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Basic Science

Liver Carbohydrate Metabolism in Rats in the Period of Recovery after Acute Heat Stress

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Key words: acute heat stress; recovery; carbohydrate metabolism; liver; rats.

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Abstract

In the present work we have estimated the effect of acute heat stress (1 hour to 41±0,5°C) and the different period of recovery at room temperature after the heat stress (0, 30 minutes, 1, 6, 12, 24, 48 and 96 hours) over the hepatic carbohydrate-related enzymes and substrates of rats.

The obtained results can be summarized in three phases: up to 6th hour, from 6th to 24th hour and form 24th up to 96th hour of recovery after the heat stress. Acute heat stress (up to 1hour of recovery) caused intensive glycogenolysis, associated with moderate, but non-significant endogenous production of glucose. The period of 6th to 24th hour of recovery after exposure to acute heat stress, is characterized with intensive rebound of glycogen stores, with decreased glucose production, and finally, from the 48th up to 96th hour of recovery, process of glycogenolysis is still evident, even though less intensive than the first hours after the heat stress.

Overall, we can conclude that acute heat stress and the period of recovery after the heat stress are followed with dynamic changes of the liver carbohydrate-relates enzymes and substrates in a course of elevated glycogenolysis, but decreased glycolysis and gluconeogenesis.

Introduction

High environmental temperature is stressful at all levels of biological organization, from cells to organism [1]. The changes of temperature are known to alter the rate of chemical and enzymatic reactions, the rate of diffusion, membrane fluidity and protein structure [2]. The adaptive ability of the organism in conditions of increased environmental temperature is accomplished through two processes: heat shock response (HSR) and heat acclimation [3]. The HSR is compatible with the development of rapid transient thermotolerance subsequent to acute heat stress [4], while acclimation is a slowly developing response, evoked by chronic exposure to moderate heat [5].

In this work, we investigated the effect of acute heat stress and the different period of recovery at room temperature after the heat stress over the carbohydrate metabolism in the liver. Acute exposure to high environmental temperature results in a reduction of blood glucose level [6, 7] or initial increase of blood

glucose, which returns to normal after normalizing the temperature [8, 9]. On the other hand, it was determined the initial decrease in the glycogen content in the liver due to the need for energy substrate in the body of rats [10].

Acute heat stress (AHS) is found to increase the concentration of adrenaline in the blood [11], which causes an increase in the activity of the enzyme glucose-6-phosphatase [12]. On the other hand, short-term exposure to high environmental temperature results with increased thyroid secretion [13], which finally contributes to the intensification of glycogen degradation. Thyroxine has a stimulatory effect on glucose-6-phosphatase activity [14], whose activity is increased during short-term exposure to heat.

It was of particular importance in this work to follow the changes in the function of duration of recovery at room temperature after acute heat stress. Given the small number of available literature data, main goal of this experiment will be examining the effects of AHS (41 ± 0,5 °C/1h) depending on the recovery period (30 ', 60', 6, 12, 24, 48 and 96 hours), on the activity of some key enzymes and substrates from carbohydrate metabolism in the liver.

The experiment was focused on the changes in enzyme activity: glycogen phosphorylase (*a* + *b* and *a)*, glucose-6-phosphatase, hexokinase, fructose-1,6-bisphosphatase and phosphofructokinase, glycogen content and concentration of glucose and glucose-6-phosphate, in the function of the recovery time after exposure to 41 ± 0.5 °C/1h.

Materials and Methods

Experimental animals and conditions

For the realization of the experiment we used white laboratory rat, Wistar strain. The experimental study was performed on adult (4-5 months old) male rats (n = 54), with an estimated weight of 250–350 g. The animals received standard diet and constant light regime of 12 hours (from 06 to 18 pm).

