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Research Article

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Psychological stress leads to hepatic iron accumulation and disturbs iron homeostasis

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ABSTRACT

The importance of psychological stress is found in the etiology and pathology of more and more diseases. It has been reported that hepcidin is up-regulated by psychological stress, however, TFR2 is a direct regulatory role of the gene on hepcidin expression. Our aim was to evaluate the regulation of TFR2 and the series of molecular mechanisms corresponding to psychological stress. We used a communication box paradigm to induce psychological stress and found that hepatic iron increased as haemosiderin, ferritin and non–transferrin-bound iron induced by quantitative iron analysis and Perl's staining after 7 d. Psychological stress down-regulated serum transferrin saturation and up-regulated hepatic transferrin receptor 2 after 3 d, then down-regulated hepatic transferrin receptor 1 and up-regulated hepatic ferritin mRNA/protein expression after 7 d. Simultaneously, the levels of hepatic non–transferrin-bound iron, malondialdehyde and superoxide dismutase activity all increased after 7 d. The present study suggested that TFR2 should be another regulator of hepcidin and contribute to hepatic iron accumulation.

Key words: Psychological Stress; Iron; Liver; Transferrin Receptor 2

INTRODUCTION

Iron is essential for DNA synthesis and the key metabolic processes in living organisms. The levels of iron in cell must be delicately balanced, as iron loading leads to free radical damage via Fenton reaction. Iron mal-regulation may be induced by infection and inflammation, and the reasons need to be studied [1, 2] further.

Recently, the social life and work is so stressful that people suffer psychological stress frequently, which is considered to be the causes of many disorders, such as hypertension, gastric ulcer, and fever [3-5]. Our previous findings have demonstrated that the repeated psychological stress exposure could decrease the serum iron level and inhibit erythropoiesis, in which hepcidin appears to be a key in iron homeostasis [6, 7]. However, the regulation of hepcidin expression under conditions of psychological stress seems to be a complex process, in which TFR2 may be involved. Because the liver plays a central role in iron metabolism, and the hepatic transferrin receptors tend to be responsible for the systemic physiologic changes. The disturbance of iron metabolism is related with many diseases, such as iron deficiency anemia and chronic hepatitis [8, 9]. So, it is important to investigate the effect of psychological stress on liver to understand the molecular mechanisms under iron metabolism.

EXPERIMENTAL SECTION

(1) Animals

Sprague-Dawley (SD) rats, male, 10 weeks old (Shanghai-BK Co., Ltd), were caged individually at room temperature with 55 ± 5% humidity in a 12-h light/dark cycle (dark cycle was from 06:00 am-18:00 pm). They were fed with a standard diet (iron content 35 mg/kg, AIN-93M) and unrestricted tap deionized water to exclude the factors related to feeding. After 7 d adaptation, the rats were divided into 2 groups randomly: the psychological stress group and the control group. Each group was subdivided into 3 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. Before perfusion, their blood was collected via the abdominal aorta while pieces from the right part of their livers were rapidly dissected and snap-frozen to be used as samples. 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.

(2) Psychological Stress Exposure

The communication box paradigm equipped with a grid floor and stainless steel rods was used as described previously [10]. The box was divided into 20 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. 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 individually in the cages only for 4 min.

(3) Liver iron and Serum Analysis

Samples were fixed in aqueous formaldehyde solution (buffered 4% vol/vol) and were embedded in paraffin. The liver sections were stained with Perls' stain. Liver iron concentration was 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. Standard and control samples were prepared in an identical manner with the experimental samples. Serum transferrin saturation was determined using the hematological analyzer (Beckman LX20, USA).

(4) ELISA and Western Blot Analysis

Liver was homogenized and lysed for ELISA and western blots. Transferrin receptor 1 (TFR1) and ferritin (R&D Systems Inc, USA) were analyzed using commercially available ELISA kits. Western blotting was performed with rabbit polyclonal anti-mouse transferrin receptor 2 (TFR2) (Santa Cruz, Inc.). Identical samples were blotted with anti-β-actin (Sigma) polyclonal antibody to keep the amount of loading protein equal. Immunoreactive bands were detected by goat polyclonal anti-rabbit-HRP antibodies (Santa Cruz, Inc.).

