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Research Paper

Geranylgeranylacetone preconditioning may attenuate heat-induced inflammation and multiorgan dysfunction in rats

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Abstract

Objectives Geranylgeranylacetone, an acyclic isoprenoid, is a non-toxic inducer of heat shock protein (HSP)70. HSP70 overproduction is associated with heat tolerance in rats. This study aimed to investigate whether geranylgeranylacetone preconditioning of rats reduced heat-induced inflammation and multiple organ dysfunction.

Methods Anaesthetised rats were given vehicle or geranylgeranylacetone (800 mg/kg) orally. After 48 h they were exposed to ambient temperature of 43°C for 70 min to induce heatstroke. Another group of rats kept at room temperature were used as normothermic controls.

Key findings Vehicle-treated rats all succumbed to heat stress; their survival time was 25 ± 4 min. Pretreatment with geranylgeranylacetone significantly increased survival time to 92 ± 15 min. Compared with normothermic controls, all vehicle-treated heatstroke rats displayed hepatic and renal dysfunction (e.g. increased plasma levels of serum urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase) and active inflammation (e.g. increased plasma and brain levels of interleukin-1β, tumour necrosis factor-α and interleukin-6). These heat-stress response indicators were all significantly suppressed by geranylgeranylacetone pretreatment. In addition, the plasma and brain levels of interleukin-10 (an anti-inflammatory cytokine) and brain levels of HSP70 were significantly increased after geranylgeranylacetone preconditioning during heatstroke.

Conclusions Geranylgeranylacetone preconditioning attenuates heat-induced inflammation and multiorgan dysfunction in rats.

Keywords pro-inflammatory; cytokine; multiorgan dysfunction; heat shock protein 70; heatstroke

Introduction

It has been documented repeatedly that heatstroke rodents display overproduction of highly reactive oxygen species (ROS) and reactive nitrogen species (RNS) in multiple organs, which trigger overproduction of interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and IL-6, a hypercoagulable state, cellular ischaemia and damage, and multiorgan dysfunction.[1–4]

It has also been demonstrated that heat shock protein 70 (HSP70) preconditioning improves heat tolerance in rodents. For example, sublethal heat shock preconditioning, in addition to stimulating HSP72 production in multiple organs, confers significant protection against heatstroke-induced arterial hypotension and brain oxidative and ischaemia damage in rats.[5] In addition, overexpression of HSP70 in transgenic mice that were heterozygous for a porcine HSP70 gene improved the outcomes of heatstroke by reducing thermoregulatory deficits, the overproduction of ROS, RNS and pro-inflammatory cytokines (e.g., IL-1β, TNF-α and IL-6) associated with circulatory shock, and multiple organ dysfunction.[6,7]

Geranylgeranylacetone (GGA), an acyclic isoprenoid, is a non-toxic HSP70 inducer, which selectively and safely induces HSP70 in guinea-pig and rat gastric mucosal cells.[8] Ischaemia–reperfusion injury in the liver,[9,10] small intestine[11] and heart[12–14] can be suppressed by GGA pretreatment. This raises the possibility that the heat-induced

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overproduction of brain pro-inflammatory cytokines and multiple organ dysfunction can be reduced by GGA preconditioning. To test this hypothesis, the present experiments were performed to assess the temporal profiles of brain HSP70 expression, indicators of multiple organ function (serum creatinine, alkaline phosphatase (ALP), serum urea nitrogen (SUN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), and plasma and brain cytokines (IL-1β, TNF-α, IL-10 and IL-6) during heat stress in rats with and without GGA preconditioning.

Materials and Methods

Animals

Adult male Wistar rats (180–220 g) were purchased from the Animal Resource Center of the Academy of Military Medical Science (Beijing, China). The animals were housed in groups of four at an ambient temperature of 24 ± 1°C and were maintained under a normal light–dark cycle. Pelleted rat chow and tap water were allowed *ad libitum*.

