Original Article

Influence of Iron Supplementation on DMT1 (IRE)-induced Transport of Lead by Brain Barrier Systems *in vivo*[*]

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Abstract

Objective To investigate the potential involvement of DMT1 (IRE) protein in the brain vascular system *in vivo* during Pb exposure.

Methods Three groups of male Sprague-Dawley rats were exposed to Pb in drinking water, among which two groups were concurrently administered by oral gavage once every other day as the low and high Fe treatment group, respectively, for 6 weeks. At the same time, the group only supplied with high Fe was also set as a reference. The animals were decapitated, then brain capillary-rich fraction was isolate from cerebral cortex. Western blot method was used to identify protein expression, and RT-PCR to detect the change of the mRNA.

Results Pb exposure significantly increased Pb concentrations in cerebral cortex. Low Fe dose significantly reduced the cortex Pb levels, However, high Fe dose increased the cortex Pb levels. Interestingly, changes of DMT1 (IRE) protein in brain capillary-rich fraction were highly related to the Pb level, but those of DMT1 (IRE) mRNA were not significantly different. Moreover, the consistent changes in the levels of p-ERK1/2 or IRP1 with the changes in the levels of DMT1 (IRE).

Conclusion These results suggest that Pb is transported into the brain through DMT1 (IRE), and the ERK MAPK pathway is involved in DMT1 (IRE)-mediated transport regulation in brain vascular system *in vivo*.

Key words: Lead; Iron; Blood-brain barrier; Divalent metal transporter 1; MAPK pathway

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INTRODUCTION

Environmental contamination with lead (Pb) from past activities remains a big problem because Pb binds tightly to certain soil types and does not go away with time. Absorption of Pb by the gastrointestinal tract is much greater in children than in adults; hence, children are more susceptible to Pb poisoning than adults. Children who are deficient in certain nutrients, such as iron (Fe), also absorb a significant percentage of an ingested Pb dose[1-4]. Previous epidemiological studies have shown that Fe deficiency and Pb poisoning are common among infants and children

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in many parts of the world, and these two problems are often associated[5-7]. Both conditions cause anemia and produce a more severe form of anemia when in combination[8-10]. Although the nature of their relationship has not been completely elucidated, characterization of a common Fe-Pb transporter and epidemiological studies among children strongly suggest that Fe deficiency may increase susceptibility to Pb poisoning. Certain human studies further suggest that high Fe intake and sufficient stored Fe may reduce the risk of Pb poisoning.

Consistent with its neurotoxic effects, Pb traverses the blood-brain barrier (BBB), accumulates in the brain, and preferentially damages the prefrontal cerebral cortex, hippocampus, and cerebellum. At high Pb exposures (blood Pb concentrations greater than 80 μg/dL), children exhibit encephalopathy symptoms and may die when exposure is not quickly reduced. If they do recover, these children may have permanent neurological damage. Recovery is accompanied by epilepsy, mental retardation, optic neuropathy, and blindness. Hence, assessing the kinetics of Pb into the brain and the effect of Fe supplementation in the prevention of Pb poisoning is necessary for future trials.

BBB is a restrictive, micro-anatomic vascular structure that is less permeable than the peripheral vasculature and tightly regulates the brain's milieu. This structure results from the unique tight junctions inherent to the BBB endothelium[11-12]. The narrow tight junctions highly reduce the transport of molecules between cells (paracellular transport). The permeability of the barrier decreases post-natally, and the barrier tightness is positively correlated with fetal brain growth[13]. The tightening of BBB reflects both a unique cytoskeletal architecture and tight junction particle density, which reaches a mature state in adults[14-15]. Very few pinocytosis vesicles have been observed, leading to a rare transcellular transport. However, these vesicles can be amplified in pathological conditions. BBB has long been known to be a target of Pb toxicity. Under chronic or acute exposure to high Pb levels, Pb-induced microvascular damage is prevalent with leaky microvessels in young animals. Pb encephalopathy probably results from the death of endothelial buds[16]. Pb may also interfere with cellular phosphorylation by acting on the protein kinase system. Pb potently activates protein kinase C (PKC) in both endothelial cells of BBB and epithelial cells of BCB[17-18].

Various substrates compete for the same transporters. Competition, especially direct competition at particular transporters, is common among metal ions. Evidence from studies on nutrition and metal transport suggests that Fe transporters may also mediate Pb uptake. The most studied candidates are the divalent metal transporter 1 (DMT1; NRAMP2, DCT1, or SLC11A2). Several studies have reported that DMT1 functions as a transporter for various metals including Fe and Pb[19-21]. DMT1 has two splice variants, which differ in the 3'-end of the message[19]. One form contains an iron responsive element (+IRE) in the 3'-untranslated region of the message that is capable of binding iron response proteins (IRPs), resulting in the stabilization of the message. Accordingly, this form of DMT1 may be similar to the transferrin receptor (TfR), which can potentially be regulated by Fe. The second mRNA form lacks IRE (-IRE) and is presumably incapable of being regulated by Fe, at least by an IRE/IRP interaction. Further studies have demonstrated that each form can be transcribed from two alternative promoters, presumably the upstream of either exon 1A or exon 1B, resulting in four predicted DMT1 mRNA isoforms that differ in both their 5'- and 3'-ends[22]. Studies have found that DMT1 is present in endothelial cells[23].

