Effects of lycium barbarum polysaccharides on neuropeptide Y and heat-shock protein 70 expression in rats exposed to heat

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Abstract. The purpose of the present study was to investigate the effects of high ambient temperature on the neuropeptide Y (NPY) mRNA level in the hypothalamus, the plasma concentration of corticotropin-releasing hormone (CRH), cortisol (Cor), heat-shock protein 70 (HSP70) and epinephrine (EPI), and the intervention of lycium barbarum polysaccharides (LBPs) in rats. Compared to the control (CN) group, the plasma levels of CRH, Cor, HSP70 and EPI were markedly increased, and the level of NPY mRNA was downregulated in the high ambient temperature (HT) group. By contrast, rats in the HT + LBP (HTL) group had: i) a significantly enhanced expression of HSP70 compared to the HT and CN groups; ii) clearly increased plasma levels of CRH, Cor and EPI compared to the CN group; and iii) a markedly upregulated expression of NPY mRNA compared to the HT group. Thus, the results showed that high-temperature environments may damage the body, and LBPs have a potentially protective function by increasing the expression of HSP70 and NPY.

Introduction

The physical factors of the human environment include temperature, humidity and noise. Living permanently in a high ambient temperature affects the daily life and work environment. More importantly, a high humidity and noisy environment cause damage to the body (1). Among these physical environmental stresses, temperature is the most significant ecological condition. As temperature is a factor that is widespread in the majority of environments, there is a lack of space or time stability (2). High ambient temperature beyond the scope of the thermal neutral zone has been considered to be the deadliest pressure source in rodents, as it triggers a wide variety of biological responses, and results in neurological, endocrine disorders and immune dysfunctions (3). Under the action of stress, a number of hormones are excreted to counteract the adverse effect on organism function. The stress hormones (catecholamines, glucocorticoid and growth hormone) play a critical role in regulating the circulation and nutritional requirement of potentially harmful situations, via the 'flight or fight' reaction (4).

Lycium barbarum belongs to the plant family *Solanaceae*. The red berries, also known as wolfberry or gou qi zi, are a well-known traditional Chinese medicine. Currently, the red berries have been extensively used to manufacture various types of healthy foods and products, including medicinal beverages and healthy soups (5). Lycium barbarum has a high level of polysaccharides, which is analogously 40% by dry mass, and therefore, studies have been devoted to the liquid fraction of the berries and the lycium barbarum polysaccharides (LBPs) (6). It has been previously reported that LBPs act against the influences of aging and oxidation (7,8,9), adjust the immune function (10,11), increase the exercise tolerance capacity, relieve fatigue and aging, and express antioxidant activity (12-14). Treatment with LBP prevented the rise of blood glucose in hypertensive rats, guarded against liver damage caused by alcohol-induced oxidative stress in rats (15-17), and reduced the side-effects of chemotherapy and radiotherapy in the treatment of cancer (18-20). Our previous study showed that LBP clearly inhibited oxidative stress, weakened the contractile response to noradrenaline, and it also enhanced the expression of endothelial nitric oxide synthase and heat-shock protein 70 (HSP70) in rats undergoing exhaustive exercise (21).

The objectives of the present study were: i) to investigate the effects of high ambient temperature on neuropeptide Y (NPY) gene expression in the hypothalamus and the concentration of corticotropin-releasing hormone (CRH), cortisol (Cor), HSP70 and epinephrine (EPI) in the plasma of rats; and ii) to examine the alleviating effects of LBP intervention under high ambient temperatures on these physiological parameters in Sprague-Dawley (SD) rats.

Materials and methods

Animals and heat exposure schedule. Five-week-old male SD rats, weighing 150-180 g, were purchased from the

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Table I. GenBank login code, primer sequences and forecast size of the amplification products.

Gene	GenBank	Forward primer	Reverse primer	bp
NPY	NM_012614	GCTCTGCGACACTACATCAATC	GCATTTTCTGTGCTTTCTCTCA	107
β-actin	NM_031144	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC	207

bp, base pair; NPY, neuropeptide Y.

