Restraint stress up-regulates expression of zinc transporter Zip14 mRNA in mouse liver

Takuya Ohashi · Tohru Matsui · Moeko Chujo ·
Masaya Nagao

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Abstract The zinc concentration in the liver was significantly higher in mice at 12 h after the onset of restraint stress than that without stress. The IL-6 protein level in the serum was transiently elevated at 3 h after the onset of restraint stress, and the IL-6-responsive zinc transporter Zip14 mRNA in the liver was expressed markedly at 6 h. These results suggest that Zip14 plays a major role in the mechanism responsible for accumulation of zinc in the liver under restraint stress.

Keywords Liver · Restraint stress · Serum · Essential trace metal · Zinc · Zinc transporter

Abbreviations

IL-6 Interleukin-6
GAPDH Glyceraldehyde-3-phosphate dehydrogenase

Introduction

Zinc is a trace nutrient indispensable for organisms (Stefanidou et al. 2006). Zinc homeostasis is mostly regulated by zinc transporters (Eide 2006). Known eukaryotic zinc transporters are largely classified into two families, the ZRT/IRT-like Protein (ZIP) and Cation Diffusion Facilitator (CDF) families, which are designated as “SLC39”, and “SLC30”, respectively (Cousins et al. 2006; Kambe et al. 2004). ZIP members facilitate zinc influx into the cytosol, while CDF members facilitate its efflux from the cytosol. Abundance of Zip14, one of the ZIP family transporters, in the plasma membrane of hepatocytes is induced after turpentine-induced inflammation and lipopolysaccharide administration (Liuzzi et al. 2005). IL-6 is considered to be the main proinflammatory cytokine regulating acute-phase genes and Zip14 expression is up-regulated through IL-6. Transfection of Zip14 cDNA into human embryonic kidney cells increased zinc uptake, suggesting that Zip14 functions as a zinc importer. Zip14 has been suggested to play a major role in the mechanism responsible for hypozincemia that accompanies the acute-phase response to inflammation and infection (Liuzzi et al. 2005).

Restraint stress elevated the zinc and metallothionein protein levels in the liver (Hidalgo et al. 1986), but the mechanism of zinc accumulation in the liver under restraint stress has not been elucidated. Nukina et al. reported that restraint stress elevated plasma

T. Ohashi · M. Chujo · M. Nagao (✉)
Laboratory of Biosignals and Response, Division of
Integrated Life Science, Graduate School of Biostudies,
Kyoto University, Kyoto 606-8502, Japan
e-mail: mnagao@kais.kyoto-u.ac.jp

T. Matsui
Laboratory of Nutritional Science, Division of Applied
Biosciences, Graduate School of Agriculture,
Kyoto University, Kyoto 606-8502, Japan

IL-6 levels both in germ-free and SPF mice, suggesting that intestinal microflora were not the cause of the stress-induced IL-6 elevation (Nukina et al. 2001).

In this study, we examined whether or not Zip14, whose mRNA expression is induced by IL-6, is involved in the movement of zinc to the liver under restraint stress.

Materials and methods

Animals

Animals were maintained and handled in accordance with the guidelines for the care and use of laboratory animals at Kyoto University. Eight-week-old ICR strain male mice (Clea) were used. The animals were exposed to the restraint stress in a 50 mL well-ventilated polypropylene centrifuge tube. Soon after the stress regimen, all mice were exsanguinated under anesthesia with pentobarbital to prevent hard stress and tissues were immediately isolated. During the stress regimen, the mice did not have access to food and water.

RT-PCR

Total RNA was extracted using commercially available solution (Sepasol-RNA I, Nacalai Tesque), and 1 µg of the RNA was reverse-transcribed followed by PCR using a PrimeScript RT-PCR kit (Takara), according to the manufacturer's instructions. PCR was performed under the following conditions. Initial denaturation at 94 °C for 10 s followed by various cycles of 94 °C for 5 s, and 60 °C for 30 s with specific primers as described in Table 1.

Quantification of IL-6 in the serum

Serum IL-6 was quantified by a commercially available ELISA kit (Endogen Mouse IL-6 Chemiluminescent ELISA kit, Pierce Biotechnology Inc.) according to the manufacturer's instruction.

Measurement of zinc levels

Zn concentrations in the serum and the liver were measured with an atomic absorption spectrophotometer (AA-6600F, Shimadzu) after digestion with concentrated nitric acid and 30% hydrogen peroxide. All data were analyzed using GLM procedures of SAS (SAS Institute Inc.).

Statistical analyses

The effect of restraint stress on serum IL-6 level and zinc concentration in the liver and the serum were statistically analyzed. Data except for basal value were submitted to a two-way ANOVA where treatment, time and their interactions served as main effects. Then differences between means were compared using the LSD test.

Results

Transient increase of IL-6 in the serum by restraint stress

Serum IL-6 was elevated after 1 h of restraint stress and peaked at 3 h, while that in the non-stressed group did not change (Fig. 1).