In light of the determination of the exact protein involved in Pb transport, the present study was undertaken to further characterize the transporter responsible for Pb transport in BBB. The function of ERK MAPK activation in enhancing the stability of the DMT1 (IRE) mRNA was also explored because other studies have shown the MAPK activation increases phosphorylation of IRP and binding of IRP to IRE. The data suggest that DMT1 (IRE) mediates Pb transport and regulated by the ERK MAPK pathway in BBB.

METHODS

Reagents

Unless otherwise stated, all chemicals were analytic grade or higher purity. SDS, acrylamide, bisacrylamide, and agarose were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Anti-phospho-ERK1/2 and anti-total-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the anti-DMT1 (IRE) was from ADI (San Antonio, TX, USA). Secondary peroxidase-labeled antibodies were purchased from Sigma-Aldrich for the anti-rabbit antibody and

anti-mouse antibody. Iron standard (1 mg iron/mL) and molecular weight standards were obtained from Alpha Products (Danvers, MA) and Pharmacia Biotech, respectively.

Animal Model and Experimental Protocol

Male Sprague-Dawley rats (littermates, the Laboratory Animal Center, Academy of Military Medical Sciences, Beijing , China), aged 20-22 d and weighing 30-50 g upon arrival, were assigned to five groups (n=12 in each group) such that the group mean body weights were comparable. The animals were housed in stainless-steel cages in a temperature-controlled, 12/12 light/dark room and allowed free access to pelleted semi-purified rat chow (solid, Vital Keao Feed Co., Beijing, China) and pre-prepared drinking water. On the third day (age of 22-24 d) after arrival, the animals were provided with Pb in their drinking water and/or Fe by oral gavage. The drinking water was prepared by dissolving Pb acetate in distilled, deionized water (342 μg Pb/mL). Three groups of rats took the drinking water with 342 μg Pb/mL as Pb acetate ad libitum. Among which, two of the groups were concurrently given 7 and 14 mg Fe/kg as $FeSO_4$ solution for six weeks and designated as the low and high Fe groups, respectively. The Fe doses were administered orally by gavage once every other day according to the body weight of the animals. For the control group, Na acetate, with an acetate concentration equivalent to the high dose of Pb acetate, was prepared in the same manner. At the same time, the group only supplied with 14 mg Fe/kg was also set as a reference. All procedures that involved animal studies were in accordance with the guidelines and approval of the local Animal Care and Use Committee. The animals were decapitated at the end of the study. Cerebral cortexes were quickly dissected and stored at -80 °C. Pb concentrations were determined by EAAS.

Preparation of Brain Capillary-rich Fraction

Brain capillary-rich fraction was prepared according to a reported method[24]. In brief, the brain was quickly isolated from the cranium after decapitation, and the cerebral cortex was removed from the excised brain. The tissue was then weighed and homogenized with a glass/Teflon Potter homogenizer (13 strokes at 1000 rpm) in a five-fold volume of 122 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 3 mmol/L KCl, 0.4 mmol/L K_2HPO_4, 1.4 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 10 mmol/L D-glucose, and 10 mmol/L HEPES (pH 7.4). Dextran solution was added to the homogenate (final concentration of dextran was 16%), and the mixture was then centrifuged at 4500 rpm for 20 min at 4 °C in an Avanti 30 Compact Centrifuge with rotor F0630 (Beckman Instruments Inc., CA, USA). The pellet was used as the brain capillary-rich fraction for western blot and RT-PCR analyses. Pb concentrations were determined by Z2000 atomic absorption spectrometer (Hitachi Ltd., Japan).

Western Blot Analysis

Brain capillary-rich fractions were homogenized in RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% SDS, 1% NP-40, 1% deoxycholate, 1% Triton X-100, 10 mmol/L PMSF) with protease inhibitor cocktail (Roche, Switzerland). The supernatant was collected after centrifugation at 12,000 rpm for 15 min at 4 °C. Afterward, the protein concentration in the supernatant was assayed. Equivalent amounts of total protein containing 10 g of protein were loaded on a single track of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred by electroblotting onto PVDF membrane overnight at 4 °C. Molecular weight standards (Amersham Pharmacia Biotech) were run in parallel. The blots were blocked using 5% blocking reagent in a solution of Tris-buffered salt with Tween-20 (TBS-T) (20 mmol/L Tris-Cl, pH 7.6, 137 mmol/L NaCl, 0.1% Tween-20) for 2 h at room temperature, and then incubated with primary antibody (1:1000) overnight at 4 °C. The blots were washed with TBS-T and incubated in secondary antibody conjugated horseradish peroxide (1:1000) for 1 h at 37 °C. After extensive washing, the complexes were visualized using West Pico chemiluminescent kit (Pierce) and quantified by transmittance densitometry using volume integration with LumiAnalyst Image Analysis software to determine the enrichment of the proteins. Similar loading and transfer of proteins to the membrane were verified by blot staining.

