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Psychological stress induces hypoferremia through the IL-6–hepcidin axis in rats

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ABSTRACT

Anemia is a widespread public health problem. The psychological stress decreases serum iron level and inhibits erythropoiesis. However, the molecular mechanisms involved, leading to iron mal-regulation are not well known. We used a communication box paradigm to induce psychological stress and found that serum iron level decreased after 3 d while liver iron storage increased after 7 d. Moreover, psychological stress up-regulated expressions of interleukin-6 (IL-6) and hepcidin, while down-regulating ferroportin expression after 3 d. These changes were blocked by the injection of IL-6 monoclonal antibody. In conclusion, the IL-6–hepcidin axis is up-regulated by psychological stress in rats, resulting in hypoferremia and increase of hepatic iron storage.

It is estimated that two billion people suffer from anemia worldwide [1]. Iron supplement has been adopted as a primary method for preventing and treating iron deficiency anemia. But more and more evidences indicate that there is no correlation between the incidence of anemia and the level of iron uptake [2,3]. Therefore, it is important to recognize its multifactorial etiology for developing effective control strategies as emphasized by WHO. Our previous study demonstrated that after repeated psychological stress exposure, the serum iron level decreases and erythropoiesis gets inhibited while iron uptake is being normal [4]. However, the involved molecular mechanisms how psychological stress leads to iron mal-regulation are not well known.

A series of iron-regulatory proteins, including iron transporters and soluble mediators have been identified the roles of which enhance our understanding of iron metabolism greatly [5,6]. Of these mediators, hepcidin plays a central role in the regulation of iron metabolism. Hepcidin knock-out mice develop iron overload in the liver, pancreas, and heart, whereas mice over-expressing hepcidin have severe iron deficiency anemia [7,8]. Iron overload increases the expression of hepcidin, while iron deficiency decreases that expression in the liver. Hepcidin is also regulated by inflammatory signals [9,10], for example by IL-6. However, the role of hepcidin under psychological stress is not well known. Understanding the molecular mechanisms of psychological stress and iron mal-regulation interaction is of considerable clinical importance, because both mental disorder and anemia are common diseases. In this study, we investigated the molecular mechanisms of iron regulation under psychological stress.

Design and methods

Animals. Sprague–Dawley (SD) rats, male, 10 weeks old, were caged individually at room temperature with 55 ± 5% humidity (Shanghai-BK Co., Ltd.). They were fed with a standard diet (iron content 35 mg/kg, AIN-93M) and unrestricted tap water. After 7 d adaptation, the rats were divided into two groups randomly: the psychological stress group and the control group. Each group was subdivided into three subgroups: 3 d group, 7 d group and 14 d group. After complete psychological stress exposure, all rats were anesthetized by intraperitoneal injection of 15% chloral hydrate, and then were perfused through portal vein with ice-cold phosphate buffered saline (PBS, pH 7.4) to flush out their blood. Prior to perfusion, their blood was collected via the abdominal aorta while their duodenums and pieces from the right part of their livers were rapidly dissected and snap-frozen to be used as samples. For anti-IL-6 antibody experiments, rats were administered with intraperitoneal injection of 2 μg/d normal saline (NS) or anti-rat IL-6 monoclonal antibody (R&D Systems, Inc., USA) daily before psychological stress exposure. All animal studies were in accordance with the institutional animal care guidelines and were approved by the animal research committee of the Second Military Medical University, Shanghai, China.

Psychological stress exposure. The communication box paradigm equipped with a grid floor and stainless steel rods was used as described previously [11]. The box was divided into 20

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compartments (A and B) by transparent plastic sheets. To prevent the electric shock, a plastic plate was placed on each of the grid floors of the compartments A. The rats (foot shock group) in compartments B, with no plastic plate on its floors, received foot shock (0.8 mA) for 10 s at intervals of 50 s through the floor by an electric shock generator. The rats had nociceptive stimulation-evoked responses, such as jumping up, defecation and crying. The rats (psychological stress group) in compartments A did not receive foot shock, but received emotional stimuli from the foot shock rats. Psychological stress was given to rats for 30 min every morning (10:00–10:30). At the end of the exposure, the rats were kept for 4 min in the cages. Animals in the control group, receiving no stress, were kept in the cages only for 4 min.

