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Original Research

Heat-Shock Protein 70–Mediated Heat Preconditioning Attenuates Hepatic Carbohydrate and Oxidative Disturbances in Rats With Type 1 Diabetes

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Key Messages

- Heat preconditioning induces heat-shock protein 70 synthesis, which is known to have protective effects against additional stress, but their production has a time-dependent dynamic after the initial heat stress.
- We found attenuation of streptozotocin-induced metabolic alterations in hepatic carbohydrate metabolism and the oxidative state in heat-preconditioned rats with diabetes.
- The preferred duration between subjection to heat shock and administration of streptozotocin is 24 h rather than 6 h, based on the evident accumulation of heat-shock protein 70 in this time frame.

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ABSTRACT

Objectives: Heat preconditioning and heat-shock protein (HSP) synthesis have significant cytoprotective effects against the development of cellular injury caused by the application of a subsequent stressor, which were found to depend on the time period between the stressors. We aimed to determine the most efficient recovery time (6 h or 24 h) following heat-stress exposure and prior application of diabetic streptozotocin (STZ) on the moderation of carbohydrate and oxidative metabolic disturbances caused by diabetes.

Methods: Experiment animals (Wistar rats) were exposed to acute heat stress at 41±1°C for 45 min, followed by 6-h or 24-h recovery times at room temperature before sacrifice or STZ administration.

Results: Our findings indicate that acute heat stress with 6-h or 24-h recovery periods results in a significant rise in the hepatic heat-shock protein 70 (HSP70) levels (even more so after 24 h), glycogen breakdown and stable glycemia, followed by reduced glycolytic and gluconeogenic activity (after 24 h) (glucose-6-phosphatase, fructose-1,6-bisphosphatase); stimulates antioxidative activity (glutathione peroxidase, glutathione reductase) (after 6 h); and decreases glutathione and catalase activity (after 24 h). Heat preconditioning (with 6-h and 24-h recovery periods) prior to STZ-induced diabetes increases HSP70 levels and causes lower serum glucose levels, higher glycogen and glucose-6-phosphate levels, lower glucose-6-phosphatase levels and glycogen phosphorylase and hexokinase levels but also elevates glutathione reductase and glutathione peroxidase activity compared to untreated STZ animals.

Conclusions: Based on our findings, heat preconditioning and HSP70 induction in rats with type 1 diabetes attenuates STZ-induced metabolic alterations in hepatic carbohydrate metabolism and oxidative states. These changes are more evident at 24 h recovery post-acute heat stress, based on the most evident accumulation of HSP70 in this time frame.

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R É S U M É

Objectifs : Le préconditionnement thermique et la synthèse des protéines de choc thermique (HSP pour *heat-shock protein*) ont des effets cytoprotecteurs importants contre le développement de lésions cellulaires causées par l'application des stresseurs subséquents, qui se sont révélés dépendants de l'intervalle de temps entre les stresseurs. Nous avions pour objectif de déterminer le temps de rétablissement le plus efficace (6 h ou 24 h) après l'exposition au stress thermique et avant l'application de la streptozotocine (STZ), agent diabétogène, sur la modération des perturbations du métabolisme des glucides et du métabolisme oxydatif causées par le diabète.
Méthodes : Nous avons exposé les animaux de laboratoire (rats Wistar) à un stress thermique aigu de 41 ± 1 °C pendant 45 min, puis à des temps de rétablissement de 6 h ou de 24 h à la température du laboratoire avant le sacrifice ou l'administration de la STZ.
Résultats : Nos conclusions montrent que le stress thermique aigu que l'on fait suivre de périodes de rétablissement de 6 h ou de 24 h entraîne une augmentation importante des concentrations de HSP70 dans le foie (encore plus après 24 h), une dégradation du glycogène et une glycémie stable, puis une activité des enzymes glycolytiques et gluconéogéniques réduite (après 24 h) (glucose-6-phosphatase, fructose-1,6-bisphosphatase); stimule l'activité antioxydante (glutathion-peroxydase, glutathion-réductase) (après 6 h); diminue l'activité du glutathion et de la catalase (après 24 h). Le préconditionnement thermique (après des périodes de rétablissement de 6 h et de 24 h) avant l'induction du diabète par la STZ augmente les concentrations de HSP70 et entraîne des concentrations sériques plus faibles en glucose, des concentrations plus élevées en glycogène et en glucose-6-phosphate, des concentrations plus faibles en glucose-6-phosphatase et des concentrations plus faibles en glycogène phosphorylase et en hexokinase, mais augmente également l'activité du glutathion-réductase et du glutathion-peroxydase par rapport aux animaux non traités par la STZ.
Conclusions : Selon nos conclusions, le préconditionnement thermique et l'induction de HSP70 chez les rats diabétiques de type 1 atténuent les altérations métaboliques induites par la STZ du métabolisme des glucides dans le foie et des états de stress oxydatif.
Ces changements sont plus évidents 24 h après le stress thermique aigu selon l'accumulation la plus évidente de HSP70 dans ce cadre temporel.

Introduction

Numerous studies have been focused on analyzing the effects of short-term exposure to sublethal temperatures on cellular mechanisms that have direct influences on the moderation of various pathologic and pathophysiologic conditions. The mechanism for coping with the deleterious effects of heat, which is activated following exposure to acute heat stress (HS), is known as *thermotolerance* (1). This phenomenon is achieved by heat preconditioning, a powerful adaptive and protective phenomenon in which the organism or cells previously exposed to sublethal acute heat stress (AHS) display greater resistance to the effects of a subsequent, more potent stress (2), such as high temperature, ischemia, hypoxia and other stressors (3,4).

The potential protective effect of heat preconditioning against injury caused by exposure to a subsequent stressor is the focus of this research. Current data indicate that preconditioning involves rapid activation of salvage kinases (the first window of protection), followed by increased transcription and translation of cytoprotective proteins, known as heat shock proteins (HSPs) (the second window of protection) (5).

