Original Paper

Heat Acclimation Regulates the Autophagy-Lysosome Function to Protect Against Heat Stroke-Induced Brain Injury in Mice

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Key Words

Heat stroke • Heat acclimation • Autophagy • Lysosome • Brain injury

Abstract

Background/Aims: The mechanisms underlying the protective role of heat acclimation (HA) in heat stroke (HS)-induced brain injury are still unclear. The autophagy-lysosome pathway is known to play an important role in protecting stressed or diseased cells from death. Nevertheless, whether autophagy and lysosomes are involved in HA-mediated neuroprotection following HS exposure remains unclear. **Methods:** The protective effects of HA were assessed by rectal temperature, hematoxylin-eosin staining, transmission electron microscopic analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining, and Fluoro Jade B staining, after mice were subjected to HS. The effects of HA on autophagy and lysosomes were assessed in the presence of the autophagy inhibitor 3-methyladenine (3MA). Autophagy and lysosome-associated proteins were analysed by Western blotting. **Results:** We found that HA protected against HS-induced death and brain injury. HS can robustly induce autophagy and impair lysosome function. HA pre-conditioning significantly modulated the autophagy level, and improved lysosome function in HS mice. Furthermore, 3MA completely abolished the neuroprotective effect of HA on HS. **Conclusion:** HS may induce brain injury through lysosomal dysfunction and impaired autophagic flux. HA protected against HS-induced brain injury via a mechanism involving the autophagy-lysosome pathway.

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Introduction

When the core body temperature increases to more than 40°C, heat stroke (HS) may occur. This elevated body temperature is accompanied by dryness of the skin and central nervous system dysfunction (delirium, confusion, coma and seizures) [1]. Because of

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sustained increases in climatic temperatures, HS is becoming one of the leading causes of mortality. Global warming may contribute to an increasing number of heat-related deaths [2, 3]. A cohort study showed that the case mortality of HS at one year was approximately 43% and that the majority of patients suffered from neurological damage after their release from the hospital [4]. The inability of modern medicine to properly diagnose and treat the long-term sequelae of HS is due to a serious limitation in our understanding of the pathophysiological mechanisms of HS-induced injury. However, exposure to moderate heat over a long period of time can induce heat acclimation (HA), which offers protection against acute HS and various other stressors [5], although the mechanisms underlying this effect remain unclear.

Autophagy is increasingly considered an important adaptive response that can protect stressed or diseased cells from death. [6]. Autophagy is predominantly involved in the digestion and reuse of macromolecular substances and damaged organelles in cells and promotes cell survival by maintaining cellular homeostasis [7]. This process includes the generation of an autophagic vesicle and fusion of the autophagosome with a lysosome, yielding an autophagolysosome. Then the contents of the autophagolysosome are degraded [8]. During autophagy, the autophagosome packages the proteins and organelles that need to be degraded and fuses with lysosomes to form an autolysosome; then, the packaged contents are digested by cathepsins and hydrolytic enzymes in the lysosome, and the products are reused by the cell. Thus, the lysosome plays a key role in autophagy. In cells with impaired autophagy due to stress, lysosomes are involved in the impairment.

Both HA and HS are induced by elevated temperatures in the external environment. However, they differ with respect to the conditions in which they occur; HA occurs following long-term and repeated mild thermal stimulation, whereas HS results from fatal, high-intensity thermal stimulation over a short period of time. Furthermore, the physiological effects of HA and HS are completely different [5]. To date, whether the function of lysosomes differs between the two types of heat stresses (HA and HS) and how these differences in lysosomal function may affect the autophagy remain unclear. Autophagy protects the nervous system from injury by removing neurotoxic protein aggregates from neurons. However, when autophagy is upregulated in an uncontrolled manner, it can be harmful to cells and lead to cell death. This process is referred to as autophagy-programmed cell death, which may occur through the activation of apoptosis [8]. Previous reports have demonstrated that HS increases the levels of autophagy which plays a protective role in neurodegeneration [9]. Thus, we hypothesized that the type of autophagy that is induced as well as its mechanism of pathological and physiological regulation may differ between HA and HS.