All protocols were approved by the Animals Ethics Committee of the Academy of Military Medical Science (Beijing, China) in accordance with the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of the Academy of Military Medical Science and the guidelines of the Animal Welfare Act.

Adequate anaesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments by a single intraperitoneal dose of pentobarbital sodium (60 mg/kg). Control rats were killed with an overdose of pentobarbital sodium.

Induction of heatstroke

When unanaesthetised rodents are exposed to high ambient temperature, the occurrence of hyperthermia (> 42°C), intracranial hypertension above 42 mmHg and hypotension (< 65 mmHg) accompanied by convulsion and coma have been taken as the time point for the onset of heatstroke.[15] In the present study, heatstroke was induced by putting the animals in a folded heating pad at 43°C controlled by circulating hot water. The onset of heatstroke was taken as the time at which mean arterial pressure (MAP) fell to about 50 mmHg and core temperature was elevated to about 42°C. After the onset of heatstroke, the heating pad was removed and the animals were allowed to recover at room temperature (24°C). This pilot study showed that the latency for onset of heatstroke in vehicle-treated rats was 70 ± 3 min (n = 15). Therefore, in the following experiments, all heat-stressed animals were exposed to 43°C for exactly 70 min and then allowed to recover at room temperature. Use of higher temperature or longer period of hyperthermia would reduce both latency for onset of heatstroke and survival time (interval between the onset of heatstroke and death).

Experimental groups

Animals were divided into two main groups. Animals in the normothermic groups were treated with an oral dose of vehicle solution (5 ml/kg) containing 6.0056% α-tocopheroal and 2% gum arabic via a gastric tube 48 h before the start of thermal experiments. Their core temperatures were maintained at around 36°C during the entire experiments using a heating pad. Animals in the heatstroke groups were treated with an oral dose of vehicle solution as above or GGA (800 mg in 5 ml emulsion per kg) 48 h before the start of the thermal experiments. GGA was from Eisai Co. (Tokyo, Japan). The dosage and time of administration of GGA were based on the reports of Fujiki *et al*.[16,17]

HSP70 analysis

Animals were killed by decapitation 70 min after initiation of heat stress. The cortex, hippocampus, hypothalamus and striatum were removed and ground to extract the total protein. The protein (100 μg) was subjected to SDS-PAGE and electroblotted onto nitrocellulose membrane. The membrane was blocked with 5% dried skimmed milk (Sigma, St Louis, MO, USA) in 0.1% phosphate-buffered saline–Tween, washed and then probed with rat monoclonal antibody to HSP70 (1 : 500, Santa Cruz, CA, USA). The membrane was washed with phosphate-buffered saline–Tween (0.1%) and incubated with antimouse-IgG–HRP conjugate (1 : 1000) for 2 h at room temperature. The membrane was then incubated with chemiluminiscent substrate (Santa Cruz) and the bands were developed using X-ray films (Kodak, New York, USA). Expression of HSP70 and actin was semiquantified using a gel densitometric scanning program.

Biochemical indicators of hepatic and renal function determinations

Blood samples were drawn from the abdominal aorta at 0 and 70 min after initiation of heat stress using a syringe. The plasma levels of SUN, creatinine, AST, ALT and ALP were determined by spectrophotometry (Hitachi 7600, Tokyo, Japan).

Determination of TNF-α, IL-1β, IL-6 and IL-10

Blood samples and different brain regions (cortex, hippocampus, hypothalamus and striatum) were taken 0 and 70 min after the start of heat stress. The blood samples were allowed to clot for 2 h at room temperature and were then centrifuged (2000g, 20 min, 4°C) and the supernatants harvested. The brain samples were disintegrated in five volumes of ice-cold Ripa buffer and the homogenates kept on ice for 30 min and then centrifuged (15 000g, 30 min, 4°C) twice. The supernatants were stored at −70°C until measurement. The concentrations of TNF-α, IL-1β, IL-6 and IL-10 in tissue lysates were determined using double-antibody sandwich ELISA (R & D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Optical densities were read on a plate reader at 450 nm. The concentrations of TNF-α, IL-1β, IL-6 and IL-10 in the samples were calculated from the standard curve multiplied by the dilution factor and are given as pg/ml.