Table 1 Primers and PCR condition

Gene	Primers	PCR cycle	Product size (bp)
Zip14	Sense	5'-CAA GGC ATC AGC ACG TCA G-3'	148
	Antisense	5'-CCA GAC CCA AAT AGC AGC AG-3'	
Zip6	Sense	5'-ACC ACC ACC ACC ACC AGA AC-3'	158
	Antisense	5'-GAA GGC AGC ACC AAT AGC AAG-3'	
GAPDH	Sense	5'-GCC TCC CGT GTT CCT ACC-3'	100
	Antisense	5'-CCT GCT TCA CCA CCT TCT T-3'

Fig. 1 Effect of restraint stress on the serum level IL-6 level. IL-6 level under restraint stress. (●—●), stressed; (○—○), control. The results are shown as the means $\pm$ standard error of the mean ($n = 4$). Effect of treatment, $P < 0.001$; effect of time, not significant; interaction, not significant. Means with different superscript letters are significantly different at $P < 0.05$

Fig. 2 Kinetics of the Zip14 mRNA levels in the liver under restraint stress estimated by semi-quantitative RT-PCR. Total RNA was prepared from the liver at the indicated time points. The mice not exposed to stress served as the control group. (a) Zip14, (b) GAPDH, as a control. The results shown are representative of four such experiments

Transient expression of hepatic Zip14 mRNA under restraint stress

Time-dependent expression of Zip14 mRNA in the liver under restraint stress was examined by semi-quantitative RT-PCR. Zip14 mRNA was expressed markedly at 6 h after the onset of stress (Fig. 2), while no significant change in Zip14 mRNA expression was detected in the non-stressed group. Expression of another zinc transporter Zip6, an ortholog of zebrafish LIV1, was examined, since LIV1 was reported to be regulated by STAT3, which is a down stream signal transducer of IL-6 (Yamashita et al. 2004). However, no significant change in the expression level of Zip6 mRNA was observed under restraint stress (Fig. 3).

Fig. 3 Kinetics of the Zip6 mRNA levels in the liver under restraint stress estimated by semi-quantitative RT-PCR. Total RNA was prepared from the liver at the indicated time points. The mice not exposed to stress served as the control group. (a) Zip6, (b) GAPDH, as a control. The results shown are representative of four such experiments

Change in the zinc concentrations in the liver and serum

The restraint stress significantly increased the zinc concentration in the liver at 12 h after the onset of stress ($P < 0.05$) (Fig. 4a). The zinc concentration in the serum slightly increased at 1 h ($P < 0.05$), slightly decreased at 6 h ($P < 0.05$) and again increased at 12 h ($P < 0.05$) after the onset of stress, but no significant change was observed in the control group during the experiment (Fig. 4b). Therefore, the zinc concentration was higher ($P < 0.05$) in the stressed group than in the control group at the end of the experiment (Fig. 4b).

Discussion

Zinc is an important trace element in organisms and it is essential as a structural or catalytic constituent of many proteins and enzymes (Stefanidou et al. 2006). Zinc is also an important element in preventing free radical formation and thus in protecting bodies from oxidative damage and in maintaining the immune functions.

In this study, we observed a transient expression of Zip14 mRNA in the liver under restraint stress (Fig. 2). Restraint stress caused transient elevation of serum IL-6 around 3 h (Fig. 1) as reported in the rat (Ando et al. 1998; Takaki et al. 1994). Nukina et al. showed that the plasma IL-6 level peaked at 1 h after the onset of restraint stress in the mouse but they did not measure the value at 3 h (Nukina et al. 2001). After this serum IL-6 elevation, mRNA of Zip14, an IL-6 responsive zinc transporter which facilitates

Fig. 4 Effect of restraint stress on the Zn level (zinc $\mu\text{g dry weight g}^{-1}$, or zinc $\mu\text{g mL}^{-1}$) in the liver (a), and the serum (b). (●—●), stressed; (○—○), control. The results are shown as the means $\pm$ standard error of the mean ($n = 4$). Zn in the liver; effect of treatment, $P < 0.05$; effect of time, $P < 0.05$;

interaction, $P < 0.05$, Zn in serum; effect of treatment, not significant, effect of time, $P < 0.05$; Interaction, $P < 0.05$. Means with different superscript letters are significantly different at $P < 0.05$

extracellular zinc into cytosol (Liuzzi et al. 2005), was induced about 12-fold at 6 h after the onset of restraint stress (Fig. 2a), followed by the increase in the zinc concentration in the liver (Fig. 4a). Expression of another zinc transporter, Zip6, an ortholog of zebrafish LIV1, was examined since STAT3, a downstream signal transducer of IL-6, was reported to induce LIV1 (Yamashita et al. 2004). However, no significant change in Zip6 mRNA expression was observed under restraint stress (Fig. 3). These results suggest that Zip14 plays a major role in the mechanism responsible for accumulation of zinc in the liver by the restraint stress. By the use of IL-6 knockout mice, the relevance of IL-6 and Zip14 to the zinc accumulation in the liver under restraint stress may be clarified. To our knowledge, this is the first report, showing evidence of the involvement of the zinc transporter in zinc movement under restraint stress in the liver.

Metallothioneins, zinc-binding proteins that act to detoxify heavy metals, act as a free radical scavenger protecting cells from reactive oxygen species (Hidalgo et al. 1988). Induction of metallothioneins in the liver by restraint stress is also mediated by IL-6 (Hernandez et al. 2000). Elevation of zinc concentration and induction of metallothionein in the liver under restraint stress may play a key role in protecting the liver from stress-inducible injury.

Recently, zinc is considered to be not only a nutrient but also a signal molecule; movement of zinc across cells triggers several biological responses.

Zinc movement through zinc transporter LIV1 plays a crucial role in zebrafish gastrula organizer cells (Yamashita et al. 2004). In dendritic cells, zinc movement caused by down-regulation of zinc importer Zip6 and up-regulation of zinc exporter ZnT-1 was involved in the maturation of dendritic cells (Kitamura et al. 2006). Zinc movement regulated by the expression of zinc transporter may have crucial roles in performing the biological function of various cells.

Approximately 20 μg zinc was accumulated in the liver during the last 6 h of stress. On the other hand, the changes in the serum zinc concentration cannot explain the accumulation of zinc in the liver because restraint stress increased the serum zinc concentration during this period and the serum zinc concentration was higher in the stressed group than in the control group at the end of the experiment (Fig. 4b). Further study is necessary to clarify the movement of zinc in the whole body during the restraint stress including the source causing the elevation of zinc in the liver.

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