Stabilization of iron regulatory protein 2, IRP2, by aluminum

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Received 17 October 1999

Abstract **Iron regulatory protein 2 (IRP2) is one of the central regulators of iron homeostasis. IRP2 regulates expression of molecules involved in iron metabolism by binding to iron responsive elements (IREs) in the transcripts of those molecules in iron depletion. IRP2 is regulated by the accelerated degradation initiated by the iron-catalyzed oxidation. Here we report that aluminum antagonizes the iron-induced decrease in IRE binding activity of IRP2. Aluminum also inhibits iron-induced oxidation of IRP2 in vitro. These results suggest that aluminum stabilizes IRP2 by interfering with the iron-catalyzed oxidation, which results in perturbation of iron metabolism.**

Key words: Iron responsive element; Iron regulatory protein; Aluminum; Iron metabolism; Metal-catalyzed oxidation

1. Introduction

The expression of proteins involved in mammalian iron metabolism are regulated post-transcriptionally through interactions between RNA stem-loop structures known as iron responsive elements (IREs) and iron regulatory proteins 1 and 2 (IRP1 and 2) (reviewed in [1,2]). The binding of IRPs to IREs in the 5′ untranslated region (UTR) prevents initiation of the translation of ferritin mRNA [3,4], and the binding of IRPs to IREs in the 3′ UTR of transferrin receptor (TfR) mRNA protects the mRNA from degradation in iron-depleted cells [5,6].

Both IRP1 and IRP2 bind IREs with high affinity only in iron-depleted cells, but the mode of regulation by iron differs between the two proteins. IRP1 is a stable bifunctional protein [7,8], whereas IRP2 is rapidly degraded in the presence of iron in cells [9,10], with the IRP2-specific domain consisting of 73 amino acids functioning as an iron-dependent degradation domain [11].

The introduction of a constitutive IRE binding IRP1 mutant perturbed the expression of TfR and ferritin and decreased cell survival possibly due to the iron toxicity [12]. Although both IRP1 and IRP2 are expressed in almost all organs and cell lines, a murine pro-B cell line, Ba/F3, has been shown to lack IRP1 and expresses only IRP2 [13]. TfR and ferritin are fully regulated by IRP2 in Ba/F3, suggesting that the presence of IRP2 is sufficient for the IRE-mediated iron regulation. Therefore, it is important to identify the inducer of IRP2. An IRP2-specific inducer, however, has not been identified to date. Nitric oxide (NO) has been claimed as an enhancer of IRP2 [14], but conflicting reports showed that IRP2 was insensitive to NO [15]. The degradation of IRP2 has been shown to be initiated by the oxidative modification of the protein by iron [16]. It is highly likely that other metals that affect the redox cellular state will have an effect on IRE binding, however, such an effect has not been shown. Here, we demonstrate that aluminum induces the IRE binding activity of IRP2 by competitively inhibiting the oxidative modification of the protein by iron, which results in elevated TfR biosynthesis and the suppression of ferritin production.

2. Materials and methods

2.1. Cell culture

A murine erythroleukemia cell line, MEL, and a murine neuroblastoma cell line, Neuro2A, were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; Gibco-BRL), 100 IU/ml penicillin G and 100 μg/ml streptomycin. A murine pro-B cell line, Ba/F3, was cultured in RPMI 1640 medium with 10% FCS, penicillin G, streptomycin, 50 μM of 2-mercaptoethanol, and 100 U/ml murine interleukin-3. These cells are cultured with $AlCl_3$, $MnCl_2$, $CuSO_4$, $ZnSO_4$ (Wako, Japan), ferric ammonium citrate (FAC), hemin, or the iron chelator desferrioxamine (Df; Ciba-Geigy).

2.2.. Preparation of cell extracts

Lysates for RNA gel shift assay and Western blot were prepared by lysing cells with lysis buffer containing 1% Triton X-100, 25 mM Tris-HCl pH 7.4, 40 mM KCl, protease inhibitors (10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice. Nuclei and membrane fractions were removed by centrifugation.

2.3. RNA gel retardation assay

Gel retardation assays were performed as described previously [11]. Briefly, 15 μg of cell lysates were incubated with 2 ng of ^{32}P-labelled IRE probe for 10 min on ice. The mixture was separated in 10% non-denaturing polyacrylamide gel.

2.4. Western blots

Lysates (50 μg) were separated in 6% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell), then incubated with rabbit affinity-purified anti-IRP2 antibody [9], followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham-Pharmacia Biotech), and visualized with the SuperSignal Western blotting detection system (Pierce).

2.5. Northern blots

RNA was extracted from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method [17]. 20 μg of total RNAs were electrophoresed, then transferred onto nylon membrane (Hybond N^+, Amersham-Pharmacia Biotech). The membrane was hybridized with radiolabelled murine TfR, ferritin H chain, and GAPDH cDNA probes. Quantitation was performed using the BAS 2000 image analyzer (Fuji Film).