For the purposes of this experiment, animals were divided into following two groups:

· C-control animals -maintained at room temperature (20 ± 2 ºC);

· Heat-stressed animals, with different period of recovery at room temperature after the acute heat stress, divided in 8 groups: HS - animals subjected to acute heat stress (41 ± 0.5°C/1h) and sacrificed just after the heat stress; HS30', HS1h, HS6h, HS12h, HS24h, HS48h and HS96h - animals subjected to acute heat stress (41 ± 0.5°C/1h), allowed to recover 30 minutes as well as 1, 6, 12, 24, 48 and 96 hours at room temperature before sacrifice.

Acute hyperthermic stress (AHS) was carried out in a special heat chamber with regulated air temperature of 41 ± 0.5°C and duration of exposure of 1 hour. All experimental animals received food and water *ad libitum* before and after the heat stress.

The animals were anesthetized with a Na-thiopental narcosis (45 mg/kg) and sacrificed using a standard laparotomic procedure. Immediately after the laparothomy, the isolated pieces of liver was washed with cold saline solution and immersed in liquid nitrogen (-196°C). Tissues were stored in liquid nitrogen until analysis.

Analytical methods

Liver glycogen content and the concentration of glucose and glucose-6-phosphate were determined in perchlorate homogenates, neutralized with 5M K_2CO_3. We measured the production of NADPH at 340 nm in a reaction catalyzed by glucose-6-phoshate dehydrogenase [15].

For determining enzyme activity, liver homogenates in appropriate medium of homogenization were prepared. The determination of the activity of the total (*a* + *b)* and active (*a)* glycogen phosphorylase (E.C. 2.4.1.1), was performed by the method of Stalmans and Hers [16]. Glucose-6-phosphatase (G-6-P-ase, E.C. 3.1.3.9) was assayed by the method of Hers [17] and the substrate mixture for this enzyme contain G-6-P (100 mM) and EDTA (2 mM), pH= 6.0-6.5. The substrate mixture for fructose-1,6-bisphosphatase (F-1,6-BP-ase, E.C. 3.1.3.11) was prepared from 5 mM fructose-1,6-bisphosphate, 2.5 mM $MgSO_4$ x 7 H_2O, 5 mM $MnSO_4$ x H_2O,30 mM L-cysteine and 20 mM L-serine [18]. In all enzyme analyses, the amount of released inorganic phosphate was measured by the method of Fiske and Subbarow [19]. For the corresponding parameters we measured the concentration of total protein by Lowry method [20] by using bovine serum albumin as a standard. The specific enzyme activity was expressed as nmol Pi/min/mg prot.

To determine the activity of phosphofructokinase

(E.C. 2.7.1.11) according to Ling et al., (21) and hexokinase (E.C. 2.7.1.1.) according to Boutemps (22), we measured produced NADH, which proportionally corresponds to the activity of the enzyme.

Statistics

The results are shown as mean ± standard deviation. We used one way ANOVA analyses with Newman-Keuls post-hoc test, to examine the significant differences between the groups. Correlation analyses for each parameter were assayed by regression analyses and only significant coefficients of correlation are presented in the figures. In all tests, a probability level of p<0,050 was used as a significant difference. Overall statistical data processing was performed using the statistical program Statgraph for Windows 3.0.

Results

Glycogen content and glucose concentration

Figure 1 and Table 1 show the changes in the hepatic glycogen content and glucose concentrations during acute exposure to 41 ± 0.5°C/1h and recovery at room temperature.

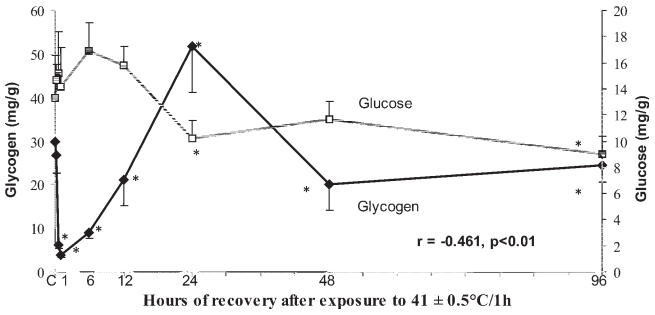

Figure 1: Glycogen content and glucose concentration in animals subjected to acute heat stress and different period of recovery at room temperature. Legend: C-control (20 ± 2°C); * - p<0.050 compared to control; r - coefficient of correlation between glycogen and glucose ($r_{Glk/Glu}$ = -0.461, p<0.01).

