Quantitative Proteomics Provided Insights into the Protective Effects of Heat Acclimation on the Rat Hypothalamus after Exertional Heatstroke

Fan Xv1,2, Li-zhen Ma3, Xin Li4, Jin-bao Zhao5, Shu-yuan Liu5, Han-ding Mao5, Jun Ma1,2, Ling Xing6, Li-feng Wang3,*; Wei-jia Zhi3,*; Qing Song2,7,*

1Postgraduate School, Medical School of Chinese PLA, 100853 Beijing, China
2Department of Critical Care Medicine, First Medical Center of Chinese PLA General Hospital, 100853 Beijing, China
3Beijing Institute of Radiation Medicine, 100850 Beijing, China
4Department of Emergency, Third Medical Center of Chinese PLA General Hospital, 100039 Beijing, China
5Department of Emergency, Sixth Medical Center of Chinese PLA General Hospital, 100048 Beijing, China
6Department of General Medicine, Beijing Shijitan Hospital, Capital Medical University, 100038 Beijing, China
7Department of Critical Care Medicine, Hainan Hospital of Chinese PLA General Hospital, 572022 Sanya, Hainan, China

*Correspondence: fangchang_14@163.com (Li-feng Wang); zhi.weijia@163.com (Wei-jia Zhi); songqing3010301@sina.com (Qing Song)

Abstract

Background: The effects of heat acclimation (HA) on the hypothalamus after exertional heatstroke (EHS) and the specific mechanism have not been fully elucidated, and this study aimed to address these questions. Methods: In the present study, rats were randomly assigned to the control, EHS, HA, or HA + EHS groups (n = 9). Hematoxylin and eosin (H&E) staining was used to examine pathology. Tandem mass tag (TMT)-based proteomic analysis was utilized to explore the impact of HA on the protein expression profile of the hypothalamus after EHS. Bioinformatics analysis was used to predict the functions of the differentially expressed proteins. The differential proteins were validated by western blotting. An enzyme-linked immunosorbent assay was used to measure the expression levels of inflammatory cytokines in the serum. Results: The H&E staining (n = 5) results revealed that there were less structural changes in hypothalamus in the HA + EHS group compared with the EHS group. Proteomic analysis (n = 4) revealed that proinflammatory proteins such as argininosuccinate synthetase (ASS1), high mobility group protein B2 (HMGB2) and vimentin were evidently downregulated in the HA + EHS group. The levels of interleukin (IL)-1β, IL-1, and IL-8 were decreased in the serum samples (n = 3) from HA + EHS rats. Conclusions: HA may alleviate hypothalamic damage caused by heat attack by inhibiting inflammatory activities, and ASS1, HMGB2 and vimentin could be candidate factors involved in the exact mechanism.

Keywords: heat acclimation; exertional heat stroke; hypothalamus; proteomic analysis; inflammatory response

1. Introduction

Heatstroke (HS), a life-threatening condition, is characterized by an unregulated increase in core body temperature above 40 °C and central nervous system (CNS) dysfunction manifested as delirium, convulsions or coma [1]. Exertional heatstroke (EHS), the most serious type of heat stroke, usually occurs when individuals are challenged by intense physical activity with/without exposure to high temperature. EHS is regarded as one of the leading causes of death among athletes and military personnel worldwide [2–4]. Excessive endogenous heat produced by muscle contraction can overwhelm the temperature regulation system and cause EHS. A hot and humid environment blunts heat transfer processes from the skin to the air and can further raise the risk of EHS [5]. Hence, the combination of physical activity with external environmental factors results in continuous body heat storage and places tremendous stress on human tissues [6]. When the thermoregulatory system is insufficient to compensate for hyperthermia, the core body temperature continues to rise, triggering direct cytotoxic effects and the inflammatory response, eventually causing multiple-organ dysfunction/injury syndrome (MODS) [7,8]. The inflammatory response plays an essential role in MODS induced by heatstroke. Heatstroke induces high levels of proinflammatory cytokines, especially interleukin (IL)-1 and IL-1β [9–11]. In heatstroke models, IL-1 is related to severe nerve damage and high mortality [12], and IL-1β may also be associated with neuroinflammation [13]. Antagonizing IL-1 or IL-1β reduces nerve damage caused by heatstroke and prolongs survival [13,14]. Moreover, it has been reported that at the onset of EHS, IL-1β and interferon-gamma (IFN-γ) are significantly increased, while IL-10 is significantly decreased [15]. Heat acclimation has been shown to abrogate the changes in IL-1β, IFN-γ and IL-10 levels caused by EHS and improve organ injury [15]. In conclusion, cytokines such as IL-1, IL-1β, IFN-γ and IL-10 have significant impacts on the development of heatstroke and can serve as therapeutic targets. Besides, like IL-10, IL-4 is a classic anti-inflammatory cytokine, likely to play a role during the inflammation caused by heat stroke.
The hypothalamus plays an important role in the CNS and is responsible for thermoregulatory responses [16,17]. The hypothalamus preoptic area receives thermal sensory information and exits signals to mediate autonomic responses to increase heat loss and thereby activate heat defense mechanisms [18]. However, extreme heat stress contributes to thermoregulatory dysfunction in the hypothalamus, which in turn drives or exacerbates the process of heatstroke. Studies have shown that direct heat stress, neurological inflammation, ischemic and oxidative damage, overload of thermoregulation and increased monoamine neurotransmitter levels are factors leading to hypothalamic impairment during severe heat shock [12,13,19,20]. In addition, some studies utilized proteomics to identify the specific proteins associated with heat stroke-mediated hypothalamic dysfunction and explored the roles of the proteins. These findings provide information on the exact molecular mechanisms underlying heatstroke-induced hypothalamic injury [19,20]. However, these results were all based on classic heatstroke models, and whether the proteomic approach is applicable for investigating the effects of heatstroke on the hypothalamus needs to be further verified.