Iron analysis. Serum and liver iron concentrations were measured with a Varian SpectrAA-220G graphite furnace atomic absorption spectrophotometer, equipped with a GTA 110 atomizer, programmable sample dispenser, and deuterium background correction. Liver samples were digested with concentrated nitric acid and were incubated at 60 °C for 24 h [12]. Standard and control samples were prepared in an identical manner with the experimental samples.

ELISA and Western blot analysis. Liver and duodenum were homogenized and lysed for ELISA and Western blots. IL-6 and IL-10 (R&D Systems, Inc., USA) were analyzed using commercially available ELISA kits. Western blotting was performed with rabbit polyclonal hepcidin-25 antibody (Abcam, Cambridge, MA), and rabbit polyclonal ferroportin 1 antibody (Abcam, Cambridge, MA). Identical samples were blotted with anti-β-actin polyclonal antibody (Sigma, USA) to keep the amount of loading protein equal. Immunoreactive bands were detected by goat polyclonal anti-rabbit-HRP antibody (Santa Cruz, CA).

Statistical analysis. SPSS 10.0 software (SPSS institute, Chicago, IL, USA) was used for statistical analysis. One-way ANOVA, correcting for differences in sample variance, was used to determine whether differences were statistically significant in groups. Data were expressed as $\overline{X} \pm SD$. $P < 0.05$ represents statistically significant difference.

Results

Hypoferremia and liver iron accumulation were induced

After 7 d psychological stress exposure, liver iron level increased (Fig. 1A). While after 3 d exposure, serum iron level decreased (Fig. 1B). No significant difference was detected between liver and serum iron levels in three control groups on 3, 7, 14 d (data not shown). In this study, 7 d control group was adopted as representative control.

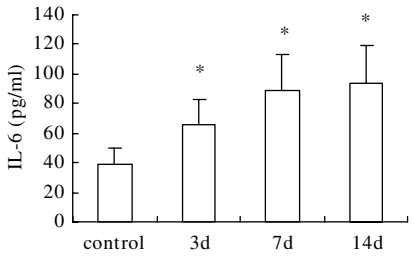

Fig. 2. Serum IL-6 concentrations of control rats and psychological stress rats on 3 d, 7 d, and 14 d. Asterisks indicate significant difference, $P < 0.05$. $\overline{X} \pm SD$ are shown ($n = 6$).

Cytokine and iron metabolism responses

After 3 d psychological stress exposure, serum IL-6 increased (Fig. 2). No significant difference was found in IL-10 levels (data not shown). Western blot analysis showed that up-regulation of hepatic hepcidin and down-regulation of hepatic and duodenal ferroportin after 3 d psychological stress (Fig. 3).

Anti-IL-6 antibody inhibited the effect of psychological stress on iron distribution and metabolism

After 3 d and 7 d psychological stress exposure, liver or serum iron levels did not change significantly after intraperitoneal injection of anti-rat IL-6 antibody compared with that in the control group. However, compared with NS-treated group, the liver iron levels decreased significantly after 7 d psychological stress exposure (Fig. 4A), and serum iron level increased after 3 d and 7 d exposures (Fig. 4B). For those rats under psychological stress, anti-rat IL-6 antibody could down-regulate hepatic hepcidin expression, while up-regulate hepatic and duodenal ferroportin expression. No significant difference was found in hepcidin and ferroportin expression between the control and IL-6 antibody treated group (Fig. 4C).

Discussion

Communication box, developed by Ogawa and Kuwabara, is a common model to investigate the physical and physiological changes under psychological stress [13–15]. Without direct physical stress, the box can produce an experimental anxiety based on intraspecies emotion. In our study, hypothalamus noradrenalin, serum corticosterone, and adrenocorticotropic hormone increased significantly after psychological stress (data not shown), which indicated that the emotional responses to foot shock activated the hypothalamic–pituitary–adrenal (HPA) axis in psychological stress rats.