Previous studies indicate that HSPs, specifically HSP70s, have preventive effects against the abnormalities that result from experimental streptozotocin (STZ)-induced diabetes, such as cellular injury caused by denaturized or misfolded proteins to which HSP70 binds (6–9). One of the most important aspects of HSPs' functions is the timeframe of their gene expression and the accumulation of newly synthesized protein molecules in the cells, which is a prerequisite for the protective effects of preconditioning itself (5,10). Taking into account the kinetics of HSP70 gene expression, Cornelussen et al (5) indicated an unstable increase in HSP gene expression 6 h following heat stress, as opposed to sustainably high HSP gene expression after 24 h, which is crucial for efficient cellular protection against the effects of a subsequent, more intensive stressor (such as STZ) when applied during a period of 24 to 48 h after preconditioning (4,11–13). Having in mind these findings, we examined the effects of heat preconditioning with 6-h and 24-h recovery periods at room temperature prior to application of the second stressor, STZ, on carbohydrate and oxidative metabolic parameters.

Diabetes mellitus is a complex metabolic disease characterized by uncontrolled oxidative stress that stimulates endogenous defense mechanisms excessively and leads to the development of diabetic metabolic alterations, such as hyperglycemia, dyslipidemia and protein and lipid modifications (14–17). Diabetes mellitus diminishes hepatic glycogen synthesis (18), suppresses peripheral glucose utilization by decreasing hexokinase (HK), fructose-1,6-bisphosphatase (F1,6Bpase) and phosphofructokinase activity (18,19), induces hepatic de novo glucose synthesis through increased activity of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (20,21) and F1,6Bpase (22) and, finally, results in pronounced hyperglycemia. Considering these findings, we aimed to determine whether heat preconditioning (45 min at 41±1°C) ameliorates the well-known diabetogenic properties of STZ regarding carbohydrate-related disturbances and activates antioxidative protective mechanisms.

We hypothesized that there are heat-preconditioning phenomena in hepatic carbohydrate metabolism and oxidative metabolic levels in heat-preconditioned animals with diabetes that depend on the time of recovery between exposure to heat stress and induction of experimental diabetes.

Methods

Experiment animals and treatment

This experiment was performed on adult (2- to 3-month-old) female Wistar rats (n=45, 250 to 350 g body weight), which were

housed under a 12-h light regimen (6 AM to 6 PM light) and fed laboratory chow and water ad libitum.

All protocols were approved by the Animal Ethics Committee of the University of Saints Cyril and Methodius, Skopje, Republic of 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 European Community Directive 86/609/EEC.

The animals were divided into 2 main groups: control (C) and diabetic (D). Further, each of the groups was divided into 3 subgroups, depending on the period of recovery after the HS. AHS was carried out for 45 min in a special heat chamber with a regulated air temperature of 41±1°C. The rectal temperature was measured with a digital rectal thermometer before and immediately after the AHS. In animals with diabetes, the experimental diabetes was induced by a single intraperitoneal injection of STZ, 65 mg/kg body weight) dissolved in 0.1M citrate buffer, pH 4.5.

The first subgroup (C) was a control group that was maintained at room temperature (20±2°C) for the whole experiment; the second group (HS 6 h) was exposed to AHS followed by 6-h recovery at room temperature before sacrifice; and the third group (HS 24 h) was exposed to AHS, followed by 24-h recovery at room temperature before sacrifice. For the animals with diabetes, the 3 subgroups were the following: D, animals kept at room temperature for the whole period of the experiment and sacrificed 7 days after STZ administration; HS 6 h D, animals exposed to AHS, allowed to recover for 6 h at room temperature, injected with STZ and sacrificed 7 days after STZ administration; and HS 24 h D, animals exposed to AHS, allowed to recover for 24 h at room temperature, injected with STZ and sacrificed 7 days after STZ administration. The organization of the experiment's groups is presented in Table 1.

All of the experiment's animals were anesthetized with Na-thiopental narcosis (45 mg/kg) and sacrificed using a standard laparotomic procedure, always at the same time of day (8 to 10 AM). Isolated liver was washed with cold saline solution, mechanically homogenized and promptly immersed in liquid nitrogen. The tissues were kept at −80°C until analysis, when tissue samples were homogenized by an ultrasonic homogenizer (Instrument 4710, Cole-Parmer, Vernon Hills, Illinois, United States) in several 7- to 10-sec cycles. The homogenization procedure was performed at 0° to 4°C (on ice).

Analytic methods

The protein levels of HSP70 were determined by an appropriate commercial kit (HSP70 EIA; Enzo Life Sciences, Farmingdale, New York, United States) with individual analyses to determine HSP70 levels, which were performed with a microplate reader (Bio-Rad, Kidlington, Oxford, United Kingdom).

Liver glycogen, glucose and glucose-6-phosphate concentrations (23) were determined in perchlorate homogenates and neutralized with 5 mol/L K_2CO_3. Production of nicotinamide adenine dinucleotide phosphate hydrogen at 340 nm was determined in a reaction catalyzed by glucose-6-phoshate dehydrogenase. The substrate concentration was expressed as μmol/g tissue. Determination of HK activity was done in a mitochondrial fraction (10,000g) by measuring the changes in the nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide hydrogen oxidation per reduction per min by using an ultraviolet spectrophotometer; they are expressed as U/g proteins (24). For determining enzyme activity, crude liver homogenates were prepared in an appropriate medium of homogenization (sucrose for G6Pase, lactic acid for fructose-1,6-bisphosphatase and NaF/glycilglycine for glycogen phosphorylase). G6Pase (EC 3.1.3.9) was assayed by the method of Hers (25), and the substrate mixture contained G6Pase (100 mmol/L) and EDTA (2 mmol/L), pH 6.5. The substrate mixture for fructose-1,6-bisphosphatase (EC 3.1.3.11) contained 5 mmol/L fructose-1,6-bisphosphate, 2.5 mmol/L $MgSO_4$, 5 mmol/L$MnSO_4$, 30 mmol/L cysteine and 20 mmol/L serine (26). For the activity of liver glycogen phosphorylase a (EC 2.4.1.1), the substrate mixture contained 50 mmol/L glucose-1-phosphate, 1% glycogen, 2.5 mmol L-1 EDTA, 0.15 mol/L NaF and 0.5 mmol/L caffeine (pH=6.0) (27). Enzyme activity was expressed as nmol Pi min^{-1} mg^{-1} protein. For all 3 enzymes, after incubation in a water bath, the enzyme activity was suppressed by 1 mol/L trichloacetic acid. Later, the amount of released inorganic phosphate was determined by Fiske and Subbarow's method (28). All analyses were performed to determine the amount of suppressed enzyme before and after incubation.