Heat acclimatization/acclimation (HA) is one of the most effective methods for reducing HS risk, especially as an achievable preventive measure for EHS, which generally occurs during planned occupational and sporting activities [21,22]. Exercise-heat acclimatization achieved by repeated heat exposure coupled with physical exercise has been widely practiced among military personnel and athletes [23,24]. HA has been shown to modulate thermoregulatory activity and thus augment heat tolerance. This process involves an increase in the heat stress response by increasing heat shock protein (HSP) expression and changes in the expression of genes associated with energy metabolism and immune responses [25–29]. It is well documented that HA training before engaging in strenuous physical exercise lowers the likelihood of acquiring EHS [30]. We successfully developed an HA rat model and verified its beneficial role in preventing EHS-mediated injury to the cerebral cortex. However, the effect of HA on the hypothalamus after EHS and its mechanism of action have rarely been reported. To solve the above problems, we first examined whether HA could mitigate HS-induced hypothalamic damage. Then, from the perspective of proteomics, we analyzed and compared the hypothalamic protein expression profiles of the EHS model rats and the EHS model rats after HA to explore the potential mechanism through which HA improved hypothalamic damage after EHS. The results might offer fresh perspectives and new targets for the development of efficient therapeutic medications and other treatment options for EHS.

In the present study, we hypothesized that HA is a protective agent that can alleviate the negative influence of EHS by modifying the hypothalamic proteome. To test our hypothesis, we established control, EHS, HA, and EHS + HA groups and detected pathological changes in the structure of the hypothalamic tissues in each group. The differentially expressed proteins between the EHS and HA + EHS groups were identified via proteome analysis. Using functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we obtained additional information regarding the mechanism underlying hypothalamic injury in EHS and the protective effects of HA on the hypothalamus after EHS. Finally, based on the proteomic analysis, we performed biological verification of these mechanisms at the tissue and serum levels and confirmed that HA might ameliorate the damage triggered by EHS to the hypothalamus by regulating proinflammatory proteins such as argininosuccinate synthetase (ASS1), high mobility group protein B2 (HMGB2) and vimentin to suppress the inflammatory response. Our study aimed to investigate the protective effects of HA against EHS-related hypothalamic disorders and the related mechanism and provide an experimental basis for therapeutic strategies that can treat organ dysfunction after EHS.

2. Materials and Methods

2.1 Animals

Pathogen-free male Wistar rats approximately 7 weeks of age with an initial weight of 200–250 g were purchased from Sipeifu (Beijing, China) Biotechnology Co., Ltd. The rats were kept at an ambient temperature of 24 ± 1 °C and a relative humidity of 50 ± 10%, and a 12 h:12 h light/dark period was implemented. Adequate food and water were provided. To rule out the effects of circadian rhythms, all the experiments were conducted at roughly the same time each day (between 12:00 and 14:00). The ambient temperature, humidity and light conditions were kept consistent. The experiments were conducted according to the ARRIVE 2.0 guidelines (https:// ARRIVEguidelines.org/arrive-guidelines) (Table 1). The experimental procedures were approved by Institutional Animal Care and Use Committee of Beijing Institute of Radiation Medicine (Ethics number: IACUCDWXZ- 2022-742) and conducted according to the guidelines and regulations for the use and care of experimental animals in China.

