adaptive metabolic responses to increased systemic lipid availability (Grimaldi, 2007, Sugden, 2009). Although mildronate by a reduction of L-carnitine concentrations in the tissues stimulates the expression of genes and proteins involved in metabolism of glucose and fatty acid (Degrace, 2007, Liepinsh, 2008), the precise molecular mechanisms and nuclear factors associated with the mildronate-induced metabolic changes previously were not clarified. The mildronate treatment-induced changes in gene expression profiles observed in previous studies suggested PPARs as possible nuclear factors promoting effects on fatty acid metabolism. To further elucidate the molecular mechanism of mildronate, the nuclear contents of

PPAR $\alpha$  and PPAR $\gamma$  in heart tissue and the expression of glucose and fatty acid metabolic genes were determined. PPAR $\alpha$  is an important regulator of fatty acid metabolism and typically responds to increased systemic lipid availability. Activation of PPAR $\gamma$  stimulates lipid uptake and adipogenesis by fat cells, regulates glucose metabolism as well as improves insulin sensitivity. Partial resistance of the PPAR $\alpha$  system in Zucker rat liver has been previously described (Satapati, 2008). Also in our study the expression of PPAR $\alpha$  and its target genes in Zucker fa/fa rat heart tissue was significantly reduced compared to that of lean animals. In contrast to metformin, treatment with mildronate and the combination of drugs increased nuclear expression of PPAR $\alpha$  and fatty acid metabolism-related gene expression in the heart tissue. Thus, the increased expression of fatty acid metabolic genes compensates for a reduction in the L-carnitine content and maintains the FAO rate in the heart tissue. In contrast, the expression of PPAR- $\alpha$  and target genes in the liver of obese Zucker rats were not significantly affected by the drug treatment. Even though the mildronate and metformin treatments slightly restored the decreased expression of PPAR $\gamma$  in the heart tissue, both drug treatments did not significantly affect PPAR- $\gamma$  target gene expression in the liver tissue. In conclusion, our results demonstrate for the first time that mildronate treatment-induced changes in fatty acid metabolism-related gene expression in the heart tissues are dependent on PPAR $\alpha$ , but not PPAR $\gamma$  activation.

As a result of an increased circulating lipid concentration in Zucker fa/fa rats, also the fatty acid influx into the mitochondria is stimulated. In turn, the increased concentration of fatty acid metabolic intermediates, in particular acetyl-CoA, inhibit the activity of the pyruvate dehydrogenase complex (PDC) and CPT I. In addition, this impairs the citric acid cycle and the respiratory chain which results in a decrease in the glucose and the FAO rate. As a result, under conditions of diabetes and insulin resistance the concentrations of circulating glucose, insulin, fatty acids, and triglycerides increase, but the capacity of the mitochondria to produce ATP is reduced. Mildronate treatment decreases the carnitine content in tissues and CPT I-mediated fatty acid mitochondrial transport, thus preventing the accumulation of fatty acid in mitochondria. In addition, insulin sensitivity is significantly improved, and concentration of blood glucose decreases. In conclusion, treatment with mildronate, an inhibitor of L-carnitine biosynthesis, activates PPAR $\alpha$  expression in the heart tissue and improves adaptation to hyperglycemia- and hyperlipidemia-induced metabolic disturbances in Zucker fa/fa rats. Our findings also indicate that the combination of mildronate and metformin has a potential therapeutic value for the treatment of obese patients with type 2 diabetes and cardiovascular diseases.

### **3.4. The molecular mechanisms of cardioprotective effect of mildronate**

A recent study demonstrated that the reduction of L-carnitine concentration is the main mechanism of action of mildronate and this decrease in cardiac tissue is necessary to induce cardioprotective effect under ischemic conditions (Kuka, 2012). In our study we discovered that mildronate treatment stimulates the expression of PPAR $\alpha$  and genes involved in fatty acid metabolism in the heart tissue of experimental model of obesity and hyperglycaemia (Zucker rats). However, the precise molecular mechanisms associated with the mildronate-induced metabolic changes and PPAR $\alpha$  activation during ischemia-reperfusion were not clarified. Therefore, we performed a study to determine the influence of decreased L-carnitine content on the FAO in mitochondria, peroxisomes and the isolated heart, as well as evaluated the role of PPAR $\alpha$  and PGC1 $\alpha$ , their nuclear contents and the expression of dependent genes in heart tissues during ischemia-reperfusion. This work is the first to describe the mildronate treatment-induced compensatory changes in gene expression and the redirection of fatty acid flux that is beneficial to preserve mitochondrial and peroxisomal function in heart tissue after ischemia.