The concentration of glutathione was determined by using a commercial kit (Glutathione Assay Kit, Sigma-Aldrich, Collegeville, Pennsylvania, United States), and the activities of glutathione peroxidase (GPx) and glutathione reductase (GR) were determined by a modification of the commercial kits (Sigma-Aldrich). Catalase (Cat) activity was assessed following Aebi's method (29). Analyses to determine GPx, GR and total glutathione were performed by using a microplate reader (Bio-Rad), and the activities of Cat and total protein were measured by an ultraviolet spectrophotometer. For the interpretation of the activity as a specific enzyme activity in the tissue extracts, the total quantity of the proteins was determined by the Lowry method using bovine serum albumin as a standard (30).

Table 1
Detailed presentation of the experiment's groups and treatments

Experiment treatment	Heat stress (41±1°C/45 min) and 6- or 24-h recovery	STZ-induced diabetes (duration of 7 days)	Sacrifice
Healthy animals			
C	—	—	x
HS 6 h	HS+6 h	—	x
HS 24 h	HS+24 h	—	x
Animals with diabetes			
D	/	STZ+7 days	x
HS 6-h D	HS+6 h	STZ+7 days	x
HS 24-h D	HS+24 h	STZ+7 days	x

C, control; *D*, with diabetes; *HS*, heat stress; *STZ*, streptozotocin.

Statistics

Results are presented as means ± SD. To examine the statistical differences between each group and the control group, we used 1 way ANOVA analyses with Newman-Keuls post hoc tests, and a probability level of $p<0.05$ was used to determine significant difference.

Results

Hepatic HSP70 concentration

Changes in hepatic HSP70 concentrations are illustrated in Figure 1. AHS exposure with a 6-h recovery significantly increased hepatic HSP70 levels (130 times), while longer recovery (24 h) resulted in 410 times higher levels than control values. STZ-induced diabetes results in significantly reduced HSP70 hepatic levels (−50%; $p<0.05$), but heat preconditioning by STZ-induced diabetes led to significantly increased HSP70 after a 24-h recovery period (85%; $p<0.05$), and these animals had ∼60% higher HSP70 values

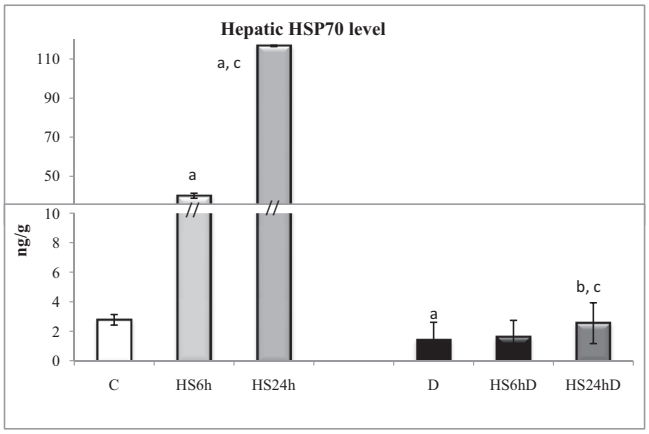

Figure 1. HSP70 levels in the livers of control and heat-preconditioned STZ animals. Significant difference ($p<0.05$): a, relative to control animals (C, HS 6 h; C, HS 24 h, C:D); b, relative to animals with diabetes (D, HS 6 h; D, HS 24 h); c, between acute heat stress-exposed animals with 6 and 24 h of recovery (HS 6 h:HS 24 h; HS 6 h D:HS 24 h D). The animals were sacrificed 7 days after administration of streptozotocin. *C,* control animals; *D,* animals with diabetes; *HS6h, HS24h,* animals exposed to acute heat stress (41±1°C/45 min) followed by 6- or 24-h recovery periods before sacrifice; *HS6hD, HS24hD,* heat-preconditioned (41±1°C/45 min) animals with diabetes and with 6- or 24-h recovery periods before administration of streptozotocin.

than corresponding animals (HS 6-h D) after a 6 h recovery (Figure 1) (Table 2).

Blood (circulating) and liver glucose concentration, G6Pase concentration and glycogen content

Changes in blood and hepatic glucose concentrations as well as changes in the G6Pase concentrations and hepatic glycogen contents are presented in Figure 2, A-D. Nonsignificant changes in circulating glucose as well as hepatic glucose and G6Pase concentrations were observed in AHS-exposed groups regardless of the recovery period when compared to control animals. Glycogen content was diminished in AHS-exposed animals, and this reduction was more pronounced after 24-h recovery periods.

STZ-induced diabetes significantly increases blood and liver glucose concentrations, followed by reductions in hepatic G6Pase and glycogen levels. Heat-preconditioned animals with STZ-induced diabetes showed significantly reduced circulating glucose levels (−15%) after 24-h recovery periods compared to shorter 6-h recovery times. No significant changes were observed regarding hepatic glucose levels. Heat preconditioning prior to STZ-induced diabetes caused a less pronounced reduction in G6Pase and glycogen levels in the liver and significantly higher substrate levels (10 times higher G6Pase and 15 to 17 times higher glycogen levels) after 6- and 24-h recovery periods compared to animals with STZ-induced diabetes at room temperature (Figure 2) (Table 3).

