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Expressions of chemokines and their receptors in the brain after heat stroke-induced cortical damage

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ABSTRACT

Despite growing evidence that cytokines and chemokines are expressed in humans and rats after heat stress, the cellular mechanisms underlying the effects on the brain after heatstroke (HS) are not fully understood. In this study, we observed time course changes of chemokines in rat brain tissues and elucidated what kinds of cortical cells were affected after HS. Male SD rats were anesthetized and randomly separated into two groups as follows: (a) normothermic sham and (b) HS rats. Rats were sacrificed at different time points (0, 1, 3, 6, and 12 h after heat exposure, n = 5 in each group) to the end of the experiment in order to extract the mRNA/proteins of cortical tissues. Cerebrospinal fluid (CSF) of sham and HS rats was also collected before sacrifice. In the HS group, an elevated body temperature (Tco > 40 °C) and abnormality of cortical cells (e.g., pyknotic nuclei) were observed. When compared to the sham group, expression levels of either mRNAs or proteins of chemokines and their receptors (including CXCL1, MIP2, MCP1, CXCR1, CXCR2, and CCR2) peaked at different time points after heat exposure. We also found that CXCR2 was expressed in the cortex of rat brain and was colocalized with neurons and microglia after HS. Hence, MCP1, MIP2, and CXCR2 might play important roles in the brain after HS, possibly indicating a new direction for treating HS.

1. Introduction

Heat stroke (HS), a leading cause of mortality when organisms encounter heat stress, is defined as a systemic disturbance that includes (a) an elevated core body temperature exceeding 40 °C (hyperthermia), (b) central nervous system (CNS) abnormalities, and (c) a systemic inflammatory response (either high cytokine expressions or disseminated intravascular coagulation) (Bouchama and Knochel, 2002; King et al., 2017; Li et al., 2013). Worldwide, patients who suffer from HS have highly mortality (ranging 10%–50%), and neuronal injury is mainly observed (Dematte et al., 1998; Sharma and Hoopes, 2003). In our previous study, an animal model of HS also showed neurological damage (Liu et al., 2010), and an inflammatory response occurred in different tissues (Leon and Bouchama, 2015). Although lots of studies indicated that CNS injury is very important in HS (Chen et al., 2008; Lin et al., 2011; Liu et al., 2010; Yi et al., 2017), the pathophysiological mechanism is still controversial.

Hyperthermia syndrome not only encompasses direct heat damage but also includes heat cytotoxicity in combination with a subsequent systemic inflammatory response syndrome (SIRS) of the host in response to tissue injury (King et al., 2017). In many clinical and pathogenic aspects, heat-induced injury resembles sepsis, and there is growing evidence that cytokines/chemokines are implicated in its pathogenesis (Biedenkapp and Leon, 2013; Bouchama et al., 2005; Lee et al., 2015). Our previous study also provided results of elevated cytokines/chemokines (e.g., interleukin (IL)-10/MCP-1) in blood of HS patients (Lu et al., 2004). Furthermore, a study reported that murine astrocytes induced chemokine expressions (e.g., MIP-2) and triggered cell survival ex vivo after heat shock (Choi et al., 2011). Recently, Audet et al. (2016) found different chemokine gene expressions in rats exposed to HS and suggested hypothalamic gene expression patterns that may be driving HS pathology and morbidity. However, it is still unknown whether protein expressions of chemokines change in the brain after HS in vivo. In order to investigate the role of chemokines after HS, we performed experiments using pathologic and biological methods to assess whether chemokines are involved in the process of

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brain tissue damage after HS.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (300 ± 30 g, Bio LASCO Taiwan Co., Ltd., Taipei, Taiwan) were used. Rats were housed in groups in a temperature - (21–25 °C) and humidity (45%–50%)-controlled room with a 12-h light/dark cycle and ad libitum access to pellet chow and water. Efforts were also made to minimize animal suffering and the number of animals used. All animals were treated in accordance with international guidelines for animal research, and the study design was approved by the animal ethics committee of Taipei Medical University.

2.2. Induction of HS

We previously established an animal model of HS (Liu et al., 2010). Briefly, an adequate anesthesia was maintained by a single intraperitoneal dose of urethane (0.6 g/kg) plus pentobarbital (30 mg/kg). A thermocouple was inserted into the rectum for continuous core temperature (Tco) monitoring, the right femoral artery was cannulated with polyethylene tubing (PE 50) for blood pressure monitoring, and a pressure transducer was inserted into the cannula for continuous monitoring of both the mean arterial pressure (MAP) and heart rate (HR). Rats were then exposed to an ambient temperature of 40 °C in a heating chamber for 1 h and then the Tco of rats reached 40.5 °C or higher (HS onset). After heat exposure, rats were transferred from the heating chamber to an animal cage (at a room temperature of 26 °C) where they were allow Tco recovery to normal. The recovery time is designated by the number of hour (s) after the onset of HS. For example, HS3 refers to conditions at 3 h after the onset of HS. HS rats and normothermic sham rats were sacrificed after various recovery periods,

2.3. Hematoxylin and eosin (H&E) staining

Brain tissues of rats were dissected, immersed in 10% neutral buffered formalin overnight, and embedded in paraffin. Blocks were cut serially (into 2-μm sections), stained with H&E, and examined under a light microscope (at 400 × magnification).