Reverse Transcription (RT)-PCR Amplification

Total RNA was prepared from the brain capillary-rich fractions using Trizol (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer. Total RNA (1 mg) was subjected to RT in a 20 mL reaction using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) with oligo dT primers according to the instructions of the

manufacturer. The partial rat DMT1 cDNA splicing IRE sequences in the 3'-UTR were amplified using primers (5'-CTGAGCGAAGATACCAGCG-3'; 5'-GGAGC CATCACTTGACCACAC-3') based on the rat DMT1 sequences obtained from GenBank, which would result in an 838 bp fragment. Amplification was performed with initial denaturation at 94 °C for 3 min, followed by 25 cycles at 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. The reaction mixture that lacked RT was used as negative control, while β-actin cDNA (sense primer 5'-GGTCACCCACACTGTGCCCATCTA-3'; antisense primer 5'-GACCGTCAGGCAGCTCACATAGCT CT-3') was amplified simultaneously as the internal control. The PCR products were analyzed on a 1.8% agarose gel using LumiAnalyst Image Analysis software (Roche, Mannheim, Germany). Gene expression values were normalized for β-actin expression and expressed in units relative to the controls.

Statistical Analysis

The results were expressed as mean±SD. The difference between means was determined by one-way ANOVA, followed by the Tukey test for multiple comparisons. A probability value of $P<0.05$ was regarded to be statistically significant.

RESULTS

Transport of Pb into the Cerebral Cortex Blocked by Fe

The levels of Pb and Fe in the cerebral cortex of the five groups were examined to test whether the transport of Pb into the brain was affected by Fe levels. The results showed that administration of lead acetate significantly increased the cortex Pb levels by 187.5% and reduced the cortex Fe levels by 31.3% compared with those in control rats (Figure 1). Only Fe supplement at high dose levels significantly increased the brain Fe levels as compared to control rats. Low dose of Fe supplementation (7 mg Fe/kg, 2 d) significantly reduced the cortex Pb levels by 52.2% and increased the cortex Fe levels by 60.7% compared with those in Pb-treated rats (Figure 1A, 1B). However, high dose of Fe supplementation (14 mg Fe/kg, 2 d) did not decrease the cortex Pb levels (Figure 1A) but increased the cortex Fe levels by 41.1% (Figure 1B). The results suggest that Fe can partially inhibit Pb transport into the brain.

Cortex Pb Levels Related with DMT1 (IRE) Expression in the Brain Capillary-rich Fraction

To further test whether the transport of Pb and Fe was related to the DMT1 (IRE)-dependent transport

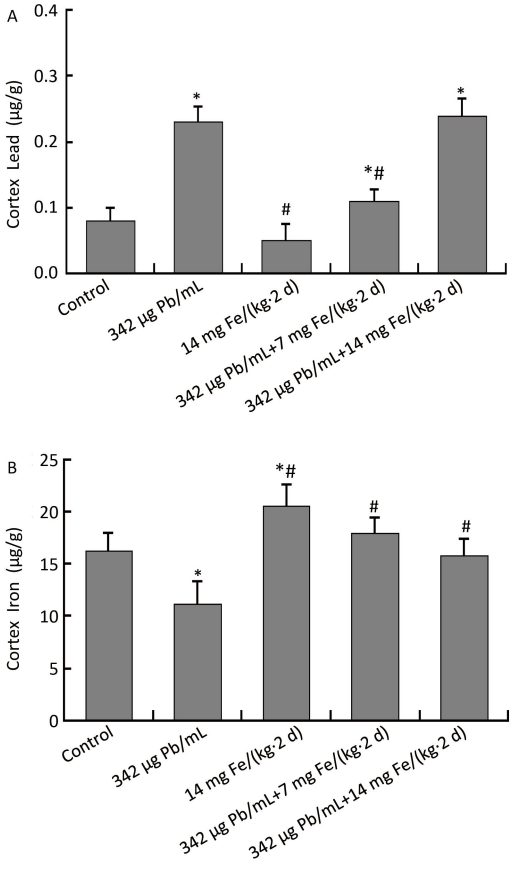

Figure 1. Analysis of Pb and Fe levels in the cerebral cortex after Pb exposure and concomitant Fe supplement treatment. Weanling male Sprague-Dawley rats were exposed to Pb as Pb acetate in drinking water (342 μg Pb/mL) daily and concomitantly administered orally by gavage with 7 (low Fe supplement group) or 14 mg Fe/kg (high Fe supplement group) as $FeSO_4$ solution, once every other day for six weeks. The group only supplied with 14 mg Fe/kg was also set as a reference. (A) Pb concentrations in the cerebral cortex after Pb exposure and concomitant Fe supplement treatment. (B) Fe concentrations in the cerebral cortex after Pb exposure and concomitant Fe supplement treatment. Data represent mean±SD (n=8), $^{*}P<0.05$ compared with control; $^{\#}P<0.05$ compared with Pb-alone group.