Based on the above findings, we hypothesized that the autophagy-lysosome pathway may play an important role in the neuroprotection that is induced by HA in response to HS exposure. We conducted this study to investigate the characteristics of HS-induced brain injury, examine HA-induced neuroprotection, and evaluate the role of the autophagy-lysosome pathway in HA-induced neuroprotection.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. The protocol was approved by the Committee for the Welfare and Ethics of Laboratory Animals, Third Military Medical University. All surgery was performed under sodium pentobarbital anaesthesia as described below, and all efforts were made to minimize suffering. Animals were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg).

Animals and experimental protocols

Adult C57BL6/J mice (8 weeks of age, male) were obtained from the Animal Centre of the Third Military Medical University (Chongqing, China). The animals were housed under standard conditions

with a temperature of $23 \pm 1^\circ\text{C}$, $55 \pm 5\%$ humidity, and a 12:12 h light/dark cycle. Food and water were provided *ad libitum*. There were three groups. Mice in the normothermia group were maintained at an ambient temperature ($23 \pm 1^\circ\text{C}$). Mice in the HS group were exposed to $43 \pm 0.5^\circ\text{C}$ for 120 min in the specific environmental control smart chamber (HOPE-MED 8150E, Tianjin, China) to achieve a core temperature (T_{co}) of $>42.5^\circ\text{C}$ (HS onset). T_{co} was indirectly measured by a closely approximate rectal temperature (T_{re}). Mice in the HA group were held under ($35 \pm 0.7^\circ\text{C}$) for 30 days; this period has been shown to establish a stable acclimated status with a lower basal metabolic rate, a lower heart rate, and improved thermotolerance [10, 11]. After HA, the mice were injected intraperitoneally with the autophagy inhibitor 3-methyladenine (3MA) (Sigma-Aldrich, St. Louis, MO, USA) at 15 mg/kg 1 h before heat stress exposure [12]. For the surgery, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). If the mice showed increases in heart rate or respiratory frequency or spontaneous movements, additional doses of 10 mg/kg were administered.

Analysis of survival rate

Adult C57BL6/J mice were randomly assigned to two experimental groups, HS and HA pre-conditioned HS. After heat exposure, mice were removed from the heat, and placed back into the cage with *ad libitum* food and water for undisturbed recovery at $25 \pm 2^\circ\text{C}$. Animals were monitored for signs of morbidity every 15 min after heat exposure. Animals were euthanized if they displayed morbid symptoms of imminent death, including reduced locomotion, no response to tail pinch, paralysis, ataxia, and altered breath frequency. Time to death was recorded based on the humane euthanasia of non-acclimated and the acclimated mice after heat stress exposure. Survival data were calculated as percent survival versus time immediately after heat exposure.

Haematoxylin and eosin stain

Fixed brain tissues were embedded in paraffin and serially sectioned ($5 \mu\text{m}$). The sections from the cerebral cortex were stained with haematoxylin and eosin and then observed under an optical microscope (Leica, Germany).

Fluoro-Jade B histochemistry

Fluoro-Jade B (FJB) is a marker of degenerating neurons. FJB staining was performed as described in a previous study [9]. Briefly, the sections were successively immersed in 1% sodium hydroxide in 80% alcohol for 5 min, 70% alcohol for 2 min and in distilled water for 2 min. The slides were then transferred to a solution containing 0.06% potassium permanganate, and after 10 min, the slides were rinsed with distilled water for 2 min and then incubated in a 0.0004% FJB (Millipore) staining solution for 30 min. Following the incubation, the slides were rinsed in distilled water three times. Then, the slides were allowed to dry. The sections were then immersed in xylene for 1 min before the slides were coverslipped with DPX (Beyotime). All sections were examined using a fluorescence microscope with 450-490 nm excitation light.

Detection of apoptosis by TUNEL staining

To detect apoptosis, the sections were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay using an *in situ* apoptosis detection kit (Roche Diagnostics Corp, Switzerland). Based on the manufacturer's instructions, sections ($20 \mu\text{m}$ thick) of the cerebral cortex were incubated with PBS containing 3% H_2O_2 for 10 min. The slides were then permeabilized by 0.1% Triton-X 100 for 5 min. After they were washed, the slides were incubated with the TUNEL reaction solution at 37°C for 1 h. Subsequently, the sections were washed three times with PBS and incubated with converter-POD at 37°C for 30 min. After they were washed, the samples were incubated with the DAB substrate solution at room temperature for 10 min. After staining, all sections were observed and photographed using a microscope.