Table 1: Statistical and percentage analysis of glycogen content and glucose concentration in the liver.

	Glycogen		Glucose	
ratio	%	P<	%	P<
C:HS	-10.4	n.s.	10.2	n.s.
C:HS30'	-79.3	0.050	14.4	n.s
C:HS1h	-86.7	0.050	6.7	n.s
C:HS6h	-70.1	0.050	14.9	n.s
C:HS12h	-29.2	0.050	18.7	n.s
C:HS24h	73.3	0.050	-23.4	0.050
C:HS48h	-32.7	0.050	-11.9	n.s
C:HS96h	-17.8	0.050	-32.3	0.050

The changes in the liver glycogen content can be summarized in three different phases: initially, significant decrease up to the 12th hour of recovery compared to the control (from -29.2% to -86.7%, p <0.050); significant increase of glycogen content at the 24th hour (C:HS24h = 73.3%, p<0.050) and significant reduction of glycogen content after the 48th hour (C:HS48h= -32.7% and C:HS96h= -17.8%, p<0.050).

Concerning hepatic glucose concentration, we found non-significant increase in the first 12 hours of recovery. In the remaining period we found a tendency of decrease of glucose concentration, which is significant at 24 and 96 hours of recovery (C:HS24h= -23.4% and C:HS96h= -32.3%, p<0.050). Statistical analysis indicated a negative correlation coefficient depending on the different duration of recovery after exposure to acute heat stress (r = - 0.611, p<0.01). Dependence between the glycogen content and glucose concentration in the liver is expressed with a moderate, but significant negative correlation coefficient (r = - 0.461, p<0.01, Fig.1).

Concentration of glucose-6-phosphate

The changes in the liver glucose-6-phosphate concentration are presented on Figure 2 and Table 2. The concentration of glucose-6-phosphate was

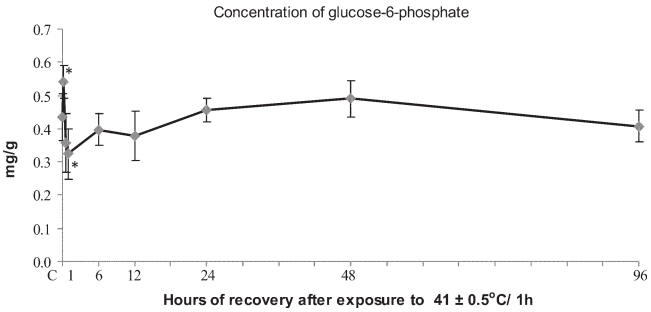

Figure 2: Concentration of glucose-6-phosphate in animals subjected to acute heat stress and different period of recovery at room temperature. Legend: C-control (20 ± 2°C); * - p <0.050 compared to control.

Table 2: Statistical and percentage analysis of glucose-6-phosphate concentration in the liver.

	Glucose-6-phosphate	
ratio	%	P<
C:HS	24.5	0.050
C:HS30'	-17.9	n.s.
C:HS1h	-25.5	0.050
C:HS6h	-8.6	n.s.
C:HS12h	-12.9	n.s.
C:HS24h	4.4	n.s.
C:HS48h	12.9	n.s.
C:HS96h	-6.1	n.s.

significantly increased immediately after the heat stress (24.5%, p <0.050), while a significant reduction compared to the control was noted at the 1st hour of recovery (C : HS1h= -25.5%, p<0.050). There were non- significant changes till the end of recovery period in hepatic glucose-6-phosphate concentration. Regression analyses showed positive correlative dependence between glucose-6-phosphate and glycogen (r = 0.440, p<0.01, results not shown).