2.2 Establishment of a 21-day Heat-exercise Acclimation Model

In contrast to the heat acclimation model, which involves only a high-temperature environment without exercise, we propose a new HA training scheme in which rats are subjected to exercise simultaneously in a hot environment and verify the feasibility of this scheme for use with
Table 1. ARRIVE Essential 10.

| Study design | 1 | (a) Control groups were included.  
(b) The experimental unit: a single animal. |
| Sample size  | 2 | (a) In 21-day HA regimen, rats were divided into the control group and the HA model group, with 5 rats in each group. After the HA model was successfully established, another 36 rats were randomly divided into a control group, HA group, EHS group and HA + EHS group, with 9 rats in each group. Four hypothalamus samples were taken from each group for proteomic analysis, and the remaining 5 intact brain tissues were taken from each group for H&E staining.  
(b) The sample size was decided according to sample size calculation, previous researches and practical conditions. GPower was used to calculate the sample size. The details were described in the statistic paragraph. |
| Inclusion and exclusion criteria | 3 | (a) Including criteria: pathogen-free male Wistar rats approximately 7 weeks of age with an initial weight of 200–250 g; excluding criteria: Rats found to have health problems or abnormal behavior and rats that died before or during the experiment.  
(b) No animals were excluded from this study.  
(c) The value of \( n \) in each group were reported exactly. |
| Randomisation | 4 | (a) Randomisation was used to allocate experimental units to control and treatment groups. All the experiments were double-blind and randomized using a random number table.  
(b) To minimize potential confounders, all rats were kept at an ambient temperature of 24 ± 1 °C and a relative humidity of 50 ± 10% with 12 h:12 h light/dark period. Adequate food and water were provided. All the experiments were conducted at roughly the same time each day (between 12:00 and 14:00). The ambient temperature, humidity and light conditions were kept consistent during each experiment, and noise was excluded as much as possible. |
| Blinding | 5 | All the experiments were double-blind. Third party personnel other than observers and study subjects was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis). |
| Outcome measures | 6 | The changes in \( T_{core} \) and body weight during training, basal \( T_{core} \) before and after HA, and heat tolerance after HA were used as the outcome measures for HA model construction. |
| Statistical methods | 7 | (a) The details of the statistical methods used for each analysis and the software used were provided in Section 2.7.  
(b) The Shapiro-Wilk test was used to determine the normal distribution of the data. Levene’s test was used to determine the homogeneity of variance. |
| Experimental animals | 8 | The details of the animals used in the study described in Section 2.1. |
| Experimental procedures | 9 | The procedures in enough detail were provided in the Materials and Methods. |
| Results | 10 | Summary statistics for each experimental group presented as the mean ± standard error (SE) were reported. |

\( T_{core} \), core body temperature; EHS, Exertional heatstroke; HA, Heat acclimatization/acclimation; H&E, hematoxylin and eosin.
°C and 55 ± 5%) for 30 min, then they ran on the chamber treadmill at a certain speed (slope 0) for 30 min and subsequently left in the chamber for 1 h while they moved freely. On the first day after HA training, the heat endurance of the rats was tested; that is, the rats in the control group and the HA group were placed in an environment of 39.5 °C for 30 minutes and then transferred to room temperature. The T<sub>core</sub> changes in the rats were monitored. The changes in T<sub>core</sub> and body weight during training, basal T<sub>core</sub> before and after HA, and heat tolerance after HA were used as the evaluation indices for HA model construction [33].

2.3 EHS Protocols

The EHS rat model was established according to our previously reported method [33]. The rats were placed in a prewarmed chamber that was maintained at 39.5 ± 0.5 °C with a relative humidity of 55 ± 5% and started to run at a speed of 5 m/min with a slope of 0 for 2 min. The speed was increased by 1 m/min per 2 min, after which the speed was maintained at 10 m/min. The rats' body temperatures were not measured during the running. Running was stopped when the rats were fatigued. At this time, the T<sub>core</sub> of the rats was closely monitored every 5 min with a rectal thermometer, and 42 °C was considered the onset of heatstroke.

2.4 Experimental Protocol

After successfully establishing the 21-day HA model, another 36 rats were randomly and double-blindly divided into 4 groups: the sham control group (CON; n = 9), exertional heat stroke group (EHS; n = 9), heat acclimation group (HA; n = 9) and heat acclimation prior to heat stroke group (HA + EHS, n = 9). The CON group was exposed to room temperature (24 ± 1 °C) freely. The 21-day heat acclimation protocol was performed directly on the HA group without temperature pill monitoring. The EHS protocol was used in the EHS group. The HA + EHS group underwent 21-day HA training and then the EHS protocol on Day 25 [33].

After the above models were established, hypothalamic tissues were collected (n = 4) under anesthesia, and blood was collected from three of the rats in each group. The rats were anesthetized under 1.5% pentobarbital sodium anesthesia (CAS:57-33-0, Sigma Aldrich, St. Louis, MO, USA) (0.3 mL/100 g) via intraperitoneal injection, the abdominal cavity was opened after disinfection, and the inferior vena cava was exposed. The blood collection needle was subsequently inserted into the blood vessel, after which the blood was collected into coagulant tubes connected to the blood collection needle. Approximately 3 mL of blood was collected per tube. Serum samples were obtained by centrifuging the blood at 3000 rpm for 15 min in a refrigerated centrifuge and then immediately stored at −80 °C. The rats were then decapitated and the brain was quickly harvested and put on ice. A vertical incision (2 mm deep) was made at the optic chiasm close to the mammillary body, enabling the removal of the hypothalamus [26]. Hypothalamic tissues from one side were placed in sterile cryotubes and immediately frozen in liquid nitrogen for Tandem mass tag (TMT)-based proteomic analysis. The hypothalamus specimen from the other side was used for Western blot (WB) analysis. Brain tissues were extracted from another 5 rats and then immersed in 4% paraformaldehyde (PFA) for fixation and subsequent hematoxylin and eosin (H&E) staining.