2.6. Biosynthetic labelling

Cells were metabolically radiolabelled with 3.7 MBq/ml of Pro-mix

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Abbreviations: IRE, iron responsive element; IRP, iron regulatory protein; Df, desferrioxamine; FAC, ferric ammonium citrate; TfR, transferrin receptor; UTR, untranslated region

0014-5793/99/$20.00
PII: S0014-5793(99)01533-1

(Amersham-Pharmacia Biotech) in methionine- and cystine-deficient DMEM containing 10% dialyzed FCS for 3.5 h in the continuous presence of the metals. Aliquots of lysates containing 4×10^7 cpm, determined by trichloroacetic acid precipitation, were immunoprecipitated with polyclonal anti-ferritin IgG (Boehringer Mannheim) or rat anti-mouse TfR antibody (R17 217) followed by rabbit anti-rat IgG (Zymed, South San Francisco, CA, USA) and separated in 7% or 13% SDS-PAGE for TfR or ferritin, respectively.

2.7. Carbonyl detection assay

Purification of baculovirus-expressed IRP2 was performed as described previously [9]. 4 μg of the recombinant IRP2 were incubated with $FeCl_3$ (Sigma), dithiothreitol (DTT) (Wako, Japan), $AlCl_3$, $CuSO_4$, or $ZnSO_4$ in 25 mM HEPES pH 7.2, 40 mM KCl at 37°C. Reactions were stopped by the addition of Df to 1 mM. The detection of carbonyl residues of the proteins was performed as described [16].

3. Results

3.1. Aluminum induces the IRE binding activity of IRP2 by antagonizing the effect of iron in MEL cells

To examine the influence of metals on the IRE binding activity of IRPs, 100 μM of aluminum, manganese, copper, and zinc were added to cultures of an erythroleukemic cell line, MEL, and an RNA gel mobility shift assay was performed. The IRE binding activities of both IRP1 and IRP2 were strongly induced by the administration of the iron-specific chelator, Df, and were strongly suppressed in the presence of FAC as reported [9,19]. The IRE binding activity of IRP2 was specifically induced in the presence of aluminum, but not in the presence of other metals (Fig. 1A). We also examined the amount of IRP2 protein by Western blot. The increase in the IRE binding activity of IRP2 coincided with the increase in the amount of IRP2 induced by aluminum (Fig. 1B), suggesting that induction of the IRE binding activity by aluminum is due to the accumulation of IRP2 protein.

To analyze the mechanism of the aluminum-induced IRE binding activity of IRP2, we treated MEL cells with aluminum chloride together with indicated concentrations of FAC (Fig. 1C,D) or hemin (Fig. 1E,F) as an iron source. The aluminum-induced IRE binding activity and amount of IRP2 decreased in a FAC or hemin dose-dependent fashion. To titrate the concentration of aluminum on IRP2, we treated MEL cells with different concentrations of aluminum in the presence of FAC. IRE binding activity of IRP2 increased in a dose-dependent fashion (Fig. 1G). Taken together, these results indicate that aluminum induces the IRE binding of IRP2 by antagonizing the effect of iron.

3.2. Enhanced IRE binding activity of IRP2 by aluminum increases the production of TfR and suppresses ferritin synthesis in MEL cells

We next analyzed the physiological significance of the aluminum-induced IRE binding activity of IRP2 in MEL cells. To examine the effect of induced IRE binding activity of IRP2 by aluminum on the amount of TfR mRNA, Northern blot analyses were performed. The induction of the IRE binding activity of IRP2 by the aluminum was confirmed by gel mobility shift analysis (data not shown). TfR mRNA was abundant in cells treated with Df, and was minimal in cells treated with FAC (Fig. 2A). The amount of TfR mRNA was increased 2.7-fold in cells treated with FAC in the presence of aluminum compared to cells treated with FAC alone. Ferritin and GAPDH mRNAs were not significantly affected by the

