Research Article

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Heatstroke induces liver injury via IL-1β and HMGB1-induced pyroptosis

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Background & Aims: Liver injury is a common complication of heat stroke (HS), and often constitutes a direct cause for patient death. The cellular and molecular mechanism underlying HS-induced liver injury remains unclear. Recent evidence indicates that inflammasome plays an important role in mediating sterile inflammation triggered by tissue damage. Using a rat HS model, we identified a novel mechanism by which inflammasome-dependent interleukin-1β (IL-1β) activation and hepatocyte pyroptosis mediate HS-induced liver injury.

Methods: To induce HS, rats were subjected to heat exposure. Inhibition of inflammasomes was achieved by RNA silencing and pharmacologic inhibitor prior to heat exposure. Inflammasome assembly, caspase-1 activation, histological changes, as well as serum levels of liver enzymes were measured.

Results: We demonstrated that the onset of HS activated inflammasome in the liver as evidenced by increased capase-1 activity and the association of inflammasome components NOD-like receptor family pyrin domain containing 3 (Nlrp3) and apoptosis speck-like protein containing a caspase-recruitment domain (ASC); and the activated inflammasome, in turn, induced IL-1β activation and hepatocyte pyroptosis, and subsequent augmented liver injury. HS-induced hepatocyte inflammasome activation seems to be high-mobility group box 1 (HMGB1) dependent. Inhibition of Nlrp3, caspase-1, or HMGB1 prevented HS-induced liver inflammation and ameliorated liver injury.

Conclusions: These findings demonstrate an important role of HMGB1 in mediating inflammasome activation in the development of liver injury following HS, and suggest that targeting inflammasome may represent a novel therapeutic strategy to limit cell death and prevent liver failure after HS.

Keywords: Hyperthermia; Nlrp3; Inflammasome; Caspase-1.

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Abbreviations: HS, heat stroke; Nlrp, NOD-like receptor family, pyrin domain containing protein; ASC, apoptosis-associated specklike protein containing a caspase activation recruitment domain; HMGB1, high-mobility group box 1; ALF, acute liver failure; DAMP, damage-associated molecular pattern; TLR, Toll-like receptor; RAGE, receptor for advanced glycation endproducts; ALT, alanine aminotransferase; SBP, Systolic blood pressure; T_c, core temperature; Ac-YVAD-cmk, AC-TYR-VAL-ALA-ASP-CHLOROMETHYLKETONE; NAC, N-acetylcysteine; LDH, lactate dehydrogenase; EP, ethyl pyruvate; sRAGE, the soluble isoform inhibitor of the RAGE receptor; ROS, reactive oxygen species; TEM, transmission electron microscopy.

Introduction

Liver injury and acute liver failure (ALF) are known complications of heat stroke (HS) [1]. Biochemical and light-microscopic evidence of liver injury is invariable in HS patients or animal models [2]. Despite adequate lowering of the body temperature and aggressive treatment, ALF still frequently occurs during HS and serves as a direct cause of patient death [2,3]. However, the mechanism underlying HS-induced ALF remains unclear.

Massive degenerative changes of hepatocytes, including cell death, are the most common pathological change of HS livers [4]. Pyroptosis is a caspase-1-dependent programmed cell death, which features cell swelling, rapid plasma membrane rupture, and release of proinflammatory intracellular contents [5–10]. A recent report suggested that in Nlrp3 knock-in mice continuative Nlrp3 inflammasome activation induced hepatocyte pyroptosis and severe liver damage [11]. Although the role of pyroptosis in the development of inflammation in response to bacterial infection has been reported [6], the role of cell pyroptosis in the ALF following HS has not yet been addressed.

Caspase-1 is synthesized as an inactive zymogen, which is activated by inflammasome, and is responsible for the maturation of pro-IL-1β and pro-IL-18. Emerging evidences suggest that

Nlrp3 inflammasome plays an important role in mediating danger signal-induced liver inflammation and injury [12,13]. HMGB1 is a prototypical damage-associated molecular pattern (DAMP) molecule [14,15], which can be released into extracellular milieu during states of cellular stress or damage [16,17]. Recent studies have shown that HMGB1 release into the circulation occurs in an early stage of HS in patients [18] and rat [19–22], and circulation HMGB1 level may serve as an indicator of the severity of illness and a mortality predictor of HS [18–22]. However, there is a significant gap in our knowledge concerning the mechanism of HS-induced HMGB1 release and subsequent effect on liver injury.

In this study, using a rat HS model, we demonstrate that HMGB1, acting through Toll-like receptor (TLR) 4 and receptor for advanced glycation end products (RAGE) signaling, mediated HS-induced activation of Nlrp3 inflammasome, which in turn, induces IL-1β activation, as well as hepatocyte pyroptosis and subsequent augmented liver injury. Inhibition of HMGB1, silencing Nlrp3, or blocking caspase-1 prevented HS-induced Nlrp3 inflammasome activation and ameliorated liver injury. These findings suggest that targeting inflammasome may represent a novel therapeutic strategy to limit cell death and prevent liver failure after HS.

Materials and methods

This report includes Supplementary material, which provides detailed information regarding reagents, cell culture and treatment, Nlrp3 knockdown, coimmunoprecipitation and immunoblot analysis, hepatocytes isolation, determination of hepatocyte pyroptosis, enzyme-linked immunosorbent assay (ELISA), Alanine aminotransferase (ALT) assay, liver histopathology, confocal microscopy, quantitative real-time polymerase chain reaction (qRT-PCR), and statistical analysis.