2.5 Hematoxylin and Eosin (H&E) Staining

The brain tissues were fixed with 4% PFA, dehydrated with gradient ethanol and cleared with xylene, and subsequently embedded in paraffin (Biological Tissue Embedding Machine, Wuhan Junjie Electronics Co., Ltd., Wuhan, Hubei, China, model JB-P7). Each brain sample was then sagittally cut (4 µm) using a rotatory microtome (RM2235, Leica, Witzler, Hesse, Germany). After sectioning, the paraffin-embedded tissues were dewaxed, rehydrated, stained with hematoxylin and eosin, dehydrated, cleared and sealed in order [34]. Ten slices of brain tissue were taken from each rat. The sections were finally observed with an Olympus optical microscope (Olympus, Shanghai, China). The sections were observed at 200× magnification.

2.6 Proteomic Analysis Protocols

The proteomic analysis steps included protein sample pretreatment, enzymolysis, TMT labeling, reversed-phase chromatography, Liquid chromatography-mass spectrometry (LC–MS) analysis and data analysis; for specific details, refer to the published literature [33].

2.7 Western Blot Analysis

A tissue grinding machine (HM-24, Huxi Industrial Co., Ltd., Shanghai, China) was used to homogenize the hypothalamus samples from all groups (n = 4) by performing three cycles of 20 seconds each at 6000 rpm. The homogenization was carried out in Radio Immunoprecipitation Assay (RIPA) Lysis buffer (R0020). The lysates were obtained from Solarbio, a biotechnology company located in Beijing, China. Phenylmethanesulfonyl fluoride (PMSE) (P0100, Solarbio, Beijing, China) was added to the lysates. After centrifugation at 12,000 rpm for 15 minutes at 4 °C, the supernatant was collected. The concentration of proteins in the collected supernatant was measured using the Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (23227, Thermo-Scientific, Waltham, MA, USA).

Protein samples (20 µg) were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and subsequently transferred to a Polyvinylidene fluoride (PVDF) membrane with a pore size of 0.22 µm (PR05505, Immobilon-P, Boston, MA, USA). After being blocked with a 5% nonfat milk solution
for 2 hours, the membrane was subsequently exposed to the following antibodies: ASS1 (16210-1-AP, rabbit, 1:1000; Proteintech, Wuhan, Hubei, China), HMGB2 (14597-1-AP, rabbit, 1:1000; Proteintech), vimentin (10366-1-AP, rabbit, 1:1000; Proteintech) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245, mouse, 1:1000; Abcam, Cambridge, UK). Overnight at the following 4 ℃, the membrane underwent incubation in a secondary antibody, which was a conjugate of horse-radish peroxidase (HRP), and specifically targeted rabbit and mouse IgG using goat anti-rabbit and goat anti-mouse IgG (1:10,000, ZB-2301, ZB-2305, ZSGB Biotechnology Co., Ltd., Beijing, China), respectively, in a concentration ratio of 1:10,000, at room temperature for 1 hour and rinsed with triethanolamine buffered saline solution with Tween-20 (TBST) three times. Protein bands were visualized using an enhanced chemiluminescence (ECL) Western Blotting Detection kit (ab133406, Abcam, UK), and X-ray imaging was used to visualize the bands. The software ImageJ 5.0 (National Institutes of Health, Bethesda, MD, USA) was used to compute the grayscale values of the bands.

2.8 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the serum levels of IL-4, IL-1, IL-8, IL-1β, IL-10, and IFN-γ (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer’s instructions (https://www.mlbio.cn/) (n = 3). Three replicate wells were used, and the experiment was repeated three times. The absorbance was measured at 450 nm with an optical absorption enzyme marker (Molecular Devices, SpectraMax 190, Sunnyvale, CA, USA). The concentrations of IL-4, IL-1, IL-8, IL-1β, IL-10, and IFN-γ were calculated from standard curves and are presented in pg/mL.

2.9 Statistical Analysis

All of the data are presented as the mean ± standard error (SE). Statistical analysis was performed using SPSS Statistics Software Version 23.0 (SPSS, Inc., Chicago, IL, USA), while GraphPad Prism Software Version 9.0 (GraphPad Software, La Jolla, CA, USA) was utilized for data visualization. Two-way repeated-measures Analysis of Variance (ANOVA) with the Bonferroni correction was used to compare $T_{\text{core}}$ and body weight. Multiple comparisons were used to compare two groups at the same point in time. Differences in WB, ELISA protein quantification and cell counts after H&E staining were evaluated via one-way ANOVA. The Shapiro-Wilk test was used to determine the normal distribution of the data. Levene’s test was used to determine the homogeneity of variance. A significance level of $p < 0.05$ was considered acceptable.