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was carried out using the SPSS 7.5 software (SPSS, Chicago, IL, USA). We assumed that data followed a Gaussian

distribution, allowing the use of one-way analysis of variance followed by Tukey's test to analyse the difference between the groups. $P < 0.05$ was considered significant.

Results

GGA improves hyperthermia, hypotension and survival during heatstroke

Figure 1 shows the effects of heat exposure on core temperature and MAP. Compared with the normothermic controls, vehicle-treated heatstroke rats had higher core temperature but lower MAP at the onset of heatstroke, and survival time was decreased from the control values of > 480 min to 25 ± 4 min (Table 1). Hyperthermia, hypotension and survival were all significantly improved in GGA-treated heatstroke rats (Figure 1).

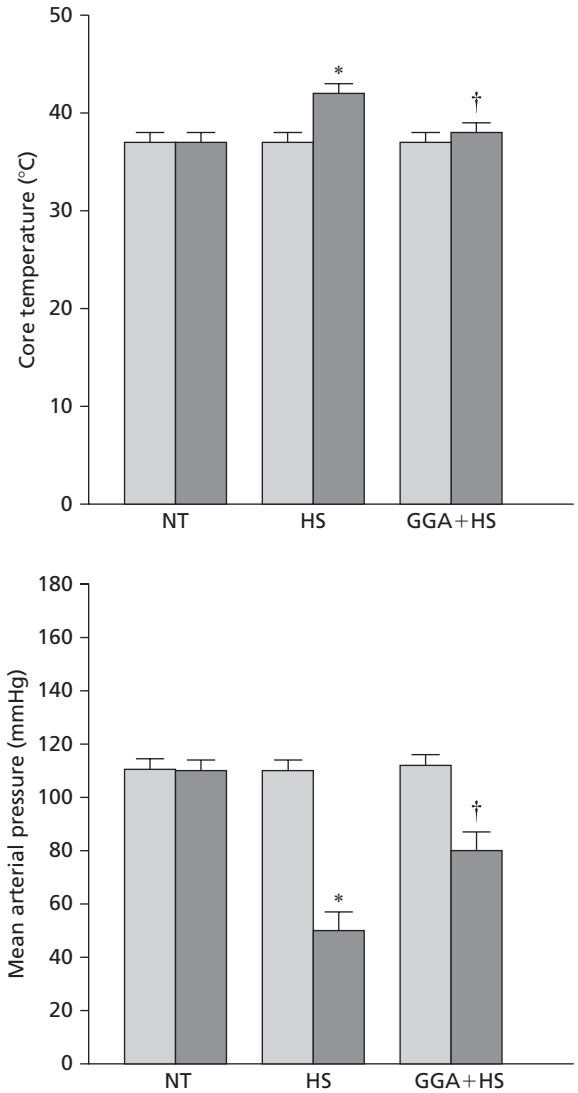

Figure 1 Effects of heat exposure on core temperature and mean arterial pressure. Core temperature and mean arterial pressure for normothermia controls (NT), vehicle-treated heatstroke rats (HS) and GGA-treated heatstroke rats (GGA + HS). Bars show means ± SD (n = 8 rats). $^{*}P < 0.05$ vs NT group; $^{\dagger}P < 0.05$ vs HS group.

GGA preconditioning increased cerebral HSP70 expression during heatstroke

Figure 2 shows the effects of heat exposure on HSP70 levels in different parts of the brain in the three groups of rats. Compared with the vehicle-treated rats and normothermic controls, GGA-treated heatstroke rats had higher levels of HSP70 in the different areas of the brain.

GGA improves renal and hepatic dysfunction during heatstroke

Figure 3 shows the effects of heat exposure on indicators of renal and hepatic function in the three groups of rats.