in BBB, the expression of DMT1 (IRE) in the brain capillary-rich fractions of the five groups was analyzed. The results showed that the changes in DMT1 (IRE) expression were consistent with those of the cortex Pb contents (Figure 2A). In Pb-treated rats, the expression of DMT1 (IRE) in the cerebral cortex was significantly increased compared with that in the control rats (Figure 2B). The over-expression of DMT1 (IRE) resulted in 187.5% increase in cortex Pb transport (Figure 2B). Thus, a high level of DMT1 (IRE) expression is necessary for a substantial increase in cortex Pb absorption. With Pb exposure, the level of DMT1 (IRE) becomes high enough to allow increased Pb absorption. During the period of low Fe supplementation, the expression of DMT1 (IRE) in the cerebral cortex, which was 1.25 folds that of the control rats, was greatly reduced by 26.5% compared with Pb-treated rats, thereby allowing significantly decreased Pb absorption but slightly increased Fe absorption (Figure 2B). After high Fe supplementation, the expression of DMT1 (IRE) in the brain capillary-rich fractions was greatly increased by 2.61 folds compared with the control rats and also increased by 53.5% compared with Pb-treated rats, thereby allowing not only increased Pb absorption but also Fe absorption (Figure 2).

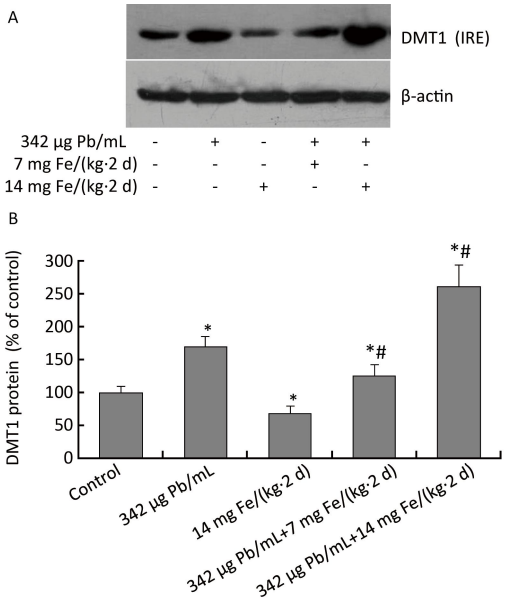

Figure 2. Effects of Fe supplementation on the Pb-induced increase in DMT1 (IRE) expression in brain capillary-rich fraction. The animal dose regimen was described in the legend in Figure 1. Brain capillary-rich fraction was prepared according to experimental procedures. (A) Representative blots of DMT1 (IRE) proteins. Antibodies directed against β-actin were used to confirm equal loading of proteins. (B) The corresponding expression levels of DMT1 (IRE) proteins are shown as the bar graphs. The DMT1 (IRE) protein of the control group was designated as 1, with which the other groups were compared. Data represent mean±SD (n=3), $^{*}P$<0.05 compared with control; $^{\#}P$<0.05 compared with Pb-alone group.

Pb or/and Fe No Effect on the Expression of DMT1 (IRE) mRNA in Brain Capillary-rich Fraction

To further determine whether the DMT1 (IRE) mRNA is expressed in the brain capillary-rich fraction, RT-PCR technique was used to examine the expression of DMT1 (IRE) cDNA. In the RT-PCR analysis, the bands of DMT1 (IRE) cDNA were found in the brain capillary-rich fractions (Figure 3A). The results showed that the specific PCR product for DMT1 (IRE) cDNA was detected from the total RNA prepared from the brain capillary-rich fraction. The bands of DMT1 (IRE) cDNA were not significantly different (Figure 3B), suggesting that Pb or/and Fe have no effect on the expression of DMT1 (IRE) mRNA. Thus, Pb most likely stabilizes the binding of IRP to DMT1 (IRE) mRNA, thereby increasing the expression of DMT1 (IRE) protein.

ERK MAPK Pathway Involved in the Modulation of DMT1 (IRE) mRNA Stability

Studies have demonstrated that Pb can induce PKC-dependent activation of the MAPK pathway[25]. PKC activation may increase the phosphorylation of IRPs and decrease the binding of IRPs with IRE to reduce the DMT1 (IRE) mRNA stability[26]. To further investigate the specific Pb-induced MAPK activation involved in the binding of IRP to IRE, western blot was used to analyze the activation of ERK1/2 in the brain capillary-rich fractions. In our experiments, western blot demonstrated the consistent changes in the levels of p-ERK1/2 with the changes in the levels of DMT1 (IRE) (Figure 4A). Pb treatment caused a significant increase in ERK activity (248% ERK1, 212% ERK2; Figure 4B). This ERK activation was suppressed after supplementation of low-dose Fe and was almost reduced to the basal level (171% ERK1, 155% ERK2; Figure 4B). Supplementation of high-dose Fe significantly promoted the activity of ERK, which was the same as the level of Pb-treated

group (Figure 4B). Only Fe treatment caused a significant reduce in ERK activity compared with that in the control rats (60% ERK1, 78% ERK2; Figure 4B). Similarly, treatment with Pb also produced an observable increase in the level of IRP1 protein (198%; Figure 5), and low Fe supplementation greatly reduced the level compared with Pb-treated rats (Figure 5B). After high Fe supplementation, the expression of IRP1 was greatly increased by 49.5% compared with Pb-treated rats (Figure 5).