The sample size was decided according to sample size calculation, previous researches and practical conditions. In 21-day HA regimen, G.Power was used to calculate the sample size, and $t$ tests were used; the effect sizes were 0.5, $\alpha = 0.05$, and $1-\beta = 0.8$, and the total sample size was 128.
Fig. 2. Structural alterations in hypothalamus. Heat acclimation (HA) can improve structural abnormalities in the hypothalamus in rats after exertional heat stroke (EHS). Representative images of hypothalamic hematoxylin and eosin (H&E) staining in the control group (A), exertional heat stroke (EHS) group (B), heat acclimation (HA) group (C) and HA + EHS group (D). Arrows indicate cell swelling and cellular vacuolation. (E) The total number of hypothalamic cells in the control, EHS, HA and HA + EHS groups. *p = 0.000 on EHS vs. Control; *p = 0.006 on EHS vs. HA + EHS; *p = 0.001 on HA vs. Control (n = 5).

However, this number is too high for animal experiments. According to the principle of reduction in the Replacement, Reduction and Refinement Principle (3Rs) of animal experiments and with reference to previous studies [35–37], we ultimately divided 10 rats into groups; the temperature data of 5 rats in each group were published, and the expression levels of heat shock proteins in various organs after the completion of heat acclimation training were also published [33]. Unpublished data on rat body temperature and body weight are presented here.

The number of animals chosen for hypothalamus tissue collection and blood withdrawal were also determined by calculation, reported researches and practical limitations. G.Power was used to calculate the sample size, and univariate analysis of variance was used to analyze the data. The results were as follows: effect size = 0.4, α = 0.05, 1-β = 0.8, and total sample size = 76. However, our experiment is limited by practical conditions. In fact, in addition to those from the hypothalamus, we also collected the cortex, hippocampus, and kidneys from each group of rats for proteomics. There were 64 protein-sequenced samples. To minimize the cost and not exceed the project budget, we chose to add one additional rat to the minimum sample size of three, that is, four rats in each group, for proteomic analysis. In a previous study [38], rats were classified into five groups, and three liver samples from each group were randomly selected for TMT-labeled quantitative proteomics. Therefore, four hypothalamic samples from each group used for proteomics was acceptable. In addition, when blood was collected from rats, the blood exhibited a hypercoagulable state after exertional heatstroke, which was also consistent with the pathological coagulation involved in heatstroke [39]. Blood collection was indeed difficult, so only three rats were collected from each group in the EHS and HA + EHS groups, and a sample size of three in each group was also used in the control and HA groups for ease of description.

3. Results

3.1 Establishment of a 21-day Heat-exercise Acclimation Rat Model

The results of this section demonstrate the feasibility of our proposed heat acclimation scheme. The changes in body weight and $T_{core}$ during the 21-day heat-exercise exposure shown in Fig. 1 were observed in some of the rats during the process of establishing the HA model, and the rest of the changes can be found in our published article [31]. The experimental scheme of establishing the HA model is shown in Fig. 1A. All of the rats (n = 5) gained weight during the HA modeling period. After 14 and 21 days of heat-exercise exposure, the increase in weight in the HA group was slower than that in the CON group (69.20 ± 3.18 vs. 103.90 ± 4.10 g ($p = 0.003$) and 101.16 ± 1.87 vs. 159.20 ± 5.70 g ($p = 0.000$), respectively) (Fig. 1B). The $T_{core}$ of the HA group rats during the training periods on Days 1, 7, 14 and 21 are presented in Fig. 1C. The rats’ core temperatures increased after they were placed in the preheated chamber, peaked at the end of the running, and
3.2 Structural Alterations

H&E staining was used to visualize the hypothalamic microstructure in each group. Compared with the hypothalamic neurons in the control group (Fig. 2A), those in the EHS group exhibited swelling, loosening of the cytoplasm, widespread vacuolation and nuclear pyknosis (Fig. 2B). In the HA group, nuclear pyknosis and cell swelling were inconspicuous (Fig. 2C). Nuclei exhibiting pyknosis and cellular edema were significantly less severe in the HA + EHS group (Fig. 2D) than in the EHS group. In addition, the number of cells in hypothalamic tissue decreased significantly after EHS ($p = 0.000$; Fig. 2E), while the number of cells in the HA + EHS group was significantly higher than that in the EHS group ($p = 0.006$; Fig. 2E).

