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Effect of single and repeated heat stress on chemical signals of heat shock response cascade in the rat's heart

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Abstract Exposure to sublethal heat stress activates a complex cascade of signaling events, such as activators (NO), signal molecules (PKCε), and mediators (HSP70 and COX-2), leading to implementation of heat preconditioning, an adaptive mechanism which makes the organism more tolerant to additional stress. We investigated the time frame in which these chemical signals are triggered after heat stress ($41 \pm 0.5°C/45$ min), single or repeated (24 or 72 h after the first one) in heart tissue of male Wistar rats. The animals were allowed to recover 24, 48 or 72 h at room temperature. Single heat stress caused a significant increase of the concentration of HSP70, NO, and PKC level and decrease of COX-2 level 24 h after the heat stress, which in the next course of recovery gradually normalized. The second heat stress, 24 h after the first one, caused a significant reduction of the HSP70 levels, concentration of NO and PKCε, and significant increase of COX-2 concentration. The second exposure, 72 h after the first heat stress, caused more expressive changes of HSP70 and NO in the 24 h-recovery groups. The level of PKCε was not significantly changed, but there was significantly increased COX-2 concentration during recovery. Serum activity of AST, ALT, and CK was reduced after single exposure and increased after repeated exposure to heat stress, in both time intervals. In conclusion, a longer period of recovery (72 h) between two consecutive sessions of heat stress is necessary to achieve more expressive changes in mediators (HSP70) and triggers (NO) of heat preconditioning.

Keywords Single and repeated heat stress · Activators · Mediators · Triggers · Rat's heart

Introduction

The heart reacts to a sublethal cellular stress (ischemia, heat stress or exercise) by mobilizing a complex sequence of cellular events that results in a shift from a naive (non-preconditioned) to a defensive (preconditioned) phenotype (Bolli 2000). The phenomena of preconditioning occurs in two phases: an early phase, which lasts 2–3 h, and a late phase, which begins 12–24 h later and provides prolonged (3–4 days) protection and tissue tolerance to a subsequent stress of the same or different nature (Bolli 1996; Joyeux-Faure et al. 2003; Das and Das 2008).

The delayed protection induced by heat PC is more extensively studied in the heart, demonstrated by the reduced myocardial damage during further exposure to stress (Joyeux et al. 1999). Specifically, the PC (both heat and ischemic PC) is a result of a complex cascade of cellular events, representing the heart response to stressful stimuli, which can be divided into three subgroups: *triggers* (like nitric oxide - NO), *signal pathways* (like epsilon isoform of protein kinase C - PKCε), and *mediators* (like heat shock protein 70 - HSP70 and cyclo-oxygenase 2 - COX-2), which confer protection 24–48 h later (Bolli 2000; Xi et al. 2001; Joyeux-Faure et al. 2003). Some studies have also shown that HSP70 is required for iNOS induction (Zhang et al. 2013), even though it is not clear whether the constitutive or inducible HSP70 have this essential role.

Numerous studies have shown that the peak of HSP70 concentration in rat heart is 24 h after exposure to HS, which later gradually decreases (48 and 72 h following HS) and after 8 days the concentration is at a level as before the stress (Arnaud et al. 2001; Patel et al. 2001; Horowitz et al. 2004;

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Diao et al. 2012). At the same period of time (24 h after exposure to HS) a noticeable increase in myocardial COX-2 protein expression is detected (Arnaud et al. 2003b). Additionally, HS induces a sharp ephemeral increase of NO production that reaches its peak 1 h after HS, 4 h later starts to decline, and after 24 h it almost does not differ from the initial level (Malyshev et al. 1995; Velkovski 2012). Moreover, it is suggested that NO production could be responsible for the activation of protein kinase C (PKC) following HS, which ultimately results in increased transcription of cardioprotective genes and realization of the delayed phase of heat preconditioning of the heart (Joyeux-Faure et al. 2003).

The cellular dynamics of the heat PC triggered mechanisms are of great importance for implementation of the protective effects of PC (Hoshida et al. 2002). It has been observed that repeated exposure of organisms to heat stress can prolong the duration of the protective effects activated by the thermal PC (Hoshida et al. 2002; Goto et al. 2005). Hoshida et al. (2002) were the first to evaluate the impact of two episodes of the same stress (physiological or pharmacological) in a 48 h time interval and reported the benefits of repeated stress, which lasted in the following 60 h. Hence, the time interval between two or more successive exposures to stress, in order to achieve the best protection of the cardiac function, is still to be determined.

Therefore, the first part of our study was aimed at monitoring the changes in the concentrations of the cell metabolites NO, PKCε, HSP70, and COX-2 at different periods of recovery (24, 48, and 72 h), following exposure to acute HS (41 ± 0.5°C/45 min). Moreover, in this study we estimated whether the two episodes of the same physiological stress could extend the duration of the protective effects activated by thermal PC. In this sense, we investigated the metabolic changes in the tested parameters in 24 h or 72 h time intervals between two sessions of HS, both of them followed by 24 h and 48 h recovery period.

Finally, in order to estimate whether the exposure to HS caused any damage to the myocardium, we determined the enzyme activity of the serum alanine aminotransaminase (ALT), aspartate aminotrasaminase (AST), and creatine kinase (CK) as indicators of the extent of tissue damage.

Materials and methods

Experimental groups and heat stress protocol

Male Wistar rats ($n = 47$, weight 250–350 g, and 3–4 months old) maintained on a 12:12 light: dark cycle, fed on rat chow and water ad libitum and bred at the Institute of Biology Faculty of Natural Science and Mathematics, Skopje, were used. All protocols were approved by the Animal Ethics Committee within the University "Ss Cyril and Methodius",

Skopje, R. Macedonia, in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for International Organizations of Medical Sciences. Anesthetics were applied according to the standards given by the guide of the EC Directive 86/609/EEC.

