Hypothermia Protects against Fulminant Hepatitis in Mice by Reducing Reactive Oxygen Species Production

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Key Words

Reactive oxygen species · Fulminant hepatitis · Hypothermia · Cold-inducible RNA-binding protein · Cold shock

Abstract

Objective: Mild hypothermia (32–33°C) shows protective effects in patients with brain damage and cardiac arrest. Although cold-inducible RNA-binding protein (CIRP) contributes to the protective effects of hypothermia through extracellular signal-regulated kinase activation in fibroblasts, the effects of hypothermia in the liver remain unclear. **Methods:** We analysed the effects of cold temperature on fulminant hepatitis, a potentially fatal disease, using the D-galactosamine (GalN)/lipopolysaccharide (LPS) and concanavalin (con) A-induced hepatitis models in mice. After GalN/LPS administration and anaesthesia, mice in the hypothermia group were kept at 25°C and those in control group were kept at 35°C. After concanavalin A (con A) administration, the mice in the hypothermia group were placed in a chamber with an ambient temperature of 6°C

for 1.5 h. **Results:** Hypothermia attenuated liver injury and prolonged survival. Activation of c-Jun N-terminal kinase and Akt, which are involved in reactive oxygen species (ROS) accumulation, was suppressed by low temperature. Hypothermia significantly decreased oxidized protein levels, and treatment with N-acetyl-L-cysteine, an antioxidant, attenuated GalN/LPS-induced liver injury. In con A-induced hepatitis, CIRP expression was upregulated and Bid expression was downregulated, resulting in decreased apoptosis of hepatocytes in the hypothermia group. **Conclusions:** These data suggest that hypothermia directly protects hepatocytes from cell death via reduction of ROS production in fulminant hepatitis.

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Introduction

Mild hypothermia has been reported to protect central neurons from ischemic damage [1–3]. Although clinical application of mild hypothermia (32–35°C) for patients with brain injury and cardiac arrest has been conducted

Fig. 1. Low temperature (32°C) reduced ROS production and cell death in TNF- α -treated cells. HeLa cells were cultured at 37 (control) or 32°C (hypothermia) in media containing TNF- α (50 ng/ml) and/or CHX (10 μ g/ml). **a, b** The cell survival rates and relative ROS levels were examined at the indicated times after initiation of TNF- α treatment. The number of viable cells was estimated by the Trypan blue assay. ROS accumulation was assessed using 5-[and-

6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA). The values are shown relative to non-treated cells; results are the mean $\pm$ SEM. * $p < 0.05$ versus culture at 37°C (control). **c** After treatment with TNF- α or TNF- α plus CHX, cell lysates were prepared and analysed by Western blotting using the indicated antibodies.

with promising results [4], the molecular mechanisms underlying the protective effects of hypothermia are unknown. In endothelial cells kept under hypothermic conditions, significant upregulation of the anti-apoptotic protein Bcl-2 has been reported. Hypothermia decreased the levels of inflammatory chemokines such as IL-8, MCP-1 and COX-2, which could lead to reduced leukocyte recruitment [5]. Low temperature protects mammalian cells from apoptosis initiated by various stimuli in vitro [6]. Cold-inducible RNA-binding protein (CIRP), a protein induced by mild hypothermia, protects against tumour necrosis factor (TNF)- α -induced apoptosis via activation of extracellular signal-regulated kinase (ERK) [7].

Fulminant hepatitis, resulting from the acute hepatitis caused by viral infection, alcohol or drugs, is associated with high mortality, and development of a new therapy is necessary. This pathophysiological disturbance is caused by excessive hepatocyte death, in which TNF- α plays an important role [8]. Reactive oxygen species (ROS) are another major mediator of inflammation and reduction of ROS levels leads to attenuation of hepatic injury [9]. Akt activation increases intracellular ROS levels [10]. ROS accumulation inhibits mitogen-activated protein kinase (MAPK) phosphatases, resulting in prolonged c-Jun N-terminal kinase (JNK) activation, which contributes to ROS accumulation and hepatocyte death [11]. Here, we analysed the effects of hypothermia on fulminant hepatitis using murine hepatitis models.

Materials and Methods

Cell Culture

Human HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum at 32 or 37°C in a humidified atmosphere of 5% CO₂ in air. For induction of cell death, confluent cultures of cells were incubated with TNF- α (50 ng/ml) in the presence of cycloheximide (CHX; 10 μ g/ml). The number of viable cells was estimated by Trypan blue assay. To assess intracellular ROS levels, TNF- α -treated cells were resuspended in PBS containing 10 μ M 5-[and-6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Invitrogen, Carlsbad, Calif., USA), maintained at 37°C in the dark for 30 min, and analysed by flow cytometry.