Laboratory Animal Center of Ningxia Medical University (Yinchuan, China). The rats were placed individually in plastic cages and were maintained on a 12-h light/dark cycle (lights on between 08:00 and 20:00 h) at a normothermic temperature (24.0±1℃), with access to food and water at liberty. The experimental procedures were approved by the Animal Ethics Committee of Ningxia Medical University and in accordance with the guidelines of the Council of the Physiological Society of China (?). Following a week-long adaptive phase, 24 rats were randomly divided into the control (CN), high ambient temperature (HT) and HT + LBP (HTL) groups. The temperature inside the climatic chamber was raised to 32.0±0.2℃ (relative humidity, 60±5%) in the HT and HTL groups, whereas the rats in the CN group were maintained at a temperature of 24.0±1℃ throughout the study. In the HTL group, the rats were treated with LBP by gavage [200 mg/kg body weight (b.w.)], whereas the rats in the HT and CN groups were treated with normal saline by gavage (1 ml/100 g b.w.). The treatments lasted for 14 days. The dose of LBP was chosen according to prophase experiments (21) and it was safe, effective and non-toxic in rats. In the course of the experiment, cages were cleaned and food and water were replaced at a random time every 2 or 3 days.

Tissue sampling and organ preparation. Following heat exposure, each rat was anesthetized by intraperitoneal injection of urethane (1.5 g/kg). Blood was extracted from the abdominal vein with a syringe, which was washed by heparin sodium solution prior to use. Subsequently, the blood was centrifuged at 3,500 x g for 15 min to obtain plasma. The plasma was stored at -80℃ until analysis.

The rats were executed by cervical dislocation, and the whole brain and hypothalamus were immediately removed and placed into liquid nitrogen. Frozen samples were reserved at -80℃ until further analysis.

RNA extraction and quantitative polymerase chain reaction (qPCR). RNA was extracted from hypothalamic tissue (n=8 in each group for the NPY mRNA assay), using an Axygen RNA kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Total RNA concentrations were measured by spectrophotometry at 260 nm. The purity of the RNA samples (260/280) was: 1.65≤purity≤2.00. cDNA was synthesized using a reverse transcription kit (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. qPCR was carried out using a Top Green qPCR SuperMix (TransGen Biotech Co., Ltd.). Primer sequences and the amplified fragment size are listed in Table I [Sangon Biotech (Shanghai) Co., Ltd., Songjiang, Shanghai,

China]. The specificity of the PCR amplified products was authenticated by analysis of a melting curve and agarose gel electrophoresis. The PCR conditions were as follows: 94℃ for 5 min (initial activation) followed by 36 cycles of amplification at 94℃ for 30 sec for denaturation; 62℃ for 30 sec for annealing; and 72℃ for 30 sec for extension. At the end of each reaction, melting curve analyses were performed to ensure the specificity of each reaction. The $2^{-\Delta\Delta Ct}$ method was used to calculate and correct the values of mRNA. In each run, the control sample was an internal standard, followed by standardization to the β-actin mRNA level. In the original experiment, the amplification efficiencies of NPY and β-actin were measured, and all amplification efficiencies were comparable. In each sample, the ΔCt value was established by measuring the difference between the Ct of NPY and β-actin as follows: ΔCt = Ct (NPY) - Ct (β-actin). Subsequently, the ΔΔCt of each sample was calculated by deducting the ΔCt value of the control: ΔΔCt = ΔCt (sample) - ΔCt (control). Each sample of RNA was tested three independent times and the average was used for the value in each sample.

Measurements of plasma CRH, Cor, HSP70 and EPI concentrations. The plasma concentrations of CRH, Cor, HSP70 and EPI were detected by an ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, Hubei, China), and all procedures were performed according to the manufacturer's instructions (n=8 in each group). Samples were arranged in replicates and the average value of each sample was used for analysis.

Statistics. The values are provided as mean ± standard deviation. Statistical comparisons were made by one-way analysis of variance. The statistical analyses were performed with SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Plasma CRH concentrations. In the plasma, the concentration of CRH significantly increased in the HT (P<0.05) and HTL (P<0.05) groups compared to the CN group. The plasma CRH level in the HTL group was not significant compared with that of the HT group (P>0.05) (Fig. 1).