*Glycogen phosphorylase (*a + b *and* a) *activity*

The dynamics of changes in the level of hepatic glycogen phosphorylase (*a + b* and *a)* depending of the different duration of recovery after exposure to 41 ± 0.5°C/1h are shown in Figure 3 and Table 3.

In the level of total glycogen phosphorylase following changes were found: in the first 6 hours of recovery non-significant changes in enzyme activity compared to control were observed, significant reduction in enzyme activity were found in the 12th and 24th hour (-17.9% to -32.6%, p <0.050), while in the 96th hour of recovery we noticed lower intensity of reduction of enzyme activity, but significant changes in terms of control (C:HS96h= -20.5%, p<0.050). There is relatively week, but significant negative coefficient of correlation depending on different recovery duration (r = - 0.458, p<0.01).

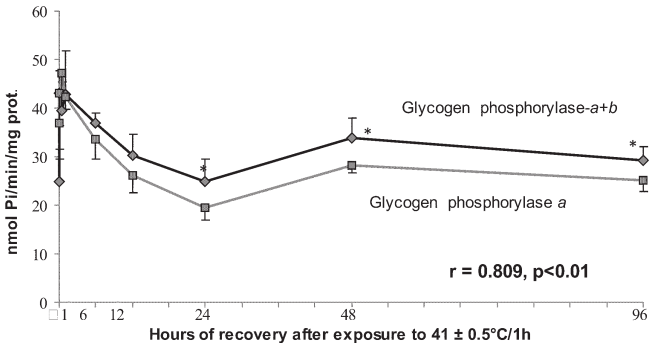

Figure 3: Glycogen phosphorylase a + b *and* a *activity in animals subjected to acute heat stress and different period of recovery at room temperature.* Legend: C-control (20 ± 2°C); * - p <0.050 compared to control; r- coefficient of correlation between glycogen phosphorylase a + b and a (r_{GPa+b} /$_{à}$ = 0.809, p<0.01).

Table 3: Statistical and percentage analysis for glycogen phosphorylase *a*+ *b* and *a* activity.

	GP *a+b*		GP *a*	
ratio	%	P<	%	P<
C:HS	16.4	n.s.	17.1	0.050
C:HS30'	7.3	n.s.	27.9	0.050
C:HS1h	16.4	n.s.	14.9	n.s.
C:HS6h	0.1	n.s.	-8.9	n.s.
C:HS12h	-17.9	0.050	-28.9	0.050
C:HS24h	-32.6	0.050	-47.1	0.050
C:HS48h	-8.6	n.s.	-23.5	0.050
C:HS96h	-20.5	0.050	-31.8	0.050

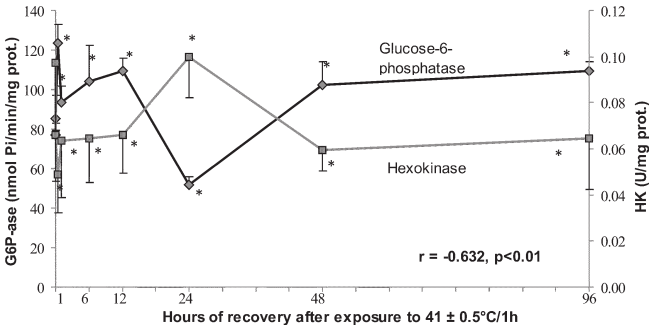

Figure 4: Glucose-6-phosphatase and hexokinase activity in animals subjected to acute heat stress and different period of recovery at room temperature. Legend: C-control (20 ± 2°C); * - p <0.050 compared to control; r- coefficient of correlation between glucose-6-phosphatase and hexokinase ($r_{G6P-ase}$ /$_{HK}$= -0.632, p<0.01).

