A potential for granulocyte-colony stimulating factor for use as a prophylactic agent for heatstroke in rats

Ming-Chi Yung,1 Chuan-Chih Hsu,1 Chien-Yi Kang, Chia-Li Lin, Shu-Ling Chang, Jhi-Joung Wang, Mao-Tsun Lin, Pei-Jarn Chen, Sheng-Hsien Chen,⁎

Abstract

Heatstroke is a form of excessive hyperthermia associated with a systemic inflammatory response that leads to multi-organ dysfunction in which central nervous system disorders predominate. Herein we determined to ascertain whether heat-induced multi-organ dysfunction in rats could be attenuated by granulocyte-colony stimulating factor (G-CSF) preconditioning. Anesthetized rats were divided into 2 major groups and given vehicle solution (isotonic saline, 0.3 ml, subcutaneously) or G-CSF (50–200 μg/kg body weight in 0.3 ml normal saline, subcutaneously) daily and consecutively for 5 days before the start of thermal experiments. They were exposed to an ambient temperature of 43 °C for 68 min to induce heatstroke. G-CSF preconditioning significantly prolonged the survival time in heatstroke rats in a dose-related way (82–98 min vs 127–243 min). The non-preconditioning heatstroke animals showed hyperthermia, arterial hypotension, increased serum levels of systemic inflammatory response molecules, increased hypothalamic apoptotic cell numbers as well as neuronal damage scores, and increased serum levels of renal and hepatic dysfunction indicators. These heatstroke syndromes could be significantly reduced by G-CSF preconditioning. Thus our results revealed a potential for G-CSF used as a prophylactic agent for heatstroke in rats.

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1. Introduction

Granulocyte colony-stimulating factor (G-CSF) is a polypeptide growth factor that stimulated the proliferation, survival and maturation of the neutrophilic granulocyte lineage (Xiao et al., 2007). G-CSF has been used for haematopoietic stem cell mobilization into the peripheral circulation (Lu and Xiao, 2006). It showed that G-CSF preconditioning decreased mortality rate, reduced infarction volume, and improved neurological behavior after cerebral ischemia in rats (Lu and Xiao, 2006).

Heatstroke is a form of excessive hyperthermia (core temperature rising above 40 °C) associated with a systemic inflammatory response that leads to multi-organ dysfunction in which central nervous system disorders predominate (Bouchama and Knochel, 2002; Chang et al., 2006). In an anesthetized rat model, the heatstroke animals display hyperthermia, hypotension, hypothalamic neuronal apoptosis and degeneration, up-regulation of systemic inflammation, and hepatic and renal dysfunction (Lin et al., 2009; Liu et al., 2009). However, it is unknown whether human recombinant G-CSF can be used as a prophylactic agent for experimental heatstroke.

To deal with the hypothesis, this study attempted to assess the temporal profiles of hyperthermia, hypotension, hypothalamic neuronal apoptosis and degeneration, hepatic and renal cell apoptosis, systemic inflammatory indicators, and bone marrow expression of endothelial progenitor cells (EPCs) during heatstroke in rats (Lin et al., 2009; Liu et al., 2009) with or without G-CSF preconditioning.

2. Materials and methods

2.1. Experimental animals

Adult male Sprague–Dawley rats (weight, 285–315 g) were obtained from the Animal Resource Center of the National Science Council of Republic of China. The animals were housed 4 in a cage at an ambient temperature of 22 ± 1 °C, with a 12-h light/dark cycle. Pellet rat
chow and tap water were available ad libitum. The experimental protocol was approved by the Animal Ethics Committee of the Chi Mei Medical Center. Animal care and experiments were conducted according to the National Science Council Guidelines. They were allowed to become acclimated for ≥ 1 week. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinching throughout all experiments (approximately 6 h) by an intraperitoneal dose of urethane (1.4 g/kg body weight). At the end of the experiments, control rats and any rats that had survived heatstroke were killed with an overdose of urethane.

The right femoral artery of rats was cannulated with polyethylene tubing (PE50), under urethane anesthesia, for blood pressure monitoring. Core temperature was monitored continuously by a thermocouple (DR130, Yokogawa, Yamanashiken, Japan) inserted into the rectum, while the mean arterial pressure and heart rate were continuously monitored with a pressure transducer and a chart recorder (2107, Gould, Valley view, OH, USA).

