Original Research

Heme oxygenase-1 aggravates heat stress-induced neuronal injury and decreases autophagy in cerebellar Purkinje cells of rats

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Abstract

We previously reported that heat stroke induces autophagy as a protection mechanism against neurodegeneration in the brain. Heme oxygenase (HO)-1 is a stress protein and can be induced by heat stress (HS). Cerebellar Purkinje cells are selectively vulnerable to heat-induced injury. In this study, we first validated an animal model of HS (38°C for 4 h) in which sustained increase of Purkinje cell injury, HO-1 expression up to 24 h post HS (HS_{24}), and hyperthermia reaching a rectal temperature 41.52 ± 0.32°C were observed. In subsequent experiments, we investigated the effects of HO-1 on HS-induced Purkinje cell injury. Rats were divided into four groups: one normothermic control group receiving saline vehicle (1 mL/kg, intraperitoneal [i.p.]) and exposed to 25°C for 4 h; and three HS groups receiving saline, or HO-1 inducer haemin (30 mg/kg, i.p.) or HO-1 inhibitor tin protoporphyrin (SnPP, 30 mg/kg, i.p.), respectively, at 12 h prior to HS. HS-induced Purkinje cell injury was further enhanced by HO-1 inducer but attenuated by HO-1 inhibitor as evaluated by immunoreactivity of apoptosis marker (active caspase-3) as well as Fluoro-Jade B histochemistry (staining for degenerating neurons), suggesting a detrimental role of HO-1. Interestingly, the protective autophagy was reduced by HO-1 inducer but enhanced by HO-1 inhibitor as demonstrated by autophagy markers including Beclin-1 and microtubule-associated protein light chain 3 in Purkinje cells. Double immunofluorescent labelling of Beclin-1 or 8-hydroxydeoxyguanosine (an oxidative DNA damage marker) with HO-1 immunoreactivity not only demonstrated their co-localization, but also confirmed that HO-1 negatively regulated Beclin-1 but increased oxidative stress in the same Purkinje cell. Taken together, our results indicate that HO-1 aggravates HS injury in cerebellar Purkinje cells. Our findings shed new light on cell damage mechanisms by HS in central nervous system and may help to provide potential therapeutic foci.

Keywords: heat stress, Purkinje cells, injury, heme oxygenase-1, autophagy

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Introduction

In view of global warming, the threat of heat waves leading to heat-related illness has attracted concern. Heat stress (HS) denotes the discomfort and physiological strain following physical exercise or daily work for long periods in the hot environment (air temperature >32°C).[1,2] Heat stroke which is severe heat illness represents high core temperature over 40°C, cease of heat dissipation, and central nervous system (CNS) abnormalities, e.g. delirium, convulsion, or coma.[2–4] More than 50% of heat stroke victims die within a short time despite lowering of the body temperature and therapeutic intervention.[5,6] Those who survive heat stroke often show a permanent neurological deficit.[7] Many studies have identified the cerebellar Purkinje cells as being particularly vulnerable to hyperthermia injury.[8–10] Purkinje cells play central roles in sensing space and distance. Hyperthermia in the range of 41–42°C per se may not be the main reason for neuronal damage,[11] but it might elicit a series of neurotoxic responses, such as super high levels of dopamine, tryptophan, glutamine, glycine, nitrous oxide, hydrogen peroxide free radical and cytokines, and/or dysregulation of the organism's metabolism and physiology.[12] In this study, we used an animal model of rats subjected to a more moderate ambient temperature of 38°C for 4 h.[13] The benefits of this model with more moderate ambient temperature of 38°C may include better survival and slowing down of reacting mechanisms for better observation. In contrast to significant neuronal damage in cerebral

cortex in our previous animal model of HS (exposed to 40°C for 1 h, anesthetized),[14] we did not observe significant morphological damage in cerebral cortex and striatum in the current model (exposed to 38°C for 4 h, unanaesthetized; data not shown). Interestingly, we observed morphological damages in cerebellar tissue sections in our pilot studies. We therefore decided to focus on cerebellar Purkinje cells because of their vulnerability to HS[8–10] and partly due to their being part of the fragile central nervous system and their own physiological importance in motor coordination and space balance. Heme oxygenase (HO), which catalyzes the breaking down of heme into carbon monoxide, ferrous ion, and biliverdin, has two isoforms: HO-1, and -2. HO-1 and HO-2 are two isoenzymes encoded by separate genes with disparate expression profiles. HO-2 is constitutively expressed in neurons and responsible for major HO activity in the brain[15,16] and HO-1 is induced and acts predominantly under stress conditions. Short-term heat exposure leading to mild hyperthermia induces an increase in HO-1 expression providing protection for subsequent various forms of stress.[17–22] Hyperthermia with core temperature reaching 41–42°C induces an intense increase in HO-1 protein in glia throughout the brain, ependyma lining the ventricles of the brain, paraventricular nuclei, Purkinje cell layer of the cerebellum, and cochlear nucleus of brainstem.[23] Although, HO-2 is virtually uninducible, its pronounced expression has been detected in several brain areas of HS rat.[24,25] Hyperthermic insults to CNS induce free radicals production and lipid peroxidation.[26–28] The contribution of HO combined with nitric oxide synthase (NOS) activities to microvascular permeability disturbances, oedema formation, and cell injury in the CNS has been reviewed by Sharma.[25,29,30]