(5) RNA Extraction and Analysis

The total RNA was extracted from tissue samples using Trizol reagent as per the manufacturer's instructions. All the RNA samples were treated with RNAse free DNAse I (IQ5 Real-Time PCR Detection System) and stored at -70 °C before using. The quality and quantity of RNA were assessed by using a spectrophotometer (Model 6300 Spectrophotometer, Jenway) before it was used. Subsequently, all the samples were diluted using nuclease free water at a concentration of 10 ng/ μ l. Two step RT-PCR method was performed using Real Time PCR Master Mix (TOYOBO Biotech Co., Ltd.). The Q-RT-PCR data were analyzed by $2^{-\triangle\triangle CT}$ method as described [11]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization.

(6) Measurement of Non–transferrin-bound Iron (NTBI) and Malondialdehyde (MDA) Concentrations, Superoxide Dismutase (SOD) Activity,

For NTBI determination, the liver tissue homogenates were analyzed using bathophenanthroline disulfonate (BPS) to chelate ferrous iron, which formed a complex that could be analyzed with spectrophotometry [12]. Commercially available BPS (4, 7-diphenyl-1, 10-phenanthroline disulfonate) and ferrous ammonium sulfate [$(NH4)_2$ Fe $(SO4)_2$] of the highest purity (Sigma) were used in the measurement. The thiobarbituric acid method was used to measure the liver MDA level with MDA-586 kit (Nanjing Jiancheng Bioengineering Institute). SOD activity was measured with kit-WST (Dojindo Laboratories).

(7) Statistical Analysis

SPSS 10.0 software (SPSS institute, Chicago, IL, USA) was used for statistical analysis. One-way ANOVA and the correcting for differences in sample variance were used to determining whether differences were statistically significant in groups. The relevant data may be expressed as $\overline{X}$ ± SD. $P < 0.05$ to represent statistically significant difference.

RESULTS

(1) Psychological Stress Led to Hepatic Iron Deposition

Hepatic iron deposition as haemosiderin was observed by Perl's staining in 7 d and 14 d psychological stress groups, which was conducted principally in hepatocytes, endotheliocyte and macrophages (Fig. 1A-C).

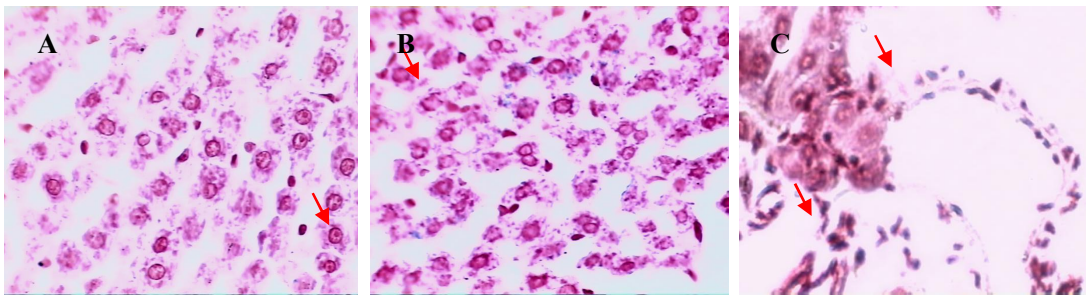

Fig. 1. Perl's staining of rat liver in control and psychological stress groups

Perl's staining of rat liver in 7d control group (A), 7d psychological stress group (B) and 14d psychological stress group (C). Arrows denote areas of Perl Prussian Blue staining (×40). Panels B and C show representaive iron deposition in hepatocytes, endotheliocyte and macrophages.

