Physiological and pharmacological inductors of HSP70 enhance the anti-oxidative defense mechanisms of the liver and pancreas in diabetic rats

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Canadian Journal of Physiology and Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjpp-2017-0394.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>07-Aug-2017</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Dimitrovska, Maja; Institute of Biology Faculty of Natural Sciences and Mathematics University &quot;St Cyrilus and Methodius&quot;, Department of Experimental Physiology and Biochemistry Dervisevik, Mirsada; Institute of Biology Faculty of Natural Sciences and Mathematics University &quot;St Cyrilus and Methodius&quot;, Department of Experimental Physiology and Biochemistry Cipanovska, Natasa; Institute of Biology Faculty of Natural Sciences and Mathematics University &quot;St Cyrilus and Methodius&quot;, Department of Experimental Physiology and Biochemistry Gerazova, Katerina; Department of Experimental Physiology and Biochemistry, Institute of Biology, Faculty of Natural Sciences and Mathematics, University &quot;St Cyril and Methodius&quot;, Skopje, R. Macedonia Dinevska – Kjovkarovska, Suzana; Institute of Biology Faculty of Natural Sciences and Mathematics University &quot;St Cyrilus and Methodius&quot;, Department of Experimental Physiology and Biochemistry Miova, Biljana; Faculty of Natural Sciences, Department of Experimental Physiology and Biochemistry, Institute of Biology</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>N/A</td>
</tr>
<tr>
<td>Keyword:</td>
<td>heat preconditioning, aspirin, experimental diabetes, HSP70, oxidative status</td>
</tr>
</tbody>
</table>

https://mc06.manuscriptcentral.com/cjpp-pubs
Physiological and pharmacological inductors of HSP70 enhance the anti-oxidative defense mechanisms of the liver and pancreas in diabetic rats

Maja Dimitrovska, Mirsada Dervisevik, Natasa Cipanovska, Katerina Gerazova, Suzana Dinevska-Kjovkarovska and Biljana Miova*

Department of Experimental Physiology and Biochemistry, Institute of Biology, Faculty of Natural Sciences and Mathematics, University “St Cyril and Methodius”, Skopje, R. Macedonia

Maja Dimitrovska, MSci
maja_bt2003@yahoo.com

Mirsada Dervisevik, MSci
mirsadadervisevik@yahoo.com

Natasa Cipanovska, MSci
natasacipan@hotmail.com

Katerina Gerazova
kgerazova@gmail.com

Prof. Suzana Dinevska – Kjovkarovska, PhD
suzanadk@pmf.ukim.mk

*Corresponding author:
Prof. Biljana Miova, PhD
Department of Experimental Physiology and Biochemistry, Institute of Biology
Faculty of Natural Sciences and Mathematics, University “St Cyril and Methodius”, Skopje, R. Macedonia
Arhemedova 3, 1000 Skopje, R. Macedonia
Phone: +389 2 3249604
Fax. +389 2 3228141
E-mail: bmiova@pmf.ukim.mk; bmiova@yahoo.com

We confirm that all authors have approved the final version of the paper.
We confirm that there is no conflict of interest between authors.

The research was performed at the Department of Experimental Physiology and Biochemistry, Institute of Biology, Faculty of Natural Sciences and Mathematics, University “St Cyril and Methodius”, Skopje, R. Macedonia

This research did not receive any specific grant from funding agencies in the public, commercial or non-profit sectors.
Abstract

Heat preconditioning (HP) is a powerful adaptive and protective phenomenon and the heat stress proteins (HSPs) it produces are an important determinant for the development of diabetic complications. Aspirin has been reported to modulate heat shock response in different organisms through increased induction of HSPs and is also known to exert anti-oxidative and radical scavenging effects in diabetes. We estimated the effect of physiological [heat stress (HS), 45min / 41±0.5°C] and pharmacological (aspirin treatment) induction of HSP70 on several parameters of oxidative state in the pancreas and liver of diabetic rats. Diabetes increased HSP70 level and decreased PARP, glutathione (GSH) and glutathione peroxidase (GPx) activities in pancreas. In liver there was reduction of HSP70 level, GSH concentration and CAT activity, while GPx and GR activity were enhanced. Heat preconditioning of diabetic rats caused an additional increase of HSP70, GSH and antioxidant enzymes in both organs. Pre-treatment of HP-diabetic animals with aspirin led to an additional increase of PARP and HSP70. In conclusion, both HP and aspirin, as physiological and pharmacological inductors of HSP70, respectively, enhance the anti-oxidative defense mechanisms of the liver and pancreas in diabetic rats.