Western Blot Analysis

Western blot analysis was performed as previously described [12]. The antibodies used were as follows: anti-phospho Akt, anti-Akt, anti-phospho-JNK, anti-JNK, anti-phospho-ERK, anti-ERK (Cell Signaling Technology, Danvers, Mass., USA), anti-Bcl-2, anti-Bad, anti-Bid (BD Transduction Laboratory, Lexington, Ky., USA), anti- β -actin (Sigma, St. Louis, Mo., USA), anti-Bcl-xL and anti-HNF-3 γ (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Rabbit polyclonal antibody recognizing C terminus of mouse CIRP was prepared as described [13]. To quantify ROS accumulation, an OxyBLOT^{TM} Protein Oxidation Detection Kit (Millipore, Billerica, Mass., USA) was used.

Fulminant Hepatitis Model

C57BL/6 mice, 4–12 weeks old, were purchased from Japan SLC (Shizuoka, Japan) and were kept at 25°C and 55% relative humidity in a 12-hour day/night cycle with free access to food and water. To induce hepatitis, D-galactosamine (GalN; 1,000 mg/kg, Sigma) and lipopolysaccharide (LPS; 0.1 or 35 μ g/kg, Sigma) were injected i.p. Thereafter, mice were anesthetized with urethane or

Fig. 2. Hypothermia ameliorates GalN/LPS-induced hepatitis. **a** Rectal temperatures of mice in the control (n = 10) and hypothermia (n = 10) groups were monitored after injection of GalN (1,000 mg/kg) and LPS (0.1 µg/kg); results are the mean. **b** Mice were injected with GalN (1,000 mg/kg) and LPS (0.1 µg/kg), and 8 h after the injection serum ALT levels were examined; results are

the mean ± SEM. * p < 0.05 versus the control. **c** After injection with GalN (1,000 mg/kg) and LPS (35 µg/kg), the survival rates of mice in the control group (n = 10) and those in the hypothermia group (n = 10) were examined. The difference in the survival rate was analysed by the Kaplan-Meier method and log-rank test. * p < 0.05 versus the control.

pentobarbital and divided into 2 groups: mice placed in a chamber with an ambient temperature of 25°C (hypothermia group) and those placed on a plate of 35°C (control group). To investigate the protective effects of N-acetyl-L-cysteine (NAC; Sigma), which is an antioxidant, mice were administered with NAC (150 mg/kg, i.p.) 30 min before GalN/LPS administration (1,000 mg/0.1 µg/kg, i.p.). NAC dissolved in PBS was neutralized before injection. The volume of insensible perspiration was 15 ml/kg/day and increased by 15% per 1°C upshift of body temperature [14]. The volume of PBS as calculated was injected i.p. into mice in the control group to reduce the effect of dehydration.

Concanavalin A (con A; Sigma) was dissolved in sterile saline and injected into the tail vein at a final volume of 200 µl. To examine the effect of hypothermia on their survival, the mice were treated with con A at a lethal dose of 35 mg/kg body weight and divided into 2 groups. One hour after con A injection, the mice in the hypothermia group were placed in a chamber with an ambient temperature of 6°C for 1.5 h. Then, all mice were observed at 22°C. For histological and gene expression analyses, mice were treated with 25 mg/kg of con A, divided into 2 groups, and euthanised at 24 h after con A injection.

This work was conducted under the Japanese Law Concerning the Care and Control of Animals and was approved by the Animal Research Committee of the Faculty of Medicine of Kinki and Kyoto University.

Histopathological Examination

The liver was removed and fixed in 10% formalin, embedded in paraffin and sliced into 5-µm sections for light microscopy. Immunohistochemistry was performed using ImmPRESS™ reagents (Vector Laboratory, Burlingame, Calif., USA) according to the manufacturer's recommendations. The number of proliferating cells was estimated by staining the sections with a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Cell Signaling Technology). TUNEL staining was performed using tissue sections with an in situ Apoptosis Detection Kit (Takara, Tokyo, Japan).

Statistical Analysis

Data are presented as the mean ± SEM. Statistical differences between sample means were calculated by analysis of variance, followed by unpaired Student's t test. To compare the survival rates between groups of mice, the log-rank test was used. p < 0.05 was considered significant.