The ability of HO-1 to alleviate oxidative stress and to block the apoptotic signalling pathways in HS has been reported.[31] In addition to classical mechanisms such as apoptosis and inflammation, several recent intriguing studies suggest that HO-1, and its byproduct CO, can possibly impact the regulation of autophagy, a vital cellular process, which may in part contribute to the cytoprotective mechanism.[32–35] Our previous study has suggested that the role of autophagy in heat stroke is neuroprotective.[14] Autophagy is a regulatory cellular pathway for the turnover of organelles and proteins by lysosomal-dependent processing.[36,37] The process of autophagy involves specific steps, including formation of a double-membrane vesicle called an autophagosome, and fusion of an autophagosome with a lysosome to become an autophagolysosome, which subsequently degrades the contents.[38] Beclin-1, an important autophagy-regulatory gene, is part of a Class III PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane.[39] Microtubule-associated protein light chain 3 (LC3) is normally located throughout the cytoplasm, but becomes concentrated in autophagosomes during autophagy through lipidation (LC3-I, unlipidated; LC3-II, lipidated). LC3-II tightly associates with the preautophagosomal and autophagosomal membrane and thus is considered a marker of autophagy.[40] There are reports indicating that hyperthermia results in enhanced autophagy in a cultured neuronal cell line[41] and in cortical tissues of rats.[14] However, the underlying mechanisms and interactions between these stress reacting factors are still poorly understood. Whether HO-1 is involved in regulation of autophagy in the response of brain cells, especially Purkinje cells, to HS is still unknown. The present experiments were, therefore, performed to assess whether HO-1 can regulate autophagy in HS rats as well as the functional role of HO-1 with a focus on the HS-induced Purkinje cell injuries.

Methods and material

Animal model of HS and experimental design

Male Sprague-Dawley rats weighing 120–140 g were purchased from the Animal Resource Center of the National Science Council of the ROC (Taipei, Taiwan). The animals were kept in a pathogen-free room with controlled temperature, humidity, and lighting (12-h light–dark cycles) and with free access to standard laboratory food and water. All protocols were approved by the Animal Use and Care Committee of the National Defense Medical Center (Taipei, Taiwan) in accordance with the National Institute of Health's guidelines for the care and use of laboratory animals. The experiments commenced between 08:00 and 09:00 to avoid circadian variations. Rats were initially assigned to HS or normothermic control (NC) groups. The HS groups were subjected to HS in a chamber at 38°C for 4 h as described by Sharma.[13] Rats in the HS group were removed from the chamber and left at room temperature immediately after termination of HS for the indicated time periods ($n = 5$ for each time point; Figure 1). For example, HS_0 represents the time point at 0 h after termination of HS, while HS_3 represents at 3 h after termination of HS (and so on for HS_6, HS_{12}, and HS_{24}). NC animals were exposed to 25°C for the same period. At the end of the experiment, rats in various time point groups were euthanized with an intraperitoneal (i.p.) overdose of urethane for analyses of brain injury and HO-1 expression in order to select an appropriate time point for subsequent studies. The time point was selected when significant brain injuries and HO-1 expression were observed. In the second series of experiments, haemin (HO-1 inducer) and tin protoporphyrin ([SnPP] HO-1 inhibitor) were used to study HO-1 implications. Based on the pharmacological doses of haemin and SnPP ranging from 15 to 50 mg/kg (i.p.) in previous studies,[42–50] we selected 15 and 30 mg/kg for haemin and SnPP to initiate the investigation. Rats were divided into four groups with one group as NC and three groups exposed to HS ($n = 5$ in each group) as follows, NC, HS group as mentioned above, haemin + HS: rats were pretreated 12 h before HS with a bolus of either 15 or 30 mg/kg (i.p.) of HO-1 inducer haemin (Sigma, St. Louis, MO, USA), and SnPP + HS group: pretreated 12 h before HS with HO-1 inhibitor SnPP (Sigma; 15 or 30 mg/kg, i.p.) and then sacrificed at the selected time point for studying roles of HO-1 in neuronal injuries and autophagy inhibition. Changes in body weight and core temperature were determined by an electronic balance and a thermocouple inserted into the

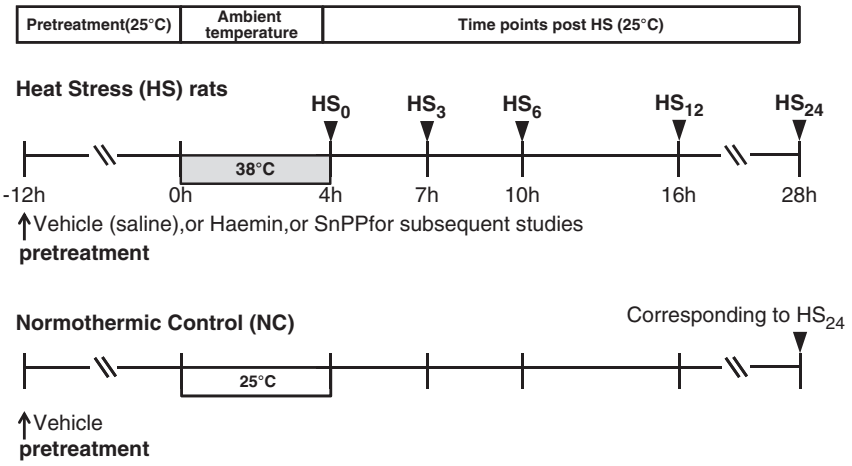

Figure 1 Schematic presentation of the study design. In HS group, rats received saline 12 h prior to HS (38°C, for 4 h) and sacrificed at the following time points as designated by their abbreviation, i.e. HS_0 represents at the time immediately (0 h) after termination of HS, while HS_3 represents at 3 h after termination of HS (and so on for HS_6, HS_{12}, and HS_{24}). Normothermic control (NC) group received saline 12 h before normothermic exposure (25°C, for 4 h) and sacrificed only at corresponding time points to HS_{24} for comparison

rectum, immediately after termination of HS and at the corresponding time point for the NC group.

Histological examination

Animals were sacrificed at various time points after HS. The cerebellum and brain were removed, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks. Sagittal sections from the midline of the cerebellum and hippocampal coronal sections at −4.5 mm from Bregma were cut (5 μm) and processed for haematoxylin and eosin (H&E) staining. Histopathological changes were examined under a light microscope by a person blinded to the experimental conditions. Injured Purkinje cells were counted by examining 100 cells/section/rat for a total of 500 cells in each group. Normal Purkinje cells were large, pyramidal, and located in the Purkinje cell layer with apical dendrites in the molecular layer. In contrast, injured Purkinje cells were small, irregular, and without dendrites.