The rats were divided into the following groups (Table 1):

- Rats exposed to single HS with 24 h, 48 h, and 72 h recovery period at room temperature before sacrifice (HS24, HS48, and HS72, respectively).
- Rats exposed to repeated HS, 24 h after the first HS, with 24 h and 48 h recovery period at room temperature before sacrifice (HS24 + HS24 and HS24 + HS48, respectively).
- Rats exposed to repeated HS, 72 h after the first HS, with 24 h and 48 h recovery period at room temperature before sacrifice (HS72 + HS24 and HS72 + HS48, respectively).
- The control group was kept at room temperature (20 ± 2 °C).

Heat stress was caused by placing the rats in a heat chamber with controlled temperature (41 ± 0.5°C) and humidity (30–40%), for a period of 45 min.

Isolating and preparing tissue for analysis

Laparotomy was performed after anesthetization of rats with sodium thiopental (45 mg/kg, i.p.). Blood was collected from abdominalis to obtain serum. The heart was washed in 0.9% saline and immediately immersed and stored in liquid nitrogen (−196°) till further analysis. Before conducting the analysis, heart samples were crushed into fine powder of which homogenates were prepared in an appropriate solution. Homogenization of tissue samples was performed on ice, with ultrasound homogenizer (Cole-Palmer Instrument-4710), for a period of 10–15 s.

Biochemical analyses

HSP70 protein level

The protein level of HSP70 in heart tissue was determined with the use of quantitative immunoassay sandwich test (Enzo Life Science International, Inc.) in accordance with the manufacturer manual. Briefly, after homogenization of heart samples with extraction buffer, in which protease inhibitor (0.1 mM PMSF) was previously added, the homogenates were centrifuged at 21000 g/10 min/4 °C and the extracts were diluted. Diluted extracts and standards were applied on microtiter plate wells, pre-coated with monoclonal antibody specific for inducible HSP70. Captured HSP70 was detected with a HSP70 specific rabbit polyclonal antibody, subsequently

Table 1 Experimental protocol and organization of the animals in experimental groups

Single heat stress

Groups	No. of animals	Heat stress (41 ± 0.5°C)	Recovery (20 ± 2 °C)		
C	5	/	/		
HS24	6	45 min	24 h		
HS48	6	45 min	48 h		
HS72	6	45 min	72 h		

Repeated heat stress

Groups	No. of animals	First heat stress (41 ± 0.5°C)	Recovery (20 ± 2 °C)	Second heat stress (41 ± 0.5°C)	Recovery (20 ± 2 °C)
HS24+HS24	6	45 min	24 h	45 min	24 h
HS24+HS48	6	45 min	24 h	45 min	48 h
HS72+HS24	6	45 min	72 h	45 min	24 h
HS72+HS48	6	45 min	72 h	45 min	48 h

bound by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The assay was developed after adding TMB substrate and the color development was stopped with acid stop solution which converts the endpoint color to yellow. The intensity of the color was in proportion to the amount of captured HSP70, which was measured in a microplate reader (Bio-Rad Model 680, Philadelphia, USA) at 450 nm. The final results were presented as ng HSP70/mg protein. For that purpose, the concentration of total proteins in heart samples was established with the use of the method by Lowry (Lowry et al. 1951), which is based on forming colored products of aromatic amino acids with Folin-Ciocalteu reagent and BSA as a standard (1 mg/ml).

Nitric oxide (NO) concentration

Concentration of NO was obtained with the use of quantitative colorimetric test (BioAssay Systems, Hayward, USA) which was performed according to the manual provided within the kit. In short, samples were homogenized in PBS puffer (pH 7.4) and centrifuged at 10000/4 °C. Working solution was added to each standard and sample (previously deproteinated) and incubated at 60 °C for 10 min. Following incubation, each reaction was added to a separate well on a microtiter plate and the optic density was read at 540 nm.

PKCε level and COX-2 concentration

The level of PKCε and the concentration of COX-2 were determined with the use of quantitative immunoassay sandwich test (Cloud-Clone Corp., Houston, USA) in accordance with the manufacturer manual. After homogenization of tissue samples in PBS (pH 7.2) on ice, the homogenates were centrifuged at 5000 g/5 min. Each supernatant and standard was applied on microtiter plate wells with a biotin-conjugated antibody specific to PKCε or COX-2, respectively. Next, conjugate was added to each well and incubated, and only those wells that contained captured PKCε or COX-2, respectively, would change color after adding TMB substrate. The enzyme-substrate reaction was stopped by adding sulfuric acid solution and the change of color was measured with reader (Bio-Rad Model 680, Philadelphia, USA) at 450 nm.

The enzymatic activity of AST, ALT, and CK

The enzymatic activity of AST, ALT, and CK in the serum was determined with kinetic biochemical reactions, with the use of automatic biochemical analyst (ChemWell® 2910, Awareness Technology) in accordance with the manual provided by the manufacturer of the reagents (Human, Germany).

Statistical analysis

All the values were presented as mean ± SD. The statistical differences between the groups were examined using one-way ANOVA with a Neuman-Keuls post-hoc test, as well as Pearson-coefficient of correlation. A probability level of $p < 0.05$ was considered to be significant. The overall statistical data processing was performed using the statistical program Statgraph for Windows.

Results

Exposure to HS caused elevation of rectal temperature (measured with digital rectal thermometer for rats) in all animals and ranged between 40.8–41.8 °C. The most evident increase of temperature, by 4.4 °C, was detected in animals exposed to second HS, 24 h after the first one (Table 2). Additionally, we observed a decrease of body mass after exposure to heat stress, which varied between 3.3–9.8 g after single and 4.7–6.7 g after repeated heat stress.