2.2. Induction of heatstroke

Before the induction of heat stress, the core temperature of the anesthetized animals was maintained at about 37 °C with a folded heating pad except in the heat stress experiment. Heatstroke was induced by patting the animals in a folded heating pad of 43 °C controlled by circulating hot water. As shown in Fig. 1, the time point (68 min) at which the mean arterial pressure dropped from the peak to a value of <50 mm Hg and core temperature over 42 °C was arbitrarily taken as the onset of heatstroke (Chen et al., 2005, 2007). Immediately after this time point (68 min), the heating pad was removed and the animals were allowed to recover at room temperature (26 °C). Seventy-eight minutes after the start of heat stress (or 10 min after the time point for the onset of heatstroke), the animals displayed both hyperthermia (~42 °C) and arterial hypotension (~48 mm Hg). The survival time was defined by the interval between the start of heat stress and the animal death.

Seventy-eight minutes after the start of heat stress for the heatstroke rats or equivalent time period for the normothermic rats, the samples were obtained for determination of neuronal damage score, number of apoptotic cells in multiple organs, serum levels of tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and soluble intercellular adhesion molecule-1 (ICAM-1), and bone marrow levels of endothelial progenitor cells (EPCs).

2.3. Experimental groups

The animals were assigned randomly to one of four groups. The first group and the second group, respectively, treated with a subcutaneous (s.c.) dose of vehicle solution (1 mL normal saline per kilogram body weight) or human recombinant G-CSF (50–200 μg/kg body weight) daily and consecutively for 5 days, were exposed to an ambient temperature of 26 °C. These two groups were used as normothermic groups. The third group and the fourth group, respectively treated with an s.c. dose of vehicle solution or G-CSF daily and consecutively for 5 days, were exposed to an ambient temperature of 43 °C for exactly 68 min and were used as vehicle-treated heatstroke group or G-CSF-treated heatstroke group. Human G-CSF was supplied by the Kyowa Hakko Kogyo Co., Ltd. This G-CSF was highly purified (99%) and was endotoxin free as restrictively confirmed by Limulus amoebocyte lysate test. For injection, G-CSF was dissolved in sterile saline. Subcutaneous injection of G-CSF (or vehicle solution) was conducted 5 days before the start of thermal experiments.

2.4. Histological verification

At the end of the experiments, the animals were killed by an overdose of urethane and the brains were fixed in situ and left in the skull in 10% neutral buffered formalin for at least 24 h before removal from the skull. The brain was removed and embedded in paraffin blocks. Serial (10 μm) sections through the hypothalamus were stained with hematoxylin and eosin for microscopic evaluation. The extent of cerebral neuronal damage in hypothalamic section was scored on a scale of 0 to 3, modified from the grading system of Pulsinelli et al. (1982), in which 0 was normal, 1 indicated that approximately 30% of the neurons was damaged, 2 indicated that approximately 60% of the neurons was damaged, and 3 indicated that 100% of the neurons was damaged. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

2.5. TUNEL assay for apoptotic cells

In situ apoptosis detection kit (Clontech, USA) was employed to assess apoptosis by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method.

Fig. 1. Values of core temperature (Tco) and mean arterial pressure (MAP) for the normothermic rats exposed to an ambient temperature of 26 °C (○) and heatstroke rats exposed to an ambient temperature of 43 °C (●). Mean arterial pressure started to drop at 68 min and reached a value of about 45 mm Hg at 78 min. The time point of (78 min) was arbitrarily defined as the onset of heatstroke. Data were means ± S.D. of 6 animals per group. *P<0.05, in comparison with (●) group.
2.6. Immunohistochemistry

For determination of glial cell line derived neurotrophic factor (GDNF)-positive or vascular endothelial growth factor (VEGF)-positive cell number at the level of hypothalamic sections, the sections were incubated with PBS containing rabbit anti-GDNF antibody (1:100) (ab18956; Abcam) or mouse anti-VEGF respectively and then detected with Fluorescein (FITC)-conjugated goat anti-rabbit (IgG) antibody (1:200) (ab6717; Abcam). The slides were examined under epifluorescence on an Olympus BX60 (Tokyo, Japan) microscope.