Key words: heat preconditioning, aspirin, experimental diabetes, HSP70, oxidative status.
Introduction

Diabetes mellitus (DM) and insulin resistance are inflammatory conditions that result in modification of cellular proteins, oxidative stress and a reduced cellular defense system (Cossins and Bowler 1987; Atalay and Laaksonen 2002; Shan et al. 2003), conditions which are strongly correlated with heat shock protein (HSPs) disorders (Bruce et al. 2003) and indicate altered heat shock response (HSR) (Hooper 2007). A remarkably low expression of HSP72 in skeletal muscle of T2DM patients with impaired glucose tolerance was observed for the first time by Kurucz et al. (2002). Low levels of HSPs contribute to an impaired stress response, protein glycation, oxidation and aggregation, free radical formation and inflammation and may be a clue to the etiology of the disease itself (Hooper et al. 2014).

The most favorable condition to increase the production of HSPs is exposure of the organism of the cells to an acute heat stress (HS) (Parsell and Lindquist 1993). Furthermore, organism / cells previously exposed to sub-lethal HS display greater resistance to the effects of higher intensity stress, including hypoxia, ischemia/reperfusion (Joyeux et al. 1999) or a strong cytotoxic agent, such as the diabetogenic agent streptozotocin (STZ) (Panchnadikar and Bhonde 2003; Najemnikova et al. 2007), a phenomenon known as a heat preconditioning (HP). In particular, enhanced cell survival in heat-exposed organisms has been linked to the expression of HSPs as specific molecular chaperones and housekeeping molecules (Zhu et al. 2009). There is some evidence pointing to the beneficial effect of HP in diabetes (Hooper 1999; Bathaie et al. 2010). Heat therapy, via hot tub immersion, improves diabetic glycemic control and diabetic neuropathy in patients with type 2 diabetes (Hooper 1999) and significantly improves lipid profile, antioxidant capacity, insulin secretion and serum HSP70 level in diabetic rats (Bathaie et
al. 2010). In rodents, heat treatment (41-41.5°C) and overexpression of HSP72 have been shown to prevent high-fat diet-induced insulin resistance (Chung et al. 2008; Gupte et al. 2009).

In addition to physiological induction by heat-preconditioning, there are also pharmacologically methods of inducing HSP production; among them, non-steroidal anti-inflammatory drugs (NSAIDs), such as sodium salicylate, aspirin and indomethacin, have been reported to modulate HSR in different organisms (Winegarden et al. 1996; Fawcett et al. 1997) and improve thermal response during hyperthermia and other toxic conditions through increased induction of HSPs (Batulan et al. 2005). Aspirin, an anti-inflammatory drug (non-specific cyclooxygenase (COX) inhibitor), is known to exert other pharmacological effects, including stimulation of insulin secretion and hypoglycaemia, as well as anti-oxidative effects, radical scavenging, diminishing of endogenous oxidant stress and activation of antioxidant defenses in a condition of diabetes (Zarich 2009; Caballero et al. 2000; Prasad and Lee 2011; Ayyadevara et al. 2013; ).

Diabetes mellitus induced experimentally by streptozotocin (STZ) causes β-cell death by alkylation of DNA (Elsner et al. 2000) and activation of the DNA-damaging enzyme poly(ADP) ribose polymerase (PARP) in pancreatic β-cells (de Murcia et al. 1997; Wang et al. 1997), which is a main reason for the increase of ROS, RNS, NO and protein carbonylation in different organs (pancreas, liver, kidney and brain) of diabetic rats (Nourooz-Zadeh et al. 1997; Raza et al. 2011; Raza and John 2012). Among these organs, the pancreas has a relatively low expression of antioxidant enzymes (Zhang et al. 1995; Lenzen and Drinkgern 1996), while the liver has the highest antioxidant capacity when compared with other tissues (Navarro-Arévalo and Sánchez-del-Pino 1998) in diabetic rats.
Taking into consideration the above, we aimed to investigate: 1. the effects of physiological induction of HSPs by heat preconditioning in diabetic animals; and 2. the pharmacological induction of HSPs by aspirin and its possible metabolic modifications in HP-diabetic animals. To do this, we evaluated changes in HSP70 levels, PARP activity and the antioxidative defense system in the liver and pancreas in STZ – diabetic rats.