A significant increase of iron concentration was found under the circumstance of 7 d and 14 d psychological stress exposure, as shown by the quantitative analysis (Fig. 2A, cited reference 7). While serum transferrin saturation (Fig. 2B) fell after 3 d psychological stress exposure.

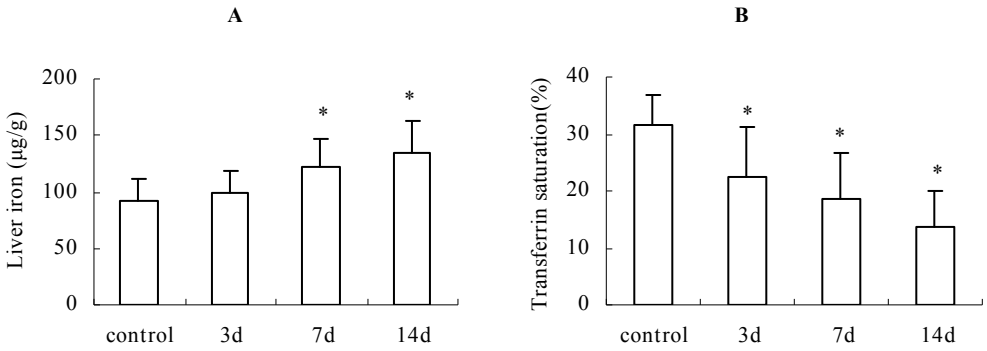

Fig. 2. Liver iron concentration and serum transferrin saturation in control and psychological stress rats

Liver iron concentration (A) and serum transferrin saturation (B) in control and psychological stress rats on 3 d, 7 d, 14 d. Asterisks indicate significant difference, $p<0.05$. $\overline{X}$ *± SD are shown (n = 6).*

No significant difference for diet uptake was detected between the control group and psychological stress group (data not shown). No significant difference was detected among liver iron levels in 3 control groups on 3, 7, 14 d (data not shown). In this study, 7 d control group was adopted as representative control.

(2) Psychological Stress Exposure Caused Changes in Ferritin, TfR1 and TfR2 mRNA/Protein

The psychological stress exposure up-regulated hepatic ferritin (Shown as Fig. 3A and Fig. 4A) but down-regulated TFR1 (Shown as Fig. 3B and Fig. 4B) expression on mRNA/protein level after 7 d, and TFR2 mRNA/protein expression (Shown as Fig. 3C and Fig. 5) was up-regulated by psychological stress exposure in liver as early as 3 days in rats.