DISCUSSION

Evidence from several studies strongly suggests that DMT1 is the major transporter of Fe in the intestine. However, specific cellular importers for Pb are unlikely because the metal is non-essential and previous experiment indicated that adequate Fe in toxic. Pb is more likely inadvertently up-taken through pathways intended for Fe. Results from our diets results in decreased Pb absorption into the blood from the intestine[27], suggesting common molecular mechanisms of Pb and Fe transport. Accordingly, in this experiment, the transport of Pb and Fe into the cerebral cortex displayed similar properties, suggesting that Fe transporters also mediate the transport of Pb into the cerebral cortex. But it should be noted that the high dose of Fe supplement (14 mg Fe/kg) did not reduce brain Pb concentrations concentrations in Pb-treated animals. This discrepancy could be due to the differential sensitivity of brain parenchyma and brain endothelia to Fe treatment. It also suggests that Pb-induced neurotoxicity seems unlikely to be solely associated with body Fe homeostasis.

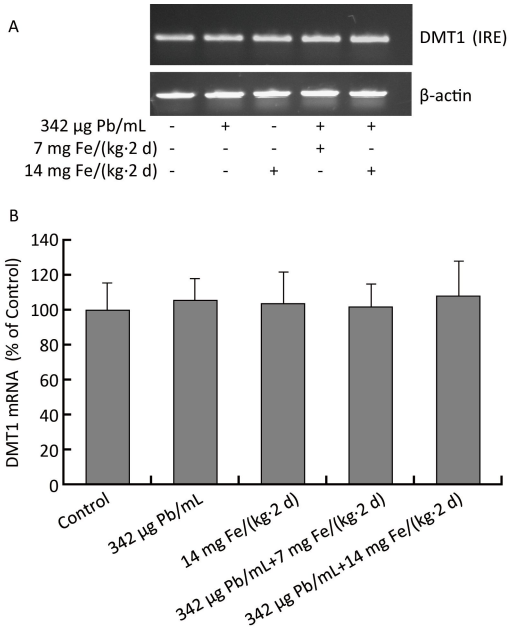

Figure 3. Levels of DMT1 (IRE) cDNA in brain capillary-rich fractions. The animal dose regimen was described in the legend in Figure 1. Brain capillary-rich fraction was prepared according to experimental procedures. (A) Representative bands of DMT1 (IRE) cDNA in the brain capillary-rich fraction. (B) The DMT1 (IRE) cDNA of the control group was designated as 1, with which the other groups were compared.

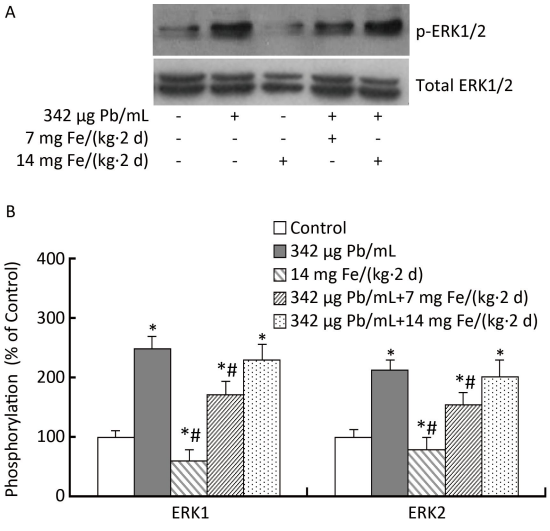

Figure 4. Effects of Fe supplement on the Pb-induced expressions of p-ERK1/2 in brain capillary-rich fraction. The animal dose regimen was described in the legend in Figure 1. Brain capillary-rich fraction was prepared according to experimental procedures. (A) Western blots for phospho-ERK1/2 MAPK (pMAPK) after experiment. (B) The corresponding expression levels of p-ERK1/2 proteins in the brain capillary-rich fraction are shown as the bar graphs. Supplementation with Fe markedly inhibited the increase of p-ERK1/2. Data represent mean±SD (n=3), $^{*}P$<0.05 compared with control; $^{\#}P$<0.05 compared with Pb-alone group.