Table 2 Changes in rectal temperature in heat-exposed groups (41 ± 0.5 °C/45 min) measured before and after single and repeated heat stress

Group	Before first HS	After first HS	Before second HS	After second HS
HS24	36.5 ± 0.4 °C	40.9 ± 0.7 °C	/	/
HS48	37.7 ± 1.1 °C	41.8 ± 0.8 °C		
HS72	37.6 ± 0.4 °C	41.8 ± 0.6 °C		
Average	**37.3 ± 0.7 °C**	**41.5 ± 0.7 °C**	**/**	**/**
After HS – before HS	**4.2 °C, p<0.05**		**/**	
HS24 + HS48	36.9 ± 0.5 °C	41.6 ± 0.3 °C	37.2 ± 0.5 °C	41.3 ± 0.4 °C
HS24 + HS48	37.4 ± 0.8 °C	41.5 ± 0.5 °C	36.9 ± 0.3 °C	41.4 ± 0.7 °C
Average	**37.2 ± 0.7 °C**	**41.6 ± 0.4 °C**	**37.1 ± 0.4 °C**	**41.4 ± 0.6 °C**
After HS – before HS	**4.4 °C, p<0.05**		**4.3 °C, p<0.050**	
HS72 + HS48	37.3 ± 0.2 °C	41.8 ± 0.4 °C	36.1 ± 0.4 °C	40.8 ± 0.2 °C
HS72 + HS48	37.4 ± 0.6 °C	41.6 ± 0.4 °C	37.2 ± 0.6 °C	40.9 ± 0.3 °C
Average	**37.4 ± 0.4 °C**	**41.7 ± 0.4 °C**	**36.7 ± 0.5 °C**	**40.9 ± 0.3 °C**
After HS – before HS	**4.3 °C, p<0.05**		**4.2 °C, p<0.05**	

After the HS and before HS between all estimated differences are presented in bold. The statistically significant changes ($p<0.05$) are presented next to the differences in values

Single heat stress with 24 h, 48 h, and 72 h recovery period

Heat stress caused a significantly increased level of HSP70 in the heart, approximately 30× after 24 h and 25× after 48 h recovery. Following 72 h of recovery, we detected a significantly reduced level of HSP70, which was still significantly higher than that of the control group (Fig. 1a). Additionally, we detected a significantly increased concentration of NO, 24 h after the HS, which in the next course of recovery gradually decreased and normalized (Fig. 1b). Moreover, at the same period of recovery there was a significant increase of PKCε levels that later on slowly reduced and normalized 72 h after HS (Fig. 1c). Concerning the COX-2 activity, we
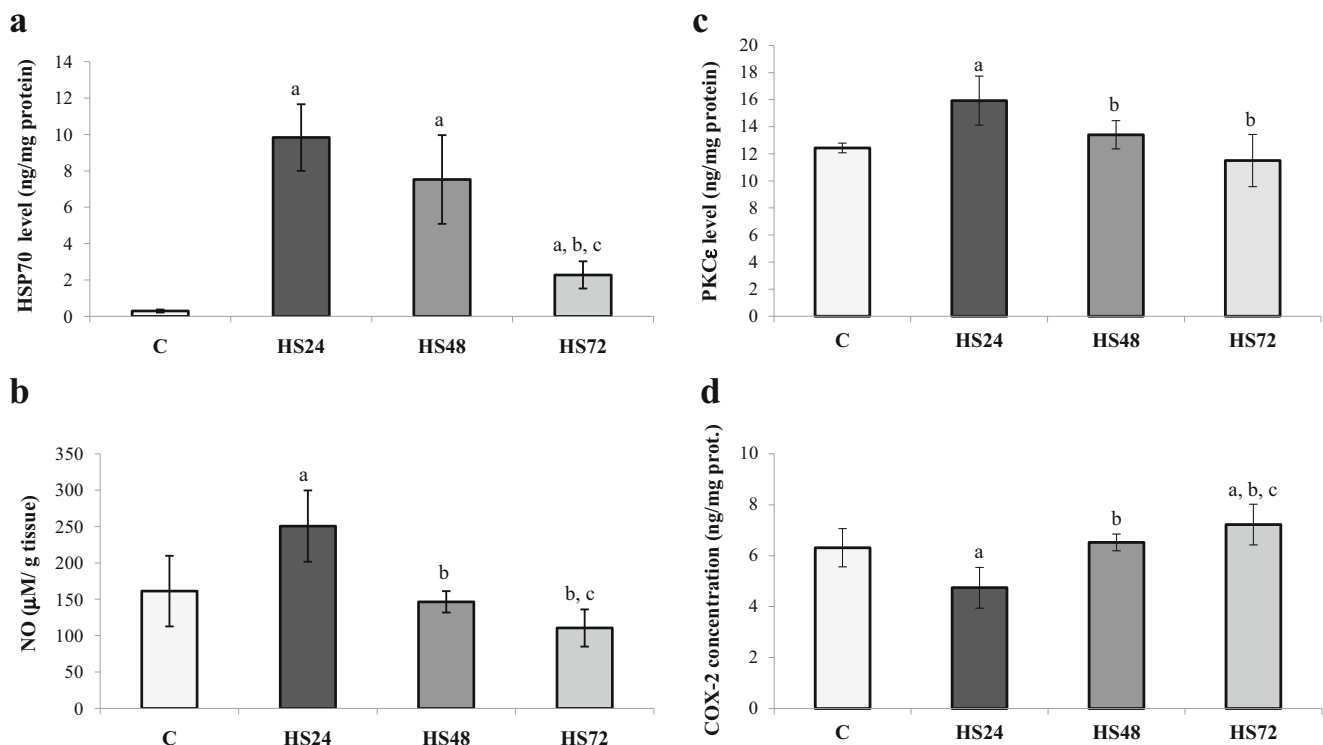