Rat HS model

Adult male Sprague-Dawley (SD) rats were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The animals were housed in the animal center of General Hospital of Guangzhou Military Command, and they received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86–23, 1985 revision). All experimental protocols involving animals were approved by the Animal Care and Use Committee of General Hospital of Guangzhou Military Command. Animals have free access to standard chow and water. Rats were habituated to an artificial climate chamber (length × width × height = 4 m × 3 m × 2 m) for two weeks before experiment. Systolic blood pressure (SBP) was determined using a non-invasive tail-cuff blood pressure measurement (BPM) system (Biowill, Shanghai, China). Core temperature (T_c) was monitored using a thermocouple (BW-TH1101; Biowill, Shanghai, China) which was inserted 6.5 cm into the rectum. To induce HS, the animals were placed in the climate chamber in the absence of food and water. The chamber temperature was then increased to 39.5 ± 0.2 °C within 30 min, with a relative humidity of 60 ± 5%. SBP and T_c were measured with an interval of ten min. The time point at which the SBP started drop down from the peak level was taken as a reference point of HS onset [23–25]. Immediately after the onset of HS, the rats were then removed from the climate chamber, weighed, and returned to their original cages with an ambient temperature of 25 °C with ad libitum food and water. The sham control animals underwent the same procedure without being heated. In order to block HMGB1, neutralizing antibody against HMGB1 (3 mg/kg body weight, Santa Cruz Biotechnologies, Santa Cruz, USA) or nonimmune control IgG was injected i.p. into the rats immediately before subjecting to heat stress. In some rats, caspase-1 inhibitor AC-TYR-VAL-ALA-ASP-CHLOROMETHYLKETONE (ac-YVAD-cmk) (0.3 mg/kg body weight; Enzo Biochem Inc., NewYork, USA) or PBS containing 2.8% DMSO as control was injected i.p. into the rats immediately before heat stress. In some experiments, antioxidant N-acetylcysteine (NAC, 300 mg/kg body weight) was injected i.p. into the rats immediately before heat stress.

Results

Thermal and SBP regulatory response to HS

High temperature (39.5 ± 0.2 °C) and humidity (60 ± 5%) induced a significant elevation of T_c and SBP in the rats (p <0.001 *vs.* sham group respectively), as shown in Table 1. By the time when SBP peaked (150.5 ± 7.2 mmHg) and started to decrease, which representing HS onset, the T_c usually rose above 43 °C (43.4 ± 0.2 °C). The average time to induce HS onset, representing by heat exposure time, is 168.1 ± 3.7 min (Table 1).

HS-induced liver inflammation and injury

Fig. 1A shows histological changes in the liver following HS. Starting from three hours after HS, we observed hepatocyte swelling and ballooning degeneration (white arrow), which indicate an increase in hepatocyte membrane permeability, and necrosis (black arrow). We also observed inflammatory infiltration (black arrowhead) and hemorrhage (white arrowhead) (Fig. 1A). Extensive hepatocyte ballooning degeneration and necrosis were observed at 9 h after the onset of HS (Fig. 1A). Histological scores are shown in Fig. 1B. Consistent with the changes in liver histology, serum ALT activity was progressively increased following HS (Fig. 1C). IL-1β and IL-18 in the liver and serum (Supplementary Fig. 1A through D) as well as myeloperoxidase (MPO) activity in the liver (Supplementary Fig. 1E) were marked increased at as early as 3 h after HS onset and were further elevated in a time-dependent manner.

Fig. 1D shows the changes in liver tissue ultrastructure at 9 h after HS. The images demonstrate: (i) hepatocytes swelling with loosen and faded cytoplasmic matrix; (ii) indistinct and ruptured outer membrane of hepatocyte (Fig. 1D a through c; black arrowhead); (iii) spilled cell contents in the sinusoid (Fig. 1D a through c; white arrow); (iv) vacuoles along the sinusoidal border (Fig. 1D b and c; black arrow); (v) mitochondria swelling and cristae vague (Fig. 1D a through c); and (vi) denuded Kupffer cells with engulfed fragments of liver cells and red cells (Fig. 1D d; white arrowhead). Notably, hepatocyte membrane formed spherical membrane bound structure, and were frequently ripped from hepatocyte surface, freed in the sinusoids (Fig. 1D a and c; black arrowhead). This ultrastructure change was believed to be one of the morphological features of pyroptosis [6].

Heat stress induces hepatocyte pyroptosis in vivo and in vitro

Pyroptosis was defined by the presence of both active caspase-1 and PI positivity [12]. To determine whether the loss of

Table 1. Thermal and SBP response in rats subjected to heat stress.

	Sham group	HS group
BW pre-heating, g	262.4 ± 6.3	260.7 ± 7.1
BW post-heating, g	248.4 ± 5.7	240.1 ± 8.3
Heat exposure time (min)	0	168.1 ± 3.7
Tc, end of heat exposure (°C)	36.7 ± 0.2	43.4 ± 0.2*
Heating rate (%)	0	4.1 ± 0.1
Maximum SBP (mmHg)	109.2 ± 4.9	150.5 ± 7.2*

Values are means ± SD. T_c is core temperatures. Heating rate is the T_c elevation rate during heat exposure, and is calculated as show in *Materials and methods*.
*Difference between groups was significant (p <0.001).