In the original studies of DMT1, the results provided evidence suggesting that DMT1 can transport other divalent metals such as Fe and Pb[19]. Other studies have shown that the inhibitory effect of Pb on Fe uptake may occur in an intracellular process rather than in a membrane-binding step, probably inhibiting the translocation of Fe across the endosomal membrane[28]. Our work showed that DMT1 (IRE) transported Pb with an affinity similar to that of Fe. The similarity in the inhibition of Pb transport by Fe in the cerebral cortex strongly suggests that Pb is partially transported by DMT1 (IRE) in this animal model. Pb transport was inhibited by the presence of 42.6% increase of Fe in the cerebral cortex. Previous studies have confirmed that

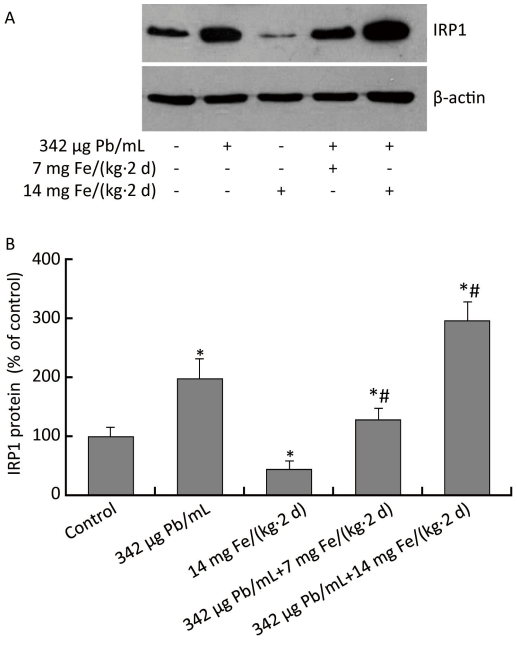

Figure 5. Effects of Fe supplementation on the Pb-induced increase in IRP1 expression in brain capillary-rich fraction. The animal dose regimen was described in the legend in Figure 1. Brain capillary-rich fraction was prepared according to experimental procedures. (A) Representative blots of IRP1 proteins. Antibodies directed against β-actin were used to confirm equal loading of proteins. (B) The corresponding expression levels of IRP1 proteins are shown as the bar graphs. The IRP1 protein of the control group was designated as 1, with which the other groups were compared. Data represent mean±SD (n=3), $^{*}P$<0.05 compared with control; $^{\#}P$<0.05 compared with Pb-alone group.

DMT1 (IRE) can transport Pb and Fe in human intestinal cells[29-30]. In human cells, DMT1 expression results in increased transport of Pb. Similarly, over-expression of DMT1 is shown to mediate the uptake of Pb in Chinese hamster ovary cells[31]. The uptake of Pb by DMT1 may explain why Pb exposure is associated with a reduction in Fe uptake. Adequate Fe intake may serve a dual function in preventing Pb absorption[30]. First, Fe intake lowers the number of Pb transporters in the gut because the DMT1 regulation in the duodenum is sensitive to the levels of Fe uptake[21,32]. Second, given that DMT1 has a much higher affinity for Fe than for Pb, the presence of Fe in the gut can competitively inhibit Pb uptake. Fe has been shown capable of inhibiting Pb uptake by DMT1[33]. They may have a similar function in BBB because DMT1 has to be present in cerebral endothelial cells[23]. The mentioned biological mechanisms are consistent with our studies, which suggest the protective effects of Fe uptake against Pb poisoning.

The mechanisms involved in the regulation of DMT1 mRNA synthesis and protein translation are not completely understood. Studies have demonstrated that DMT1 mRNA synthesis and protein translation in the small intestine are both negatively regulated by the Fe status[21,34-35]. However, our findings and the results reported by others imply that DMT1 mRNA expression and translation in response to Fe status in different organs or tissues might be different[34,36]. Our results show that Fe status has a significant effect on DMT1 protein translation but not on the synthesis of DMT1 (IRE) mRNA in brain capillary-rich fraction. The treatment with Pb and/or Fe had no effect on the expression of DMT1 (IRE) mRNA. DMT1 protein in brain capillary-rich fraction increased in Pb-treated and high Fe-supplemented rats and decreased in low Fe-supplemented rats, showing a positive post-transcriptional regulation by Pb.

The increase in the expression of DMT1 (IRE) protein was likely due to a decrease in the degradation rate of DMT1 mRNA, as no evidence of an increase in DMT1 mRNA transcription was found. Very little is known about the other mechanisms of the regulation of DMT (IRE) protein synthesis in addition to IRE-IRP interaction. Hence, the interaction of RNA-binding proteins that recognize specific consensus sequences at 3'-UTR[37] is a possible mechanism for stabilizing mRNA. For example, transferrin mRNA is stabilized through the interaction between the IRE located at 3'-UTR and an

IRP[33,38]. PKC has been shown to phosphorylate the IRP, resulting in increased binding between IRP and IRE. This mechanism may explain why the activation of PKC increased the level of DMT1 (IRE) but does not explain the effects on DMT1 (-IRE)[21]. Certain evidence suggests that several different stimuli can activate the MEK/MAPK pathway in a PKC-dependent manner[39-40]. PKCα can activate Raf-1 kinase either by direct phosphorylation[41] or by modulating its membrane association[42]; Raf-1, in turn, can phosphorylate MEK, which then activates the ERK1/2 MAPK[43-44]. The present study investigates whether Pb would activate the ERK MAPK pathway to gain a good understanding of the signal transduction pathway that may be activated by Pb and may underlie the increased DMT1 (IRE) protein translation. Western blot demonstrated the consistent changes in the levels of p-ERK1/2 with the changes in the levels of DMT1 (IRE) *in vivo*. So we presumed that Pb specifically induced the dysregulation of IRP1 protein by activating the ERK1/2 signaling pathway, and then regulated the translation of DMT1 (IRE) mRNA.