Fig. 1 Changes in cell metabolites in the heart tissue of animals exposed to single heat stress (41 ± 0.5 °C/45 min), with 24 h, 48 h, and 72 h recovery period: **a**. Level of HSP70; **b**. Concentration of NO; **c**. Level of PKC; **d**. Concentration of COX-2. Significant difference $p < 0.05$: a – compared to C; b – compared to HS24; and c – compared to HS48

observed a significantly reduced concentration 24 h after the heat stress, which in the next course of recovery slowly increased, and after 72 h recovery became significantly elevated (Fig. 1d). The exposure to single heat stress caused significant reduction of AST, ALT, and CK serum activity during the first 48 h recovery at room temperature and in the next period of recovery it gradually normalized (Fig. 2a–c).

Repeated HS (24 h after the first heat stress), with 24 h and 48 h recovery period

The results obtained showed a significant decrease of the HSP70 only after 48 h recovery from the second heat stress (Fig. 3a). Exposure of the rats to the second heat stress, 24 h after the first one, caused a significantly reduced concentration of NO, both at 24 h and 48 h recovery period, compared to animals exposed to single heat stress (Fig. 3b). Concerning PKCε levels, a significant decrease of the values was detected only after 48 h recovery from the second heat stress (Fig. 3c). Conversely, we measured a significant increase of COX-2 concentration both at 24 h and 48 h after the second heat stress (Fig. 3d).

All serum enzymes activities (AST, ALT and CK) showed similar dynamics of changes after the second heat stress: increased activities after 24 h recovery and decrease or tendency of normalization after 48 h recovery (Fig. 4a–c).

Repeated HS (72 h after the first heat stress), with 24 h and 48 h recovery period

The second heat stress, 72 h after the first, caused a significant increase of HSP70 level and NO concentration in 24 h-recovery groups, while after the 48 h recovery period, there was a decrease or tendency of normalization of both parameters (Fig. 3a, b). The level of PKCε was not significantly changed during the period of recovery after the second heat stress, 72 h from the first (Fig. 3c). On the other hand, concerning the animals exposed to second heat stress, we measured a significantly increased COX-2 concentration only in the group allowed to recover for 48 h (Fig. 3d).

Concerning the serum enzyme activities, we measured a significant increase of AST and CK only in the animals with 48 h recovery after the second heat stress (Fig. 4a, c). Conversely, the enzyme activity of ALT showed a significant decrease, both at the 24 h and 48 h period of recovery (Fig. 4b).

The significant coefficients of correlation between the estimated groups on the level of estimated parameters are presented in Table 3.
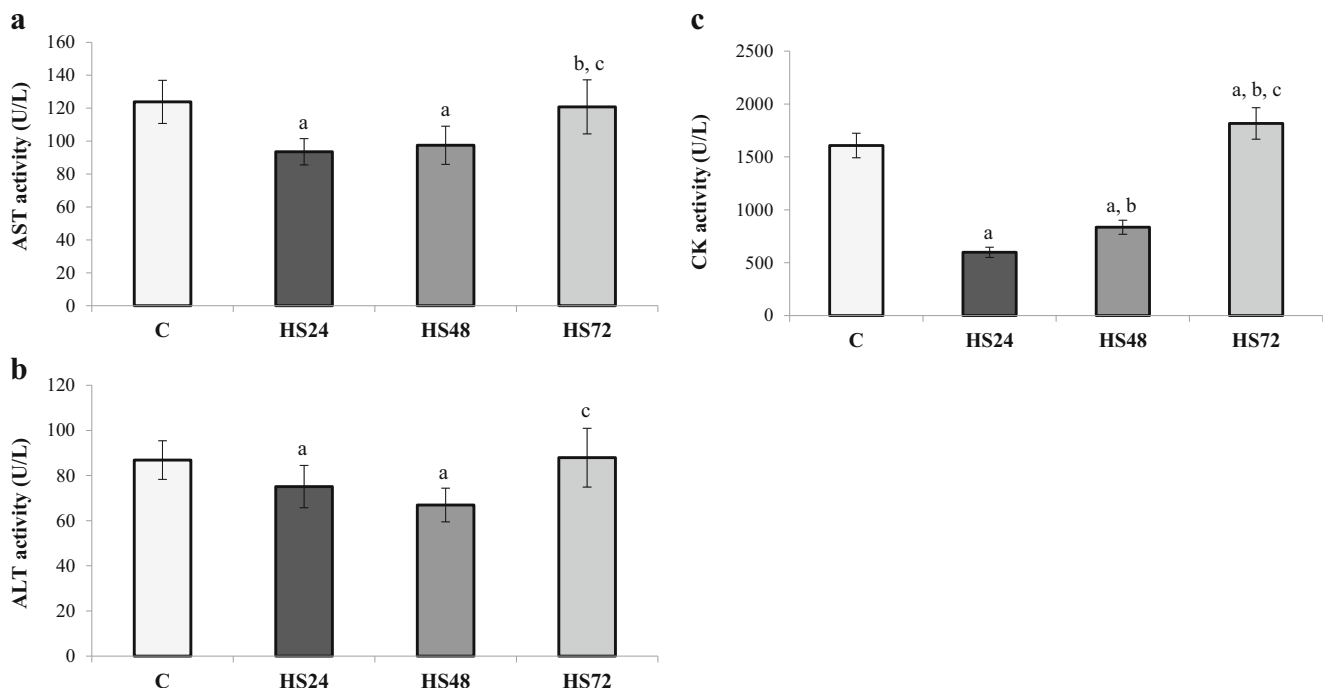