**Material and methods**

**Animals and tissue procedures**

This experimental study was performed with adult (3 - 4 months old) male Wistar rats (n=80) with a weight of 250 - 300g, which were housed under a 12-hour light regime (6 a.m. - 6 p.m. light) and fed laboratory chow and water *ad libitum*. All experimental animals were anaesthetized with Na-thiopental narcosis (45 mg / kg) and sacrificed using a standard laparothomic procedure, always at the same time of day (9-10 a.m.). The isolated pancreas and liver were washed with cold saline solution and immersed in liquid nitrogen. The tissues were kept at -80°C until analysis and were converted into tissue powder before analysis (at liquid N₂–temperature). The tissue powder was homogenized with an ultrasonic homogenizer (Cole-Parmer Instrument - 4710) in several 7-10 sec. cycles. The whole procedure was performed at 0 - 4°C (on ice). All procedures followed the Canadian Council on Animal Care.

**Study design and treatments**

The animals were divided into two general groups: healthy animals and diabetic ones. The first group was divided into three subgroups: control animals (C), heat-preconditioned control animals (HC) and heat-preconditioned control animals pretreated with ASA (AHC). The
diabetic group was divided into six subgroups: control diabetic groups (D2 and D14 - sacrificed 2 or 14 days after STZ-administration, respectively); heat-preconditioned diabetic groups (HD2 and HD14 - sacrificed 2 or 14 days after STZ-administration, respectively) and heat-preconditioned diabetic groups, pretreated with ASA (AHD2 and AHD14 - sacrificed 2 or 14 days after STZ-administration, respectively).

Heat preconditioning (HP) was carried out by placing animals in special temperature-controlled chambers (45 min at 41±0.5ºC), followed by 24h recovery at room temperature (20 ± 2ºC).

The duration of diabetes in our experiment was defined by the following criteria: minimum period for manifestation of the effect of administrated STZ (48h) and optimal period for the development of diabetic complications (14 days); abundant HSP70 protein level in the first 24-48h after the single HS, and further decrement after the 72h after the HS [both in cells (Miova et al. 2015) and in rats (Horowitz 2003)]. Experimental diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg body weight) and was freshly dissolved in 0.1M citrate buffer, pH=4.5. All animals with clear diabetic symptoms (fasting glycemia levels higher than 15 mmol/L) 24-48 hours after the induction of the experimental diabetes were included in the experiment.

Aspirin (ASA - acetylsalicylic acid, Sigma-Aldrich) was freshly dissolved in water and administrated to the animals in a concentration of 100 mg / kg b.w. Subsequently, sodium carbonate was slowly added until the ASA crystals had dissolved (the pH of the solution remained just below 7.0 (Kelton et al. 1978) and was administrated intraperitoneally in a 0.5-mL volume (Fawcett et al. 1997; Locke and Atance 2000), one hour before exposure to heat stress.

Table 1.
Biochemical analyses

The protein level of HSP70 was determined with an appropriate commercial kit (HSP70 EIA, Enzo), and PARP activity was measured with a colorimetric commercial kit (TREVIGEN). GSH concentration was determined with a commercial kit (Glutathione Assay Kit, Sigma-Aldrich), while the enzyme activity of glutathione peroxidase (GPx) and glutathione reductase (GR) was determined by a modification of the commercial kits (Sigma-Aldrich). Catalase activity was assessed following Aebi’s method (Aebi 1984). Analyses to determine GPx, GR, total GSH, HSP70, PARP were performed with a microplate reader (BioRad), while the activity of catalase and total protein was measured with a UV-spectrophotometer (Cary 50, Varian).