CPT I has long been suggested as a rate-limiting enzyme for the mitochondrial oxidation of long chain fatty acids. However, a recent study has shown that the uptake and oxidation of long chain fatty acids in the heart is independent of the activity of CPT I (Luiken, 2009). In addition, a considerably large decrease of L-carnitine content does not influence FAO in the whole heart (Degrace, 2004). The results of the present study also indicate that a long term decrease in L-carnitine concentration and CPT I activity induced by mildronate treatment does not alter the total metabolism of long chain fatty acids in the heart. Although mildronate treatment induces a decrease in CPT I activity which leads to decreased CPT I-driven fatty acid oxidation rate in mitochondria (Kuka, 2012, Simkhovich, 1988), at the same time mildronate treatment promotes the increase in gene expression involved in peroxisomal  $\beta$ -oxidation and thus preserves the peroxisomal and mitochondrial function and recovery after heart ischemia.

During ischemia the availability of oxygen is significantly diminished and FAO rates considerably decrease. This shortage leads to fatty acid accumulation-induced damage to the mitochondria and subsequent disturbances in energy metabolism. The available mechanisms to preserve the energy metabolism during ischemia are very limited. In contrast, after oxygen supply is restored during reperfusion, a better functional recovery of the ischemic myocardium after transient ischemia can be achieved by stimulating glucose oxidation (Doenst, 1999, Ussher, 2012). In this study, we show that the redirected fatty acid metabolism could also reduce the lipid overload-induced

lipotoxicity and protect the mitochondria against fatty acid accumulation. Mitochondrial fatty acid overload could be prevented by the initiation of complex changes in the transport and oxidation of long chain fatty acids. Mildronate treatment increases the expression of genes involved in mitochondrial long chain fatty acids metabolism in heart tissue, particularly CPT I, but this do not fully compensate for the reduced L-carnitine content. Therefore, the CPT I-dependent transport and the oxidation rate of long chain fatty acids in cardiac mitochondria are significantly decreased (Kuka, 2012). In turn, the peroxisomal  $\beta$ -oxidation rate of fatty acid is upregulated to compensate for the decreased mitochondrial FAO, and long chain fatty acids are, to a large extent, shortened to acetyl-CoA and octanoyl-CoA, which are not harmful for mitochondria (Korge, 2003). Thus, simultaneous reduction of long chain fatty acids transport into mitochondria and activation of peroxisomal FAO protect mitochondria from the accumulation of long chain fatty acids in the ischemic heart. In addition, in this study we observed that mildronate treatment induces a marked increase in mitochondrial  $\beta$ -oxidation. Therefore, the facilitated mitochondrial  $\beta$ -oxidation could be another mechanism that directly protects mitochondria against the deleterious effects of long chain fatty acid accumulation. Thus, mildronate treatment induces the increase in peroxisomal number and mitochondrial  $\beta$ -oxidation rates, which reduces lipid accumulation and ameliorates long chain fatty acid-induced damage in the ischemic heart.

PPARs are activated by endogenous ligands and transcriptional coactivators and function as nutrient sensors and regulators of energy metabolism (Duncan, 2011). One of the well-studied coactivators of PPARs is PGC1 $\alpha$ . The interaction between PGC1 $\alpha$  and PPAR $\alpha$  in the heart plays an important role in regulating expression of enzymes involved in FAO and uptake and may also be involved in regulation of mitochondrial biogenesis (Duncan, 2011). In the present study, we observed an increase in PPAR $\alpha$  and PGC1 $\alpha$  nuclear contents and the expression of genes related to FAO. However, the mechanisms that may promote mildronate-induced increase in PPAR $\alpha$  and PGC1 $\alpha$  levels in heart tissue remain to be discovered. In this study, we observed that the PPAR $\alpha$  /PGC1 $\alpha$  pathway did not respond to an increased long chain fatty acid level in the area at risk of control hearts either in ischemia or reperfusion. Moreover, in control hearts PPAR $\alpha$ -dependent gene expression in reperfusion was significantly diminished. In contrast, in heart tissue mildronate treatment stimulated PPAR $\alpha$  expression even in the presence of low long chain fatty acid concentrations in heart tissues. Thus, under the conditions of decreased L-carnitine in heart tissues the PPAR $\alpha$  activation is independent of the content of long-chain fatty acids.