Results

Low Temperature (32°C) Reduced ROS Production and Cell Death in TNF-α-Treated Cells

Treatment with TNF-α and CHX induced death of HeLa cells cultured at 37°C within 6 h (fig. 1a). The number of surviving cells was significantly higher when cells were cultured at 32°C (hypothermia) than when they were cultured at 37°C (control). Similar results were obtained with the human hepatoma cell line HuH-7 [7]. Decreased H₂O₂ accumulation in cells cultured at 32°C was detected using the ROS indicator 5-[and-6]-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; fig. 1b). Akt activation promotes ROS production [10]. At 60 min after treatment with TNF-α, the protein level of phospho-Akt was lower in HeLa cells cultured at 32°C than in cells cultured at 37°C (fig. 1c).

Hypothermia Ameliorates GalN/LPS-Induced Hepatitis

The mean rectal temperature was kept at approximately 30°C in the hypothermia group and at 37°C in the control group (fig. 2a). As shown in fig. 2b and c, hypothermic treatment significantly reduced hepatic injury and improved the survival rate in GalN/LPS-induced hepatitis.

Fig. 3. Hypothermia reduces ROS accumulation in GalN/LPS-treated livers. Mice in the control (n = 8) and hypothermia (n = 8) groups were injected with GalN (1,000 mg/kg) and LPS (0.1 µg/kg), and 24 h after the GalN/LPS injection, tissue lysates were extracted. **a** ROS accumulation was assessed using the OxyBLOT™ Protein Oxidation Detection Kit; the results are the mean ± SEM. * p < 0.05 versus the control. **b** Tissue lysates were analysed by Western blotting using the indicated antibodies. **c** Mice in the con-

trol group (n = 8) and hypothermia group (n = 8) were injected with GalN (1,000 mg/kg) and LPS (0.1 µg/kg). Mice received NAC (150 mg/kg, i.p.) in the control group (Cont+Nac; n = 8) and the hypothermia group (Hypo+Nac; n = 8) 30 min before GalN/LPS administration (1,000 mg/0.1 µg/kg, i.p.). Eight hours after the GalN/LPS injection, the mice were euthanised and serum ALT levels were examined; results are the mean ± SEM. * p < 0.05 versus the control.

Fig. 4. Hypothermia improves the survival rate in con A-induced hepatitis. **a** Rectal temperatures of mice in the control (n = 14) and hypothermia (n = 11) groups were monitored after injection of con A (35 mg/kg); results are the mean. **b** After injection with con A

(35 mg/kg), the survival rates of mice in the control group (n = 14) and those in the hypothermia group (n = 11) were examined. The difference in the survival rate was analysed by the Kaplan-Meier method and log-rank test.

Hypothermia Reduces ROS Accumulation in GalN/LPS-Treated Livers

Mice in the hypothermia group were found to have lower levels of oxidized protein than those in the control group (fig 3a). ROS accumulation inhibits MAPK phosphatases, resulting in prolonged JNK activation, which contributes to hepatocyte death [11]. Accordingly, Akt and JNK activity were decreased in hypo-

thermia-treated livers (fig. 3b). To evaluate the contribution of oxidative stress to GalN/LPS-induced liver damage, we injected the antioxidant NAC. NAC-treated mice showed a significant reduction in GalN/LPS-induced liver injury (fig. 3c). Thus, hypothermia reduces GalN/LPS-induced hepatocyte death through mechanisms that may depend on attenuated ROS accumulation.

Fig. 5. Hypothermia upregulates CIRP in con A-induced hepatitis. **a–f** Liver histology. Mice were injected with 25 mg/kg body weight of con A. One hour later, the ambient temperature was changed to 25°C (**a, c, e**) or 6°C (**b, d, f**) for 1.5 h, and then returned to room temperature. Twenty-four hours after the injection, the mice were euthanised and liver sections were obtained after fixation. Haematoxylin and eosin staining (**a, b**); TUNEL staining (**c, d**); immunohistochemical staining with mouse anti-PCNA antibody and

horseradish peroxidase-conjugated anti-mouse antibody (**e, f**). Arrows indicate PCNA-positive cells. Scale bar = 50 μm. **g–i** Gene expression. Groups of mice were treated as above, and the expression of CIRP and HNF-3γ (**g**), Bcl-2 family members (**h**) and ERK (**i**) in the livers of control mice and hypothermic mice (Hypo) was analysed by Western blotting using the indicated antibodies. Results with representative samples are shown.