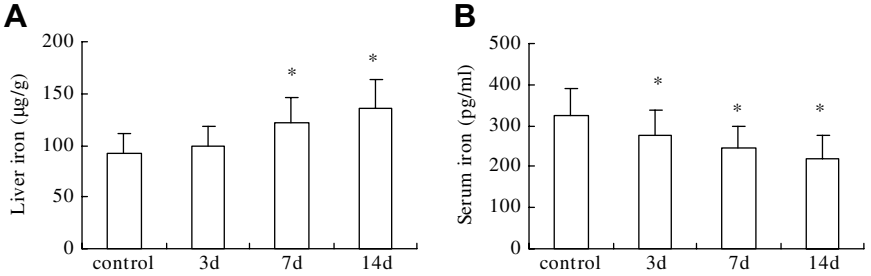

Fig. 1. Iron concentrations in liver (A) and serum (B) in control rats and psychological stress rats on 3 d, 7 d, and 14 d. Asterisks indicate significant difference, $P < 0.05$. $\overline{X} \pm SD$ are shown ($n = 6$).

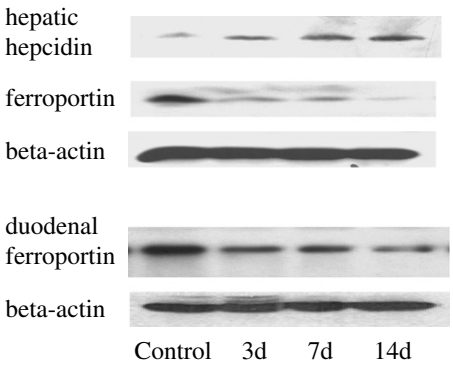

Fig. 3. Western blot of hepatic hepcidin and ferroportin, duodenal ferroportin protein in control and psychological stress rats on 3 d, 7 d, and 14 d. One of six representative experiments is shown.

Overload exercise and spaceflight can lead to the decline of serum iron level [16,17]. In our study, serum iron level was decreased after 3 d repeated psychological stress exposure before the decline of red cell count and hemoglobin (7 d) [4]. However, body iron stores in those rats increased as elevated hepatic iron concentrations, indicating that psychological stress changed iron distribution, limited the transportation and utilization of iron. Liver iron accumulation might be one of the reasons to aggravate hypoferremia and inhibit erythropoiesis. The repeated psychological stress exposure increased the risk of anemia under the situation of normal iron uptake. In this study, a standard diet was given to exclude the factors related to feeding. No significant difference for diet uptake was detected between the control and psychological stress group (data not shown). Only male adolescent rats were chosen to be subjects for our study to exclude the factors related to blood loss due to menstruation.

It is already well known that increased expression of IL-6 can be found as a result of tissue injury, infection, and inflammation [18,19]. In our study, IL-6 increased after repeated psychological stress exposure, which was accordance with the report of Goebel MU' speech task experiments [20]. After psychological stress exposure, IL-6 increased through a mechanism independent of tissue injury or inflammation, while through the activation of the HPA axis and sympathetic nervous system. Moreover, it is to note that the pituitary and adrenal glands are capable of producing IL-6 [21,22]. It is recognized that IL-6 may function as a hormone, to induce production of acute-phase proteins from hepatocytes [23], and to regulate secretion of hormones from the hypothalamus as well as from pituitary and adrenal glands [24,25]. Using Western blot, we observed up-regulation of hepcidin and down-regulation of ferroportin, following psychological stress. Hepcidin is synthesized in the liver as a central regulator of iron metabolism which regulates the absorption of intestinal iron and the release of iron from macrophages [26,27]. After binding to hepcidin, ferroportin is internalized and degraded, leading to decreased export of cellular iron [27]. When iron release from macrophages and absorption of dietary iron from the intestine are inhibited, serum iron concentrations will decrease rapidly and cause hypoferremia.