Fig. 2 Changes in serum enzyme activities in animals exposed to a single heat stress (41 ± 0.5 °C/45 min), with 24 h, 48 h, and 72 h recovery period: **a**. Concentration of AST; **b**. Concentration of ALT; **c**. Concentration of CK. Significant difference $p < 0.05$: **a** – compared to **c**; **b** – compared to HS24; and **c** – compared to HS48
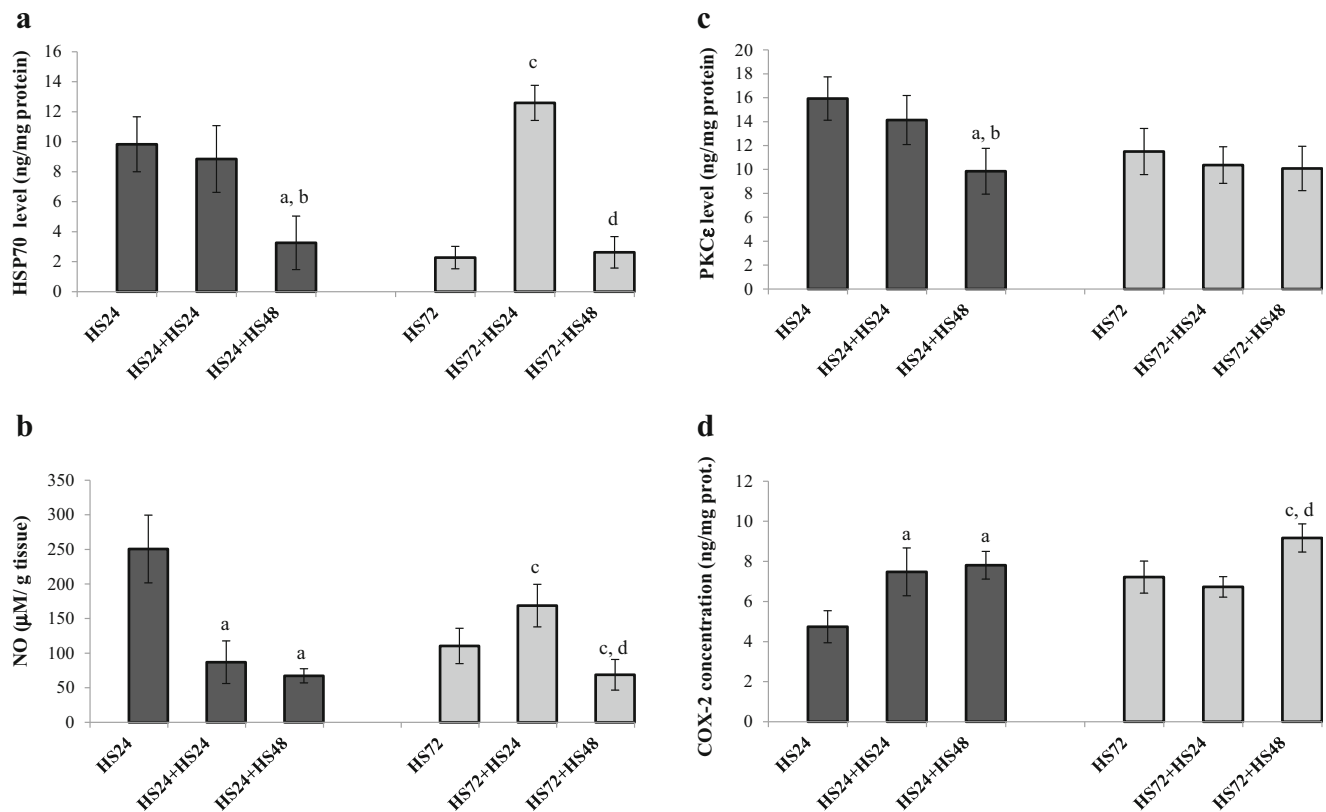
Fig. 3 Changes in cell metabolites in the heart tissue of animals exposed to repeated heat stress (24 h and 72 h after the first), with 24 h and 48 h recovery period: **a**. Level of HSP70; **b**. Concentration of NO; **c**. Level of PKC; **d**. Concentration of COX-2. Significant difference $p < 0.05$: **a** – compared to HS24; **b** – compared to HS24+HS24; **c** – compared to HS72; and **d** – compared to HS72+HS24

Discussion

Single heat stress with 24 h, 48 h, and 72 h recovery periods

In the first part of this study, we estimated the effect of single heat stress over some cellular metabolites and serum enzymes. In this sense, our results reveal that single heat stress caused a significant increase of the concentration of HSP70, NO, and PKC level and decreased COX-2 level 24 h after the heat stress, which in the next course of recovery gradually normalized. These changes were followed by reduction of AST, ALT, and CK serum activities during the first 48 h recovery and gradually normalized in the next period of recovery.