Table 1 Survival times following induction of heatstroke

Treatment group	Survival time (min)
Normothermic controls	>480
Vehicle-treated heatstroke rats	25 ± 4*
GGA-treated heatstroke rats	92 ± 15†

Values are means ± SD (n = 8). $^{*}P < 0.05$ vs normothermic control; $^{\dagger}P < 0.05$ vs vehicle-treated heatstroke rats (analysis of variance followed by least squares difference test).

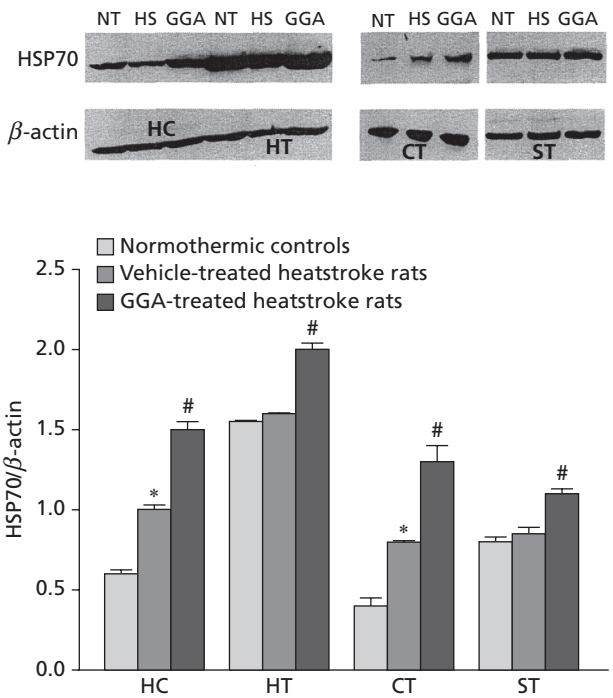

Figure 2 Effects of heat exposure on HSP70 levels. HSP70 expression in the hippocampus (HC), hypothalamus (HT), cerebral cortex (CT) and striatum (ST) at 70 min and a representative Western blot of extracts from different groups. HSP70 levels were quantified densitometrically and expressed as relative density (HSP70/β-actin). Bars show means ± SD (n = 8). $^{*}P < 0.05$ vs normothermia controls; $^{\#}P < 0.05$ vs vehicle-treated heatstroke rats.