Statistics

Results are presented as means ± SD. Statistical differences between groups were examined using one-way ANOVA with a Neuman-Keuls post-hoc test. 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

Rectal temperature (effects of HP, ASA and ASA+HP)

Our measurements showed a significant increase of rectal temperature of about 3.7°C in HP-animals, which returned to basal values after 24h recovery at room temperature (Table 2). Aspirin (ASA) treatment alone produced a rise of rectal temperature of about 1°C one hour after the treatment, while a combination of ASA+HP led to an additional increase of rectal
temperature (total increment of about 4.4°C, Table 3). Finally, when animals had recovered for 24h at room temperature, their rectal temperature was found to return to normal.

**Pancreas**

Figure 1 shows the results obtained for HSP70 protein level (Fig. 1A), PARP activity (Fig. 1B), total glutathione (GSH) concentration (Fig. 1C) and glutathione peroxidase (GPx) activity (Fig. 1D) in the pancreas of our rats.

Twenty-four hours after HS there was a significant increase in the level of pancreatic HSP70 level and GPx activity and a significant decrease in PARP activity and total GSH concentration compared to control animals (C:HC). Pretreatment of HP–control animals with ASA (HC:AHC) resulted in an additional increase of HSP70 (p<0.050), a significant increase of PARP activity and GSH concentration and a significant decrease of GPx activity compared to heat-preconditioned control animals (HC).

Our results show there was a significant increase in pancreatic HSP70 level and significant decreases in PARP activity, total GSH concentration and GPx activity, both 2d- and 14d after STZ administration (C:D2, C:D14). Heat preconditioning of diabetic animals (D2:HD2, D14:HD14) led to a significant increase in HSP70 level in 2d-diabetic animals and a significant decrease in HSP70 level in 14d-diabetic animals. Heat preconditioning led to an increase of GSH concentration only in 14d-diabetic animals and an increase of GPx activity regardless of the duration of diabetes. Moreover, pretreatment of HP–diabetic animals with ASA (HD2:AHD2, HD14: AHD14) resulted in a further increase in HSP70 level (only in 2d-diabetic animals), a significant increase in PARP activity, and non-significant changes in GSH concentration and GPx activity.
Finally, time-dependent changes were manifested with respect to HSP70 level and GSH concentration – significantly lower HSP70 and significantly higher GSH in 14d-diabetic animals versus 2d-diabetic animals - regardless of whether or not there was aspirin treatment (HD2:HD14 and AHD2:AHD14, p<0.050).

Figure 1.

Liver

Figure 2 shows the results obtained for the parameters studied in the liver: HSP70 protein level (Fig. 2A), total GSH concentration (Fig. 2B), GPx activity (Fig. 2C), GR activity (Fig. 2D) and CAT activity (Fig. 2E).

We observed that HP led to a significant 10-fold increase in hepatic HSP70 protein level, a significant increase of GPx and GR activity, and a significant reduction in GSH concentration compared to control animals (C:HC). Pretreatment with ASA caused an additional increase in HSP70 level (p<0.050) and an almost total reversal of antioxidative parameters in HP-animals.

Our results showed that STZ-diabetes (C:D2, C:D14) led to a significant reduction in hepatic HSP70, total GSH and CAT, and a significant increase of GPx and GR in the liver. Heat preconditioning of diabetic rats resulted in a significant increase in HSP70 level and total GSH concentration, as well as an increase in GR and CAT activity compared to diabetic rats (D2:HD2, D14:HD14). Treatment with ASA led to an additional increase of HSP70 in both 2d- and 14d-diabetic animals, an additional increase of CAT activity in 2d-diabetic animals, and a decrease of GR-activity in both 2d- and 14d-diabetic animals and GPx activity in 2d- diabetic animals (HD2:AHD2, HD14: AHD14) with respect to HP-diabetic animals.
In diabetic animals time-dependent changes were manifested only with respect to HSP70 level – significantly lower HSP70 in 14d-diabetic animals versus 2d-diabetic animals, regardless of whether or not there was aspirin treatment (HD2:HD14 and AHD2 : AHD14, p<0.05).