2.7. Plasma concentrations of inflammatory and intercellular adhesion molecules and cytokines

Blood samples were taken at 78 min after the start of heat exposure for determination of TNF-α, IL-10 and ICAM-1 (Intercellular adhesion molecule) levels. For measurement of serum cytokines, 5 mL of blood was withdrawn from the femoral vein of each rat. The amounts of the cytokines including TNF-α, IL-10 and ICAM-1 in serum were determined by double antibody sandwich enzyme-linked immunoabsorbent assay (R&D Systems, Minneapolis, Minn) according to the manufacturer’s instructions. Optical densities were read on a plate reader set at 450 nm for TNF-α, IL-10 and ICAM-1. The concentration of TNF-α, IL-10 and ICAM-1 in the serum samples was calculated from the standard curve multiplied by the dilution factor and was expressed as picograms per milliliter.

2.8. Isolation of endothelial progenitor cells (EPCs)

Bone marrow was harvested by isolating the femurs of rats which were carefully cleaned from adherent soft tissue. The marrow cavity was inserted by a syringe needle (23-gauge) and flushed with phosphate buffered saline. The marrow cells were harvested and filtered through a 40-μm nylon cell strainer (BD Falcon, USA). The viable lymphocyte populations of blood and marrow cells were analyzed for CD133 (ab15898; Abcam) conjugated with the corresponding FITC-labeled secondary antibody (ab6717; Abcam) and VEGFR-2 (ab10972; Abcam) conjugated with the corresponding phycoerythrin-labeled secondary antibody (ab7004; Abcam). Isotype-matched antibodies served as controls in every experiment.

2.9. Statistical analysis

Data for immuno-reactive cell counting and serum markers were evaluated for Gaussian (normal) distribution, and if P<0.05, followed by Neumann–Keuls post-hoc test. The Wilcoxon test was used for histological assessment. Significant differences were established at P<0.05. All data were analyzed with Sigma Plot for windows version 11.0 (Systat Software, Inc. San Jose, California, USA).

3. Results

3.1. G-CSF improved survival and attenuated hypotension during heatstroke

The survival time (interval between the heat stress onset and animal death) values during heatstroke for vehicle-treated rats was decreased from the control values of 480±2 min (n=6) to new values of 82–98 min (n=6). When G-CSF-treated rats were exposed to the same heat regimen, their survival time values were significantly increased to new values of 127–243 min significantly and dose-related (Table 1).

Fig. 2 depicted the effects of heat exposure (43 °C for 68 min) on both core temperature and mean arterial pressure in the vehicle-
treated heatstroke rats, or G-CSF-treated heatstroke rats at 78 min after the start of heat stress. Seventy-eight minutes after the start of heat stress (or 10 min after the onset of heatstroke) in the vehicle-treated heatstroke group, the values of mean arterial pressure were significantly lower than those of the normothermic rats (~50 mm Hg vs ~100 mm Hg; P<0.05). In contrast, the values of core temperature in the vehicle-treated heatstroke group were significantly higher than those of the normothermic rats (~40 °C vs 37 °C; P<0.05). Heatstroke-induced arterial hypotension, but not hyperthermia, was significantly attenuated by treatment with G-CSF (P<0.05).

![Fig. 2. Values of core temperature and mean arterial pressure for the normothermic rats (□), vehicle-treated heatstroke rats (■), and G-CSF (100 μg/kg)-treated heatstroke rats (●). Data were obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups or the equivalent time period for the normothermic group. Data were means±S.D. of 6 animals per group. *P<0.05, in comparison with □ group. †P<0.05, in comparison with ■ group.](image)
3.2. G-CSF reduced hypothalamic apoptosis and neuronal damage during heatstroke

Figs. 3 and 4 showed that the hypothalamic values of both neuronal damage score and TUNEL-positive cells for vehicle-treated heatstroke rats were significantly higher at 78 min after the start of heat exposure than they were for the normothermic rats. The increased amounts of neuronal damage score and TUNEL-positive cells in the hypothalamus that occurred during heatstroke were significantly reduced by G-CSF pretreatment (Table 2 and Fig. 4).