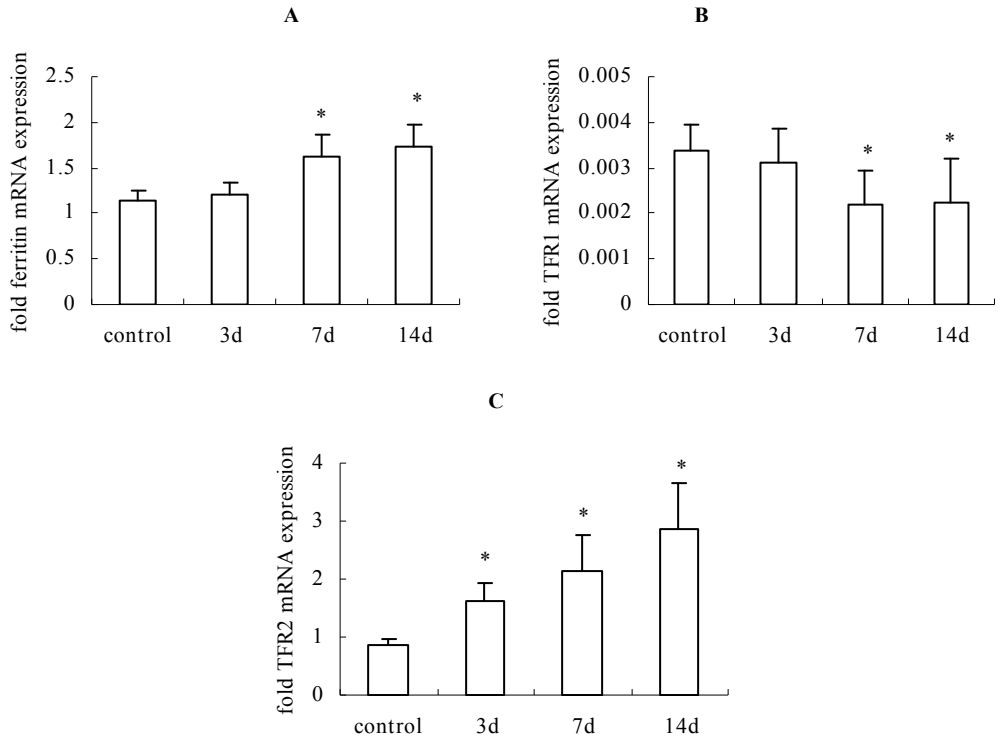

Fig. 3. Hepatic fold ferritin, TFR1 and TFR2 mRNA expression in control and psychological stress rats

Hepatic fold ferritin (A), TFR1 (B) and TFR2 (C) mRNA expression in control and psychological stress rats on 3 d, 7 d, 14 d. Asterisks indicate significant difference, $p<0.05$. $\overline{X}$ ± SD are shown (n = 6).

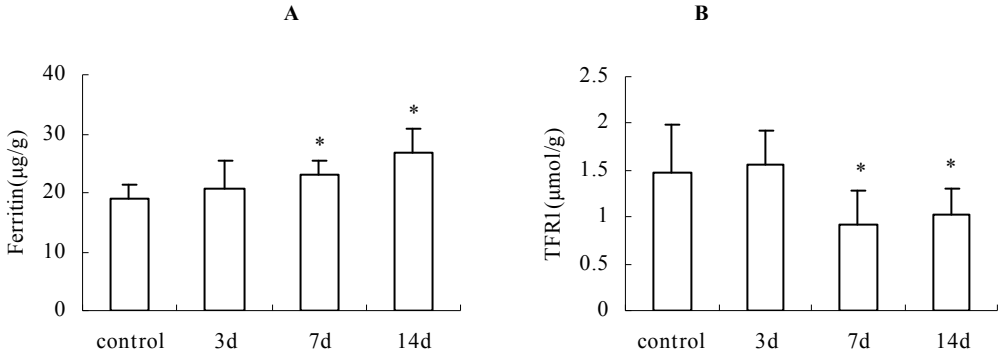

Fig. 4. Hepatic ferritin and TFR1 concentrations in control and psychological stress rats

Hepatic ferritin (A) and TFR1 (B) concentrations in control and psychological stress rats on 3 d, 7 d, 14 d. Asterisks indicate significant difference, $p<0.05$. $\overline{X}$ ± SD are shown (n = 6).

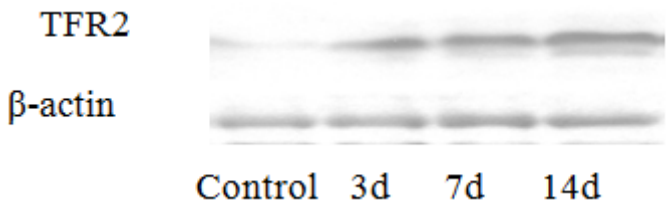

Fig. 5. Western blot of hepatic TFR2 in control and psychological stress rats

Western blot of hepatic TFR2 in control and psychological stress rats on 3 d, 7 d, 14 d. One of 6 representative experiments is shown.

(3) Psychological Stress Exposure Intensified the Oxidative Reaction

After 7 d psychological stress exposure, hepatic NTBI level was enhanced obviously (Shown as Fig. 6A), simultaneously, hepatic MDA level and SOD activity were increased (Shown as Fig. 6B and Fig. 6C).