Immunofluorescence staining

Cerebellar sections (5 μm) were deparaffinized in xylene and rehydrated in a descending, graded series of alcohol. Antigen retrieval was achieved by treating specimens with sodium citrate buffer (10 mmol/L sodium citrate and 0.05% Tween 20 at pH 6.0) at 95°C for 20 min. After blocking unspecific binding sites with phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 0.1% Triton X-100 for at least 1 h at room temperature, sections were incubated overnight with the primary antibody raised against active caspase-3 (1:50; Millipore, Billerica, MA, USA), HO-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Beclin-1 (1:200; Abcam, Cambridge, UK), and LC3 (1:500; Abcam). After washing with PBS, sections were incubated with a rhodamine isothiocyanate-conjugated secondary antibody diluted 1:300 in blocking buffer for 2 h. For double labelling, sections were incubated overnight at 4°C using primary antibodies specific for either Beclin-1 or 8-hydroxyquanosine (8-OHdG, Abcam Corp., Cambridge, MA, USA) combined with HO-1. After several washes in 0.1 mol/L PBS, sections were incubated in DyeLight 549 anti-rabbit IgG (1:300, 2 h, room temperature, Jackson ImmunoResearch, West Grove, PA, USA), washed again, and incubated in Alexa 488-conjugated anti-sheep IgG (1:300, 2 h, room temperature, Invitrogen, Carlsbad, CA, USA), or Alexa 488-congugated anti-mouse IgG (Invitrogen), respectively. Sections were mounted with Vecta-shield mounting medium (Vector Labs, Burlingame, CA, USA). All sections were observed and photographed under a fluorescence microscope. The percentage of immunopositive cells was calculated by counting and analysing 100 cells in a section per animal and five animals in each group were used. To ensure comparable immunostaining, sections were processed together at the same time under the same conditions. To assess non-specific immunostaining, alternating sections from each experimental group were incubated without the primary antibody. Fluorescent photos were captured with an Olympus microscope (BX-51) with an attached DP71 digital camera and processed with DP Controller and DP Manager software (Olympus).

Fluoro-Jade B (FJB) histochemistry

FJB (Chemicon International, Temecula, CA, USA) is a polyanionic fluorescein derivative that sensitively and specifically binds to degenerating neurons. Staining was performed according to the manufacturer's recommendations. After staining, slides were washed and mounted on coverslips with Vecta-shield mounting medium. All sections were observed and photographed under a fluorescence microscope with blue (450–490 nm) excitation light.

Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used for comparison between

multiple groups. A value of $P < 0.05$ was considered statistically significant.

Results

HS induced time-dependent cerebellar Purkinje cell injury

Following HS, Purkinje cells were histologically examined. Figure 2 shows morphological features of normal Purkinje cells with larger size, pyramidal shape, and apical dendrites in the molecular layer in cerebellar cortex of the NC rat in contrast to injured cells with smaller size, irregular morphology, and without dendrites in HS rats sacrificed at various time points (HS_0 to HS_{24}). Degree of injury as estimated by counting 100 cells/section/rat for a total of 500 cells in each group. Injured Purkinje cells showing small and irregular shapes were 2.1 ± 1.6 % in NC rats, 37.0 ± 6.7 % at HS_0 (immediately after HS), 53.2 ± 6.9% at HS_3, 62.1 ± 5.8% at HS_6, 70.2 ± 7.4% at HS_{12}, and 75.3 ± 7.6% at HS_{24}. The degree of injury in NC rats remained low throughout the corresponding observation periods. The number of injured Purkinje cells in the HS groups significantly increased (75.3 ± 7.6% in HS_{24} group vs. 2.1 ± 1.6% in NC group, $P < 0.01$) as compared to the NC group.

HS induced time-dependent HO-1 expression

Immunofluorescence staining using an antibody against HO-1 indicated a significant increase in HO-1-expressing Purkinje cells in the cerebellums from HS rats compared to those from NC rats. Quantitative analysis of HO-1-positive cells indicated that the cell number significantly increased at HS_0 and continued to increase up to HS_{24} (Figure 3). Because that evident Purkinje cells injuries and significant HO-1 expression were seen at HS_{24}, this time point was selected for subsequent studies on the roles of HO-1 in Purkinje cells injuries and autophagy.

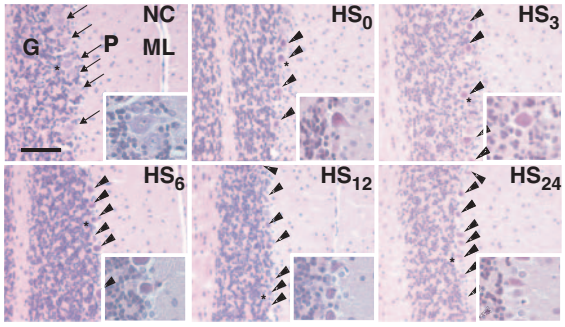

Figure 2 Histopathological evidence of Purkinje cell injury. Representative photomicrographs of H&E-stained cerebellar sections from NC or HS rats sacrificed at various times (HS_0, HS_3, HS_6, HS_{12}, and HS_{24}) post HS. Purkinje cells in NC rats showed the normal morphology of a bulbous cell body and extending dendrites, forming a pyramidal shape (arrows). Injured Purkinje cells (arrowheads) in HS rats appeared small and irregularly shaped at HS_0 (0 h post HS). The degree of injury progressively increased with time at HS_3, HS_6, HS_{12}, and HS_{24}. Insets represent high power magnifications of the indicated cells (*). G: granular layer; ML: molecular layer. Scale bar 100 μm. (A color version of this figure is available in the online journal)

HO-1 aggravated HS-induced Purkinje cell injury as evaluated by counting apoptotic and degenerating neurons

Immunofluorescence staining with anti-active caspase-3 and FJB histochemistry were performed for the identification of apoptotic subpopulations and degenerating neurons. Both indicated significant cellular injuries induced by HS were with similar patterns at HS_{24} as compared to the NC group (Figures 4 and 5). Pretreatment with the HO-1 inducer haemin at the dose of 15 mg/kg (i.p.) did not show significant difference (data not shown) while haemin (30 mg/kg, i.p.) administered at 12 h prior to HS resulted in a higher number of active caspase-3 and FJB-positive cells at HS_{24} compared to the group pretreated with vehicle (1 mL/kg, i.p.). In contrast, pretreatment with the HO-1 inhibitor SnPP at dose of 30 mg/kg (i.p.), but not 15 mg/kg, significantly reduced the HS-induced cell damage.