3.3. G-CSF increased hypothalamic number of GDNF- and VEGF-positive cells

Figs. 5 and 6 showed that the amounts of both GDNF-positive cells and VEGF-positive cells in the hypothalamus for vehicle-treated heatstroke rats were significantly higher at 78 min after the start of heat stress than they were for the normothermic control rats. However, the increased amounts of hypothalamic GDNF and VEGF-positive cells that occurred during heatstroke were significantly decreased by G-CSF treatment.
3.4. G-CSF decreased systemic inflammatory response indicators

Fig. 7 showed that the serum levels of systemic inflammatory response indicators such as TNF-α and ICAM-1 for vehicle-treated heatstroke rats were significantly higher at 78 min after the start of heat exposure than they were for the normothermic rats. The increased serum levels of both TNF-α and ICAM-1 that occurred during heatstroke were significantly reduced by G-CSF pretreatment.

3.5. G-CSF decreased EPS and IL-10

Figs. 7 and 8 showed that the levels of both serum IL-10 and bone marrow EPCs expression for vehicle-treated heatstroke rats were significantly higher at 78 min after the start of heat exposure than they were for the normothermic rats. The increased serum levels of both TNF-α and ICAM-1 that occurred during heatstroke were significantly reduced by G-CSF pretreatment.

3.6. G-CSF decreased the amounts of renal and hepatic apoptotic cells during heatstroke

Figs. 9 and 10 showed that the number of TUNEL-positive cells of both liver and kidney for vehicle-treated heatstroke rats was significantly higher at 78 min after the start of heat exposure than they were for the normothermic rats. Again, the increased number of TUNEL-positive cells in both the liver and kidney that occurred during heatstroke was significantly reduced following G-CSF pretreatment.

4. Discussion

It is generally believed that the anterior hypothalamic preoptic area is an essential thermoregulatory center in the brain. When unanesthetized, unrestrained mice exposed to severe heat stress displayed hypothalamic neuronal apoptosis and cell degeneration accompanied by thermoregulatory deficits (Chatterjee et al., 2005; Shen et al., 2008). In the current results, we further demonstrated the heatstroke-induced hypothalamic neuronal apoptosis and degeneration in anesthetized rat models could be prevented by G-CSF preconditioning in rats. In transient focal ischemia of mice, G-CSF exerted a neuroprotective effect through the direct activation of the anti-apoptotic pathway by up-regulating STAT3 (signal transducer and activation of transcription 3), STAT3 and Bcl-2 (Komine-Kobayashi et al., 2006) and the JAK (Janus kinase)/STAT (Harada et al., 2005). It was also found that the expression of Bcl-2 protein and MAP-2 (microtubule-associated protein-2) protein within brain after ischemia was associated in rats receiving G-CSF treatment compared with control rats (Komine-Kobayashi et al., 2006). Thus, it is likely that G-CSF may mediate the anti-apoptotic pathway in the hypothalamus of the heatstroke animals through the JAK/STAT signaling and subsequent activation of Bcl-2. Of course, the contention requires further verification in future studies.
Our previous (Liu et al., 2009) and present results demonstrated that after the onset of heatstroke, the animals displayed up-regulation of systemic inflammatory response molecules including serum TNF-α, ICAM-1 and E-selectin. The serum levels of TNF-α, E-selectin, and ICAM-1 were also upregulated in patients with heatstroke (Bouchama and Knochel 2002). During inflammation, endothelial cells expressed ICAM-1, which initiated adhesion and transendothelial migration of circulating leukocytes (Menger and Vollmar, 1996). The serum TNF-α and ICAM-1 levels could be considered markers for the systemic inflammatory response because they indirectly reflected the whole body production of TNF-α and ICAM-1 in various organs (Kuzu et al., 2002; Olanders et al., 2002; Wyble et al., 1996). The present results further showed that the increased serum levels of these molecules during heatstroke in a rat model could be significantly reduced by G-CSF treatment. In fact, G-CSF has been used as an anti-inflammatory agent in murine endotoxemia (Lu and Xiao, 2006). G-CSF may have protected against ischemia/reperfusion injury via reducing production of TNF-α or inhibiting inducible nitric oxide synthase activity (Gorgen et al., 1992; Squadrito et al., 1997). The plasma levels of inflammatory cytokines such as TNF-α and interleukin-1β were elevated in humans (Chang, 1993; Hammami et al., 1998) or rodents (Lin et al., 1994, 1997) attendant with heatstroke. The increase in the plasma levels of these proinflammatory cytokines is believed to be associated with heatstroke. Other lines of evidence indicated that interleukin-10 may have had a therapeutic potential in inflammatory diseases. For example, IL-10-knockout mice had an increased likelihood of inflammatory illness (Rennick et al., 1997) and higher mortality rates after experimental sepsis (Berg et al., 1995). Exogenous injection of recombinant IL-10 protected mice from lethal endotoxemia by reducing TNF-α release (Gerard et al., 1993). Neutralization of endogenously produced IL-10 increased proinflammatory cytokines and enhanced mortality in endotoxemic mice (Standiford et al., 1995). In the current study, we showed that G-CSF preconditioning increased the serum levels of IL-10 and decreased the levels of TNF-α, and prolonged the survival time during heatstroke in rats.