Immunofluorescence

Brain sections ($20 \mu\text{m}$ thick) were incubated with 1.5% blocking serum at room temperature for 1 h, and then with an anti-LC3 (Cell Signaling Technology; rabbit, monoclonal, 1:100) antibody at 4°C overnight. Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Life Technology; 1:200) was used to visualize immunoreactivity. Then, all sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 3 min to stain the nuclei. All sections were observed and photographed under a

Immunoblotting

Cerebral cortex tissues were collected from the mice. The tissues were lysed in RIPA lysis buffer (Beyotime Company, Jiangsu, China) containing a cocktail of phosphatase and protease inhibitors (Roche Diagnostics Corp). The protein lysates were separated on 4% to 12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen Corp, Carlsbad, CA, USA), and the proteins were then electrophoretically transferred onto polyvinylidene fluoride membranes (Invitrogen). Each membrane was blocked in 5% nonfat powdered milk in PBS for 1 h. After blocking, the membrane was incubated with an anti-LC3 (Cell Signaling Technology; rabbit, monoclonal, 1:1,000), anti-Beclin-1 (Cell Signaling Technology; rabbit, monoclonal, 1:1,000), anti-cathepsin B (Santa Cruz; rabbit, monoclonal, 1:1,000) or anti-lysosomal-associated membrane protein 1 (LAMP-1, Santa Cruz; rabbit, monoclonal, 1:1,000) antibody at 4°C overnight. After washing, the membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody (Life Technology; diluted 1:10,000 in blocking buffer) for 1 h. After washing, the blots were developed using an enhanced chemiluminescence (ECL) system (Amersham Corp., Cardiff, UK).

Cathepsin B Activity Assay

The CTSB/cathepsin B assay was conducted using a CTSB fluorometric assay kit (ab65302, Abcam). Briefly, mice were anesthetized, perfused intracardially with heparinized saline, and decapitated. Damaged cortical tissue was dissected and homogenized in ice-cold cell lysis buffer. After centrifugation at 4°C at 15,000 g for 5 min, the protein concentration was determined by a BCA kit (Beyotime). Then, 50 ng protein was incubated with the CTSB substrate mixture at 37°C for 1 h. Fluorescence intensity was examined by a fluorescence plate reader (Synergy Hybrid, Bio-Tek) at Ex/Em=328/460 nm.

Evaluation of oedema

Whole brains were obtained after the mice were sacrificed following heat exposure. First, the wet weight of each brain was determined. Second, the brains were fully dried in an oven at 105°C, and their dry weight was measured. The water content was calculated as a percentage according to the following formula: $100 \times (\text{wet weight} - \text{dry weight}) / \text{wet weight}$.

Transmission electron microscopic (TEM) examination

After the mice were anesthetized, they were perfused intracardially with heparinized saline, followed by PBS containing 4% paraformaldehyde. The brains were removed and stored overnight in 2% paraformaldehyde. The next day, the brains were cut into 50-μm-thick slices with a vibratome. The parietal lobe cortex was postfixed in 1% osmium tetroxide for 2 h, dehydrated in graded propanone, and embedded in epoxy resin. Polymerization was conducted at 80°C for 24 h. A Reichert ultramicrotome was used to cut blocks into ultrathin sections (60–70 nm), which were then post-stained by uranyl acetate and lead citrate and viewed under a TECNAI10 electron microscope (FEI). To quantify the number of autophagosomes, three mice in each group and 10 fields for each mouse were examined using a previously described protocol [13].

Statistical analysis

The relative intensities of the bands from Western blot were evaluated by the NIH Image 1.62 software. SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used to perform one-way ANOVA of multiple variables, and unpaired Student's t-test was used to compare two groups. Values are expressed as the mean ± standard error of the mean (SEM). A *P*-value < 0.05 was considered statistically significant.

Results

HA protected against HS-induced death and brain injury

To confirm the protective role of HA, rectal temperature (Tre), mortality, brain water content, HE staining, TEM ultrastructure, FJB staining, and TUNEL staining were assessed in C57BL6/J normal control mice and HA pre-conditioning mice after they were exposed to 43°C for 120 min and then allowed to recover at 25°C for 24 h. Consistent with previous studies of classic adaptations after HA, the Tre profile of HA mice showed an initial overshoot,