In summary, DMT1 does transport Pb across BBB into the brain. Remarkably, although the expression of DMT1 (IRE) protein in the brain capillary endothelial cell showed a positive post-transcriptional regulation by Pb, treatment with Pb and/or Fe had no effect on the expression of DMT1 (IRE) mRNA. The increase in DMT1 (IRE) protein may be attributed to the IRP/IRE system. Finally, ERK MAPK activation may have an integral function in the regulation of DMT1 (IRE). Pb may indirectly regulate the expression of the DMT1 (IRE) possibly by modulating the RNA-binding activity of IRP.

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REFERENCES

1. Petrone LR. Iron deficiency, lead poisoning, and development. Arch Pediatr Adolesc Med, 2007; 161, 523-4.
2. Jeonga KS, Park H, Ha Eunhee, et al. Evidence that cognitive deficit in children is associated not only with iron deficiency, but also with blood lead concentration: A preliminary study. J Trace Elem Med Bio, 2015; 29, 336-41.
3. Kim Y, Park S. Iron deficiency increases blood concentrations of neurotoxic metals in children. Korean J Pediatr, 2014; 7, 345-50.
4. Ye JP, Du C, Wang L, et al. Relationship of Blood Levels of Pb with Cu, Zn, Ca, Mg, Fe, and Hb in children aged 0-6 years from Wuhan, China. Biol Trace Elem Res, 2015; 164,18-24.
5. Muwakkit S, Nuwayhid I, Nabulsi M, et al. Iron deficiency in young Lebanese children: association with elevated blood lead levels. J Pediatr Hematol Oncol, 2008; 30, 382-6.
6. Rahman MA, Rahman B, Ahmad MS, et al. Blood and hair lead in children with different extents of iron deficiency in Karachi. Environ Res, 2012; 18, 94-100.
7. Rondó PH, Conde A, Souza MC. Iron deficiency anaemia and blood lead concentrations in Brazilian children. Trans R Soc Trop Med Hyg, 2011; 105, 525-30.
8. Shah F, Kazi TG, Afridi HI, et al. Environmental exposure of lead and iron deficit anemia in children age ranged 1-5 years: a cross sectional study. Sci Total Environ, 2010; 408, 5325-30.
9. Turgut S, Polat A, Inan M, et al. Interaction between anemia and blood levels of iron, zinc, copper, cadmium and lead in children. Indian J Pediatr, 2007; 74, 827-30.
10. Choi JW, Kim SK. Association between blood lead concentrations and body iron status in children. Arch Dis Child, 2003; 88, 791-2.
11. Krueger M, Härtig W, Reichenbach A, et al. Blood-brain barrier breakdown after embolic stroke in rats occurs without ultrastructural evidence for disrupting tight junctions. PLoS One, 2013; 8, e56419.
12. Pfeiffer F, Schäfer J, Lyck R, et al. Claudin-1 induced sealing of blood-brain barrier tight junctions ameliorates chronic experimental autoimmune encephalomyelitis. Acta Neuropathol, 2011; 122, 601-4.
13. Keep R, Ennis S, Beer M, et al. Developmental changes in blood-brain barrier potassium permeability in the rat: relation to brain growth. J Physiol, 1995; 488, 439-48.
14. Utsumi H, Chiba H, Kamimura Y, et al. Expression of GFRalpha-1, receptor for GDNF, in rat brain capillary during postnatal development of the BBB. Am J Physiol Cell Physiol, 2000; 279, C361-8.
15. Roux KJ, Amici SA, Notterpek L. The temporospatial expression of peripheral myelin protein 22 at the developing blood-nerve and blood-brain barriers. J Comp Neurol, 2004; 474, 578-88.
16. Hossain MA, Russell JC, Miknyoczki S, et al. Vascular endothelial growth factor mediates vasogenic edema in acute lead encephalopathy. Ann Neurol, 2004; 55, 660-7.
17. Xu SZ, Bullock L, Shan CJ, et al. PKC isoforms were reduced by lead in the developing rat brain. Int J Dev Neurosci, 2005; 23, 53-64.
18. Behl M, Zhang Y, Shi Y, et al. Lead-induced accumulation of beta-amyloid in the choroid plexus: role of low density lipoprotein receptor protein-1 and protein kinase C. Neurotoxicology, 2010; 31, 524-32.
19. Ngueta G. Racial disparities in children's blood lead levels: possible implication of divalent metal transporter 1. Med Hypotheses, 2014; 82, 71-3.
20. Zhu G, Fan G, Feng C, et al. The effect of lead exposure on brain iron homeostasis and the expression of DMT1/FP1 in the brain in developing and aged rats. Toxicol Lett, 2013; 216, 108-23.
21. Wang Q, Luo W, Zhang W, et al. Involvement of DMT1 +IRE in the transport of lead in an in vitro BBB model. Toxicol *In Vitro*, 2011; 25, 991-8.
22. Hubert N, Hentze MW. Previously uncharacterized isoforms of divalent metal transporter (DMT)-1: implications for regulation and cellular function. Proc Natl Acad Sci USA, 2002; 99, 12345-50.
23. Burdo JR, Menzies SL, Simpson IA, et al. Distribution of divalent metal transporter 1 and metal transport protein 1 in the normal and Belgrade rat. J Neurosci Res, 2001; 66, 1198-207.
24. Ohtsuki S, Asaba H, Takanaga H, et al. Role of blood-brain barrier organic anion transporter 3 (OAT3) in the efflux of indoxyl sulfate, a uremic toxin: its involvement in neurotransmitter metabolite clearance from the brain. J