Discussion

Heat stress / heat preconditioning

The first evident manifestation of acute HS (45min, 41±0.5°C) that we observed was a significant increase of rectal temperature of approximately 3.7°C, which returned to control values after 24h recovery at room temperature. This elevated rectal temperature in intact animals led to a significant increase of HSP70 protein levels in both organs: up to 3- and 10-fold in the pancreas and the liver, respectively. A number of studies suggest that HSP70 has a protective function in the prevention of cellular damage in thermal injury (Horowitz 2002) and that its synthesis is a crucial part of the whole physiological response to the stressor (Feder and Hofmann 1999).

The glutathione (GSH) metabolic network also exhibits profound disruption in response to heat stress, since free radicals and other ROS are neutralized by GSH in redox regulation cycles (Ippolito et al. 2014). The pancreas is particularly vulnerable to the harmful effects of ROS due to its lower expression of antioxidant enzymes compared to other tissues (Zhang et al. 1995; Lenzen and Drinkgern 1996). We observed that 24h after HS there was a significant reduction of total GSH concentration in the pancreas with respect to control animals. By contrast, the liver is the organ with the highest antioxidant capacity (Navarro-Arévalo and Sánchez-del-Pino 1998).
The decreased concentration of GSH and elevated activity of GPx, GR and CAT observed in our rats clearly manifest an activated anti-oxidative defense mechanism of the liver.

Increased ROS may also lead to apoptosis, DNA damage and disruption of the metabolism (Trachootham et al. 2008). Our research reveals a significant decrease of PARP activity in the pancreas 24h after exposure to HS. Interestingly, with its catalytic activity, PARP can also affect the transcription of specific genes, including genes for HSP70 (Tulin and Spradling 2003; Juet al. 2006; Krishnakumar and Kraus 2010). Little is known of how PARP activates transcription of HSP70 after HS, but research by Petesch and Lis (2012) in *Drosophila melanogaster* indicates that PARP is associated with the 5’ end of HSP70 and that its enzymatic activity is rapidly induced by HS, which causes PARP to redistribute throughout HSP70 loci. It is possible that the reduced activity of PARP as a result of HS involves binding of strongly elevated HSP to PARP or to the DNA molecule (Bellmann et al. 1995).

**Experimental diabetes**

PAPR is a constitutively expressed nuclear enzyme which can be activated by damaged DNA (Kim et al. 2004; Pinnola et al. 2007), and a single application of STZ leads to expression of cleaved PARP in the first 6 hours in rats and for a shorter period in isolated cells (3h after STZ application) (Ku et al. 2012). In our experiments, the reduced activity of PARP might have accounted for the decreased PARP activity in a time-delay course (2d- and 14d- after STZ administration). Pancreatic β-cells are vulnerable to oxidative stress as they possess reduced antioxidant levels, including glutathione peroxidase (GPx) and catalase (Newsholme et al. 2012). Our results show that the induction of experimental diabetes, regardless of its duration, leads to a significant reduction in GSH concentration in the pancreas, accompanied by a significant
decrease in GPx activity. Interestingly, islet cells have been reported to have a disturbed GSH metabolism, with a 10-fold lower GPx activity compared with other cell types (Malaisse et al. 1982). In our experiments, the diabetic livers manifested a significant reduction in GSH levels and a significant increase in GR and GPx activity. Indeed, it is known that GPx gene expression is regulated by the concentration of H$_2$O$_2$ and other oxidants (Matés et al. 1999), and the increased production of ROS during diabetes can inhibit certain antioxidant enzymes, such as CAT (Sindhu et al. 2004), which we observed in our experiments.

Finally, our results showed tissue-specific regulation and expression of HSP - there was a significant increase of protein levels in the pancreas and a decrease in the liver. Experimental monkeys have manifested a similar response to DM - increased HSP70 protein expression and HSF (heat shock factor) in pancreatic tissue versus a reduction of both parameters in the liver (Kavanagh et al. 2009). Namely, in organs with high secretory capacity, such as the liver and pancreas, HSPs are vital for a normal physiology of the organism (Hooper 2007), and the vulnerability of diabetic animals is due heavily to disturbances of HSPs.