Fig. 1. The protective role of HA against HS-induced injury in mice. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to a 35°C for 30 days. Rectal temperature during acclimation (A) and HS-recovery (B). (C) Survival curves of HS mice and HA pre-conditioned HS mice (n = 12–14 per group). (D) Brain water content at the indicated time points in control and HA mice with or without HS treatment. (E) Representative HE staining and TEM photomicrographs of brain sections at 12 h post-HS (n = 6 per group, 8–10 histological sections per brain). Data are expressed as the mean ± SEM (n = 6–8 per group). *P < 0.05 vs. CON. # P < 0.05 vs. HA (-) at the same time point post-HS. N, nucleus; CON, normal mice with or without HA pre-conditioning; HS, heat stroke; HA, heat acclimation; HE, haematoxylin and eosin; TEM, transmission electron microscope. Scale bar, 50 µm and 1 µm.

followed by a slow and stable change to a Tre that was significantly decreased, compared with the control mice (Fig. 1A). Following exposure to 43°C for 120 min, the majority of the HS mice were unconscious or in a coma, whereas the HA pre-conditioned HS mice were still conscious, although their activity level was reduced (data not shown). A prevention of the depth of hypothermia was observed at the time points of 60, 90, 120, and 180 min in HA mice after HS (Fig. 1B). After 24 h of recovery, the mortality rate was 35.7% (5/14) in the HS group, whereas no mice died in the HA pre-conditioned HS group (Fig. 1C). The brain water content of the HA pre-conditioned HS mice was significantly lower than that of the HS

Fig. 2. Effect of HA on HS-induced neuronal injury and cell death. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. (A) Representative photomicrographs from FJB and TUNEL-stained brain sections at 12 h post-HS. (B) Quantification of the FJB-labelled cells was calculated from (A). The percentage is expressed as the number of FJB-stained cells divided by the total number of DAPI-stained cells. (C) Quantification of TUNEL-labelling was calculated from (A). The percentage is expressed as the number of TUNEL-stained cells divided by the total number per field of view. Data are expressed as the mean $\pm$ SEM ($n = 6$ per group, 8–10 histological sections per brain). CON, normal mice with or without HA pre-conditioning; HS, heat stroke; HA, heat acclimation; FJB, Fluoro-Jade B; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling. Scale bar, 25 μ m. ** $P < 0.01$ vs. CON. *** $P < 0.05$ vs. HA (-) post-HS.

mice and, reached its peak value after 12 h of recovery (Fig. 1D). Because the most serious injury occurred at 12 h, we chose this time point to examine the histopathological injuries. As demonstrated in Fig. 1E, HE staining of brain sections from HS mice showed substantial cell swelling; cytoplasmic rarefaction; chromatin concentration and edge accumulation; pyknotic nuclei; and cell pyknosis, rupture, dissolution, and vacuolation. Furthermore,

Fig. 3. Effect of HA on HS-induced autophagy in mice. Representative photomicrographs of LC3 immunostaining and representative TEM images of mouse brains at 12 h post-HS. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. n = 6 per group, 8–10 histological sections per brain. N, nucleus; CON, normal control mice; HS, heat stroke; HA, heat acclimation; TEM, transmission electron microscope. Scale bar, 25 µm and 1 µm.

TEM examination revealed endoplasmic reticulum shortening, scattering and disordered arrangement; enlarged mitochondria; large quantities of pyknotic mitochondrial DNA; vacuolation; and reduced numbers and fusion of synaptic vesicles (Fig. 1E).

To determine whether apoptotic mechanisms contribute to the neuronal injury caused by HS, brain sections were labeled with FJB and TUNEL. The fluorescence intensity of FJB staining, which indicates neuronal degeneration, was notably higher in HS mice than in control mice, and TUNEL staining showed increased TUNEL-positive cells in HS mice (Fig. 2A). However, none of the histopathological changes described above were found in the control and HA mice (Fig. 1E and Fig. 2A). In addition, these histopathological changes were substantially attenuated by HA (Fig. 1E and Fig. 2A). Statistical analysis revealed that the numbers of FJB- and TUNEL-positive cells were significantly lower in the HA pre-conditioned HS mice than the HS mice (Fig. 2B and 2C).