IL-6 can stimulate hepcidin expression *in vitro* and *in vivo* [9,10]. M.V.V. Falzacappa reported that IL-6 could induce hepcidin expression through STAT3 [28], however, the expression of hepcidin mRNA in macrophages is not induced by IL-6 [29]. Experimental and clinical findings find such a role of IL-6 in various diseases, which provides a rationale for targeted therapeutic investigations using anti-rat IL-6 monoclonal antibody or its receptors [30–32]. To examine whether the IL-6–hepcidin axis was necessary for the development of hypoferremia induced by psychological stress, we performed anti-IL-6 antibody experiments and found that anti-IL-6 antibody inhibited the up-regulation of hepcidin and down-regulation ferroportin, while also inhibiting liver and serum iron level changes. Our study suggests that IL-6 is required for the induction of hepcidin

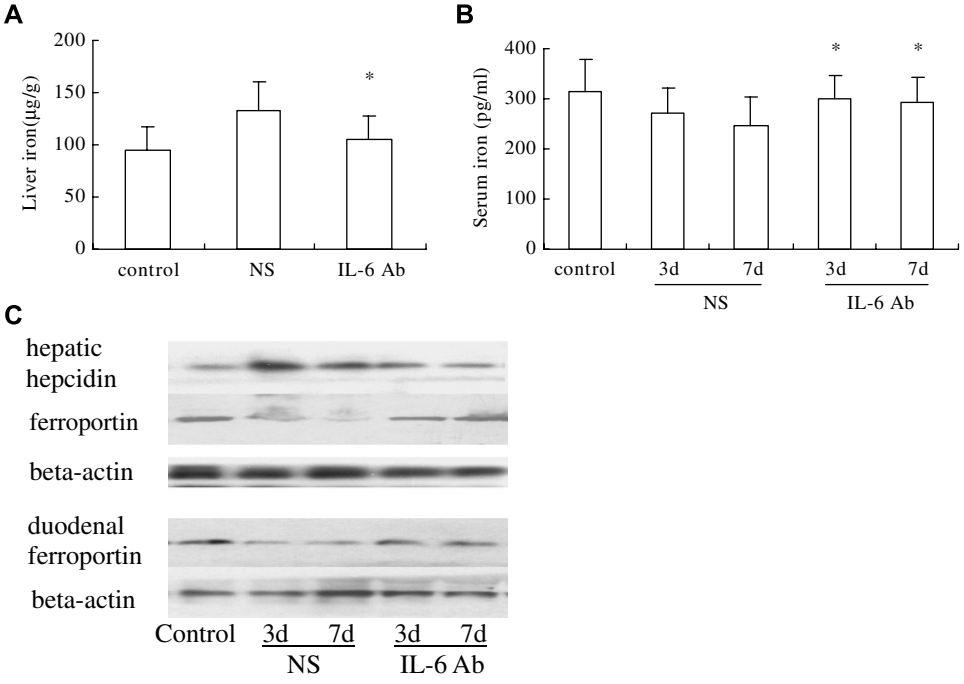

Fig. 4. Serum iron concentrations (A) on 3 d and 7 d in control, NS and anti-IL-6 antibody treated psychological stress rats. Liver iron concentrations (B) on 7 d in control, NS and anti-IL-6 antibody treated psychological stress rats. Asterisks indicate significant differences from NS-treated psychological stress rats ($P < 0.05$) and no significant differences from control rats ($P > 0.05$). $\overline{X} \pm$ SD are shown ($n = 6$). Hepatic and duodenal protein expression (C) of hepcidin and ferroportin in control rats, NS and anti-IL-6 antibody treated psychological stress rats on 3 d and 7 d. One of six representative experiments is shown.

activity leading to hypoferremia, and IL-6–hepcidin axis can mediate hypoferremia and hepatic iron storage. The anemia of the psychological stressed rats is consistent with the anemia because of chronic diseases except for the differences related to serum erythropoietin and ferritin [32,33]. However, in case of thalassemia, very low hepcidin and high serum ferritin levels are found [34]. Psychological stress, anemia of inflammation and thalassemia all lead to the decline of circulating iron and accumulation of iron stores.

In conclusion, the repeated psychological stress exposures change iron distribution in rats, and up-regulate IL-6–hepcidin axis, which is the main pathway for understanding iron metabolism disturbances such as hypoferremia and increased hepatic iron storage, etc.

Acknowledgments

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