Generally, heat stress exposure activates the cascade reaction of heat shock response (HSR), displayed by increased synthesis of heat shock proteins (HSPs), followed with important changes in the expression pattern of stress associated genes (Horowitz et al. 2004; Horowitz 2007), thus providing a delayed protection from additional insult of the same or different nature (Somji et al. 1999, Joyeux-Faure et al. 2003; Maslov et al. 2010; Hsu et al. 2013). The most evident result that we obtained was the enormous increase of HSP70 (approximately 30× higher than the one of the control group) 24 h

after exposure to heat stress (Fig. 1a), and in the next course of recovery the concentration of HSP70 slowly reduced, but was maintained at a significantly high level 48 h after HS (25× higher than control group). The peak in the first 24 h which coincides with the period of maximum protection of the rat's heart from ischemia-reperfusion injuries as demonstrated previously (Joyeux et al. 1999; Arnaud et al. 2002; Diao et al. 2012). Decline of HSP level, during the later hours of recovery, is correlated with diminished cardiac protection (Qian et al. 1998; Arnaud et al. 2002). On the other hand, some researchers have detected that maximum cardioprotection is afforded 48 h and 72 h following whole-body hyperthermia (42 °C/15 min), and this discrepancy might be due to different methods used to induce hyperthermia (Yamashita et al. 1998; Patel et al. 2001). In vivo studies on rats have shown that prior heat shock has a protective effect over heatstroke-induced hyperthermia, hypotension and bradycardia, and it correlated with HSP72 expression at 16 h, but at 48 h, when HSP72 expression returned to basal values, the above parameters were indistinguishable between the two groups (0 h versus 48 h) (Yang and Lin 1999). Further studies of the above authors showed that pre-inducing HSP70 attenuates heat-stimulated cell autophagy, apoptosis, and damage in the heart, but this requires in vivo confirmation (Hsu et al. 2013).
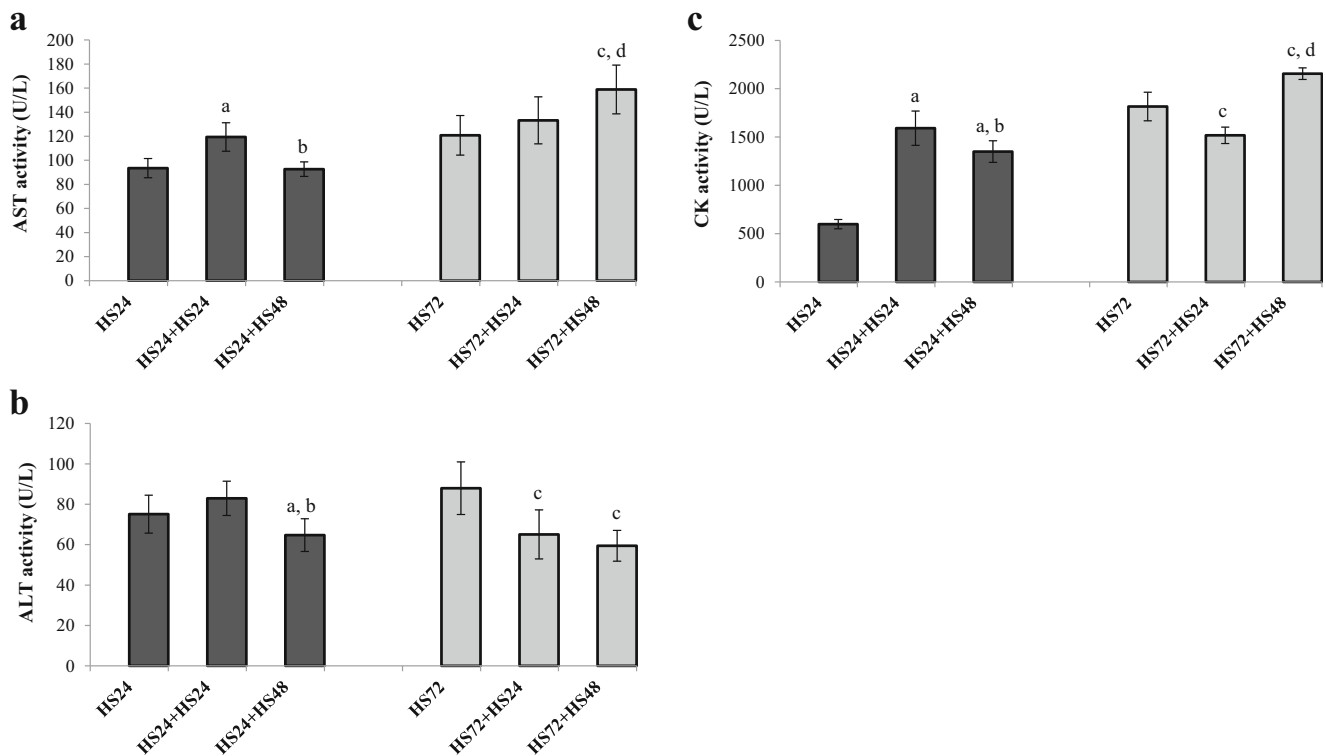
Fig. 4 Changes in serum enzyme activities in animals exposed to repeated heat stress (24 h and 72 h after the first), with 24 h and 48 h recovery period: **a**. Concentration of AST; **b**. Concentration of ALT; **c**. Concentration of CK. Significant difference $p < 0.05$: **a** – compared to HS24; **b** – compared to HS24+HS24; **c** – compared to HS72; and **d** – compared to HS72+HS24

Table 3 Coefficient of correlation (r) with a significance $p < 0.05$, between tested parameters after single and repeated exposure to heat stress