HA modulated HS-activated autophagy in the mouse brain

To investigate HS-induced autophagy and the potential protective role of HA, we first used immunofluorescence to evaluate the expression of LC3, which is a marker of autophagy [14]. In normal control mice, there was little LC3 staining, whereas in HA and HS mice, the intensity of LC3 fluorescence was notably increased (Fig. 3). Although LC3 expression was

Fig. 4. Effect of HA and HS on the expression of autophagy-associated proteins, LC3I/II and Beclin-1 at the indicated time points in mice. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. (A) Representative immunoblots of LC3I/II and Beclin-1 in brain tissue lysates. β-Actin antibody was used as a control for equal protein loading. The LC3I/II (B) or Beclin-1(C)/actin ratio was determined by densitometric analysis. Data are expressed as the mean ± SEM (n = 6 per group). *P < 0.05, **P < 0.01 vs. CON. #P < 0.05 vs. HA (-) at the same time point post-HS. CON, normal mice with or without HA pre-conditioning; HS, heat stroke; HA, heat acclimation.

detectable in HA pre-conditioned HS mice, it appeared to be expressed at a level lower than that in HS mice (Fig. 3). Moreover, TEM examination enabled direct observation of autophagosomes; typical autophagosomes are double- or multiple-membrane structures (Fig. 3), which contain cytoplasm or undigested organelles such as mitochondria [15]. No autophagosomes were observed in control mice, whereas many autophagosomes were found in the HA, HS, and HA pre-conditioned HS mice, especially in HS mice (Fig. 3). Furthermore, immunoblotting was used to quantitatively analyse the levels of autophagy-associated proteins. We detected the expression of LC3II, which is the autophagosome membrane form of LC3, and Beclin-1, which is a component of the phosphatidylinositol-3-kinase complex required for autophagy [16]. As shown in Fig. 4A-C, both HA alone and HS exposure increased the expression of Beclin-1 and the conversion of LC3I to LC3II; expressions of both proteins in the normal control and HA-pre-conditioning mice peaked at 12 h after HS exposure, which is consistent with our previous observations. As expected, the expression of HS-elevated proteins was significantly attenuated by HA pre-conditioning (Fig. 4A-C).

HA modulated lysosome function in both normal and HS-exposed mice

Autophagosomes are degraded in lysosomes by lysosomal hydrolases; thus, lysosomal function plays a key role in autophagy [6]. Therefore, lysosome function was evaluated by detecting the expression of sequestosome 1 (SQSTM1)/p62, lysosomal-associated membrane protein-1 (LAMP-1) and cathepsin B as well as the activity of these enzymes. p62 delivers damaged organelles and toxic protein aggregates to autophagosomes, and these proteins and organelles can accumulate within cells when autophagic clearance is impaired [17]. In our study, the expression of p62 was significantly increased in a time-dependent manner following HS exposure, especially at 12 h and 24 h post-HS exposure; however, these increases were abolished by HA pre-conditioning (Fig. 5A and 5B). These results indicated

Fig. 5. Effect of HA and HS on the expression of lysosome-associated proteins, p62, cathepsin B, and LAMP-1 at the indicated time points in mice. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. (A) Representative immunoblots of p62, cathepsin B, and LAMP-1 in brain tissue lysates. β-Actin antibody was used as a control for equal protein loading. The p62 (B), cathepsin B (C), or LAMP-1 (D) /actin ratio was determined by densitometric analysis. (E) Enzymatic activity of cathepsin B. Data are expressed as the mean ± SEM (n = 6 per group). **P* < 0.05, ***P* < 0.01 vs. CON. #*P* < 0.05, ##*P* < 0.01 vs. HA (-) at the same time point post-HS. CON, normal mice with or without HA pre-conditioning; HS, heat stroke; HA, heat acclimation.

that the impaired autophagic clearance was due to lysosome dysfunction. Cathepsin B, a lysosomal cysteine protease, is a molecular link between autophagy and apoptosis [18]. The protein expression and enzymatic activity of cathepsin B were significantly reduced in HS mice, and these reductions were largely prevented by HA pre-conditioning (Fig 5A, 5C, and 5E). Interestingly, the expression of the lysosome activity marker LAMP-1 was significantly increased by HA alone, and the increased LAMP-1 expression in HS mice at 24 h post-HS exposure was not affected by HA pre-conditioning (Fig. 5A and 5D).