Table 4: Statistical and percentage analysis for *liver glucose-6-phosphatase and hexokinase activity.*

	G6P-ase		HK	
ratio	%	P<	%	P<
C:HS	10.7	n.s.	-32.4	0.050
C:HS30'	60.7	0.050	-49.9	0.050
C:HS1h	21.3	0.050	-35.1	0.050
C:HS6h	35.3	0.050	-33.8	0.050
C:HS12h	42.6	0.050	-32.1	0.050
C:HS24h	-32.4	0.050	2.5	n.s.
C:HS48h	32.7	0.050	39.1	0.050
C:HS96h	41.8	0.050	33.9	0.050

Significant increase of glycogen phosphorylase *a* activity was observed during the first 30 minutes (C:HS30'= 27.9%, p<0.050), followed with non-significant changes in the 1st and 6th hour of recovery. In the remaining period of recovery after exposure to acute heat stress we found a significant reduction in the activity of glycogen phosphorylase *a* (from -23.5% to -47.1%, p<0.050). Correlation analysis for the entire recovery period is represented by a significant negative correlation coefficient (r = - 0.549, p<0.01). The obtained results showed significant positive coefficient of correlation recovery compared to the control, with exception in the 24th hour where we found non-significant increase in heksokinase activity.

The correlation coefficient (r = - 0.632, p<0.01, Fig. 4) indicates a moderately strong relationship between glucose-6-phosphatase and hexokinase activity.

Fructose-1,6-bisphosphatase and phosphofructokinase activity

The changes in the fructose-1,6-bisphosphatase and phosphofructokinase activity depending on the different duration of recovery after exposure to 41 ± 0.5° C/1h are presented on Figure 5 and Table 5.

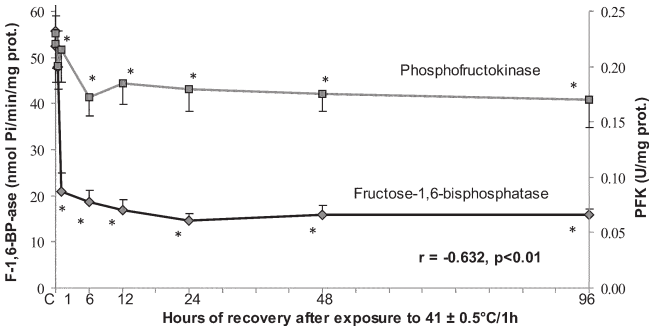

Figure 5: Fructose-1,6- bisphosphatase and phosphofructokinase activity in animals subjected to acute heat stress and different period of recovery at room temperature. Legend: C-control (20 ± 2°C); * - p <0.050 compared to control; r- coefficient of correlation between fructose-1,6- bisphosphatase and phosphofructokinase ($r_{F\text{-}1,6\text{-}BF\text{-}ase/PFK}$ = 0.522, p<0.01).

Table 5: Statistical and percentage analysis for liver fructose-1,6- bisphosphatase and phosphofructokinase activity.

	F-1,6-BP-ase		PFK	
ratio	%	P<	%	P<
C:HS	-5.7	n.s.	-3.9	n.s.
C:HS30'	-13.9	n.s.	-12.8	n.s.
C:HS1h	-62.6	0.050	-6.2	n.s.
C:HS6h	-66.7	0.050	-24.8	0.050
C:HS12h	-69.5	0.050	-19.3	0.050
C:HS24h	-73.8	0.050	-21.8	0.050
C:HS48h	-71.3	0.050	-23.7	0.050
C:HS96h	-71.6	0.050	-25.9	0.050

The obtained results showed non-significant changes in the fructose-1,6-bisphosphatase activity during the first 30 minutes, while in the remaining recovery period, (from 1st to 96th hour) there was decline in the enzyme activity in terms of control (from -62.6% to -73.8%, p <0.050). Correlation analyses for the whole period of recovery for fructose-1,6-bisphosphatase are represented by significant negative coefficient of correlation (r= -0.510, p<0.01).

Concerning phosphofructokinase activity, non-significant changes in enzyme activity compared to control were found during the first hour of recovery, while in the remaining period, from 6th to 96th hour, there is a significant reduction in the activity of phosphofructokinase (from -19.3% to -25.9%, p <0.050). Statistical analysis indicated a negative correlation coefficient depending on the different duration of recovery after exposure to acute heat stress (r = - 0.449, p<0.01). The analyses showed positive correlative dependence between fructose-1,6- bisphosphatase and phosphofructokinase activity (r = 0.522, p<0.01, Fig. 5).