HO-1 aggravated HS-induced injury in hippocampus

To delineate whether the role of HO-1 is region-specific in cerebellum, we also examined another brain area. Hippocampus, another vulnerable brain region to hyperthermia, was histologically examined at HS_{24} in each group. Significant neuronal damage was observed in the CA 4 region of hippocampus in vehicle + HS group as compared to NC group (Figure 6). The degree of injury was attenuated by HO-1 inhibitor, SnPP while enhanced by its inducer, haemin. This result indicated that HO-1 also aggravated HS-induced hippocampal neuronal injury in a pattern similar to what we observed in cerebellar Purkinje cells. In subsequent studies in this paper, we focused mainly in cerebellar Purkinje cells.

Lack of effects of HO-1 modulations on hyperthermia and dehydration induced by HS at HS_0

Table 1 shows the changes of rectal temperature and body weight among four study groups. While rectal temperature remained 37.42 ± 0.32°C in the NC group, rats in the vehicle + HS group reached 41.52 ± 0.32°C in rectal temperature, which was even higher than ambient temperature exposed (38°C for 4 h; Table 1). Body weights of HS rats were significantly lower as compared to the NC group (128 ± 9.2 vs. 119 ± 9.3 g). HO-1 modulation by either haemin or SnPP had no significant effects on hyperthermia and dehydration induced by HS (Table 1).

HO-1 down-regulated protective autophagy in Purkinje cells of HS animals

The effect of HO-1 on protective autophagy was evaluated by immunofluorescent staining of autophagic markers Beclin-1 and LC3 in sections of cerebellums from each group of rats. High levels of Beclin-1 were detected in Purkinje cells in HS rats with the fluorescence showing a granular, homogeneous patterns found almost exclusively in the perikaryon regions (Figure 7). Fewer Beclin-1-positive Purkinje cells were observed in HS rats pretreated with haemin (30 mg/kg, i.p.) as compared to the vehicle + HS

rats whereas SnPP (30 mg/kg, i.p.) pretreatment increased Beclin-1-positive Purkinje cells. LC3 in rats subjected to HS significantly increased in Purkinje cells and displayed numerous bright, punctate dots as compared to those of NC rats (Figure 8). The results showed that haemin markedly down-regulated LC3 punctate dot formation in Purkinje cells whereas SnPP up-regulated them at HS_{24}. The LC3 data were consistent with the Beclin-1 findings and revealed that HO-1 negatively regulated autophagy in cerebellar Purkinje cells of HS rats. Furthermore, results of double labelling not only demonstrated the co-localization of HO-1 and Beclin-1 in the same Purkinje cells but also confirmed that HO-1 negatively regulates Beclin-1. HO-1 inducer haemin decreased Beclin-1 levels whereas HO-1 inhibitor SnPP enhanced Beclin-1 levels (Figure 9).

HO-1 potentiated oxidative stress in Purkinje cells of HS animals

To further investigate whether heat exposure produces oxidative stress in Purkinje cells, we examined 8-OHdG, a marker of oxidative DNA damage at HS_{24}. Purkinje cells displayed increased 8-OHdG immunoreactivity in vehicle + HS group as compared with NC group (Figure 10). Double-labelling analyses showed co-localization of 8-OHdG and HO-1 in the same Purkinje cell. The co-localization was more prominent in haemin + HS group as compared to vehicle + HS group. By contrast, in SnPP + HS group only HO-1 immunoreactivity could be seen, the intensity of 8-OHdG was very low in Purkinje cell.

Discussion

We employed HS model in this study according to previously described in which the animal does not develop neurological symptoms of acute heat stroke.[13] Within 1 h of heat exposure, the rats are no longer able to maintain their body temperature effectively, resulting in hyperthermia.[51] The core temperature increases linearly with exposure time during the 4 h experimental periods. Rats in this model, exhibit signs of prostration without losing their righting reflex that is quite comparable to the human cases seen in clinical situations.[52] The validity of the HS model was demonstrated by the neuropathological observation that rat cerebellar Purkinje cells were injured after HS (38°C for 4 h), and the injury gradually progresses for up