Conceivably, the decreased L-carnitine-induced activation of PPAR $\alpha$  and facilitated fatty acid oxidation in the ischemic heart may involve the AMPK pathway, which contributes to the adaptive regulation of heart glucose

and particularly fatty acid oxidation. AMPK is activated by an increase in the AMP/ATP ratio, a sensitive indicator of cellular energy, and it functions to restore ATP levels by enhancing the oxidation of glucose and fatty acid (Beauloye, 2011). AMPK activation is essential for the ischemic myocardium which is characterized by an altered ATP level (Kim, 2011). In this study, we detected decreased levels of phosphorylated AMPK in response to the decreased L-carnitine content. This finding indicates that the mildronate treatment-induced decrease in L-carnitine content leads to changes in the energy metabolism which are beneficial because the AMP/ATP ratio is preserved even during ischemia. This result agrees with previous findings that demonstrated a reduced AMP/ATP ratio in mildronate-treated ischemic hearts (Asaka, 1998, Hayashi, 2000). Overall, our findings indicate that mildronate treatment induces activation of the PPAR $\alpha$ /PGC1 $\alpha$  pathway in heart tissue which is not related to AMPK phosphorylation.

In conclusion, the long term inhibition of CPT I activity and decrease in L-carnitine content induced by mildronate treatment is compensated for by the increase in the expression of genes involved in peroxisomal and mitochondrial fatty acid metabolism, resulting in an overall fatty acid oxidation rate that is similar to that in control animals. The mildronate-induced decrease in L-carnitine content activates the PPAR $\alpha$ /PGC1 $\alpha$  pathway and redirection of fatty acid metabolism to protect mitochondria against long chain fatty acid overload.

### **3.5. Association of blood L-carnitine levels with the development of late complications of DM**

Under physiological conditions L-carnitine is an essential factor for the transport of long chain fatty acid into mitochondria. In addition, it has been shown that L-carnitine as pharmacological agent stimulates glycolysis by increasing PDH activity and glucose metabolism-related protein expression (Foster, 2004, Stephens, 2007). The previous studies of blood L-carnitine levels in diabetes have provided inconsistent results (Okuda, 1987, Poorabbas, 2007, Pregant, 1991, Pregant, 1993). Several studies reported decreased plasma L-carnitine levels in patients with type 1 and type 2 diabetes and L-carnitine supplementation for patients with diabetes have been suggested (Capaldo, 1991, Mingrone, 2004). In contrast, other studies have shown that reduction of L-carnitine prevents development of late complications in diabetic patients and animal models with diabetes (Liepinsh, 2009a, Statsenko, 2008). To evaluate the relationship of L-carnitine concentration in blood with the prevalence and severity of late diabetic complications in patients with DM, we determined free L-carnitine levels in control subjects and individuals with type 1 and type 2 diabetes. The diabetic patient subgroups diagnosed as having various late complications and control subjects were characterized by similar average L-



carnitine concentrations. The present study suggest that higher L-carnitine concentrations do not reduce the occurrence or severity of late diabetic complications in type 1 and 2 diabetic patients, therefore obtained results leads one to question the rationale of the supplementation with L-carnitine to prevent late complications.