Parameters	r	Parameters	r
Single heat stress			
HSP70:COX-2	−0.530; p < 0.05	AST:ALT	0.551; p < 0.05
HSP70:NO	0.598; p < 0.05	AST:CK	0.746; p < 0.05
HSP70:PKCε	0.698; p < 0.05	ALT:CK	0.677; p < 0.05
NO:PKCε	0.652; p < 0.05		
NO:COX-2	−0.837; p < 0.05		
PKCε:COX-2	−0.709; p < 0.05		
Repeated HS (24 h after the first heat stress)			
HSP70:COX-2	−0.505; p < 0.05	AST:ALT	0.626; p < 0.05
HSP70:NO	0.655; p < 0.05	AST:CK	0.650; p < 0.05
HSP70:PKCε	0.674; p < 0.05	ALT:CK	0.580; p < 0.05
NO:PKCε	0.614; p < 0.05		
NO:COX-2	−0.849; p < 0.05		
PKCε:COX-2	−0.668; p < 0.05		
Repeated HS (72 h after the first HS)			
HSP70:NO	0.799; p < 0.05	AST:CK	0.526; p < 0.05
HSP70:COX-2	−0.600; p < 0.05		
NO:COX-2	−0.822; p < 0.05		

One of the mechanisms that ensures enhanced, heat-induced synthesis of HSP70 is nitric oxide (NO) (Malyshev et al. 1995). Bolli et al. (1998) promoted the "NO hypothesis of late PC", which postulates that NO plays a prominent role both in initiating and in mediating this cardioprotective response. Its role as a trigger of heat stress-induced delayed cardioprotection was demonstrated by Arnaud et al. (2001) who later also showed the role of NO as a mediator of heat stress preconditioning (Arnaud et al. 2003a). Results from our previous study (Velkovski 2012), as well as some other data (Malyshev et al. 1995), showed the most evident increase of the concentration of NO one hour after the heat stress. Still, its accumulation is transient, which after 4 h leads to reduction, gradually reaching the level of the control group 24 h after heat stress (Malyshev et al. 1995).

Furthermore, NO activates the intracellular signal pathway of PKCε. The results that we obtained give insight into the dynamics of changes of the PKCε level in the rat's heart during a prolonged period of recovery after a single exposure to heat stress—a significant increase of PKCε levels in the first 24 h that later on slowly reduced and normalized 72 h after heat stress (Fig. 1c). There was a parallel manner of changes between these two parameters ($r = 0.614$; $p < 0.05$, Table 3). Bolli et al. (1998) reported that NO and ROS produced by cellular stress activates a complex of signal transduction

cascade, which involves mostly PKCε. By using two different pharmacological donors for NO, Ping et al. (1999) managed to induce translocation and activation of PKCε, thus demonstrating that NO-induced activation of PKCε.

Finally, we obtained significant coefficient of correlation between PKCε and HSP70 ($r = 0.674$; $p < 0.05$) as well as with COX-2 ($r = -0.668$; $p < 0.05$), which confirms that NO, HSP70, and COX-2 are a part of the PKCε signal pathway (Table 3). Our results show that 24 h after heat stress the concentration of COX-2 is significantly decreased and during later hours of recovery it gradually reaches and exceeds the levels of the control group (Fig. 1d). According to Salvemini et al. (1993), NO interacts with iron-containing enzymes, such as COX, leading to either a stimulation or inhibition of their enzymatic activity. Stress-induced expression of iNOS is associated with concomitant induction of COX-2 in different cell types, including smooth muscle cells and cardiomyocytes in rats. Its role as a mediator of the cardioprotection, afforded by heat stress preconditioning, was demonstrated with the use of two COX-2 selective inhibitors after which the heat stress-induced reduction in infarct size was abolished (Arnaud et al. 2003b). The highest expression of COX-2 in a rat's heart was detected 24 h after exposure to heat stress (Arnaud et al. 2003b), while Shinmura et al. (2000) observed a rapid increase of COX-2 mRNA levels after brief exposure of rabbits to myocardial ischemia/reperfusion.

Various forms of stress can cause a leakage of cytoplasmic enzymes into the blood in humans and laboratory animals by causing cell injury or increasing cell membrane permeability (Arakawa et al. 1997). Our results showed that 24 h after exposure to heat stress the serum activity of AST, ALT, and CK was significantly reduced and during the next course of recovery it gradually normalized. It is important to mention that all of them have similar dynamics of changes, confirmed by the significant coefficient of correlation between the groups (AST: ALT, $r = 0.551$, $p < 0.05$; AST: CK, $r = 0.746$, $p < 0.05$; ALT: CK, $r = 0.677$, $p < 0.05$, Table 3). Taking into consideration that biochemical changes, such as an increase of the concentration of serum transaminases found in skeletal and cardiac muscle, generally reflects the extent of tissue damage due to thermal injury (Franesoni and Mager 1978), the reduced activity of the enzymes found in our experiment might suggest single exposure to heat stress does not cause damage to the myocardium. Moreover, our study showed that these enzymes, in addition to the other obtained results, rather improve some kind of protection of myocardial cells. These results are in accordance with the previous findings of Diao et al. (2012) which detected that of all estimated groups (24, 48, and 96 h after the heat stress), the 24-h group had the lowest lactate dehydrogenase and creatine kinase activity. Additionally, the same autors found the highest HSP70 expression in the 24-h group which decreased gradually in the 48- and 96-h groups. Our results manifested almost the same dynamics of changes.

Oka et al. (2013) showed that serum AST and ALT were reduced in mice that were pre-exposed to heat stress (42 °C/20 min) and underwent partial hepatectomy, suggesting that heat shock reduced liver damage.

Repeated heat stress (24 h and 72 h after the first heat stress)

The time interval between two or more successive exposures to stress, in order to achieve the best protection of the cardiac function, is still to be determined. To our knowledge, this is the first report that estimated the time interval between two sessions of whole-body hyperthermia in rats. Our results reveal that exposure of rats to a second heat stress, 24 h after the first one, caused a significant reduction of the concentration of NO, PKCε and HSP70 levels and a significant increase of COX-2 concentration. A second heat stress-exposure, 72 h after the first one, caused more expressive changes of HSP70 and NO in the 24 h-recovery groups. The level of PKCε was not significantly changed, but there was significantly increased COX-2 concentration during recovery after the second heat stress (Fig. 3 a, b, c, d). All serum enzymes showed increased activities after repeated exposure to heat stress, in both time intervals (Fig. 4 a,b,c).