Hepatic HK, glycogen phosphorylase a, G6Pase and fructose-1,6-bisphospatase activity

Changes in hepatic HK, glycogen phosphorylase a, G6Pase and fructose-1,6-bisphospatase activity are presented in Figure 3, A-D. AHS with a 6-h recovery period caused significantly reduced HK activity, while no significant alterations, regardless of the recovery times, were observed in GP *a* and G6Pase activity. G6Pase as well as F1,6BPase activities were significantly reduced after a 24-h recovery period.

STZ-induced diabetes in rats left at room temperature caused significantly reduced hepatic HK and glycogen phosphorylase a GP *a* activity, as well as increased G6Pase activity. Except for the F16BPase, all of the enzymes were significantly lower in heat-preconditioned rats with diabetes, regardless of recovery duration (Figure 3) (Table 4).

Table 2
Statistical and percentage analyses of hepatic HSP70 levels

Liver HSP70 levels (ng/g)					
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	0.05	1338.6	C:D	0.05	−50.5
C:HS 24 h	0.05	4101.9	D:HS 6 h D	ns	16.8
HS 6 h:HS 24 h	0.05	192.1	D:HS 24 h D	0.05	85.1
			HS 6 h D:HS 24 h D	0.05	58.5

C, control; *D,* with diabetes; *HS,* heat stress; *HSP70,* heat-shock protein 70.

Hepatic glutathione concentration, glutathione peroxidase, glutathione reductase and Cat activity

Changes in hepatic glutathione concentration, as well as GPx, GR and Cat activity, are presented in Figure 4 (A-D). Levels of total glutathione and Cat activity were significantly reduced after 24-h recovery periods following AHS, while GPx and GR activities were increased only in the first 6 h of recovery, followed by nonsignificant changes after prolonged recovery periods (24 h).

STZ-induced diabetes in rats left at room temperature caused reduced glutathione concentration and Cat activity along with elevated hepatic GPx and GR activity. Heat preconditioning prior to STZ-induced diabetes reduced liver glutathione concentrations in the first 6 h after HS exposure by 41%, but no significant changes were observed after 24-h recovery periods. Both GPx and GR activities were significantly increased in heat-preconditioned STZ-induced diabetes in rats with 6- and 24-h recovery periods (GPx: 44% and 47%; GR: 31% and 49%, respectively; $p<0.05$), whereas no significant changes were observed in Cat activity, regardless of recovery periods following heat stress (Figure 4) (Table 5).

Discussion

AHS exposure and different periods of recovery

Changes in hepatic HSP70 levels, depending on recovery times, were examined in terms of key carbohydrate and oxidative metabolic parameters in the livers of heat-exposed rats. The results of this study indicate that exposure to AHS (41±1°C/45 min) results in multiple increases in hepatic HSP70 concentrations, with this increase being 192% higher after 24 h of recovery time (410 times control values) compared to 6-h of recovery time (130 times control values) (Figure 1). In our previous work (31), we found that HSP70 mRNA expression peaked (about a 50- to 200-fold increase) just after HS, was maintained until 2 h post-HS (0 h, 1 h, 2 h), and subsequently waned, reaching control values or even slightly lower. On the other hand, the increase in HSP70 levels manifested a certain time delay and followed a more gradual dynamic than mRNA; it peaked from 6 h to 24 h (approximately a 6- to 14-fold increase), and a slow decrease took place thereafter without reaching control values (untreated cells), even at 48 h after treatment. Moreover, according to Wang et al, the initial HSP70 expression peak is unstable, followed by stable and sustainable HSP70 expression after 24 h, which correlates with the most effective tissue protection by HSPs found previously by others (9,32,33).

Our results indicate a substantial depletion of hepatic glycogen stores in rats exposed to HS, especially after a 24-h recovery time, followed by stable blood and hepatic glucose, G6P levels and glycogenolytic (GP *a*) activity, regardless of recovery time, as well as reduced gluconeogenic (G6Pase, F6BPase) activity after 24-h recovery periods (Figures 2 and 3). The observed results are