The concentration of L-carnitine in blood samples varied up to 3-fold within the control group and in patients with diabetes. Therefore, in studies with smaller numbers of participants, the risk of selection of specific subgroups might be relatively high. The lower L-carnitine concentrations found in diabetic patients in previous reports could also be the result of lower L-carnitine intake in the diet. It has been calculated that the average non vegetarian adult diet provides approximately 75% to 100% of daily L-carnitine requirements (Liepinsh, 2009b, Longo, 2006). The main source of L-carnitine is red meat, particularly beef (Demarquoy, 2004). The dietary recommendations for diabetic patients suggest avoiding red meat to reduce saturated fat and cholesterol intake (ADA, 2011, Hodge, 2011). There is substantial evidence that replacing red meat with poultry, beans, or nuts is effective in the prevention of cardiovascular diseases and that avoiding red meat lowers the risk of type 2 diabetes (Aune, 2009, Craig, 2010, Psaltopoulou, 2010). If diabetic patients follow these recommendations and significantly reduce their red meat intake, they could, in turn, also reduce their L-carnitine intake, resulting in lower L-carnitine concentrations in blood plasma. In a recent study, type 2 diabetic women were characterized by lower serum free L-carnitine levels and lower dietary intake of protein compared with controls (Poorabbas, 2007). The same study also found a positive significant correlation between free L-carnitine concentration and protein intake (Poorabbas, 2007). Because meat products are an important source of dietary proteins, diabetic patients might be exposed to a diet containing less L-carnitine, which explains the reduced L-carnitine concentration observed in diabetic patients. In this study we did not control the long-term dietary habits before blood sampling and without dietary data we could not directly link L-carnitine levels with meat intake. However, variations in subjects' day-to-day diets should not significantly influence the measured L-carnitine concentrations in fasted blood samples, which can serve as a marker for longterm body content of L-carnitine (Bain, 2006).

The kidneys are the most important organs for the regulation of L-carnitine homeostasis. According to the results of the present study, the L-carnitine concentrations in the plasma of diabetic patients with microalbuminuria and macroalbuminuria were similar to those observed in the nondiabetic subjects, and nephropathy was not associated with a lower concentration of L-carnitine. In patients undergoing long-term dialysis, lower plasma free L-carnitine levels were shown to be related to increased clearance of L-carnitine (Vernez, 2006, Verrina, 2007). Clinical studies evaluating the benefits of carnitine supplementation in adult uremic patients have generated

inconsistent results, as well as evidence that L-carnitine alone cannot explain the disturbed hematologic parameters or lipid profile (Hurot, 2002, Verrina, 2007). To our knowledge, alterations in L-carnitine excretion rates in diabetic patients have not been studied. In the present study, the patients with lower L-carnitine concentrations in the blood did not have more severe late complications than did those with higher L-carnitine levels. Similarly, in vegetarians, the L-carnitine concentration in the serum is 20% to 30% lower than normal (Delanghe, 1989), as is the prevalence of type 2 diabetes (Craig, 2010). In addition, a calorie-restricted vegetarian diet had a greater capacity to improve insulin sensitivity compared with a conventional diabetic diet (Barnard, 2009, Kahleova, 2011). For this study patients supplemented with L-carnitine and vegetarians were not recruited. Overall, the evidence suggests that decreased L-carnitine concentrations in blood might not indicate an L-carnitine deficit, but rather reflects a reduction of red meat consumption.

Obtained results suggest that low L-carnitine levels in diabetic patients with did not have increased occurrences or severity of late diabetic complications (peripheral neuropathy, nephropathy, retinopathy, and hypertension). In addition, patient subgroups with higher average L-carnitine concentrations did not have decreased prevalence of late diabetic complications. Our results provide evidence that reduction of L-carnitine concentrations could be useful approach to delay the development of late complications of DM.

## 4. CONCLUSIONS

1. Our results demonstrate that Glo1 activity is lower in patients with both types of diabetes mellitus who were diagnosed with painful diabetic neuropathy. Obtained data support the hypothesis that Glo1 activity modulates the phenotype of diabetic neuropathy.
2. Glo1 activity does not reflect early disturbances in vascular reactivity; however, the measurement of blood Glo1 activity might be an useful approach to study diabetic complications in advanced stage.
3. A decrease of L-carnitine levels induced by long term mildronate administration activates the PPAR $\alpha$ /PGC1 $\alpha$  pathway and induces redirection of fatty acid metabolism thus protecting mitochondria against long chain fatty acid overload in ischemia and carbohydrate metabolism-related disorders.
4. Lower concentrations of blood L-carnitine in patients with diabetes mellitus is associated with reduced incidence of diabetic neuropathy and hypertension.