Fig. 6. Effect of 3MA on the expression of autophagy and lysosome-associated proteins, LC3I/II, p62, cathepsin B, and LAMP-1, in normal control and HA-pre-conditioned mice at 12 h after HS exposure. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. (A) Representative immunoblots of LC3I/II, p62, cathepsin B, and LAMP-1 in brain tissue lysates. β-Actin antibody was used as a control for equal protein loading. The LC3I/II (B), p62 (C), cathepsin B (D), or LAMP-1 (E) /actin ratio was determined by densitometric analysis. Data are expressed as the mean ± SEM (n = 6 per group). *P < 0.05, **P < 0.01 vs. CON. #P < 0.05 vs. 3MA (-) post-HA+HS. CON, normal mice with or without 3MA pre-conditioning; HS, heat stroke; HA, heat acclimation; HA+HS, heat acclimated-mice subject to heat stroke.

Inhibition of autophagy abolished HA-mediated neuroprotection

To further confirm the involvement of autophagy in HA-mediated neuroprotection, we examined the effects of 3MA, a specific inhibitor of the early stages of autophagy. The 3MA pre-treatment significantly inhibited LC3II expression in normal control mice (Fig. 6A and 5B). Moreover, the increased LC3II expressions in HA, HS, and HA pre-conditioned HS mice were also significantly inhibited by 3MA pre-treatment (Fig. 6A and 6B). However,

Fig. 7. The 3MA treatment completely abolished the neuroprotective effects of HA on HS. HS mice were exposed to a 43°C ambient temperature for 2 h, and HA mice were exposed to 35°C for 30 days. (A) Representative photomicrographs from FJB and TUNEL-stained brain sections at 12 h post-HA+HS. (B) Quantification of the FJB-labelling was calculated from (A). The percentage is expressed as the number of FJB-stained cells divided by the total number of DAPI-stained cells. (C) Quantification of TUNEL-labelling was calculated from (A). The percentage is expressed as the number of TUNEL-stained cells divided by the total number per field of view. Data are expressed as the mean $\pm$ SEM ($n = 6$ per group, 8–10 histological sections per brain). Scale bar, 25 μ m. ** $P < 0.01$ vs. CON. # $P < 0.05$ vs. 3MA (-) post-HA+HS. (D) Brain water content at 12 h post-HS in control and HA pre-conditioned HS mice with or without 3MA pre-treatment. Data are expressed as the mean $\pm$ SEM ($n = 6$ –8 per group). * $P < 0.05$ vs. CON. # $P < 0.05$ vs. 3MA (-) post-HA+HS. CON, normal mice with or without 3MA pre-conditioning; HA+HS, heat acclimated-mice subject to heat stroke; 3MA, 3-methyladenine; FJB, Fluoro-Jade B; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling.

the increased p62 and LAMP-1 expressions in HA and HA pre-conditioned HS mice, but not in HS mice, were also substantially reduced by 3MA pre-treatment (Fig. 6A, 6C and

6E). Furthermore, 3MA did not affect the expression of cathepsin B in HA and HS mice, but decreased the expression of cathepsin B in HA pre-conditioned HS mice (Fig. 6A and 6D).

Next, we evaluated the effect of 3MA pre-treatment on HA-mediated neuroprotection and found that the percentages of FJB- and TUNEL- positive cells were significantly increased in HA pre-conditioned HS mice (Fig. 7A-C). Moreover, the brain water content of HA pre-conditioned HS mice was also significantly enhanced by 3MA pre-treatment (Fig. 7D).

Discussion

In this study, we first established a HS mouse model by exposing mice to a 43°C ambient temperature for 2 h. We then established a HA mouse model by exposing mice to a 35°C ambient temperature for 30 days. We demonstrated that HA protected against HS-induced animal death and brain injury, and these effects involved HA-mediated modulation of HS-induced autophagy and lysosome dysfunction. However, HA-mediated neuroprotection was abolished by an autophagy inhibitor. Our results indicate that the autophagy-lysosome pathway may play an important role in HA-mediated neuroprotection following HS exposure.

In humans, hyperthermia (generally $T_{co} > 40^{\circ}\text{C}$) and central nervous system dysfunction are required for the diagnosis of HS [1]. In our HS mouse model, body temperature gradually increased and reached a T_{re} of $> 42.5^{\circ}\text{C}$, and this elevated body temperature was accompanied by brain oedema and swelling. Subsequently, a hypothermia and an elevation were observed in T_{co} . Similarly, the biphasic thermoregulatory response, having two peaks, was observed in a well-known heat stroke mouse model, previously [19]. We also found morphological abnormalities in the brain tissues, including neuronal shrinkage and pyknotic nuclei in the cerebral cortex, indicating that the HS mouse model was successfully established. Mice that were maintained at 35°C for 30 days were shown to achieve a stable HA state [10]. Therefore, we next determined whether HA protected against brain injury following HS exposure. After the mice were exposed to 43°C for 2 h, the T_{re} , mortality and brain water content of the HA pre-conditioned HS mice were lower than those of the HS mice, and the morphological abnormalities, neuronal apoptosis and neuronal degeneration observed in the HA pre-conditioned HS mice were less than those observed in HS mice. Neuronal degeneration was based on FJB staining, which is an anionic fluorescein derivative that has been shown to be a specific marker of degenerating neurons [20]. Therefore, in our study, the observation of FJB-positive neurons following HS exposure indicated that neuronal degeneration had been induced.