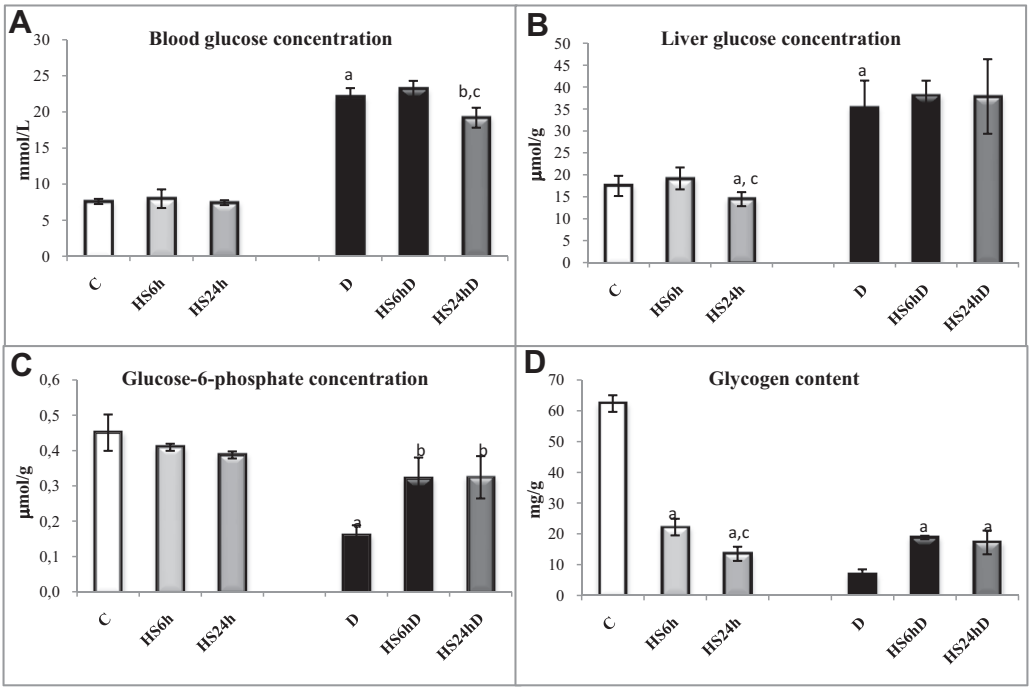

Figure 2. A, Blood glucose concentration. B, liver glucose concentration. C, glucose-6-phosphate concentration. D, glycogen content in the livers of control and heat-preconditioned STZ animals. Significant difference ($p<0.05$): a, relative to control animals (C: HS6h, C: HS24h, C:D). b, relative to diabetic animals (D: HS6hD, D: HS24hD). c, between acute heat stress-exposed animals with 6- and 24-h recovery periods (HS6h: HS24h; HS6hD:HS24hD). *C,* control animals: *D,* animals with diabetes; *HS6h, HS24h,* animals exposed to acute heat stress (41±1°C/45 min) followed by 6- or 24-h recovery periods before sacrifice; *HS6hD, HS24hD,* heat-preconditioned (41±1°C/45 min) animals with diabetes with 6- or 24-h recovery periods before administration of streptozotocin.

indicators of stimulated peripheral glucose utilization as a result of stress-induced prompt hypothalamo-pituitary-adrenal axis activation (34) and adrenalin release (35,36). Stimulated glucose influx and peripheral glycolytic activity, mediated by increased glucose transporter 1 activity, insulin-independent glucose transport and stimulated phosphofructokinase activity, have been found after exposure to stress (37–39). Additionally, the significantly lower hepatic HK activity found in the first 6 h following AHS (−42%) is probably a result of stimulated glucose production by glycogen breakdown, so that the hepatic cells do not require additional glucose uptake, and excess glucose can be directed toward peripheral tissues and used as a source for adenosine triphosphate. Similar reduction in hepatic HK activity following AHS has been observed previously (33,40) in the first hour after AHS on an average of ∼40%. Unlike shorter periods of recovery (1 to 6 h), our results indicate that HK activity normalizes 24 h following AHS, which indicates an increased hepatic glucose demand in order to replenish exhausted glycogen stores. Reduction in hepatic gluconeogenic activity—a 40% reduction in G6Pase, a 93% reduction in G6P and a 20% reduction in liver F1,6BPase after 24 h of recovery—has been observed previously (41) as has a 60% decrease in hepatic F1,6BPase 1 h following heat stress (41±0; 5°C/1 h) (42). Inhibited gluconeogenic flux is most probably due to activation of the hypothalamo-pituitary-adrenal axis following exposure to HS, which results in intensified glycogenolysis as the primary source of glucose molecules, which are used as cellular energy sources because of reduced de novo glucose production.

Table 3
Statistical and percentage analyses of blood and liver glucose concentration, G6P concentration and glycogen content

Blood glucose concentration (mmol/L)			Liver glucose concentration (µmol/g)		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	ns	5.0	C:HS 6 h	n.s.	9.8
C:HS 24 h	ns	−2.2	C:HS 24 h	0.05	−17.2
HS 6 h:HS 24 h	ns	−6.9	HS 6 h:HS 24 h	0.05	−24.6
C:D	0.05	190.4	C:D	0.05	102.5
D:HS 6 h D	ns	5.0	D:HS 6 h D	ns	7.5
D:HS 24 h D	0.05	−15.0	D:HS 24 h D	ns	6.9
HS 6 h D:HS 24 h D	0.05	−17.2	HS 6 h D:HS 24 h D	ns	−0.5
G6P concentration (µmol/g)			**Glycogen content (mg/g)**		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	ns	−9.2	C:HS 6 h	0.05	−64.3
C:HS 24 h	ns	−14.0	C:HS 24 h	0.05	−78.3
HS 6 h:HS 24 h	ns	5.3	HS 6 h:HS 24 h	0.05	−39.1
C:D	0.05	−64.6	C:D	0.05	−89.1
D:HS 6 h D	0.05	101.0	D:HS 6 h D	0.05	177.7
D:HS 24 h D	0.05	103.5	D:HS 24 h D	0.05	154.1
HS 6 h D:HS 24 h D	ns	1.3	HS 6 h D:HS 24 h D	ns	−8.5

C, control; *D,* with diabetes; *G6P,* glucose-6-phosphatase; *HS,* heat stress; *ns,* nonsignificant.

Regarding hepatic oxidative metabolism, glutathione concentration (Figure 4A) is higher immediately after HS exposure in order to ensure more effective cellular oxidative protection. Prolonged recovery time results in reduced glutathione, most probably due to the reduced amount of reactive oxygen species, so that glutathione released from the liver circulates to the kidneys, which are the only organs able to accept intact glutathione (42). Increased GR and GPx activity in the first hours following HS exposure is important for efficient oxidized glutathione reduction and glutathione renewal as well as free-radical scavenging, which is an asset for ensuring adequate cellular oxidative balance. Using malondialdehyde as an accurate marker for lipid peroxidation, Mladenov et al and Hadzi-Petrushev et al (43,44) have confirmed the finding that exposure to acute heat induces lipid peroxidation in the liver, which is correlated with loss of antioxidant capacity (45). Additionally, heat-induced oxidative stress initially stimulates Cat activity as a protective mechanism, whereas in the following period of 24 h, Cat