Discussion

During this experiment we estimated the changes in the activity of some key carbohydrate-related enzymes and substrates in the liver of rats exposed to acute heat stress (41 ± 0.5°C/1h), depending on the different period of recovery at room temperature (short-term - 0, 30, 60 minutes and long-term - 6, 12, 24, 48 and 96 hours) after the heat stress.

The obtained results showed that carbohydrate metabolism undergoes significant changes in the designed experimental conditions. In addition we found: a significant reduction in glycogen content, reduction of glycogen phosphorylase (*a* + *b* and *a*), fructose-1,6-bisphosphatase, hexokinase and phosphofructokinase activity, while there is significant increase in glucose-6-phosphatase activity. During the whole period we found non-significant changes in the concentration of glucose and glucose-6-phosphate.

The changes in the estimated parameters can be summarized in three different phases: up to 6th hour, from 6th to 24th hour and form 24th up to 96th hour of recovery after the heat stress.

The first phase of the recovery period is characterized with significant reduction in hepatic glycogen content, which is most intensive during the first 60' of recovery after exposure to 41 ± 0.5°C/1h (C:HS60'= -86.7%, p<0.050). The obtained results are consistent with some previous studies [23, 10]. We assume that such a intensive depletion of glycogen stores occurs as a result of the increased need for energy substrates during the exposure on heat stress [6, 8]. Given that glycogen is the most mobile and the best available energy reservoir in the body, the emergence of intensive glycogenolysis is most obvious. Spending of glycogen stores is followed with increased activity of the total and the active glycogen phosphorylase (Fig. 3). Glycogen phosphorylase is the key glycogenolytic enzyme and has significant role in the regulation of glycogen metabolism [24, 25]. This enzyme catalyzes the first step in the breakdown of glycogen [26], which could be the main reason for decreased glycogen stores in our results.

In addition to intensive glycogenolytic processes,

there was increased G-6-P-ase activity during the first 60' of recovery, which is most evident in the point 30' after the heat stress (C:HS30'= 60.7%, p<0.050). This enzyme plays an important role in regulation of glucose homeostasis [27], both as a glucogeneogenic and glycogenolitical enzyme, catalyzing the critical step of the glucose synthesis [28]. Our results are consistent with previous research from Mithieux [29], who suggest that intensified glycogenolysis contributes to increased activity of glucose-6-phosphatase. During the same period there is significant reduction in the concentration of the intermediate substrate, glucose-6-phosphate (C : HS1h= -25.5%, p<0.050). Our assumption is that during intensified glycogenolysis in hepatocytes, the spended glycogen and glucose-6-phosphate stores resulted in moderate production of hepatic glucose in the first 60 minutes after the heat stress (Fig. 1). On the other side, the significant reduction of fructose-1,6 -bisphosphatase activity indicates for a lower intensity of gluconeogenesis in the short period of recovery after acute heat stress. Namely, on the level of fructose-1,6 -bisphosphatase, which is typical key regulatory enzyme in gluconeogenesis [30, 31], there is significant reduction regarding to the control in the 60th minute of recovery (C:HS1h= -62.6%, p<0.050).

At the same time (60' after the heat stress) we found a significant reduction in the hexokinase activity (from -32.4% to -49.9%, p <0.050), and non-significant decrease in the phosphofructokinase activity, both indicated for a reduction in the intensity of the process of glycolysis. Similar studies that indicate reduced glycolysis, by reducing the phosphofructokinase activity, in short-term exposure to high temperatures are made by Inomoto et al. [32].