Plasma Cor concentrations. Plasma Cor levels were significantly increased in the HT and HTL rat groups that were exposed to the high ambient temperature (P<0.05), compared to the rats in the CN group. However, there were no clear differences in the plasma Cor levels between the HT and HTL groups (P>0.05) (Fig. 2).
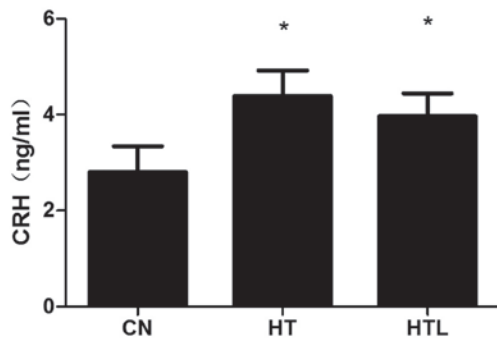
Figure 1. Effects of high ambient temperature and LBP intervention on the level of CRH (ng/ml) in the plasma of rats. Data are shown as mean ± standard deviation (n=8).*P<0.05 compared with CN. LBP, lycium barbarum polysaccharides; CRH, corticotropin-releasing hormone; CN, control group; HT, high ambient temperature group; HTL, high ambient temperature + LBP group.
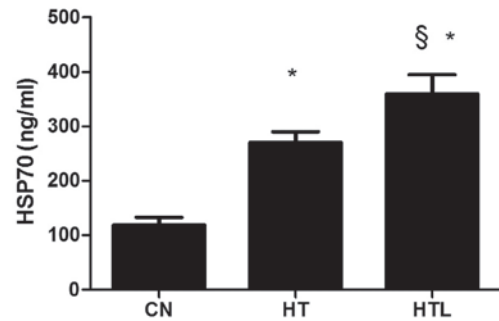

Figure 3. Effects of high ambient temperature and LBP intervention on the level of HSP70 (ng/ml) in the plasma of rats. Data are shown as mean ± standard deviation (n=8). *P<0.05 compared with CN, §P<0.05 compared with HT. LBP, lycium barbarum polysaccharides; HSP70, heat-shock protein 70; CN, control group; HT, high ambient temperature group; HTL, high ambient temperature + LBP group.
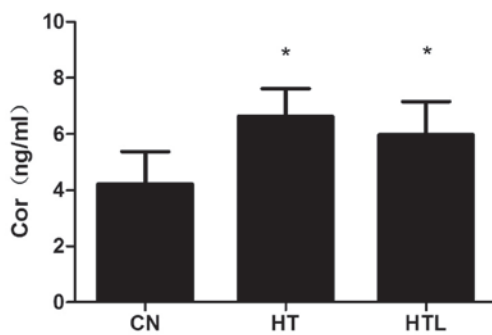

Figure 2. Effects of high ambient temperature and LBP intervention on the level of Cor (ng/ml) in the plasma of rats. Data are shown as mean ± standard deviation (n=8). *P<0.05 compared with CN. LBP, lycium barbarum polysaccharides; Cor, cortisol; CN, control group; HT, high ambient temperature group; HTL, high ambient temperature + LBP group.
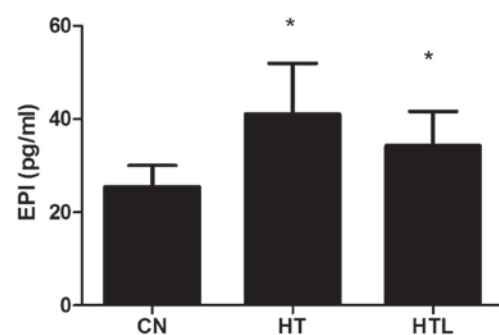

Figure 4. Effects of high ambient temperature and LBP intervention on the level of EPI (pg/ml) in the plasma of rats. Data are shown as mean ± standard deviation (n=8). *P<0.05 compared with CN. LBP, lycium barbarum polysaccharides; EPI, epinephrine; CN, control group; HT, high ambient temperature group; HTL, high ambient temperature + LBP group.