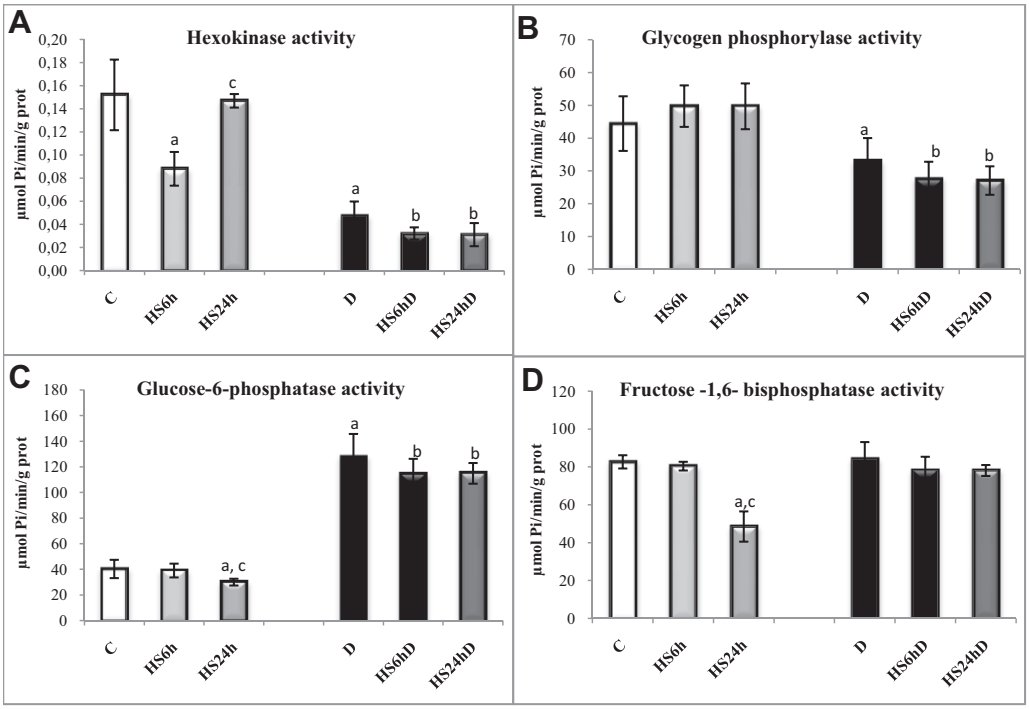

Figure 3. Hepatic hexokinase (HK). A, glycogen phosphorylase a; B, glucose-6-phosphatase; C, fructose-1,6-bisphosphatase activity; D, in the liver of control and heat-preconditioned STZ animals. Significant difference ($p<0.05$): a, relative to control animals (C:HS6h; C:HS24h; C:D). b, relative to diabetic animals (D:HS6hD, D:HS24hD). c, between acute heat stress-exposed animals with 6- or 24-h recovery periods (HS6h:HS24h; HS6hD:HS24hD). *C,* control animals; *D,* animals with diabetes; *HS6h, HS24h,* animals exposed to acute heat stress (41 ± 1°C/45 min) followed by 6- or 24-h recovery periods before sacrifice; *HS6hD, HS24hD,* heat-preconditioned (41 ± 1°C/45 min) animals with diabetes with 6- or 24-h recovery periods before administration of streptozotocin.

activity progressively decreases, with concomitant activation of auxiliary oxidative protection (GPx, GR). Similarly, nonsignificant changes in Cat activity 24 h after HS were found in rats' kidneys (46) as well as in rats' livers (47,48).

Finally, our results indicate that unlike the transient changes observed after 6-h recovery periods, intensive HSP70 hepatic accumulation after 24-h recovery periods contributes more efficiently to the normalizing of hepatic carbohydrates and oxidative metabolic alterations caused by AHS exposure.

Table 4
Statistical and percentage analyses of hepatic enzyme activities of HK, GP *a*, G6Pase and F16BPase

HK activity (µmol Pi/min/g prot)			GP *a* activity (µmol Pi/min/g prot)		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	0.05	−42.1	C:HS 6 h	ns	12.0
C:HS 24 h	ns	−3.2	C:HS 24 h	ns	11.9
HS 6 h:HS 24 h[c]	0.05	67.1	HS 6 h:HS 24 h	ns	−0.1
C:D	0.05	−69.0	C:D	0.05	−25.8
D:HS 6 h D	0.05	−32.3	D:HS 6 h D	0.05	−16.4
D:HS 24 h D	0.05	−35.0	D:HS 24 h D	0.05	−17.9
HS 6 h D:HS 24 h D	ns	−4.1	HS 6 h D:HS 24 h D	ns	−1.8
G6Pase activity (µmol Pi/min/g prot)			F1,6BPase activity (µmol Pi/min/g prot)		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	ns	−3.0	C:HS 6 h	ns	−2.7
C:HS 24 h	0.05	−25.5	C:HS 24 h	0.05	−41.3
HS 6 h:HS 24 h	0.05	−23.1	HS 6 h:HS 24 h	0.05	−39.7
C:D	0.05	218.5	C:D	ns	1.7
D:HS 6 h D	0.05	−11.0	D:HS 6 h D	ns	−7.0
D:HS 24 h D	0.05	−10.5	D:HS 24 h D	ns	−7.1
HS 6 h D:HS 24 h D	ns	0.6	HS 6 h D:HS 24 h D	ns	−0.1

C, control; *D,* with diabetes; *F16Bpase,* fructose-1,6-bisphosphatase; *G6Pase,* glucose-6-phosphatase; *GP* a, glycogen phosphorylase *a*; *HK,* hexokinase; *HS,* heat stress; *Pi,* inorganic phosphate; *Prot,* protein.

Heat preconditioning and STZ-induced diabetes

One of the goals of this study was to examine the possible protective effects of heat preconditioning on the development of STZ-induced disturbances in rats' hepatic carbohydrate and oxidative metabolism. Our results indicate that STZ-induced diabetes reduces the concentration of the principal mediator molecules, HSP70, in this protective effect, compared to control animals left at room temperature (−50%; $p<0.05$) (Figure 1). It might be that insulin deficiency, which leads to inhibited phosphorylation of PI3K and PKB and stimulates GSK-3, results in reduced HSF-1 in insulin-sensitive tissues and reduced HSP synthesis (49). However, we found that previous heat exposure with longer periods of recovery (24 h) significantly increases HSP70 levels in heat-stressed rats with diabetes (85%; $p<0.05$). These results are most likely correlated with previous data concerning decreased pancreatic poly (adenosine diphosphate [ADP] ribose) polymerase (PARP) activity 24 h following HS exposure (50) because PARP's catalytic activity affects the transcription of specific genes such as HSP70 genes (51,52). HS suppresses STZ-induced nicotinamide adenine dinucleotide cellular depletion, a main cause of pancreatic beta-cell death, by inhibiting PARP activity, adenosine triphosphate loss and cell lysis (5), and PARP inhibition activates the HSP70 gene, with which PARP is associated in a histone complex, and stimulates increased HSP70 gene expression (53).