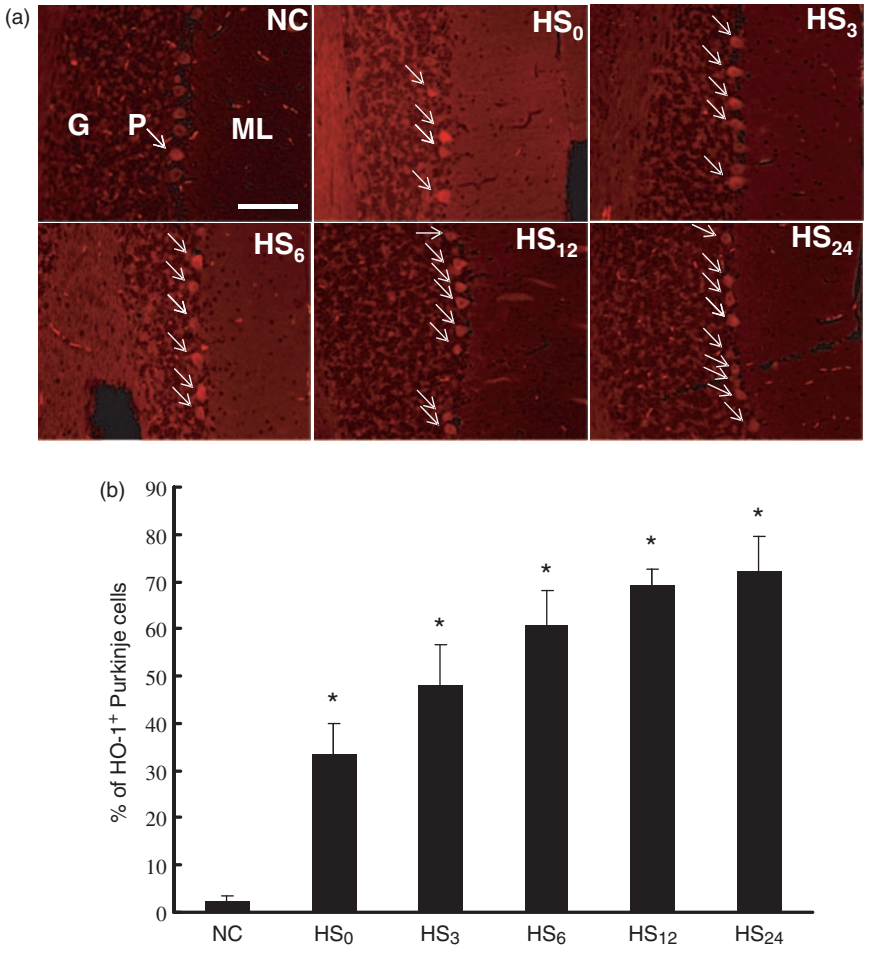

Figure 3 Representative immunostaining images showing the time course of changes in heme oxygenase (HO)-1 expression in cerebellar tissues from NC or HS rats. (a) White arrows indicate Purkinje cells in the cerebellar cortex exhibiting HO-1 immunoreactivity (HO-1^+) Purkinje cells in cerebellar sections from NC or HS rats sacrificed at HS_0, HS_3, HS_6, HS_{12}, and HS_{24}. (b) Bar graphs showing significantly higher numbers of HO-1-positive cells in the HS_0, HS_3, HS_6, HS_{12}, and HS_{24} groups. *$P < 0.05$, compared to the NC group. G: granular layer; ML: molecular layer. Scale bar 100 μm. (A color version of this figure is available in the online journal)

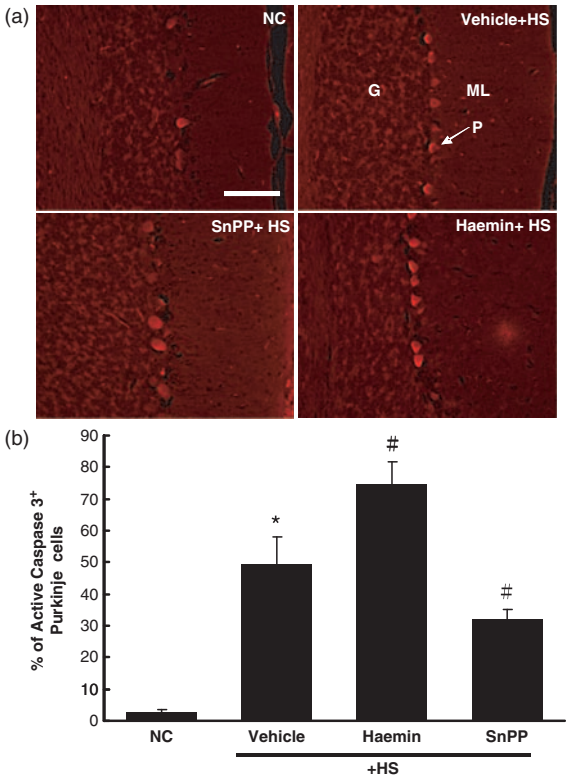

Figure 4 Effects of HS and HO-1 modulation on apoptosis as shown by immunoreactivity of active caspase-3 in Purkinje cells in the four study groups. Normal saline was given at 12 h prior to experiment on NC rats. Vehicle (normal saline; 1 mL/kg, i.p.), haemin (30 mg/kg, i.p.), or tin protoporphyrin (SnPP, 30 mg/kg, i.p.) was administered, respectively, 12 h before HS. Cerebellar tissues were collected at HS_{24} and immunostained for the apoptosis marker active caspase-3. (a) Sections of the cerebellar cortex from the vehicle + HS group displayed more active caspase-3^+ Purkinje cells (arrow) than those from the NC group. In the haemin + HS group, the HS-induced positive Purkinje cell number was elevated, but was decreased in the SnPP + HS group. (b) Bar graphs showing the number of active caspase-3^+ Purkinje cells in rats subjected to various treatments. Haemin pretreatment further elevated the number of active caspase-3^+ cells while SnPP pretreatment decreased it. $^*P < 0.05$, compared to the NC group. $^{\#}P < 0.05$, compared to the vehicle + HS group. G: granular layer; ML: molecular layer. Scale bar 100 μm. (A color version of this figure is available in the online journal)

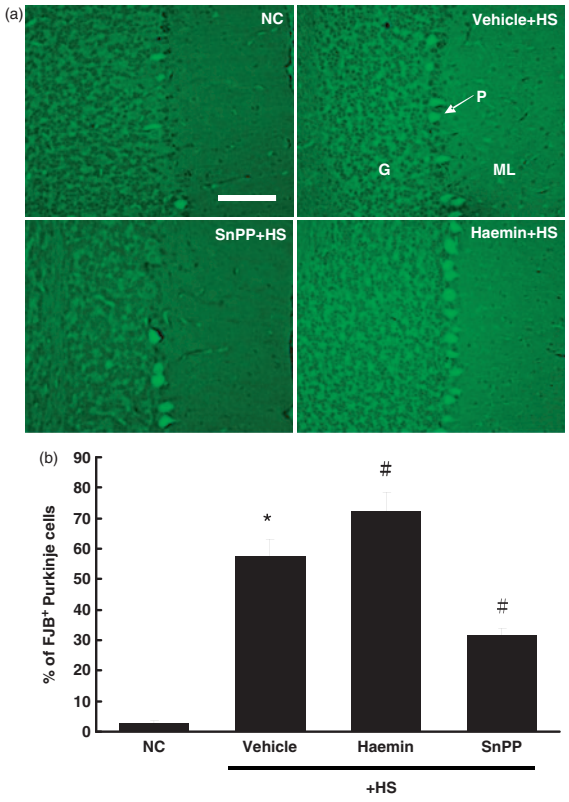

Figure 5 Effects of HS and HO-1 modulations on neurodegeneration in Purkinje cells. Photomicrographs of Fluoro-Jade B $(FJB)^+$ Purkinje cells in the four study groups showing an increase of the number of Purkinje cells staining positively for FJB, an index of neurodegeneration, following HS. HS animals were given saline (vehicle + HS), the HO-1 inducer haemin (haemin + HS), or HO-1 inhibitor SnPP (SnPP + HS) 12 h prior to HS. (a) More FJB^+ cells (arrow) were observed following HS as compared to the NC group at HS_{24}. (b) Quantitative analysis of FJB-positive Purkinje cells in each group is depicted. Pretreatment with haemin significantly increased the number of FJB^+ cells as compared to the vehicle + HS group, whereas SnPP markedly decreased the HS-induced positive cell number. $^*P < 0.05$, compared to the NC group. $^{\#}P < 0.05$ compared to the vehicle + HS group. G: granular layer; ML: molecular layer. Scale bar 100 μm. (A color version of this figure is available in the online journal)