According to our results from the effects of single heat stress, the highest level of HSP70 was observed 24 h after the first heat stress, with a slight but significant decrease after 48 h and a tendency of normalization 72 h after the first heat stress. Our previous investigations conducted on HepG2 cells showed that heat stress caused a major up-regulation of HSP70 mRNA in the first 2 h, while HSP70 protein gradually increased and was especially abundant from 2 h to 24 h and declined later on (Miova et al. 2015). In the present study, we observed that the exposure of organisms to a second heat stress, 24 h after the first (when the concentration of HSP70 reached its peak), does not cause additional synthesis of HSP70 in the 24 h recovery period and declines in the 48 h recovery period. We assume that an abundant level of HSP70 can be observed if the time interval between two sessions of consecutive heat stress is longer, i.e., when the increased level of HSP as a result of the first initiation is reduced. We thought that this might be because of energetic resources for initiation of a new session of synthesis, but this needs to be confirmed.

The first evidence about the benefits of repeated physiological or pharmacological stress (extended cardioprotective effect against an ischemic injury) was presented in the work of Hoshida et al. (2002). The authors evaluated the two episodes of the same stress (ex. whole-body hyperthermia) in a 48 h interval and detected a prolonged protection from an ischemic injury of the heart that lasted up to 60 h after two sessions of heat stress.

According to Kregel (2002), the primary function of HSP during cellular stress is to preserve translation and protein

integrity, causing thermally tolerant cells to produce less HSP during the second stress, which suggests that the regulation of HSP synthesis is dependent on their level in the cell. On the other hand, if the time delay after the first heat stress is longer (i.e., 72 h in our study, at a time when the increased synthesis of HSP70 is normalized), 24 h later the levels of HSP70 are significantly increased by almost six times compared to the HS72-group. Based on the results of Moseley (1997) that thermotolerance is transient (24–72 h) and its duration is proportional to the concentration of HSP, the exposure to the second heat stress, 72 h after the first one, causes more intensive HSP70 synthesis. However, these elevated levels of HSP70 are not stable, so in the next course of recovery (48 h after the second heat stress) we found a sharp decrease of HSP70 level (Fig. 3a).

Furthermore, our results show the same dynamics of changes for NO with a high coefficient of correlation (HSP70: NO, $r = 0.655$, $p < 0.05$, Table 3). In animals exposed to the second heat stress 24 h after the first one, we observed a continuous decrease of NO in the period of recovery. On the other hand, in animals exposed to the second heat stress 72 h after the first one, initially (after 24 h recovery) we observed an increased concentration of NO, which later (at 48 h recovery) significantly decreased. Malyshev et al. (1995) point out that one of the mechanisms involved in heat stress-induced synthesis of HSP70 is related to the NO-synthase and increased NO production, since the blockade of NO synthase decreases the level of HSP70, 24 h after the heat stress. However, on the other hand, some recent results (Zhang et al. 2013) have shown that HSP70 is required for iNOS induction and modulates iNOS gene transactivation by either participating in signal transduction or influencing transcriptional factor function, both in mouse macrophages and in endotoxemic mouse in vivo. Some studies have shown that HSP70 overexpression attenuates iNOS induction (Demarco et al. 2004; Howard et al. 2010), suggesting that inhibition of HSP70 augments iNOS induction.

To our knowledge, no previous research has monitored the effect of repeated exposure to heat stress on the content of PKCε and COX-2 in the heart. Previous studies suggested that NO also takes part in the activation of PKCε, either by direct oxidative modification or by activation of phospholipase (Bolli et al. 1998; Bolli 2000). On the other hand, NO might inhibit COX activity (Salvemini et al. 1993), which in our study led to an increased content of COX-2 in the heart after the second heat stress, regardless of the period of recovery. This was confirmed by the significant coefficient of correlation between NO and COX-2 ($r = -0.849$; $p < 0.05$, Table 3).

Contrary to the single heat stress, which caused a decrease of the activities of serum enzymes, the second heat stress caused normalization or even an increase of all the analyzed enzymes (Fig. 4a, b, c). Even though these enzymes are used as biomarkers of tissue damage, we think these changes are not sufficient to confirm myocardial damage taking into consideration all obtained results in this work. Thus, we think further investigations are needed regarding biomarkers of tissue damage. Moreover, according to Hoshida et al. (2002), repeated hyperthermic stress can have a beneficial effect and can cause a decrease of the infarct size.

Conclusion

To our knowledge, this is the first investigation that gives insight into the dynamics of changes of the level of PKCε and COX-2 concentration during recovery after single exposure to HS. In this sense, we found that the most evident changes in the estimated parameters were detected 24 h after exposure to the single heat stress.

Moreover, this study provides the first evidence of changes in the concentration of chemical signals of heat shock response cascade after repeated exposure to heat stress in two different time intervals between two stressors, 24 h and 72 h. In conclusion, a 24 h time interval between two sessions of the same heat stress (41 ± 0.5 °C/45 min) does not cause significant changes in HSR cascade. On the other hand, a longer period of recovery (72 h) between two consecutive sessions of heat stress resulted in a marked increase of the chemical signals. We consider that 72 h recovery is necessary to achieve more expressive changes in mediators (HSP70) and triggers (NO) of heat stress-preconditioning.

Compliance with ethical standards

Conflict of interest We confirm that there is no conflict of interest between authors.

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