3.3 Overview of Differentially Expressed Proteins Based on TMT-based Proteomic Analysis

The differentially expressed proteins (DEPs) in the hypothalamus from rats in the EHS and HA + EHS groups were analyzed using TMT-based proteomic analysis. The threshold fold change (FC) values were above 1.2 or below 0.8, respectively, with a statistically significant $p$ value $\leq 0.05$. Compared with those in the EHS group, 153 upregulated proteins and 161 downregulated proteins were
Fig. 4. Bioinformatics analyses of the differentially expressed proteins. (A) Gene Ontology (GO) enrichment analysis of biological process (BP), cell component (CC), and molecular function (MF) terms between heat acclimation followed by exertional heat stroke (HA + EHS) group and EHS group are shown. (B) Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways identified in the HA + EHS vs. Control (CON) comparison are shown.
Fig. 5. Heatmaps and Gene Ontology (GO) enrichment analysis between exertional heat stroke (EHS) and heat acclimation (HA) + EHS revealed the key differential proteins involved in inflammation. (A) Heatmap of the HA + EHS group vs. the EHS group shows the relative abundances of the differentially expressed proteins involved in inflammation. The downregulated proteins included metalloproteinase inhibitor 2 (TIMP2), programmed cell death protein 10 (PCDP10), S100 calcium-binding protein A13 (S100A13), argininosuccinate synthetase (ASS1), high mobility group protein B2 (HMGB2) and vimentin (VIM). (B) GO enrichment analysis showed that the BPs enriched in these differentially expressed proteins were associated with the response and regulation of cytokines such as interferon-gamma and tumor necrosis factor. (C) The results of the molecular function (MF) analysis are shown. The enriched MFs in the differentially expressed proteins were associated with cell adhesion molecular binding, the receptor for advanced glycation end products (RAGE) binding and integrin binding related to immune inflammation. (D) The cell component (CC) enrichment results are shown. The CCs associated with these differentially expressed proteins were synapses, cell junctions, and neural projections.

observed in the HA + EHS group. The differences between the groups are listed in Supplementary Table 1 and visualized in volcano plots (Fig. 3A). Volcano plots revealed significant differences in protein expression between the HA + EHS and EHS groups. The blue points on the left represent the underexpressed proteins, and the overexpressed proteins are indicated by red points on the right. Hierarchical clustering analysis of protein expression was further performed to determine the protein expression profiles (Fig. 3B). Notably, among these DEPs,
proinflammatory proteins such as argininosuccinate synthetase (ASS1; gene name: Ass1), high mobility group protein B2 (HMGB2; gene name: Hmgb2), metalloproteinase inhibitor 2 (TIMP2; gene name: Timp2), programmed cell death protein 10 (PCDP10; gene name: Pdcd10), S100 calcium-binding protein A13 (S100A13; gene name: S100a13), and vimentin (VIM; gene name: Vim) [40–42] were expressed at lower levels in the HA + EHS group. These findings suggested that EHS could disturb hypothalamic function by altering the abundance of certain proteins that regulate the inflammatory response and that HA could reverse some of the changes caused by EHS, thereby exerting a protective effect on organ function.

3.4 Bioinformatics Analyses

Subsequently, Gene Ontology (GO) functional annotation clustering and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted on the hypothalamus of the HA + EHS vs. EHS groups to further explore the biological functions of the DEPs. The results of the GO analysis of hypothalamic proteins are shown as follows. Biological process (BP) analysis revealed that the differentially expressed proteins in the HA + EHS and EHS groups were involved in nervous system development, regulation of cell homeostasis, and cyclic adenosine monophosphate (cAMP) biosynthetic process. The enriched molecular functions (MF) were structural molecule activity, calcium-dependent protein binding, and the receptor for advanced glycation end products (RAGE) receptor binding. The enriched cellular component (CC) terms included cell junctions, cell projections and plasma membrane-bounded cell projections (Fig. 5A; Supplementary Table 2). Notably, the proinflammatory differential proteins are showed in Fig. 5A. ASS1 and HMGB2 mentioned above are involved in BP (Fig. 5B) related to the regulation of the response to cytokine stimuli and inflammatory reactions, and they also participate in MF (Fig. 5C) associated with RAGE receptor binding, cell adhesion molecule binding and integrin binding, which mediate cytokine activities and the immune response process. The CC terms were synapses, cell junctions, and neural projections (Fig. 5D). KEGG pathway analysis revealed that the hypothalamic DEPs in HA + EHS vs. EHS rats were associated with spliceosome pathways (Fig. 4B; Supplementary Table 3). Protein–protein interaction network analysis (PPI network analysis) (Supplementary Fig. 1) revealed the predicted functional associations between these different proteins and inflammation.

Thus, controlling inflammatory conditions could be a key strategy employed by HA to mitigate the hypothalamic dysfunction induced by EHS. ASS1, HMGB2 and VIM may be candidate proteins involved in the mechanism underlying the protective effect of HA against hypothalamic injury after EHS.