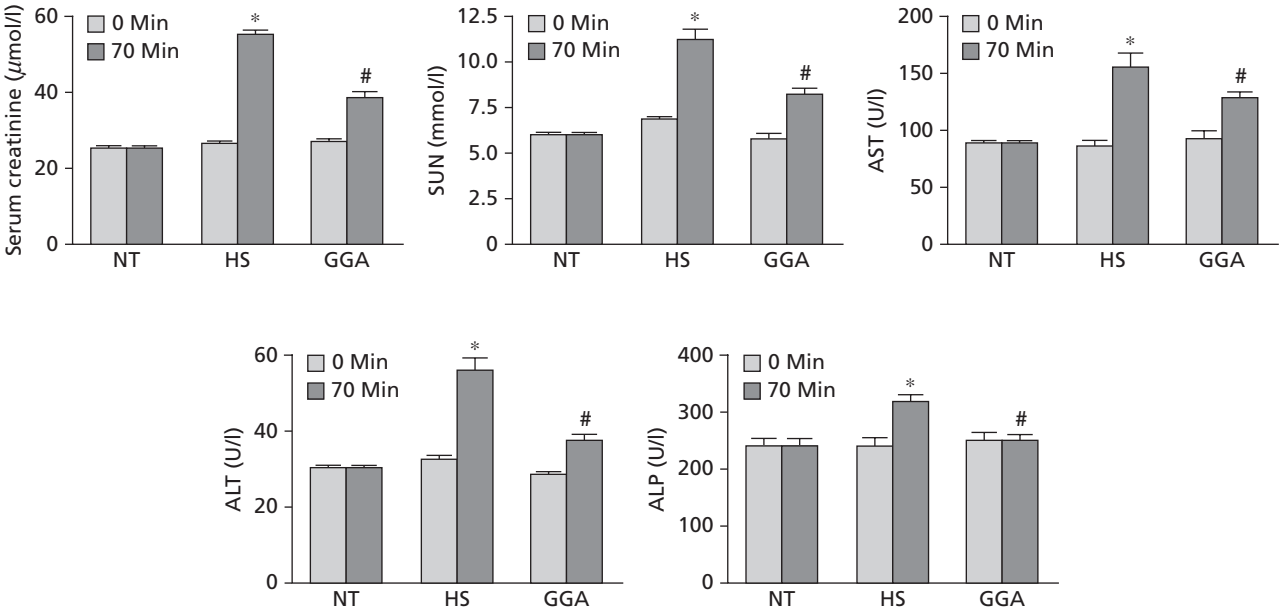

Figure 3 Effects of heat exposure on indicators of renal and hepatic function. Values of serum creatinine, serum urea nitrogen (SUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in normothermic controls (NT), vehicle-pretreated heatstroke rats (HS) and GGA-pretreated heatstroke rats (GGA) at time 0 and after 70 min. Bars show means ± SD ($n = 8$). $^{*}P < 0.05$ vs NT group; $^{\#}P < 0.05$ vs HS group.

Compared with normothermic controls, vehicle-treated heatstroke rats had higher levels of serum creatinine, SUN, ALT, AST and ALP. The increased levels of these renal and hepatic function indicators were significantly reduced in GGA-treated heatstroke rats compared with vehicle-treated heatstroke rats.

GGA attenuates IL-1β, TNF-α and IL-6 overproduction but stimulates IL-10 production during heatstroke

Figures 4 and 5 show the effects of heatstroke on serum and brain TNF-α, IL-1β, IL-6 and IL-10 in the three groups of rats. Serum and brain levels of TNF-α, IL-1β and IL-6 of

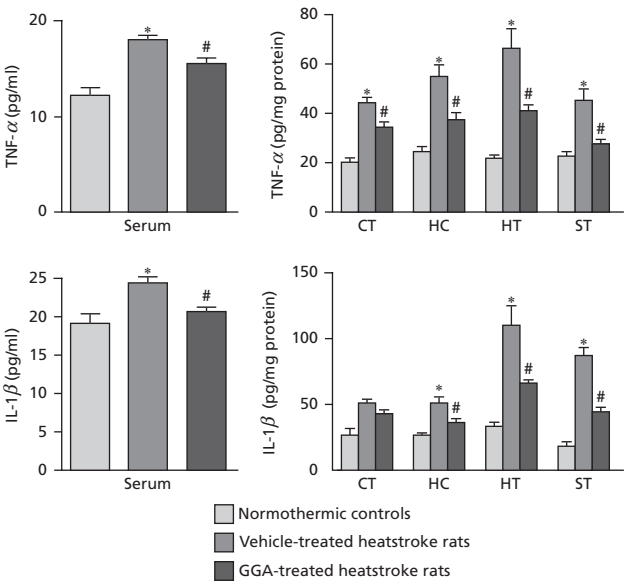

Figure 4 Effects of heat exposure on tumour necrosis factor α and interleukin 1β in serum and different brain regions. Levels of tumour necrosis factor (TNF)-α and interleukin (IL)-1β in the serum, cerebral cortex (CT), hippocampus (HC), hypothalamus (HT) and striatum (ST) after 70 min. Bars show means ± SD ($n = 8$). $^{*}P < 0.05$ vs NT group; $^{\#}P < 0.05$ vs HS group.