**Heat preconditioning of diabetic animals**

The main mechanism of heat preconditioning is based on production of cellular HSPs, which have strong cytoprotective effects and speed up recovery from additional stress. Our results show that HP of diabetic animals causes a significant increase compared to diabetic controls in HSP70 levels in both organs, especially the liver. Heat-stressed diabetic animals show an increased HSP72 and HSP25 content in the heart, kidney, and liver compared to the same tissues from heat-stressed, non-diabetic animals (Najemnikova et al. 2007). Nevertheless, a significantly lower HSP70 expression has been reported in 14d- versus 2d-diabetic animals.
which is in accordance with other reports showing that HSP72 synthesis flourishes in the first 24-48 following HS and declines later on (Horowitz 2003). In HepG2 cells, abundant level of HSP70 and BCl-2 was observed 24-48h after the single HS, which indicate that HS might be a “physiological conditioner” and might obtain cytoprotection against an additional stress (Miova et al. 2015).

Additionally, we found that HP led to an improvement in the redox potential of the diabetic pancreas and reduced oxidative damage, evident in the increased total GSH level in 14d-diabetic animals, followed by a significant increase in GPx activity.

On the other hand, the liver is organ-guard during thermal stress (Hall et al. 1994; Kregel et al. 1995) and is also sensitive to changes in diabetic conditions. According to Raza and John (2012), STZ treatment of human hepatocellular cells causes cytotoxicity, formation of ROS, and oxidative stress by increasing lipid peroxidation and altering the GSH-dependent antioxidant metabolism. Our results show that HP-diabetic animals have a significantly higher concentration of total GSH and increased GR and CAT activity in the liver compared to normothermic diabetic animals. This is in accordance with Panchnadikar and Bhonde (2003), who reported that thermoresistent cells are generally resistant to environmental stress and death. In the same line, results obtained in HeLa cells have shown that hyperglycaemia and oxidative stress up-regulate HSP60 and HSP70 expression concomitantly to the increase in the intracellular levels of ROS (Hall and Martinus 2013).

**Aspirin pretreatment of HP- control and diabetic animals**

We observed that aspirin administration given as a pre-treatment to acute HS enhanced the rise of rectal temperature by about 4.5°C in ASA-treated HP-control rats (Table 3). Our
assumption is that this is a main trigger for additional production of HSP70 in both the liver and pancreas with respect to non-ASA-treated HP-control animals (Fig. 1A and 2A). It is known that aspirin treatment causes a slight rise in body core temperature in rats, even in an absence of heat, but treatment with aspirin prior to HS markedly enhances heat-induced HSP70 levels (Fawcett et al. 1997). Aspirin can also reduce the temperature threshold of the HSR (Koo et al. 2000), acting as a co-inducer of HSP and emphasizing HSP expression (Fawcett et al. 1997). According to our results, increased HSP70 synthesis due to ASA pretreatment was accompanied by a significantly higher PARP activity (about 32.6%) with respect to non-treated HP-animals, but enzyme activity was still significantly lower than that of control animals. Furthermore, our results demonstrate that ASA pre-treatment reversed both GSH concentration and enzyme activity in the liver and pancreas of HP-control animals to almost control levels.

In HP-diabetic rats, ASA-pretreatment produced an additional increase of HSP70 concentration in both the liver and pancreas, and a significant increase in PARP activity in the pancreas. It is important to note that these changes were evident only in 2d-diabetic animals, indicating that time-dependent changes in HSP production are related to the duration of the recovery period after the HS. We believe that the increased synthesis and accumulation of HSP70 in the pancreas during ASA pretreatment resulted in a lower reduction of PARP activity in diabetic animals. In terms of oxidative enzymes, ASA pretreatment did not cause significant changes in the pancreas, while in the liver, there was an additional increase of CAT activity and a decrease of GR-activity and GPx activity compared to non-treated HP-diabetic rats. There is evidence that aspirin abolishes accumulation of TBARS in a long term assay and partially reverts catalase activity in diabetic mice (Caballero et al. 2000), and that it has a nitric oxide radical-scavenging potential in atherogenic-diet induced DM in rats (Sethi et al. 2011). Still, more
analyses should be performed to confirm the antioxidative potential of aspirin during diabetes conditions.