3.5 Validation of Candidate Proteins Using Western Blotting

To validate the above differential proinflammatory proteins, we performed Western blotting. Compared with those in the CON group, the expression of ASS1 (p =
Fig. 7. The ability of heat acclimation (HA) to regulate the inflammatory response in exertional heat stroke (EHS) was validated by measuring interleukin (IL)-1, IL-1β, IL-8, IL-4, IL-10 and interferon-gamma (IFN-γ) levels via Enzyme-linked Immunosorbent Assay (ELISA). (A–C) The expression levels of IL-1β, IL-1 and IL-8 in the EHS and HA samples were greater than those in the control samples, and the cytokine levels in the EHS samples were more obviously elevated. However, the levels of these cytokines in the heat acclimation followed by exertional heatstroke (HA + EHS) group were evidently lower than those in the EHS group. (D–F) IL-4, IL-10 and INF-γ levels in the EHS and HA groups were reduced compared with those in the control group, and cytokine levels in the EHS group presented a more visible decrease. IL-4 and IL-10 levels in the HA + EHS group were evidently greater than those in the EHS group, although there was no change in INF-γ. *p values were calculated by one-way analysis of variance (ANOVA); ns, not significant; *, p value indicates statistical significance; the data show one representative experiment from at least 3 independent experiments with triplicate biological replicates in each (mean ± SE).

0.024), HMGB2 (p = 0.009) and VIM (p = 0.009) in the EHS group was significantly greater, and the protein levels of ASS1 and HMGB2 in the HA group were not significantly different, while the expression of VIM in the HA group was significantly greater (p = 0.000). Compared with those in the EHS group, the relative expression levels of ASS1 (p = 0.047), HMGB2 (p = 0.000) and VIM (p = 0.009) were decreased in the HA + EHS group (0.71-, 0.67-, and 0.64-fold, respectively; Fig. 6). Supplementary Figs. 2,3,4,5 present the full-length blots/gels of HMGB2, ASS1, VIM and GAPDH.

3.6 Cytokine Level Verification via ELISA

Proinflammatory cytokines such as IL-1 and IL-8 are involved in the inflammatory response promoted by ASS1, HMGB2 and VIM [43–45]. Thus, we examined the expression of IL-1β, IL-1, IL-8, IL-4, IL-10 and IFN-γ in the serum samples obtained from the rats in each group. IL-1, IL-1β and IL-8 expression levels were greater in the EHS and HA samples than in the control samples (IL-1: p = 0.000 EHS vs. CON, p = 0.017 HA vs. CON; IL-1β: p = 0.000 EHS vs. CON and HA vs. CON; IL-8: p = 0.000 EHS vs. CON and HA vs. CON), with the EHS sample exhibiting a more pronounced increase. However, there the total cytokine level in the HA + EHS group was lower than that in the EHS group (p = 0.000) (Fig. 7A–C). The expression levels of IL-4, IL-10, and INF-γ were lower in the EHS and HA groups than in the control group (p = 0.000), with the EHS group showing a more substantial decline. Although there was no change in INF-γ, the IL-4 and IL-10 levels in the HA + EHS group were noticeably greater than those in the EHS group (p = 0.017 and p = 0.001, respectively) (Fig. 7D–F). These data suggested that HA could improve the hypothalamic proinflammatory reaction provoked by heatstroke by regulating the activities of ASS1, HMGB2, VIM, and related cytokines.

4. Discussion

When the thermoregulatory system is unable to maintain the core body temperature within a normal range during heat stress, hyperthermia occurs. The cytotoxic effects of hyperthermia (>41 °C) induce tissue damage and multiple
organ failure [46–48]. Heat acclimation is generally known to improve thermoregulation and lower the risk of heatstroke. However, the molecular mechanisms underlying the protective effects of HA against HS have not been elucidated. The hypothalamus is the dominant region of the thermoregulatory system. Therefore, we hypothesized that HA plays essential roles in alleviating hypothalamic dysfunction caused by heatstroke. In this study, pathological analysis, TMT-based proteomic analysis, protein immunoblotting and enzyme-linked immunosorbent assays were used to determine the effect of HA on ameliorating hypothalamic damage from EHS and the related mechanism.

Based on the findings of previous studies, the relationship between hypothalamic injury and heatstroke can be summarized as follows: extreme heat exposure leads to decreased mean arterial pressure (MAP), increased intracranial pressure (ICP), and reduced cerebral perfusion pressure (CPP = MAP – ICP), which may trigger hypothalamic ischemia and oxidative stress [49], in turn causing hypothalamic tissue inflammation and neuronal damage [50]. These changes not only result in thermoregulation deficits but also affect the hypothalamic–pituitary–adrenal axis (HPA axis) and exacerbate heat intolerance, eventually leading to multiple-organ dysfunction or failure and promoting the occurrence of heatstroke [51,52]. Systemic inflammatory response syndrome and coagulation abnormalities accompany the progression of heatstroke [1,53], which further exacerbate hypothalamic inflammatory insult. Therefore, hypothalamic inflammation is closely associated with the occurrence and progression of heatstroke.