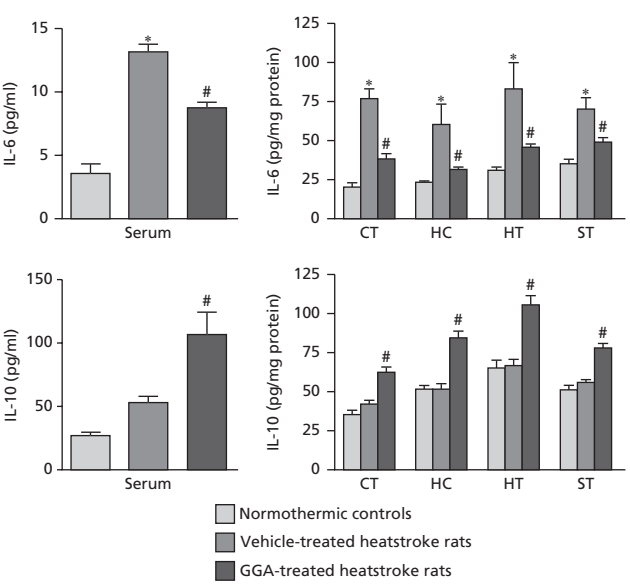

Figure 5 Effects of heat exposure on interleukin 6 and 10 in serum and different brain regions. Levels of interleukin (IL)-6 and IL-10 in the serum, cerebral cortex (CT), hippocampus (HC), hypothalamus (HT) and striatum (ST) levels. Bars show means ± SD ($n = 8$). $^{*}P < 0.05$ vs NT group; $^{\#}P < 0.05$ vs HS group.

vehicle-treated heatstroke rats at 70 min after initiation of heat exposure were all significantly higher than those of normothermic controls. The heat-induced increase in levels of these three pro-inflammatory cytokines were significantly reduced by GGA pretreatment, as shown in GGA-treated heatstroke rats. Compared with normothermic controls and vehicle-treated heatstroke rats, the GGA-treated heatstroke rats had higher serum and brain levels of IL-10 ($P < 0.05$).

Discussion

Unanaesthetised rodents exposed to severe heat stress display hyperthermia, hypotension, raised intracranial hypertension and central nervous system disorders (e.g. convulsion and coma).[15] In the present study, the time point at which both hyperthermia (> 42°C) and hypotension (MAP ~50 mmHg) occurred was taken as the onset of heatstroke for anaesthetised rats. The current results demonstrate that an oral dose of GGA (800 mg/kg) administered 48 h before the start of thermal stress increased expression of HSP70 in the brain and significantly prolonged the survival time of heatstroke animals. Both dosage and time of administration of GGA are based on the reports of Fujiki *et al.*[16,17] With this dosage and time of administration of GGA, the heat-induced activated inflammation (evidenced by increased production of pro-inflammatory cytokines), multiple organ dysfunction and decreased survival time were all significantly attenuated in our rats under general anaesthesia. Although the survival time during heatstroke was significantly prolonged by GGA preconditioning, all the animals nevertheless died. It should be stressed that our animals were under general anaesthesia. Anaesthesia impairs body temperature regulation and has potential effects on the study of heatstroke pathophysiology in this model. Nevertheless, this potential source of variance should have been accounted for by the appropriate controls in the present study. The cause of death in our heatstroke animals may be related to anaesthesia state. To ascertain whether the improvement in survival is durable, experiments need to be performed in unanaesthetised and unrestrained animals, with and without GGA treatment. In a recently reported study,[7] unanaesthetised and unrestrained transgenic mice that were heterozygous for a porcine HSP70β gene (+HSP70) and transgene-negative littermate controls (−HSP70) were exposed to heat stress (ambient temperature 42.4°C for 1 h). Mice that survived to day 4 after heat stress were considered survivors. When exposed to the same heat stress, none of the 12 −HSP70 mice survived, whereas all of the 12 +HSP70 mice survived. Together, our previous and present results suggest that GGA preconditioning may improve outcomes of heatstroke in rodents by increasing HSP70 expression.