Interestingly, the HS-induced brain injury was concomitant with the induction of autophagy and lysosome dysfunction, which can be modulated by HA. During autophagy, the conversion of cytosolic LC3I to LC3II is required for autophagic membrane recruitment, and the LC3II levels are related to the extent of autophagosome formation; therefore, LC3II protein expression can be determined by immunoblotting and used as a measure of autophagy induction [21]. Our data suggested that LC3II protein levels were significantly increased following HS exposure. A previous study showed that exposure to heat stress resulted in autophagosome formation and increased levels of endogenous LC3II [22]. Beclin-1, a Bcl-2-interacting protein, is generally used to evaluate the neurodegenerative process [23]. In the present study, we also observed an increase in Beclin-1 expression that paralleled the increases in LC3II, providing additional evidence of the induction of autophagy by HS exposure. Recent reports have shown that autophagy may play a defensive role in chronic neurodegenerative diseases such as Parkinson's disease [24], Alzheimer's disease [25], and Huntington's disease [26]. Moreover, previous studies have reported that HS-induced autophagy provided protection against neurodegeneration in the brain [9]. In the present study, not only HS but also HA induced autophagy to different extents. HS exposure induced over-activation of autophagy, which may induce subsequent neuronal injury and autophagic cell death. In contrast, HA moderately activated autophagy, which may contribute to neuroprotection. Lysosome function plays a key role in autophagy [27], and previous studies

have reported that impairment of the lysosome system can disrupt autophagy and promote neuronal cell death [28]. Thus, we examined the expression of LAMP-1 and cathepsin B as well as the activity of cathepsin B to investigate whether lysosome function is disrupted by the two heat stresses, HA and HS, and whether lysosome disruptions were associated with autophagy regulation. HS inhibited the expression of LAMP-1 and cathepsin B as well as the activity of cathepsin B, suggesting the major dysfunction is lysosomal impairment. Decreased autophagic flux via the lysosome would account for the increased accumulation of LC3-II, Beclin-1 and p62. However, HA enhanced lysosomal function and, maintained the autophagic flux during extreme heat stimulation, which may be responsible for HA-mediated neuroprotection following HS exposure.

In this study, we used a specific inhibitor of autophagy (3MA) to evaluate the role of the autophagy-lysosome pathway in HA-mediated neuroprotection following HS exposure. After 3MA treatment, LC3II expression was inhibited in the 3MA, HA+3MA and HA+3MA+HS mice, but LC3II expression remained high in the 3MA+HS mice. In addition, we found that after the mice were exposed to 43°C for 2 h, the brain water content of the HA+3MA+HS mice was higher than the HA pre-conditioned HS mice, and the morphological abnormalities, neuronal apoptosis and neurodegeneration were also increased relative to the those in the HA pre-conditioned HS mice. These results indicate that the induction of moderate autophagy by HA played a protective role in HS-induced brain injury. In contrast to our results, pretreatment with 3MA significantly reduce ischaemic neuronal damage, which suggested that autophagy may be harmful in the brain [29]. These contradictory results suggest that the role of autophagy can be both protective and harmful. Moreover, the fatal thermal stimulation of HS impaired lysosome function, whereas the mild stimulation of HA enhanced lysosome function, which could explain the protective role of HA-induced autophagy in HS-induced brain injury. However, 3MA treatment did not alter lysosome function, and the reason for this requires further investigation.

In summary, our results demonstrated that HS induces lysosomal dysfunction and impaired autophagic flux consequent to brain injury. HA protects against HS-induced brain injury via a mechanism involving the autophagy-lysosome pathway. Autophagy intervention may be a promising therapeutic strategy for heat-related diseases and requires further investigation.

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Disclosure Statement

The authors declare that there are no conflicts of interests.

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