Neurochem, 2002; 83, 57-66.
25. Cheng YJ, Yang BC, Liu MY. Lead increases lipopolysaccharide-induced liver-injury through tumor necrosis factor-alpha overexpression by monocytes/macrophages: role of protein kinase C and P42/44 mitogen-activated protein kinase. Environ Health Perspect, 2006; 114, 507-13.
26. Fillebeen C, Caltagirone A, Martelli A, et al. IRP1 Ser-711 is a phosphorylation site, critical for regulation of RNA-binding and aconitase activities. Biochem J, 2005; 388, 143-50.
27. Wang Q, Luo WJ, Zheng W, et al. Iron supplement prevents lead-induced disruption of the blood-brain barrier during rat development. Toxicol appl pharmacol, 2007; 219, 33-41.
28. Qian ZM, Xiao DS, Wang Q, et al. Inhibitory mechanism of lead on transferrin-bound iron uptake by rabbit reticulocytes: a fractal analysis. Mol Cell Biochem, 1997; 173, 89-94.
29. Tallkvist J, Bowlus CL, Lonnerdal B. Functional and molecular responses of human intestinal Caco-2 cells to iron treatment. Am J Clin Nutr, 2000; 72, 770-5.
30. Bannon DI, Abounader R, Lees PS, et al. Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells. Am J Physiol Cell Physiol, 2003; 284, C44-50.
31. Bannon ID, Portnoy ME, Olivi L, et al. Uptake of lead and iron by divalent metal transporter 1 in yeast and mammalian cells. Biochem Biophys Res Commun, 2002; 295, 978-84.
32. Morgan EH, Oates PS. Mechanisms and regulation of intestinal iron absorption. Blood Cells Mol Dis, 2002; 29, 384-99.
33. Elsenhans B, Janser H, Windisch W, et al. Does lead use the intestinal absorptive pathways of iron? Impact of iron status on murine ^{210}Pb and ^{59}Fe absorption in duodenum and ileum *in vivo*. Toxicology, 2011; 284, 7-11.
34. Trinder D, Oates PS, Thomas C, et al. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut, 2000; 46, 270-6.
35. Yeh KY, Yeh M, Watkins JA, et al. Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression. Am J Physiol Gastrointest Liver Physiol, 2000; 279, G1070-9.
36. Wardrop SL, Richardson DR. The effect of intracellular iron concentration and nitrogen monoxide on NRAMP2 expression and non-transferrin-bound iron uptake. Eur J Biochem, 1999; 263, 41-9.
37. Day DA, Tuite MF. Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. J Endocrinol, 1998; 157, 361-71.
38. Kühn LC. Iron regulatory proteins and their role in controlling iron metabolism. Metallomics, 2015; 7, 232-43.
39. Yu XT, Wang PY, Shi ZM, et al. Up-regulation of urotensin II and its receptor contributes to human hepatocellular carcinoma growth via activation of the PKC, ERK1/2, and p38 MAPK signaling pathways. Molecules, 2014; 19, 20768-79.
40. Wang H, Ferraris JD, Klein JD, et al. PKC-α contributes to high NaCl-induced activation of NFAT5 (TonEBP/OREBP) through MAPK ERK1/2. Am J Physiol Renal Physiol, 2015; 308, F140-8.
41. Wang CY, Wang YT, Tzeng DW, et al. Lead acetate induces EGFR activation upstream of SFK and PKCalpha linkage to the Ras/Raf-1/ERK signaling. Toxicol Appl Pharmacol, 2009; 235, 244-52.
42. Schonwasser DC, Marais RM, Marshall CJ, et al. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol Cell Biol, 1998; 18, 790-8.
43. Yu Z, Ye S, Hu G, et al. The RAF-MEK-ERK pathway: targeting ERK to overcome obstacles to effective cancer therapy. Future Med Chem, 2015; 7, 269-89.
44. Chen JC, Chen YJ, Lin CY, et al. Amphiregulin enhances alpha6beta1 integrin expression and cell motility in human chondrosarcoma cells through Ras/Raf/MEK/ERK/AP-1 pathway. Oncotarget, 2015; 6, 11434-46.