**Conclusion**

In conclusion, both HP and aspirin, as physiological and pharmacological inductors of HSP70, respectively, cause alterations in the diabetes-induced oxidative state in the liver and pancreas. We confirm enhanced HSP70 concentrations in the liver and pancreas 24h after HS, with an additional increase when there is pretreatment with aspirin. These abundant HSP levels enhance the anti-oxidative defense mechanisms of the liver and pancreas in diabetic rats, suggesting a cross-tolerance between heat preconditioning and STZ-induced diabetes. Moreover, this effect is more expressed in combination with pre-treatment with aspirin, which is known to have a potential HSP-inducing effect.

**Acknowledgements**

The authors thank to Dr Nadezda Apostolova for her critical reading and assistance with the preparation of the manuscript and to Brian Normanly for his English language editing of the MS (both of them form Deparment of Pharamacology, Faculty of Medicine, University of Valencia, Valencia, Spain)
References:


de Murcia J.M., Niedergang C., Trucco C., Ricoul M., Dutrillaux B., Mark M., Oliver F.J., Masson M.,
poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc. Natl. Acad.
Elsner M., Guldbakke B., Tiedge M., Munday R., Lenzen S. 2000. Relative importance of transport and
tolerance and prevents skeletal muscle insulin resistance in rats fed a high-fat diet. Diabetes 58:
567–578.
Hall D.M., Buettner G.R., Matthes R.D., Gisolfi C.V. 1994. Hyperthermia stimulates nitric oxide
formation: electron paramagnetic resonance detection of NO-heme in blood. J. Appl. Physiol. 77:
548–553.
Hall L. and Martinus R.D. 2013. Hyperglycaemia and oxidative stress upregulate HSP60 and HSP70
expression in HeLa cells. Springer Plus 2: 431
Hooper P.L. 2007. Insulin Signaling, GSK-3, Heat Shock Proteins and the Natural History of Type 2


Table 1. Organization of the experimental groups according the treatments timing.

<table>
<thead>
<tr>
<th>Group</th>
<th>ASA - treatment</th>
<th>Recovery</th>
<th>Heat stress (HS)</th>
<th>Recovery</th>
<th>Duration of diabetes</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sacrifice</td>
</tr>
<tr>
<td>HC</td>
<td></td>
<td></td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td></td>
<td>Sacrifice</td>
</tr>
<tr>
<td>AHC</td>
<td>ASA single dose</td>
<td>1h/ 20±2ºC</td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td></td>
<td>Sacrifice</td>
</tr>
<tr>
<td>D2</td>
<td></td>
<td></td>
<td></td>
<td>STZ administration + 2 days</td>
<td></td>
<td>Sacrifice</td>
</tr>
<tr>
<td>HD2</td>
<td></td>
<td></td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td>STZ administration + 2 days</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>AHD2</td>
<td>ASA single dose</td>
<td>1h/ 20±2ºC</td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td>STZ administration + 2 days</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>D14</td>
<td></td>
<td></td>
<td></td>
<td>STZ administration + 14 days</td>
<td></td>
<td>Sacrifice</td>
</tr>
<tr>
<td>HD14</td>
<td></td>
<td></td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td>STZ administration + 14 days</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>AHD14</td>
<td>ASA single dose</td>
<td>1h/ 20±2ºC</td>
<td>45 min/ 41±0.5ºC</td>
<td>24h/ 20±2ºC</td>
<td>STZ administration + 14 days</td>
<td>Sacrifice</td>
</tr>
</tbody>
</table>
Table 2. Rectal temperature in a function of acute heat stress (45min / 41±0.5°C). The temperature was measured just after (0’) or 24h after the heat stress. The measurement present the average values for all groups of animals (HC, HD2 and HD14) which were exposed to heat stress.

Legend:
C- rectal temperature in control animals;
HS +0’- rectal temperature in animals exposed to acute heat stress, just after (0’) the heat stress;
HS +24h- rectal temperature in animals exposed to acute heat stress, 24h after the heat stress.
Significant difference p<0,05: a- compared to control; b- compared to HC (0’)

<table>
<thead>
<tr>
<th>Rectal temperature in a function of acute heat stress (45min / 41±0.5°C)</th>
<th>C</th>
<th>HS +0’</th>
<th>HS +24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average ± SD</td>
<td>37,0± 0,7</td>
<td>40,7± 0,7a</td>
<td>36,9± 0,7b</td>
</tr>
</tbody>
</table>
Table 3. Rectal temperature in a function of aspirin treatment (100 mg/ kg b.w.) and exposure to acute HS (45min / 41±0.5°C). The temperature was measured 1h after the aspirin treatment, as well as just after (0’) or 24h after the HS. The measurement present the average values for all groups of animals (AHC, AHD2 and AHD14) which were pre-treated with aspirin and exposed to HS 1h later.