In the present study, we first developed a stable HA rat model, providing the basis for establishing a rat model of EHS following HA. Consistent with these earlier findings, our data showed that HA decreased body weight and basal core temperature as well as improved heat tolerance.

Our pathological test results showed that the hypothalamus had evident pathological structural changes after heat shock, which was consistent with the results of some reported studies [19,20,54]. The differentially expressed proteins revealed by TMT-based proteomic analysis were related to several cellular activities. In particular, we found that some downregulated proteins between the HA + EHS group and the EHS group were associated with cytokine activity and the inflammatory response. These genes included ASS1, HMGB2, and VIM. Activation of ASS1 plays a central role in mediating host innate immune defense against bacterial infection in vivo by controlling inflammatory macrophage activity [40]. Additionally, as an immunomodulatory molecule, ASS1 modulates the immunological microenvironment and cytokine response via C-X-C motif chemokine ligand 8 (CXCL8) signaling [55]. HMGB2 is known for its proinflammatory role in mobilizing innate immunity and is involved in signaling pathways that are associated with various inflammatory disorders [56]. VIM is critical for nucleotide-binding oligomerization domain (NOD)-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome activation, and its loss leads to decreased levels of proinflammatory cytokines, subsequently protecting against harmful inflammation [57]. Moreover, VIM induces Golgi body stress and neuroinflammation as a component of the NLRP3 pathway [57]. These findings suggested that the harmful effect of EHS on the hypothalamus might be caused by excessive inflammation. Proteomic analysis suggested that HA might ameliorate hypothalamic abnormalities after heat attack by modulating inflammatory responses. Proteomic analysis indicated that proinflammatory proteins such as ASS1, HMGB2 and VIM may be involved in the regulatory effect of HA on hypothalamic inflammation after EHS.

Previous findings have demonstrated that HS induces an inflammatory response in the hypothalamus [19,20,54], and it has been shown that HA regulates hypothalamic metabolic and inflammatory processes [26]. However, few studies have examined how HA affects the hypothalamus following the onset of heatstroke. We found that HA could reduce the hypothalamic damage caused by EHS, and this protective effect could be accomplished by reducing inflammation promoted by ASS1, HMGB2, and VIM.

Additionally, proinflammatory cytokines such as IL-1 are engaged in the proinflammatory activities of ASS1, HMGB2, and VIM [43–45]. We further measured the concentrations of important immuno-inflammatory mediators. Heat acclimation reduced the levels of cytokines, indicating that inflammatory activation was triggered by EHS. Therefore, ASS1, HMGB2, and VIM may be potential targets through which HA improves hypothalamic inflammation after EHS.

Our study has several limitations. First, in addition to controlling body temperature, the hypothalamus is also responsible for coordinating multiple physiological homeostasis pathways, such as the stress response, electrolyte and water balance, and energy metabolism. These results may lack specificity because we did not conduct additional studies to determine whether these unique proteins and pathways are involved in the regulation of other processes in the hypothalamus in addition to body temperature regulation. Additionally, the molecular mechanism by which HA protects against EHS was not thoroughly investigated. For example, the particular pathways involved were not investigated. Third, only serum cytokine levels were examined; the amounts of cytokines in hypothalamic tissue were not. However, the detection of serum cytokines is easier and more practical for clinical applications. Fourth, changes in other classic inflammatory proteins, such as tumor necrosis factor-alpha (TNF-α), IL-6, IL-18, HGBM1, NLRP3, inducible nitric oxide synthase (iNOS), macrophage Inflammatory Protein-1 alpha (MIP-1α) and cyclooxygenase-2 (COX2), were not investigated. Further studies are needed to fill these gaps.
5. Conclusions

In conclusion, HA improved the anatomical abnormalities of the hypothalamus caused by EHS. This could be attributed to the fact that HA regulates proinflammatory proteins such as ASS1, HMGB2, and VIM, which in turn affects the inflammatory response to EHS.

Abbreviations

HA, heat acclimatization/acclimation; EHS, exertional heat stroke; AT, ambient temperature; RH, relative humidity; T_core, core body temperature; CNS, central nervous system; HSP, heat shock protein; DEPs, differentially expressed proteins; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FC, fold change; BP, biological process; MF, molecular function; CC, cellular component; HPA axis, hypothalamic–pituitary–adrenal axis; ASS1, argininosuccinate synthetase; HMGB2, high mobility group protein B2; VIM, vimentin.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article and its Supplementary information files.

Author Contributions

WJZ, LFW and QS conceptualized and designed the study. FX, LZM, XL, JBZ and JM performed the research. SYL, HDMS and LX provided help and advice on the methods. FX, LZM, XL, JBZ and JM analyzed the data. FX, JBZ and JM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The experimental procedures were approved by Institutional Animal Care and Use Committee of Beijing Institute of Radiation Medicine (Ethics number: IACUC-DWZX-2022-742) and conducted according to the guidelines and regulations for the use and care of experimental animals in China.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2306116.

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