to 24 h after termination of HS as examined. Our results were consistent with a previous neuropathological study in which clear cerebellar atrophy after hyperpyrexia, pronounced cell loss and degeneration of Purkinje cell axons in dentate nuclei were found.[8] In addition to Purkinje cell damage, HO-1 expression level was up-regulated in a time-dependent manner during the 24 h observation period after heat treatment. Consistent with other reports we found indeed HO-1 was significantly elevated by HS. In the present study, we further demonstrated that HO-1 is an important factor aggravating HS-induced Purkinje cell injuries as examined by both anti-active caspase-3 immunostaining (apoptosis marker) and FJB staining (neurodegeneration indicator) in which pretreatment with haemin, an HO-1 inducer, increased the numbers of Purkinje cells with apoptotic or degenerative patterns whereas pretreatment of SnPP, an HO-1 inhibitor, decreased injured cell numbers. However, concomitant with the significant

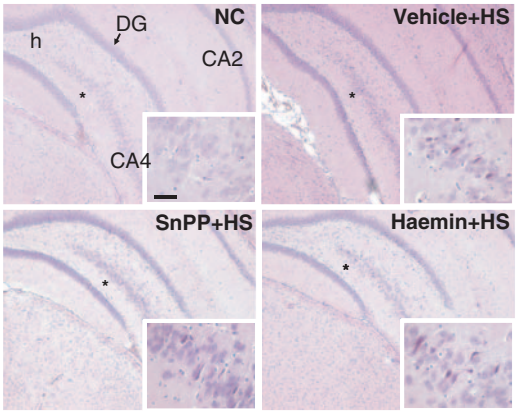

Figure 6 Representative images of hippocampal histomicrograph in four groups at HS_{24}. Photomicrographs showed apparent cell damage in CA4 area (*) in HS groups. Compared to the HS + vehicle group, pretreatment with HO-1 inducer haemin aggravated the injury level whereas HO-1 inhibitor SnPP attenuated it, indicating HO-1 exerted an adverse effect on HS-induced hippocampus neuronal injury. Insets represent high power magnifications of the indicated cells (*). DG: dentate gyrus; CA: cornu ammonis areas; h: hilus. Scale bar 20 μm. (A color version of this figure is available in the online journal)

elevation of HO-1 expression was significant aggravation of cell injury, as evidenced by the increase in the number of cells possessing pathological morphology, and increase in neurodegeneration and apoptotic death. Plausible explanations for HO-1 being unexpectedly detrimental might be biphasic actions of being beneficial at low or moderate levels and becoming detrimental at high levels with the excessive ferrous iron catalyzing oxidative reactions to produce excessive deleterious oxygen radicals. It has been shown that breakdown of the blood–brain barrier (BBB) is apparent in hyperthermia-induced brain injury[53] and causes significant behavioural/locomotor changes.[54] One main mechanism underlying BBB breakdown would be the oxidative stress derived from HO and nNOS expression following hyperthermic brain injury.[24,30] CO and NO can induce signal transduction mechanisms leading to an increase in cyclic guanosine monophosphate levels in the both neural and non-neural cells[55] which are associated with increase in the breakdown of the BBB permeability and edema formation.[56,57] Presumably, increased CO by HS-induced HO-1 enzyme activity might exacerbate the damage extent. Although we did not perform the experiment examining leakage of serum proteins or breakdown of the BBB, we did try to measure the water content by wet–dry weight ratio. Albeit that oedema formation was evident by increased water content following HS, we could not

Table 1 Comparison of rectal temperature and body weight among study groups[a]

Groups	Rectal temperature (°C)	Body weight (g)
1. NC (vehicle, 1 mL/kg, i.p.)	37.42 ± 0.32	128 ± 9.2
2. Vehicle + HS	41.52 ± 0.32[b]	119 ± 9.3[b]
3. Haemin (30 mg/kg, i.p.) + HS	41.46 ± 0.29[b]	121 ± 8.9[b]
4. SnPP (30 mg/kg, i.p.) + HS	41.48 ± 0.30[b]	120 ± 9.7[b]

[a]Rats were subjected to heat stress (HS) at 38°C for 4 h; normothermic controls (NC) were kept at 25°C. Vehicle (normal saline), tin protoporphyrin (SnPP) or haemin was administered 12 h before HS. Values are the means ± SEM of five rats per group and measured immediately at the termination of HS or at corresponding time point in NC group; group 1.
[b]$P < 0.05$, compared with the values of group 1 (one-way ANOVA, followed by Tukey test).