Plasma HSP70 concentrations. After 14 days of heat exposure, the plasma level of HSP70 in the HT group was significantly (P<0.05) higher compared with the CN group, whereas the plasma HSP70 level in the HTL group was markedly (P<0.05) higher compared with the HT group (Fig. 3).

Plasma EPI concentrations. After 14 days of heat exposure, the plasma EPI level in the HT (P<0.05) or HTL (P<0.05) group was significantly higher compared with the CN group. The EPI level in the plasma of the HTL group was not significantly different compared with the HT group (P>0.05) (Fig. 4).

NPY mRNA expression. The mRNA expression level of NPY was significantly downregulated in the HT group compared with the CN group (P<0.05). The expression of NPY mRNA markedly increased in the HTL group compared with the HT group (P<0.05). However, there were no significant differences in the levels of NPY mRNA between the CN and HTL groups (P>0.05) (Fig. 5).

Discussion

In the present study, the primary purpose was to investigate the effects of high ambient temperature on the physiological parameters in SD rats. Our previous study showed that the
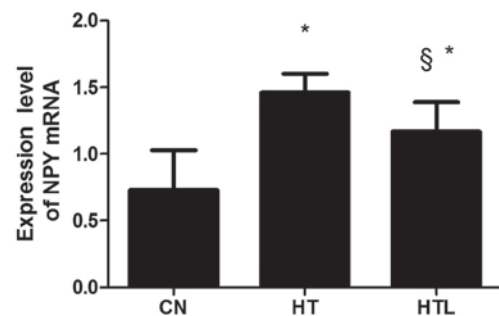

Figure 5. Effects of high ambient temperature and LBP intervention on the expression of NPY mRNA in the hypothalamus of rats. Data are shown as mean ± standard deviation (n=8). *P<0.05 compared with CN, §P<0.05 compared with HT. LBP, lycium barbarum polysaccharides; NPY, neuropeptide Y; CN, control group; HT, high ambient temperature group; HTL, high ambient temperature + LBP group.

mean abdominal temperature (Tab) in the light and dark phases of day in the HT and HTL groups was slightly higher than those of the CN group, but Tab did not differ between these groups (22). High ambient temperature has a negative impact on the performance of the neuroendocrine system. The hypothalamic-pituitary-adrenal (HPA) axis is an important part of the neuroendocrine system and its main action is to connect the functions of the hypothalamus, pituitary

gland and adrenal cortex (23). The HPA axis, not only regulates and controls the reaction to stress, but also has a number of other functions, including body heat regulation, the regulation of metabolism and immune function. The HPA axis plays a key role in the process of stress response by regulating the secretion of Cor. Cor is widely accepted as a standard for stress response as secretion of Cor has been shown to increase when animals are exposed to a stress state (24). The stress-induced activation of the HPA axis is dependent on CRH, which is the primary regulator of stress. CRH activates the HPA axis by inducing the release of the adrenocorticotropic hormone (ACTH) from the pituitary gland, which in turn stimulates the adrenocortical release of Cor (25).

In humans, CRH is the major hormone of the HPA axis. CRH is a 41-amino-acid neuropeptide, which is produced and released by the hypothalamus. When the body is exposed to a stressful environment, CRH is released by the hypothalamus (26-29). A study by Elias *et al* (30) showed that the concentration of Cor and CRH was increased immediately, following a long-time high-intensity exercise. Results of the present study have shown that exposure to high ambient temperature increased the level of CRH in plasma. When the secretion of CRH increases, CRH stimulates the cells of the anterior pituitary to synthesize and release the ACTH, which in turn stimulates the cells of the adrenal cortex to synthesize and release Cor (31,32). A previous human study has shown that Cor is the end result of the HPA-axis activation (33). Cor can also be considered a reliable indicator of the initial strength of nauseous or harmful events (34). Megahed *et al* (35) showed that in Upper Egypt, the summer heat stress induced the increase of Cor in the serum of Buffalo-Cows. The present study has confirmed that exposure to high ambient temperature caused a significant increase in the Cor plasma level. Therefore, the study has shown that high ambient temperature exposure causes a clear activation of the HPA axis, manifested as the increased secretion of CRH and Cor. Based on these data, there was no significant difference between the HTL and HT groups in the rat plasma levels of Cor and CRH. This finding may indicate that LBP had no evident effect on the alleviation of the increased plasma levels that were caused by heat stress.