2.4. Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from brain tissues using the TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA (3 μg) was reverse-transcribed to complementary (c)DNA using the Rever Tra Ace-α First-strand cDNA Synthesis Kit (Toyobo Life Sciences, Osaka, Japan). The resulting cDNA was incubated with SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and primers (Table 1) for chemokines and their receptors (CXCL1, MIP2, MCP1, CXCR1, CXCR2, and CCR2). For the quantitative analysis, we performed 40 amplification cycles (denaturation at 95 °C for 15 s; annealing at 60 °C for 30 s; and elongation at 72 °C for 35 s) on an ABI 7500 PCR Detection System (Applied Biosystems). Melting curve and sequencing data were used to confirm the specificity of the PCR products. Messenger (m)RNA levels of chemokines and their receptors were normalized to those of β-actin and were then expressed as values relative to the control using the comparative threshold cycle (Ct) method.

2.5. Measurement of chemokines in cerebrospinal fluid (CSF)

CSF samples of sham and HS rats were collected from the cisterna magna, immediately separated, and stored at −80 °C until they could be assayed. We used commercially available enzyme-linked immunosorbent (ELISA) kits to determine levels of MIP2 and MCP1, according to the manufacturer's instructions (Quantikine, R&D System, Minneapolis, MN).

2.6. Immunoblotting

Brain tissues of sham and HS rats were collected. Tissues were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 1% NP-40, and 10% Complete Protease Inhibitor solution (Roche Molecular Biochemicals, Mannheim, Germany). Twenty micrograms of protein lysate was run on 4%–12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). Proteins were transferred from the gels to polyvinylidene difluoride (PVDF) membranes (Invitrogen) by electro-blotting. Each membrane was incubated in blocking buffer containing phosphate-buffered saline (PBS, 120 mM NaCl, 2.7 mM KCl, and 0.01 M PB), 3% bovine serum albumin (BSA; Sigma, St. Louis, MO), and 0.04% Tween 20 for at least 1 h, blocked, incubated with an anti-CXCR2 antibody (1:1000; Novus Biologicals) at 4 °C overnight, washed in PBS containing 0.04% Tween 20 (three times, 10 min each) to remove unbound primary antibodies, incubated with a horseradish peroxidase-conjugated secondary antibody ($1{:}10^4$ diluted in blocking buffer, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h, and washed in PBS (three times, 10 min each). Labeled proteins were visualized by chemiluminescence (ECL Plus kit, Amersham, Arlington Heights, IL).

2.7. Immunofluorescence staining

Brain tissues of normothermic sham and HS rats were immersed in 10% neutral buffered formalin overnight and then embedded in O.C.T. compound (Tissue-TeK, Sakura Finetek, Torrance, CA). Cryosections (10 μm) were mounted onto slides, incubated with blocking buffer (PBS containing 3% BSA and 0.1% Tween 20) for 30 min, incubated with a primary antibody against CXCR2 (rabbit anti-CXCR2, 1:200; Abcam) overnight, washed three times with PBS, incubated with an FITC-conjugated secondary antibody (1:500 in blocking buffer for 2 h), then incubated with a another primary antibody against Neu N (mouse anti-1:300; Millipore) or OX42 (mouse anti-OX42, 1:100; Abcam) overnight, washed three times with PBS, incubated with Texas red-conjugated secondary antibody (1:300 in blocking buffer for 2 h), covered with mounting media containing DAPI (5 μg/ml, Vector Laboratories, Burlingame, CA), and observed and photographed under a fluorescence microscope.

Table 1
Primers used for the analysis of chemokines and its receptor expression.

Gene name	Forward	Reverse
CXCL1	5′-GAAGATAGATTGCACCGATG-3′	5′-CATAGCCTCTCACACATTTC-3′
MIP2	5′-GGGGGAGTTGGGTACTGACT-3′	5′-CCTTGAAAGCCCTCTGACTG-3′
MCP1	5′-TTCACTGGCAAGATGATCCC-3′	5′-TGCTTGAGGTGGTTGTGGAA-3′
CXCR1	5′-CATCTTCCGCCAGGCATATAAA-3′	5′-GGGACAGACCACGCAATGTT-3′
CXCR2	5′-CAGCAGTGTTCTGTTGCTAGCCT-3′	5′-CCAAGTGTCTCTTCTGGATCAGTGT-3′
CCR2	5′-CGCAGAGTTGACAAGTTGTG-3′	5′-GCCATGGATGAACTGAGGTA-3′
β-actin	5′-TGTCACCAACTGGGACGATA-3′	5′- GGGGTGTTGAAGGTCTCAAA-3′