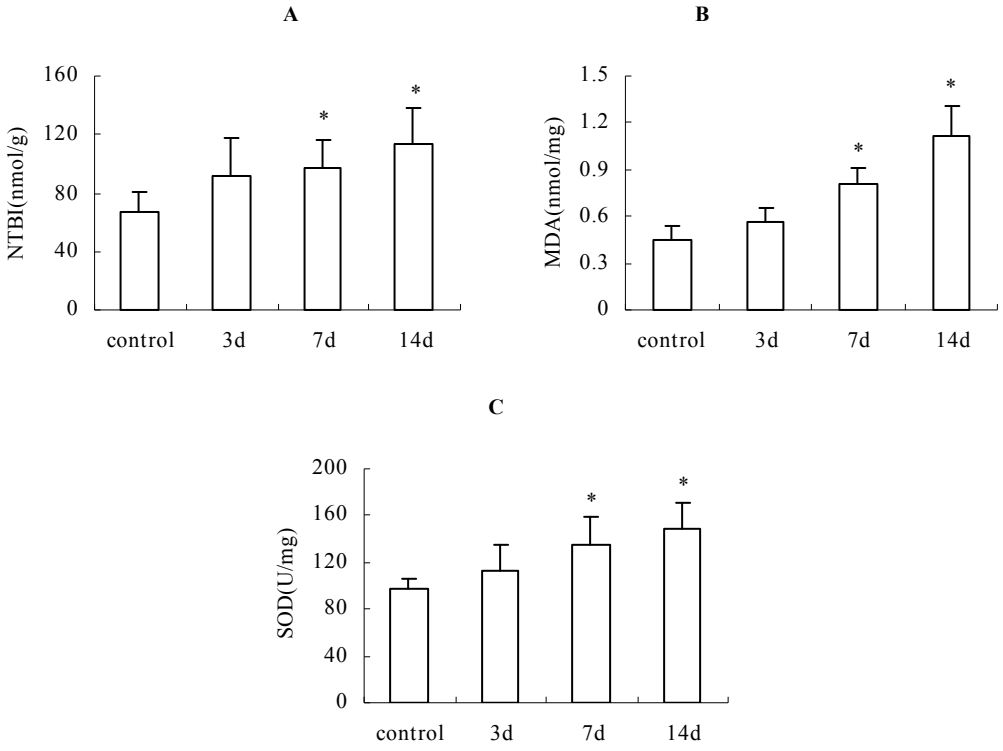

Fig. 6. Hepatic NTBI and MDA concentrations, SOD activity of control and psychological stress rats

Hepatic NTBI (A) and MDA (B) concentrations, SOD activity (C) of control and psychological stress rats on 3 d, 7 d, 14 d. Asterisks indicate significant difference, $p<0.05$. $\overline{X}$ ± SD are shown (n = 6).

DISCUSSION

Communication box model had been used for a common method to study the small animal under psychological stress, because it can produce an experimental anxiety based on intraspecies emotional communication without the direct physical stress interference. In our study, the psychological stress was induced by exposure to emotional responses from foot shocked rats [8, 10]. Hypothalamus noradrenalin, serum corticosterone and adrenocorticotropic hormone increased significantly after psychological stress [7], which indicated that the emotional responses to foot shock activated the hypothalamic-pituitary-adrenal (HPA) axis in psychological stress rats. In some other reports, the psychological stress exposures could cause behavioral and physiological changes in human and animals [3-5, 10, 13, 14].

In our study, the increased iron concentration was mainly related to the iron uptake of hepatocytes according to hepatic iron distribution. Psychological stress changed iron distribution and transportation and limited the iron acquisition from diet and utilization in blood. Serum iron level [7], transferrin saturation, hepatic and duodenal ferroportin [7] were decreased, and hepatic hepcidin [7] as well as TFR2 were up-regulated following 3 d repeated psychological stress exposure before the increase of liver iron content [7], NTBI and ferritin. Furthermore, the decline of hepatic TFR1, red cell count, hemoglobin and serum iron ferritin [6] (7 d), indicated that liver iron accumulation might be one of the reasons for aggravating hypoferremia in addition to the fall of iron absorption. The repeated psychological stress exposure increased the risk of anemia under the situation of normal iron food intake.