Hypothermia Upregulates CIRP Expression and Improves the Survival Rate in con A-Induced Hepatitis

TNF-α has been suggested to be a crucial factor in fulminant hepatitis [8]. In con A-induced hepatitis, a mouse model of fulminant hepatitis, the intrahepatic levels of cytokines, including TNF-α, maximally increase 1 h after con A administration [15]. As shown in figure 4, hypothermic treatment improved the survival rate, but the improvement was not significant ($p = 0.096$).

Histological examinations revealed that con A induced severe morphological changes in the liver (fig. 5a). Dilatation of veins and bile ducts was prominent. Massive de-

generative lesions consisting of dead parenchymal cells were observed in the midlobular area. The liver of mice in the hypothermia group showed smaller areas of the lesion than the liver of the mice in the control group (fig. 5b). Cells positive for TUNEL staining were localized in the parenchymal cells in the midlobular area and adjacent to the degenerative lesions, and the areas with TUNEL-positive cells were smaller in the hypothermia group (fig. 5c, d). Cold exposure slightly reduced the number of PCNA-positive cells in mice without con A challenge (4.5 ± 1.2 vs. 3.4 ± 0.8 per 1,000 cells). As shown in figure 5e and f, PCNA immunoreactivity was decreased after con A administration in the control and hypothermia groups

to equivalent levels (1.4 ± 0.5 vs. 1.8 ± 0.6 per 1,000 cells). These results suggest that in con A-induced hepatitis, hypothermia improves the survival of mice not by enhancing hepatocyte regeneration but rather by suppressing apoptosis.

The stress response protein CIRP protects cells by activating the ERK pathway [7]. HNF-3 γ shows a hepatoprotective effect in acute liver injury [16]. Hypothermia induced the expression of CIRP, but not HNF-3 γ , in the livers of con A-treated mice (fig. 5g). As shown in figure 5h, Bid was downregulated in livers in the hypothermia group. There was no difference in the protein levels of XIAP/ILP and other Bcl-2 family members, including Bcl-2, Bcl-xL, Bad and Mcl-1 (fig. 5h and data not shown). The level of phosphorylated ERK was increased in 2 out of 3 examined mice in the hypothermia group (fig. 5i).

Discussion

Fulminant hepatitis is a devastating liver disease with a progressive course and a high mortality rate [17]. Although several studies have shown that mild hypothermia has a protective effect against the encephalopathy resulting from severe liver injury [18], the direct effect of hypothermia on the liver has not been determined. In the present study, we found that hypothermia inhibited apoptosis in the liver and increased the survival rate in mice with con A-induced hepatitis and GalN/LPS-induced hepatitis, which are considered to be relevant to human fulminant hepatitis [19]. Apoptosis is essential for the homeostasis of organs such as the liver [20]. In both human fulminant hepatitis and its animal models, apoptosis of hepatocytes is mediated by death receptors such as Fas (CD95) and the TNF- α receptor [20, 21]. In the death receptor pathway, the pro-apoptotic protein Bid is processed, and its translocation to the mitochondria activates the mitochondrial apoptotic pathway [22]. Bid is required, at least in some cells, for death receptor activation to initiate the apoptosis cascade. Here, we showed that in the livers of mice treated with con A, the Bid protein level was lower in the hypothermia group than in the control group (fig. 5). These results suggest that the protective effect of hypothermia in mice is mediated, at least partly, by the decrease in the Bid protein level in the liver. CIRP blunts TNF- α -mediated apoptosis via ERK activation [7] and inhibits H₂O₂-induced apoptosis through upregulation of thioredoxin expression [23]. CIRP, which was upregulated by hypothermia in

this study, may contribute to the anti-apoptotic effects of hypothermia by regulating ERK activity and ROS accumulation in the liver.

Several mechanisms have been proposed to explain the increased resistance of humans and animals to tissue damage as body temperature is reduced. Hypothermia suppresses the production of superoxide anions, nitric oxide and TNF- α in ischemic cells [24]. The hepatic inflammatory response after ischemia is suppressed by hypothermia through selective inhibition of JNK and activator protein-1 [25]. ROS promote TNF- α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases [11, 26]. Akt activation increases intracellular ROS levels through increased oxygen consumption and by inhibition of the expression of ROS scavengers downstream of FoxO, particularly *sestrin 3* [10]. In the present study, we demonstrated that hypothermia suppressed liver injury and the Akt and JNK pathways in the livers of GalN/LPS-treated mice (fig. 3). Furthermore, mice treated with an antioxidant showed a significant reduction in the severity of liver injury. These data suggest that the attenuation of ROS accumulation is involved in the cyto-protective effect of hypothermia. Further elucidation of the underlying mechanisms of these protective effects will lead to the future development of novel therapeutic modalities.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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