Legend:
C- RT in control animals;
ASA+ 1h – RT in animals 1h after aspirin treatment;
ASA+1h+HS+0’- RT in animals pre-treated with aspirin, 1h later exposed to HS and recovered 0’ at room temperature.
ASA+1h+HS+24h- RT in animals pre-treated with aspirin, 1h later exposed to HS and recovered 24h at room temperature.

Significant difference p<0,05: a- compared to control; b- compared to ASA+1h; c- compared to ASA+1h+HS+0’

| Rectal temperature in a function of aspirin treatment and acute heat stress (45min / 41±0.5°C). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | C               | ASA+ 1h         | ASA+1h+HS+0’   | ASA+1h+HS+24h   |
| Average ± SD                   | 36,9± 0,8       | 37,7 ± 0,6a     | 41,4 ± 0,9b    | 37,1 ±0,8c     |
Figure legends:

**Fig. 1.** Changes in pancreas of heat-preconditioned intact and diabetic rats treated with aspirin. 
A. HSP70 level; B. PARP activity; C. Total glutathione level; D. Glutathione peroxidase activity.

Legend:

C - Control (intact) animals;
HC - Control animals exposed to HS (45 min / 41±1°C), allowed to recover 24h at room temperature;
AHC - Control animals treated with aspirin (100 mg/kg b.w), 1h before HS (45 min / 41±1°C), allowed to recover 24h at room temperature.
D2, D14 - Diabetic animals (sacrifice 2 or 14 days after STZ-application);
HD2, HD14 - Diabetic animals exposed to HS (45 min / 41±1°C), allowed to recover 24h at room temperature before induction of diabetes (sacrifice 2 or 14 days after STZ-application);
AHD2, AHD14 - diabetic animals treated with aspirin (100 mg/kg b.w), 1h before HS (45 min / 41±1°C), allowed to recover 24h at room temperature before induction of diabetes (sacrifice 2 or 14 days after STZ-application).

Significant difference (p <0.050):

a – relative to control animals (C:HC, C:D2, C:D14);
b - relative to diabetic animals (D2:HD2, D14:HD14, respectively);
c - relative to control animals exposed to HS (HC:AHC).
d - relative to diabetic animals exposed to HS (HD2:AHD2, HD14:AHD14, respectively).

**Fig. 2.** Changes in liver of heat-preconditioned intact and diabetic rats treated with aspirin. 
A. HSP70 level; B. Total glutathione level; C. Glutathione peroxidase activity; D. Glutathione reductase activity; E. Catalase activity.

Legend:

C - Control (intact) animals;
HC - Control animals exposed to HS (45 min / 41±1°C), allowed to recover 24h at room temperature;
AHC - Control animals treated with aspirin (100 mg/kg b.w), 1h before HS (45 min / 41±1°C), allowed to recover 24h at room temperature.
D2, D14 - Diabetic animals (sacrifice 2 or 14 days after STZ-application);
HD2, HD14 - Diabetic animals exposed to HS (45 min / 41±1°C), allowed to recover 24h at room temperature before induction of diabetes (sacrifice 2 or 14 days after STZ-application);
AHD2, AHD14 - diabetic animals treated with aspirin (100 mg/kg b.w), 1h before HS (45 min / 41±1°C), allowed to recover 24h at room temperature before induction of diabetes (sacrifice 2 or 14 days after STZ-application).

Significant difference (p <0.050):

a – relative to control animals (C:HC, C:D2, C:D14);
b - relative to diabetic animals (D2:HD2, D14:HD14, respectively);
c - relative to control animals exposed to HS (HC:AHC).
d - relative to diabetic animals exposed to HS (HD2:AHD2, HD14:AHD14, respectively).