Stimulated HSP70 synthesis contributes to some improvement in key hepatic carbohydrate enzyme and substrate activities and levels in heat-preconditioned STZ rats. Thus, we found that a longer recovery (24 h) after HP results in less pronounced changes regarding circulating glucose levels, glycogen depletion, G6P content (Figure 2) and G6Pase activity (Figure 3) compared to heat-preconditioned STZ rats with 6-h recovery periods and STZ rats remaining at room temperature. This might be a result of the higher

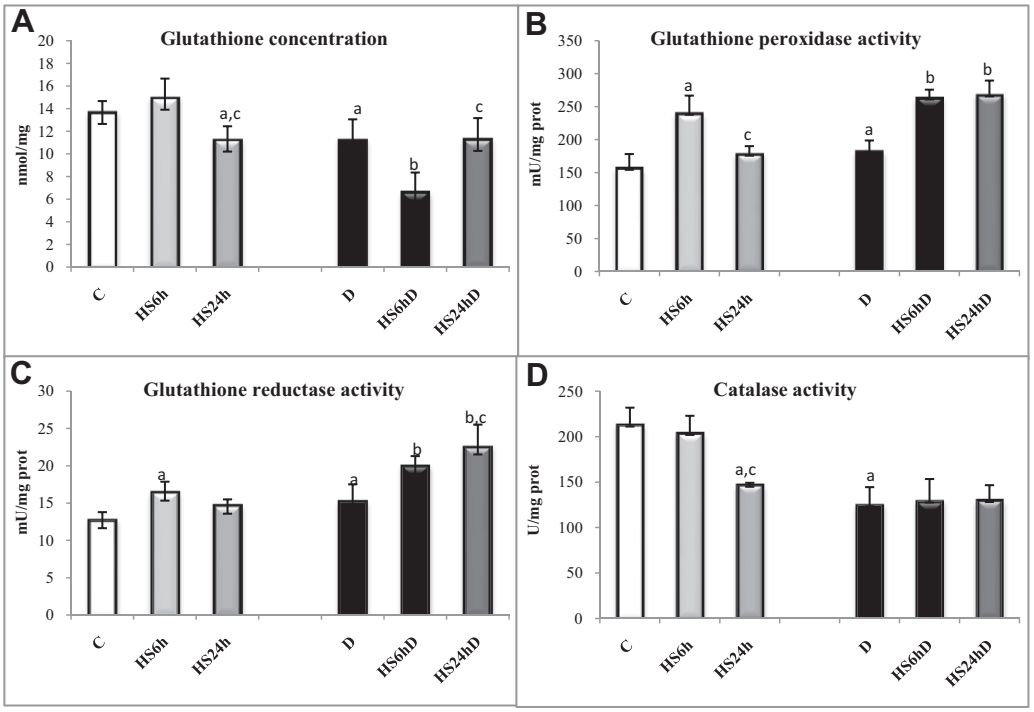

Figure 4. Hepatic total glutathione levels. A, glutathione peroxidase. B, glutathione reductase. C, catalase activity. D, in the livers of control and heat-preconditioned STZ animals. Significant difference ($p<0.05$): a, relative to control animals (C:HS6h, C:HS24h, C:D). b, relative to diabetic animals (D:HS6hD, D:HS24hD). c, between animals exposed to acute heat stress with 6- or 24-h recovery periods (HS6h:HS24h, HS6hD:HS24hD). *C,* control animals; *D,* animals with diabetes; *HS6h, HS24h,* animals exposed to acute heat stress (41 ± 1°C/45 min) followed by 6- or 24-h recovery periods before sacrifice; *HS6hD, HS24hD,* heat-preconditioned (41 ± 1°C/45 min) animals with diabetes with 6- or 24-h recovery periods before administration of streptozotocin.

HSP accumulation 24 h after AHS that has been found in previous studies of heat-exposed cell cultures (10) and rats' livers (54).

Our previous results (40) have also found less intensive glycogen depletion in heat-preconditioned STZ rats with 1 h and 24 h of recovery after AHS compared to STZ rats left at room temperature (176% and 205%, respectively), followed by decreased GP *a* acitivity. In line with these findings, we found less intensive glycogen breakdown in heat-preconditioned STZ rats (6 h and 24 h), which is probably due to decreased GP *a* activity and higher hepatic glucose compared to non-STZ rats with diabetes after AHS, a possible protective mechanism against severe glycogen loss and the occurrence of intensive hyperglycemia during stress and diabetes mediated by preserved islet insulin activity.

Table 5
Statistical and percentage analyses of enzyme activities of glutathione concentration, GPx, GR and Cat activity

Glutathione concentration (nmol/mg prot)			GPx activity (mU/mg prot)		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	ns	9.6	C:HS 6 h	0.05	53.8
C:HS 24 h	0.05	−18.1	C:HS 24 h	ns	13.8
HS 6 h:HS 24 h	0.05	−25.3	HS 6 h:HS 24 h	0.05	−26.0
C:D	0.05	−18.3	C:D	0.05	16.7
D:HS 6 h D	0.05	−41.3	D:HS 6 h D	0.05	44.5
D:HS 24 h D	ns	1.0	D:HS 24 h D	0.05	47.1
HS 6 h D:HS 24 h D	0.05	71.9	HS 6 h D:HS 24 h D	ns	1.8
GR activity (mU/mg prot)			**Cat activity (U/mg prot)**		
Compared groups	p<	%	Compared groups	p<	%
C:HS 6 h	0.05	29.2	C:HS 6 h	ns	−4.4
C:HS 24 h	n.s	15.3	C:HS 24 h	0.05	−31.2
HS 6 h:HS 24 h	n.s	−10.8	HS 6 h:HS 24 h	0.05	−28.1
C:D	0.05	19.6	C:D	0.05	−41.4
D:HS 6 h D	0.05	31.3	D:HS 6 h D	ns	3.4
D:HS 24 h D	0.05	49.0	D:HS 24 h D	ns	3.9
HS 6 h D:HS 24 h D	0.05	13.5	HS 6 h D:HS 24 h D	ns	0.5

C, control; *Cat,* catalase; *D,* with diabetes; *GPx,* glutathione peroxidase; *GR,* glutathione reductase; *HS,* heat stress; *prot,* protein.