Most of the enzymatic changes are regulated by hormones. In short-term hypothermic exposure it is found increased ACTH, corticosterone [33] and prolactin as a stress hormone [34, 9]. ACTH stimulates the adrenal cortex to secrete hormones especially glucocorticoids [35]. It's known that glucocorticoids have exhilarating effect on glucose-6-phosphatase activity [36], and also they activate gluconeogenetic enzymes. Catecholamines also have an important role in glucose regulation and stress response [37, 38]. Short-term exposure on heat stress results in increased concentration of adrenaline in the blood [11, 39]. According to Bady et al. [12], the adrenaline increases glucose-6-phosphatase activity. Short-term exposure to high temperature results with increased thyroid secretion [13], which contributes to the intensification of glycogen degradation, since thyroxine has a stimulatory effect on glucose-6-phosphatase activity [14].

From the above mentioned, it could be summarized intensive degradation of glycogen stores to glucose in the first 1h of recovery after exposure to 41 ± 0.5°C/1h. The intensive glycogenolysis is associated with moderate, but non-significant endogenous production of glucose concentration, which could be due to the increased peripheral consumption of this energetic substrate.

In the subsequent period of recovery, particularly in the 6th and 24th hour of recovery after exposure to acute heat stress, we noticed an attempt for rebound of hepatic glycogen with significant increase of glycogen content at 24th hour (C:HS24h= 73.3%, p<0.050). Opposite to glycogen, the glucose concentration showed tendency of decrease, with a significant changes at 24th hour point (C:HS24h= -23.4%, p<0.050). It is important to stress that the lowest values of glycogen phosphorylase and glucose-6-phosphatase (C:HS24h= -32.4%, p<0.050) were also noticed after 24 hour of recovery period, while hexokinase, like opposing enzyme of glucose-6-phosphatase was moderately increase at this point. Namely, the lower glycogen phosphorylase resulted with increased glycogen stores, which from the other side, caused decrease of glucose-6-phosphatase activity and subsequently, decreased glucose concentration. In the same period there is expressed decline of fructose-1, 6 – bisfosfatase activity. We assume that such a significant reduction in enzyme activity occurs as a result of the reduced intensity of gluconeogenesis.

All these data give us assumption that the period of 6th to 24th hour of recovery after exposure to acute heat stress, is characterized with intensive rebound of glycogen stores, with decreased glucose production.

In the remaining recovery period (48th and 96th hour), again there is significant reduction on hepatic glycogen content, but less intensive that in the first 6 hours (C:HS48h= -27.8%, C:HS96h= -27.6%, p<0.050). In the same time period we found significant reduction in the enzyme activity of the total (*a* + *b*) and the active (*a*) glycogen phosphorylase. The obtained results indicate a significant increase in the glucose-6-phosphatase activity for about 32-42%. Namely, it is important to stress that there is significant negative correlation between glycogen and glucose-6-phosphatase activity during whole experimental period (r= -0.702, p<0.01) as well as positive correlation with glycogen phosphorylase a (r= +0.549, p<0.01). The

decrease of glycogen is followed with still lower values of glucose and almost normal values for glucose-6-phosphate.

During the same period there is significant reduction in fructose-1,6-bisfosfatase activity (C:HS48h= -71.3% and C:HS96h= -71.6, $p<0.050$). Concerning glycolytic enzymes, hexokinase and phosphofructokinase, we found further significant reduction in the enzyme activity regarding the control.

From the above data, it can be concluded that in the 48th and 96th hour of recovery, as in previous hours of recovery after exposure to 41 ± 0.5°C/1h, the process of glycogenolysis is still evident, even though less intensive than the first hours after the heat stress. This give us a possibility to propose that the metabolic processes in the liver are not completely returned to the control values even after 96 hour of recovery at room temperature.

Overall, based on the results of the tested parameters, we can conclude that the acute heat stress (41 ± 0.5°C/1h) and the recovery period after the heat stress are followed by dynamic changes in the activity of some key enzymes and substrates from the carbohydrate metabolism. This is manifested with inhibiting of glycolytic processes and stimulation of glycogenolysis in hepatocytes, as well as by increased endogenous glucose production that is required for peripheral consumption in heat stress conditions.

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