## 5. APROBATION OF THE STUDY – PUBLICATIONS AND THESIS

### Doctoral thesis is based on following SCI publications:

1. Skapare E, Riekstina U, Liepinsh E, Konrade I, Makrecka M, Maurina B, Dambrova M, Flow cytometric analysis of glyoxalase-1 expression in human leukocytes, *Cell Biochemistry and Function*, **2011**, 29(2):171-174.
2. Liepinsh E, Skapare E, Svalbe B, Makrecka M, Cirule H, Dambrova M, Anti-diabetic effects of mildronate alone or in combination with metformin in obese Zucker rats, *European Journal of Pharmacology*, **2011**, 658(2-3):277-283.
3. Liepinsh E, Skapare E, Vavers E, Konrade I, Strele I, Grinberga S, Pugovics O, Dambrova M, High l-carnitine concentrations do not prevent late diabetic complications in type 1 and 2 diabetic patients, *Nutrition Research*, **2012**, 32(5):320-327.
4. Skapare E, Konrade I, Liepinsh E, Makrecka M, Zvejniece L, Svalbe B, Vilskersts R, Dambrova M, Glyoxalase 1 and glyoxalase 2 activities in blood and neuronal tissue samples from experimental animal models of obesity and type 2 diabetes mellitus, *The Journal of Physiological Sciences*, **2012**, 62(6):469-478.
5. Skapare E, Konrade I, Liepinsh E, Strele I, Makrecka M, Bierhaus A, Lejnieks A, Pirags V, Dambrova M, Association of reduced glyoxalase 1 activity and painful peripheral diabetic neuropathy in type 1 and 2 diabetes mellitus patients, *Journal of Diabetes and its Complications*, **2012**, in press.

### Prepared and submitted manuscript:

1. Liepinsh E, Skapare E, Kuka J, Makrecka M, Cirule H, Vavers E, Sevostjanovs E, Grinberga S, Pugovics O, Dambrova M, Activated peroxisomal fatty acid metabolism improves cardiac recovery in ischemia-reperfusion, submitted to journal.

### Non SCI publications:

1. Konrāde I, Škapare E, Makrecka M, Gulbe L, Liepiņš E, Lejnieks A, Dambrova M, Glioksalāzes II aktivitātes metodes izstrāde cukura diabēta komplikāciju molekulāro mehānismu pētījumiem, *RSU Zinātniskie raksti, 2010. gada medicīnas nozares pētnieciskā darba publikācijas*, **2011**, 1.sējums, 70.-76. lpp.
2. Tīrzīte D, Konrāde I, Segliņa D, Škapare E, Dambrova M, Āboli, florizīns un cukura diabēts, *Latvijas ārsts*, **2011**(10), 59-61.

3. Konrāde I., **Škapare E.**, Lejnieks A., Makrecka M., Dambrova M., Neenzimātiska glikēšanās: problēma ne tikai cukura diabēta pacientiem, *Latvijas Ārsts*, **2010**(11), 34.-37. lpp.
4. Konrāde I, **Škapare E.**, Liepiņš E, Kūka J, Pētersone I, Lejnieks A, Bierhaus A, Dambrova M. Microplate assay of glyoxalase I activity in blood samples, *Proc. Latvian Acad. Sci.*, **2007**, 61(5), P.149-154.

### Results are reported in following conferences

1. Svalbe B, **Škapare E.**, Vavers E, Zvejniece L, Dambrova M. Investigation of cognitive function and pain perception in animal models of obesity and type 2 diabetes mellitus, *The International Nutrition and Brain Summer School*, Bordeaux, France, September 3-14, **2012**. Abstract Nr.21.
2. **Škapare E.**, Kuka J, Makrecka M, Dambrova M, Liepinsh E, The lower L-carnitine availability is cardioprotective through the compensatory activation of PPAR/PGC-1 pathway and redirection of fatty acid metabolism, *6. EPHAR Congress*, Granada, Spain, July 17-20, **2012**, Final Programme, P. 56.
3. Svalbe B, **Škapare E.**, Liepinsh E, Vilskersts R, Vavers E, Zvejniece L, Dambrova M. Investigation of macro- and microvascular complications in animal models of obesity and type 2 diabetes mellitus, *8th FENS forum of Neuroscience*, Barcelona, Spain, July 14-18, **2012**. Abstract, Nr.2295.
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