Another interesting feature of the hepatic carbohydrate metabolic activity of heat-preconditioned STZ rats is the significantly decreased activation of the common gluconeogenic/glycogenolytic enzyme, G6Pase, regardless of the recovery time (−11%, 6 and 24 h) (Figure 3) compared to STZ rats left at room temperature. Reduced G6Pase activity as a result of partially preserved islet insulin activity is another contributing factor for maintaining hepatic glycogen stores in heat-preconditioned STZ rats. Preserved insulin activity contributes to a decreased level of de novo glucose production, higher G6P hepatic level (by 103% at both 6 h and 24 h of recovery) and the subsequent redirecting of G6P toward glycogen resynthesis. In addition to this remark, Gomis et al (55) indicated that G6P derived from gluconeogenic precursors, as well as G6P produced by glucose phosphorylation in cultivated hepatocytes, makes a mutual cellular pool and stimulates glycogen synthesis activity toward glycogen accumulation. On the other hand, there is insignificantly reduced HK activity in heat-preconditioned STZ rats, regardless of recovery time (6 h or 24 h), but these changes did not affect hepatic glucose content.

Although the STZ model that we used is related to type 1 diabetes, some comparisons can be made with different models of rat diabetes or isolated cells. Namely, maintaining high HSP levels is correlated with the most efficient protectiveness of preconditioning (9) and is probably related to the prevention of pancreatic beta-cell damage and subsequent insulin loss due to STZ (5). Namely, the last study clearly indicates that heat exposure at 43°C for 90 min reduces beta-cell lysis resulting from STZ by 90%, suppresses nicotinamide adenine dinucleotide depletion and preserves islet insulin activity (5). In addition to this presumption, previous studies in obese rodents and humans with diabetes reveal that

thermal therapy improves tissue insulin sensitivity (56–58), increases basal and circulating insulin levels and decreases adipose tissue mobilization in a variety of species (59). Stimulated insulin action might explain the increase in whole-body glucose utilization observed during HS (60). Additionally, Li et al (61) previously noted that insulin administration increases myocardial HSP70 expression and protein levels and potentiates HSP synthesis in response to HS, and this HSP response is independent of nitric oxide signaling and is secondary to HSF-1 activation.

Regarding changes in oxidative metabolism, we found that heat preconditioning of STZ diabetes initially decreased hepatic glutathione concentration by 41% in the first 6 h of recovery but not after 24 h compared to STZ rats left at room temperature (D: HS24D, n.s.). We assume that reduced glutathione in the first 6 h is a defense mechanism against stress-induced accumulation of reactive oxygen species, which depletes cellular glutathione, but the level increases after 24 h to that established in STZ rats left at room temperature. However, reduced glutathione is accompanied by stimulated activities of GPx and GR, both after 6 h and 24 h of recovery, as a hepatic defense reaction to overproduction of reactive oxygen species induced by STZ and heat exposure. Previous data (46) also indicate higher glutathione, along with increased GR and Cat activity and lower GPx activity in kidneys of heat-preconditioned STZ rats after 2 and 14 days. However, stimulated hepatic glutathione metabolism, in our study, was accompanied by nonsignificant changes in Cat activity in heat preconditioned STZ rats regardless of the recovery time, which indicates that previous heat exposure does not influence Cat activity in STZ-induced diabetes. Namely, the primary cellular oxidative defense mechanism in STZ-induced diabetes, regardless of previous HS exposure, is activation of the glutathione metabolism on account of Cat activity.

Investigations made by Bathaie et al (62) concerning exposure of rats with STZ-induced diabetes to hot-tub therapy showed significant improvements in lipid profiles, antioxidant capacities, insulin secretions and serum HSP70 levels, as well as significant decreases in advanced glycation end product formation compared to untreated rats with STZ-induced diabetes. Still, according to the author, hot-tub therapy had a significant borderline effect on weight and fasting blood glucose levels.

Although our research study was done in an animal model for type 1 diabetes, our findings hold a possibility for further clinical investigations of the beneficial effect of HSP induction and synthesis in patients with lifestyle-related illnesses such as diabetes. Regarding the possible therapeutic use of heat preconditioning in humans, previous studies of patients with type 2 diabetes who were hot-tub immersed for 30 min a day, 6 days a week during a period of 3 weeks, indicate significantly reduced circulating glucose, a 1% drop in glycated hemoglobin levels, a trend toward weight loss and improvement in diabetes-related neuropathic symptoms. An explanation for the beneficial effects of hyperthermia centres upon their ability to induce the cellular stress response (the heat-shock response) and restore cellular homeostasis (56,63).

Conclusions

Based on our findings, we can conclude that heat preconditioning of rats with STZ-induced diabetes and 6-h and 24-h recovery periods attenuate STZ-induced metabolic disturbances. These changes are more evident in heat-preconditioned rats with diabetes and with 24-h recovery periods, as manifested by lower serum glucose levels, higher glycogen and G6P levels, lower G6Pase levels as well as lower GP *a* and HK compared to control rats with diabetes. Also, there was stimulation of glutathione metabolic oxidative defense. We consider that higher HSP70 concentrations after 24-h recovery periods are the key physiologic conditioners for these alterations.

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Author Disclosures

Conflicts of interest: None.

Author Contributions

KGE was a PhD student and this is part of her doctoral thesis. SDK and BM are mentor and co-mentor of the thesis and principal investigators in the lab. We confirm that all authors have approved the final version of the paper.

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