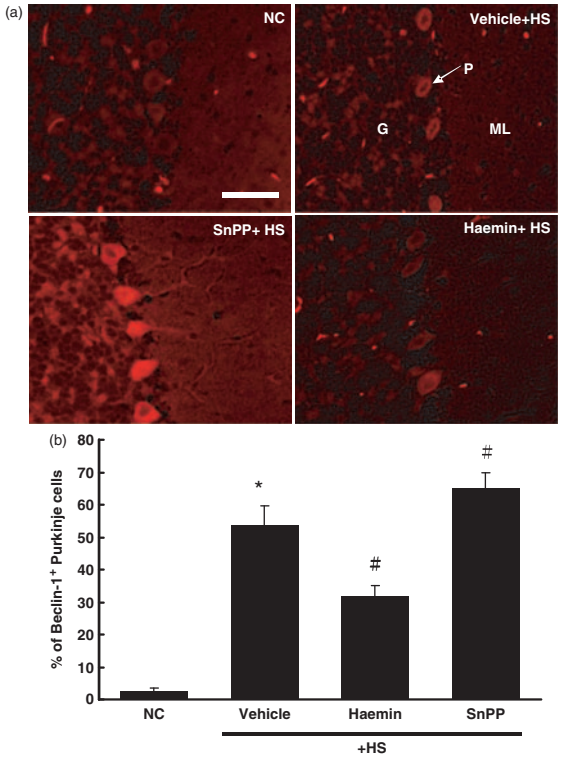

Figure 7 Effects of HS and HO-1 modulation on autophagy. Immunostaining of the cerebellar sections with anti-Beclin-1 in four study groups. (a) Photomicrographs of immunostaining in cerebellar sections showing a significant increase in the number of cells staining positively for the autophagic marker Beclin-1 with granular fluorescence in perikaryons (arrow) at 24 h after HS compared to NC group. (b) Quantitative assessment of Beclin-1-positive cells of cerebellar sections in each group is depicted. Note that pretreatment with haemin significantly decreased the number of positive cells as compared to vehicle + HS group while SnPP markedly increased the cell numbers. *$P < 0.05$ as compared with the NC group. #$P < 0.05$ as compared with the vehicle + HS group. G: granular layer; ML: molecular layer. Scale bar 50 μm. (A color version of this figure is available in the online journal)

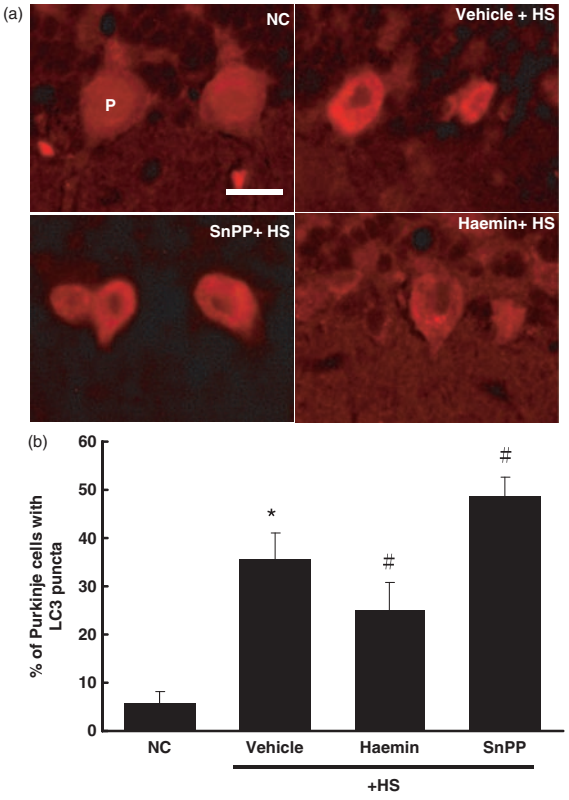

Figure 8 LC3 immunoreactivity of cerebellar sections in four study groups. (a) Photomicrographs showing a significant increase in concentrated fluorescent dots in Purkinje cell at HS_{24} in vehicle + HS group as compared to NC group. Note the redistribution of LC3 with punctate fluorescence in Purkinje cells was enhanced by pretreatment with SnPP as compared to vehicle + HS group but was decreased in the haemin + HS group. (b) Quantitative assessment of Purkinje cells with punctate LC3 fluorescence in each group is depicted. *$P < 0.05$ as compared with the NC group. #$P < 0.05$ as compared with the vehicle + HS group. P: Purkinje cell; G: granular layer; ML: molecular layer. Scale bar 20 μm. (A color version of this figure is available in the online journal)

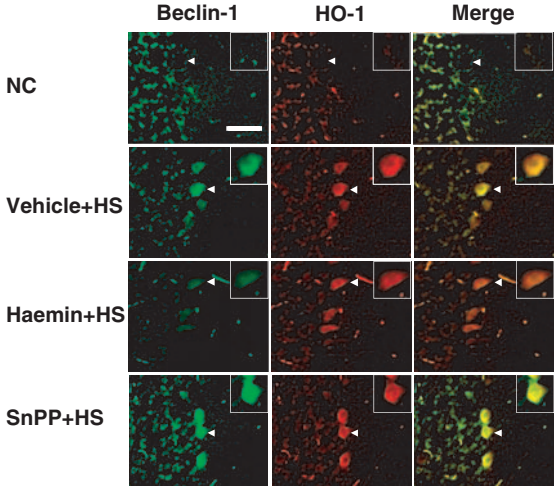

Figure 9 Representative images of double labelling for Beclin-1 and HO-1 at HS_{24}. Photomicrographs showing co-localization of Beclin-1 and HO-1 in the same Purkinje cell in HS groups. Compared to the HS + vehicle group, pre-treatment with HO-1 inducer haemin down-regulated Beclin-1 expression whereas HO-1 inhibitor SnPP up-regulated it, suggesting HO-1 modulation had an effect on HS-induced autophagy. Insets represent high power magnifications of the indicated cells (arrowhead). Scale bar 50 μm. (A color version of this figure is available in the online journal)