Previous studies (Shyu et al., 2004; Six et al., 2003) showed that G-CSF significantly decreased infarct volumes and enhanced survival.

**Fig. 6.** Values of VEGF-positive cells in the hypothalamic section for the normothermic rats (□), vehicle-treated heatstroke rats (■), and G-CSF (100 μg/kg)-treated heatstroke rats (▲). Samples were obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups or the equivalent time period for the normothermic group. †P<0.05, in comparison with (□) group. Photographs showing VEGF staining for the normothermic rats (A), vehicle-treated heatstroke rats (B), and G-CSF-treated heatstroke rats (C).

**Fig. 7.** Values of serum levels of TNF-α, IL-10, and ICAM-1 for the normothermic rats (□), vehicle-treated heatstroke rats (■), and G-CSF (100 μg/kg)-treated heatstroke rats (▲). Samples were obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups or the equivalent time period for the normothermic group. †P<0.05, in comparison with (□) group. •P<0.05, in comparison with (■) group.
rates in stroke rats by mobilizing autologous hemopoietic stem cells (HSCs) from the bone marrow into the peripheral blood (Shyu et al., 2004; Six et al., 2003). Additionally, subcutaneous injection of G-CSF increased the mobilization of circulating CD34+ cells that were seen in the ischemic hemisphere (Yanqing et al., 2006). Indeed, in a rat model, heatstroke-induced hypotension, hepatic and renal dysfunction, hyper-coagulable state, activated inflammation, and cerebral ischemia and injury could be ameliorated by preconditioning with human umbilical

![Image](image1.png)

**Fig. 8.** Values of EPC expression for the normothermic rats (□), vehicle-treated heatstroke rats (●), and G-CSF (100 μg/kg)-treated heatstroke rats (▲). Samples were obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups of the equivalent time period for the normothermic group. *P<0.05, in comparison with (□) group. †P<0.05, in comparison with (●) group.

![Image](image2.png)

**Fig. 9.** Values of liver TUNEL-positive cells for the normothermic rats (□), vehicle-treated heatstroke rats (●), and G-CSF (100 μg/kg)-treated heatstroke rats (▲). Samples were obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups or the equivalent time period for the normothermic group. *P<0.05, in comparison with (□) group. †P<0.05, in comparison with (●) group. Top panels showing the TUNEL staining for a normothermic rat (A), a vehicle-treated heatstroke rat (B), and a G-CSF-treated heatstroke rat (C).
obtained 78 min after the start of heat exposure (43 °C for 68 min) for the heatstroke groups or the equivalent time period for the normothermic group.

Through: (A) Anti-inflammation; (B) Anti-apoptosis; (C) The mobilization of autologous hematopoetic stem cells; (D) Angiogenesis; and (E) Neuronal differentiation.

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