TFR2, as a regulator of the hepcidin production, might be a sensor of transferrin saturation in vitro [15], while hepcidin expression preceded the decline in transferrin saturation [16]. Psychological stress exposure changed TFR2, hepcidin, and transferrin saturation as early as 3 days in rats. In liver, iron is stored partly as ferritin, and the transfusion of iron in liver cellular membrane was related to transferrin receptor. The TFR1with iron responsive element (IRE) was down-regulated by excess iron [17]; however the TFR2without IRE was up-regulated through another mechanism [18-20]. The major site for iron storage is liver, and transferrin bound iron is absorbed into the cells by receptor-mediated endocytosis via the classical TfR1 (presumably mainly via homologous TfR2 [18]). TfR2 mRNA is highly expressed in the liver. Andin erythroid precursors as well as in erythroleukaemic cells [21], TfR2 may mediate the uptake of transferrin-bound iron although its affinity for transferrin is approximately 30-fold

less in comparison with TfR1 [22]. In our study, the up-regulation of hepatic TFR2 (3 d) was prior to the decline of TFR1 (7 d), so we suggested that TFR2 might be responsible for liver iron accumulation. In consistent with the observation of a direct correlation between hepcidin and TFR2 expression in the liver [23], the role of TFR2 is as a regulator of hepcidin production and required for hepcidin to respond appropriately to changes in body iron levels [20], which suggests that TFR2 has dual roles in iron metabolism: one is signaling, the other is iron transportmediating uptake of transferrin-bound iron and NTBI [24]. Thus the increase of hepatic hepcidin, ferritin and NTBI might be related to the increase of TFR2 in psychological stress rats. In fact, TFR2 is a modulatory role on hepcidin expression more than a direct contribution to cellular iron uptake [23].

Iron is potentially toxic owing to its catalytic role in the formation of extremely reactive hydroxyl radicals via Fenton chemistry. The mechanisms for iron uptake into hepatocytes as transferrin-bound iron and NTBI have been well characterized [25，26]. We found that hepatic NTBI level increased as hepatic iron deposition. It is reported that NTBI could enhance the symptoms of oxidative stress, support bacterial growth, or increase incidence of infectious diseases. Compared with ferritin and transferrin-bound iron, NTBI is more readily available for catalyzing free radical formation and causing cellular damage through various mechanisms [27-30]. After repeated psychological stress, hepatic MDA level and SOD activity were increased, and obvious lipid peroxidation was found. As an endogenous free radical scavenger, SOD represents a first line of defense against toxic reactants by metabolizing them to innocuous byproducts.

Due to its direct importance in bacterial growth, excess iron plays a crucial role in impairment of the host immune system [31-33]. In response to psychological stress, rats developed its own mechanisms of withholding iron to reduce iron related damage, including the reduced serum iron level, the increased hepatic ferritin and the haemosiderin accumulation. The increased hepatic iron might be protective for instant, but definitely had a bad effect on physical requirements of body for long, especially under the situation of decreased serum iron level and the inhibition of erythropoiesis.

In conclusion, TFR2 could be another regulator of hepcidin, which binds to ferroportin, accordingly inhibites the export of cellular iron and iron absorbed from dietary, eventually leads to hypoferremia. We speculate that TFR2 contributes to hypoferremia and hepatic iron accumulation as haemosiderin, ferritin and NTBI, which increases products of lipid peroxidation. Hepatic iron accumulation in psychological stress rats was not primary and seemed to be secondary to the TFR2 and/or IL-6-hepcidin axis. These interactions play an important role in the pathogenesis of several iron disorders, including anemia of inflammation, thalassemia and hemochromatosis. However, for the mechanism, how the psychological stress induces iron mal-regulation, which still needs to be investigated further.

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