The hypothesis is in part supported by the findings of several investigators. For example, healthy volunteers with a core temperature of 39°C displayed elevated serum HSP70 levels whereas heatstroke patients with a core temperature of 42°C and central nervous system disorders did not have increased serum levels of HSP70.[18] Administration of L-arginine to heat-stressed mice increases the expression of HSP70 and contributes to the protective effect of L-arginine in mice subjected to heatstroke.[19,20] Glutamine has also been shown to increase the expression of HSP70 and to reduce cytokine release, gut permeability and mortality in a rat model of heatstroke.[21] In addition to influencing gut permeability, HSP70 overexpression may exert its protective effect during heatstroke by reducing cerebrovascular dysfunction.[22]

When rodents are exposed to high ambient temperature, hypoxia resulting from splanchnic ischaemia induces generation of RNS and ROS in the gut, which increase intestinal mucosal permeability to endotoxins.[4,23] Endotoxaemia can stimulate the production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL6, which may trigger systemic inflammation. Activated inflammation, when exaggerated, can cause inadequate coagulation, cellular ischaemia and damage, and multiple organ dysfunction or failure.[3,24,25] Indeed, as shown in the present results, plasma and brain levels of IL-1β, TNF-α, and IL-6 were all elevated in heatstroke rats. These heatstroke reactions lead to multiple organ dysfunction and death. Our findings further show that prior administration of GGA, in addition to stimulating production of HSP70, causes significant reduction of heat-induced inflammation and multiple organ dysfunction, and prolongs survival time.

Human heatstroke victims[26] and rodents with heatstroke[27] have elevated concentrations of both IL-1β and TNF-α in the plasma. These pro-inflammatory cytokines induce production of IL-6, which is positively associated with the severity of the heatstroke.[28] By contrast, accumulated evidence indicates that IL-10 may have therapeutic potential in acute and chronic inflammatory diseases. For example, IL-10-knockout mice have an increased likelihood of inflammatory illness[29] and higher mortality after experimental sepsis.[30] Injection of IL-10 protects mice from lethal endotoxaemia by reducing TNF-α production.[31] In mice with endotoxaemia, neutralisation of endogenously produced IL-10 results in an increased production of pro-inflammatory cytokines and enhances mortality.[32] In the current findings, we further show that GGA preconditioning increased the production of IL-10 and decreased the production of TNF-α, IL-1β and IL-6 in both the plasma and the brain, and prolonged survival during heatstroke.

The present studies were conducted in rats under sodium pentobarbital anaesthesia. Anaesthesia impairs normal body temperature regulation and has potential effects on the study of heatstroke pathophysiology in this model. Nevertheless, this potential source of variance should have been accounted for by the appropriate controls in the present study.

It is believed that dehydration and hypovolaemia impair cardiovascular and thermoregulatory adjustments to heat stress.[33] When unanaesthetised mice were exposed to heat stress, approximately 13% of body weight was lost after reaching a core temperature of 39.5°C.[34] This body weight loss, which served as an indirect measure of dehydration, was not observed in our anaesthetised heat-stressed rats. Our previous results have also shown that heat-stressed rats did not display hypovolaemia during experimentation, as reflected by an insignificant change in the haematocrit and

plasma levels of sodium and chloride.[35] There is no published evidence that fluid and electrolyte abnormalities are critical determinants of exercise-related[36] or classic heatstroke. In our rodent model, dehydration was not observed during heatstroke. This does not however negate firm evidence that dehydration has important physiological effects that impair heat loss. Rather, it shows that classic heatstroke requires a powerful initiating factor in addition to dehydration. In this study, there were insignificant differences in haematocrit and plasma sodium and chloride concentrations between the two experimental groups (data not shown).

Conclusions

Our results suggest that GGA preconditioning may improve heat tolerance in rats by increasing brain levels of HSP70 and decreases systemic inflammation (evidenced by decreased production of IL-1β, TNF-α, and IL-6 and increased production of IL-10) and multiple organ dysfunction (evidenced by increased levels of SUN, creatinine, AST, ALT and ALP).

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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