HSPs are molecular chaperones, that play a crucial role in protein transport, aggregation, degradation, folding and unfolding (36). Furthermore, these highly-conserved proteins belong to the stress-responsive protein family, and play a key role in protecting cells against stress and apoptosis (37). Hsp70 is one of the largest and most conserved families that participate in cytothesis and other protective mechanisms (38). In addition, various types of environmental stresses and toxic chemical substances are able to upregulate the expression of Hsp70 (39-41). It is known that HSP play important physiological actions in situations involving both pantosomatous and cellular stress. HSP reacts quickly to environmental stress, and also protects cells from it. Additionally, all manner of environmental stresses and nocuous chemical materials induce the expression of HSP70. Yu and Bao (42) reported that heat stress induced an increase in HSP70 protein and mRNA levels in the heart and liver of broiler chickens. Thus, in the present study, the plasma level of HSP70 was detected in rats exposed to high ambient temperature. The data show that the levels of HSP70 in the HT and HTL groups were significantly increased compared to the CN group. The data also show that the level of HSP70 was markedly increased in LBP-treated animals compared to the HT group. In addition, the upregulation of NPY mRNA expression was observed in the LBP-treated rats. Therefore, HSP70 may participate in this upregulation by protecting the cells from the deleterious effects of heat stress.

A large-scale stress-antileptic physiological reaction refers to a complicated neural endocrine, and the interaction between the immune system to preserve internal homeostasis and to respond to life-threatening unexpected events (43). The first sign of the stress response is the activation of the sympathetic nervous system. This activation increases the release of catecholamines and the release of co-located substances, including neuropeptides at the sympathetic neuroeffector juncture into the blood. For a number of years, it has been known that stress induces a cascade of adaptive neuroendocrine responses, which generally includes the synthesis and release of Cor and EPI from the adrenal gland (44,45). Melin *et al* showed that when rats were exposed to heat stress followed by exhaustive exercise, the levels of EPI and norepinephrine were increased (46). In the present study, it was demonstrated that high temperature exposure increased the plasma level of EPI, and that there was no significant change in the HTL group compared with the HT group. This suggests that LBP had no obvious effect on alleviating the increased plasma EPI level that was caused by heat stress.

NPY is a 36-amino acid peptide, which is widely distributed in the central and peripheral nervous system of mammals. NPY plays a decisive role in maintaining the homeostasis of the internal environment (47). Whether in humans or animals, stressors, including cold, electroshock and exercise, can increase the plasma NPY levels has also been investigated (48). The NPY gene expression was elevated by a acute stress, whereas the NPY gene expression was different in a reduplicated stress (49). NPY is a primary neurotransmitter activated by stress that has been widely studied in the field of anxiety and stress. In the present study, in the rats exposed to high ambient temperature, a significant downregulation in the mRNA expression of NPY was observed. This may indicate that prolonged heat stress may cause injury to the body. The downregulation was entirely reversed by the intervention of LBPs in the HTL group.

The present study has shown that LBP enhanced the expression of HSP70 in plasma. LBP treatment was also shown to reverse the downregulation of NPY mRNA expression. This may indicate that LBP alleviates the harm caused by prolonged heat stress. However, the mechanism of the increased NPY mRNA expression in LBP-treated rats has not examined thoroughly in the study, and further investigation is required to investigate this mechanism.

In conclusion, results of the present study have shown a significant increase in the plasma concentration of CRH, Cor, HSP70 and EPI in rats exposed to high ambient temperature. By contrast, LBP significantly increased the plasma level of HSP70 and the mRNA expression of NPY, to protect the body from the damage caused by heat stress.

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