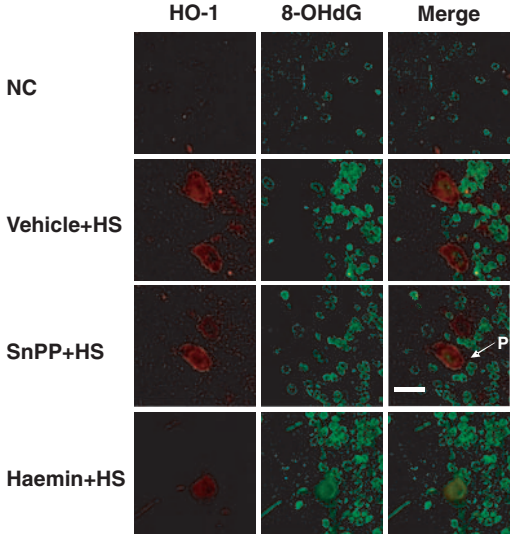

Figure 10 Representative images of double labelling for 8-OHdG and HO-1 at HS_{24}. Purkinje cells displayed increased immunoreactivity for 8-OHdG which was co-localized with HO-1 in vehicle + HS group as compared to NC group. The co-localization of 8-OHdG and HO-1 was more prominent in haemin + HS group whereas the intensity of 8-OHdG was very low in SnPP + HS group, suggesting increased HO-1 could potentiate the levels of oxidative stress to HS-induced Purkinje cells. Scale bar 20 μm. (A color version of this figure is available in the online journal)

detect significant alteration by HO-1 modulating drugs (data not shown). Autophagy is another factor stimulated by stress. We previously provided evidence that autophagy was neuroprotective in the context of HS.[14] How might autophagy interact with HO-1 might be of interest. We attempted to establish cause–effect relationships by modifying HO-1 expression with the inducer haemin and inhibiting the HO-1 activity by SnPP and tracking changes in cell injury, autophagy, and apoptosis. We further found that pharmacological modulation of HO-1 could significantly change the profile of autophagy to HS. Immunostaining with the autophagic markers beclin-1 and LC3 indicated induction of HO-1 with haemin led to autophagy down-regulation whereas inhibition of HO-1 resulted in up-regulation. These findings collectively provide evidence that HO-1 exhibits an inhibitory effect on autophagy involved in the cascade of events leading to HS-induced early Purkinje cell injury including apoptosis and degeneration. However, it is unlikely that in the brain the heat inducibility of HO-1 reflects an increased cellular demand for augmented haeme-degradation activity, since the brain already possesses high levels of such activity from a ubiquitous and constitutive form of HO-2.[56,58] HO-1 immunoreactivity was found to be selectively expressed in rat neurons of the cerebellum, hippocampus, hypothalamus, and brainstem,[59] and sparsely expressed throughout the cortex[60] and other areas, such as the thalamus.[61] After HS, an intense increase in HO-1 protein was observed in both Purkinje cells of the cerebellum and epithelial cells lining the cerebral aqueduct of the brain.[62] The hyperthermic inducibility of HO-1 in highly selected regions and only in certain neuronal populations suggests the pathophysiological importance of the protein in special cell populations such as Purkinje cells. Our result confirmed that HO-1 was indeed harmful in HS-induced Purkinje cell injuries. Recent studies reported that HO-1 can down-regulate autophagy, a process which engulfs long-lived or misfolded proteins, and then degrades and recycles them.[63] The role of HO-1 in controlling autophagy under conditions of various stimuli and cell types was observed. Exposure of human bronchial epithelial cells to cigarette smoke extract induced autophagy which was significantly down-regulated by HO-1 through adenoviral-mediated gene expression.[32] In animal models of nephrotoxicity with cisplatin, HO-1-deficient mice had significantly more autophagosomes than did wild-type mice, even in saline-treated animals, and ecdysone-induced overexpression of HO-1 in cells led to a delay in autophagy progression.[64] In the present study, our results are consistent with those studies revealing that HS induction of HO-1 dampens autophagy and is correlated with Purkinje cell injuries. Previous studies have shown that HO-1 up-regulation plays a protective role in many neuronal pathological conditions.[65–67] However, HO-1 involved in the progression of neuronal injuries is seldom disclosed. It heretofore was not well known that what mechanisms are mediated by HO-1 in HS-insulted neurons. We suggest that mechanisms for HO-1 aggravating HS-induced Purkinje cell injury might involve autophagy. Increasing evidence has proposed that loss of basal autophagy by either deletion of autophagy-related gene or inhibition of autophagic clearance in neurons caused disruption of axonal transport leading to axonal dystrophy.[68–71] In addition, cerebellar degeneration was observed in a study with neural-specific deletion of FIP200 involved in autophagosome biogenesis causing neuronal cell death and axon degeneration.[72] Our work previously demonstrated autophagy to be protective in heat-induced brain damage,[14] exhibiting that increases in both autophagy-related proteins, LC3II and Beclin-1, in the

heat-exposed group were observed compared to the NC group. In addition, pretreatment with 3-methyadenine, an autophagy inhibitor, suppressed levels of LC3II and Beclin-1, and such inhibition of autophagy aggravated HS-induced neurodegeneration. HO-1 is protective in moderate levels when confronted with stress. However, at high levels, it leads to toxic impact due to ferrous iron byproduct of enzymatic reaction and ensuing extremely oxidative pressure.[73] Both HO-1 in our study and HO-2 from previous work[24,25] were shown to be markedly up-regulated and considered to be unfavourable for neuronal survival under HS insult. In the present study, we propose additional detrimental action of HO-1 in down-regulating autophagy which further potentiates Purkinje cell injury. Autophagy plays an important role in maintaining physiological homeostasis but its high influx can bring about cell damage due to excessive consumption of cellular components.[37] Herein, we demonstrated that the induction of autophagy in HS conditions was insufficient to protect Purkinje cell from injury due to the induction of HO-1 as enhancement of autophagy by SnPP resulted in higher cell survival. Furthermore, double immunostaining confirmed that HO-1 and autophagic elements were indeed co-localized in the same cells. Thus, in addition to the possibility of excessive high levels of HO-1 breakdown products contributing to cell injury, we provided evidence of an addition mechanism accounting for such deleterious effects in inhibition of the protective autophagy. To date, heat-induced neuronal injury is still a critical problem that remains to be solved. This study is the first to show that HS-induced HO-1 expression is detrimental resulting in attenuated autophagy and more apoptotic and degenerating Purkinje cells. In conclusion, our results suggested that HO-1 augments HS-induced neuronal injury and suppression of the protective effects of autophagy. Targeting HO-1 may provide a therapeutic strategy for the treatment of heat-related illness.

Author contributions: CWL conducted the majority of the experiments and wrote the manuscript. YFL was responsible for the funding, study design, and discussion. TTL assisted in animal experiment and tissue collection. JYW supervised the experimental design, provided funding, and held primary responsibility for final content. All authors read and approved the final manuscript.

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