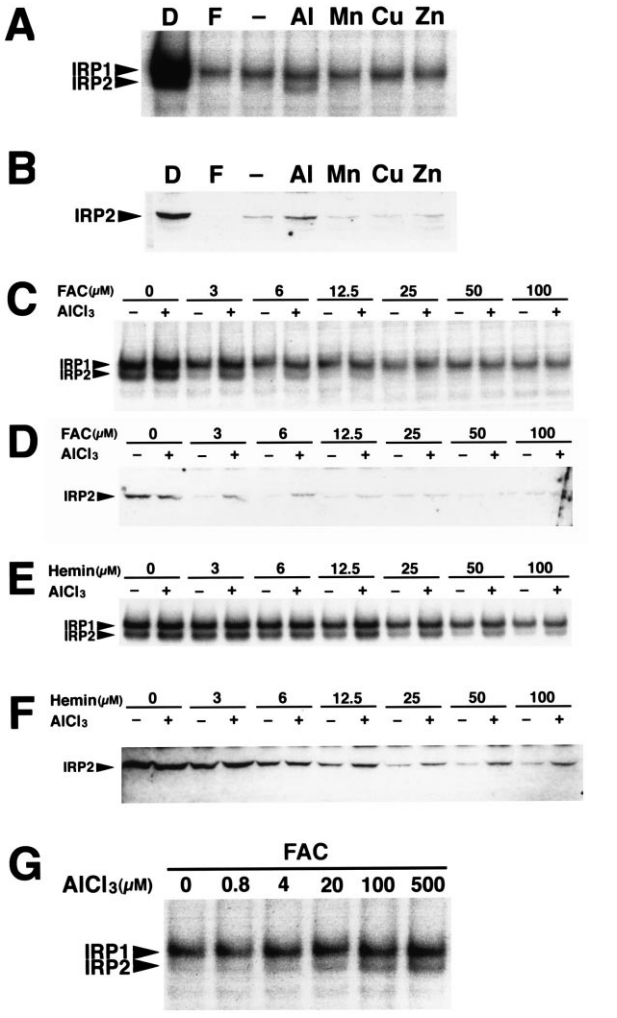

Fig. 1. Aluminum induces the IRE binding activity of IRP2 by competing with iron in MEL cells. MEL cells were treated with 300 μM FAC (F), 100 μM $AlCl_3$ (Al), 100 μM $MnCl_2$ (Mn), 100 μM $CuSO_4$ (Cu), or 100 μM $ZnSO_4$ (Zn) for 48 h, together with non-treated control (−) and cells treated with 100 μM Df for 16 h (D). A: Gel retardation assay. Lysates (15 μg) were incubated with radiolabelled IRE and electrophoresed in 10% native polyacrylamide gel. B: Western blot analysis of IRP2 using rabbit affinity-purified anti-IRP2 antibody. MEL cells were treated with the indicated concentrations of FAC (C, D) or hemin (E, F) in the presence (+) or absence (−) of 100 μM of $AlCl_3$ for 48 h. The same samples were assessed by gel retardation assays (C, E) and Western blots for IRP2 (D, F). MEL cells were treated with the indicated concentrations of $AlCl_3$ in the presence of 6 μM FAC for 48 h, and lysates were assessed by gel retardation assays (G).

presence of aluminum (Fig. 2A). We also followed the effect of aluminum on the biosynthesis of TfR in MEL cells (Fig. 2B). TfR synthesis was strongly induced by Df and was suppressed by FAC. However, the suppression of TfR synthesis by iron was significantly reversed by the addition of aluminum (1.6-fold). Biosynthesis of ferritin was monitored after the treatment of MEL cells with FAC in the presence or absence of aluminum (Fig. 2C). Ferritin production was induced in the presence of FAC. This induction by FAC was significantly suppressed by the presence of aluminum (0.6-fold). These results show that aluminum suppresses the production of ferritin, and stimulates TfR biosynthesis by stabilizing its mRNA through the induction of the IRE binding activity of IRP2.

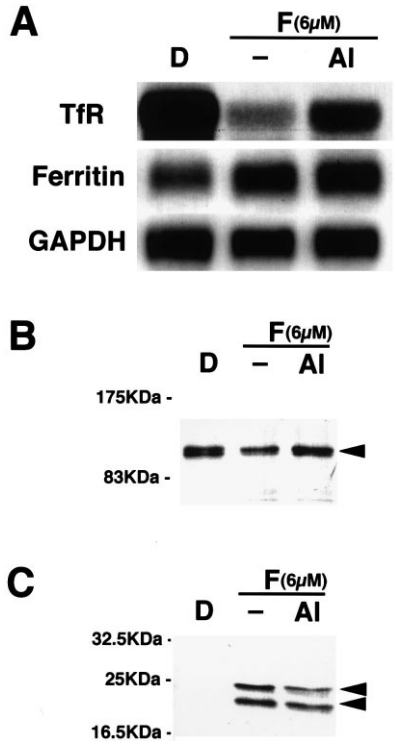

Fig. 2. Aluminum stimulates TfR production by stabilizing its mRNA and suppresses ferritin biosynthesis in MEL cells. A: MEL cells were treated with 6 μM FAC (F) in the presence (Al) or absence (−) of 100 μM $AlCl_3$ for 48 h. Control cells were treated with 100 μM Df for 16 h (D). Northern blot for TfR, ferritin H chain, and GAPDH RNA levels in MEL cells were performed. B, C: MEL cells were treated under the same condition as in A. Cells were labelled metabolically, and biosynthesis of TfR or ferritin was assessed as described in Section 2. Arrows indicate TfR (B) or ferritin H and L chains (C).

3.3. Induction of the IRE binding activity of IRP2 by aluminum in neuronal cells and pro-B lymphoid cells

To exclude the possibility that the effect of aluminum is a unique feature of iron metabolism in an erythroid cell line, MEL, we analyzed the effect of aluminum on IRP2 in a murine neuroblastoma cell line, Neuro 2A, or an IRP1-defective murine pro-B cell line, Ba/F3. Increasing concentrations of FAC were added to the cultures of Neuro 2A or Ba/F3 cells along with 500 μM aluminum (Fig. 3A,B). Gel mobility shift assays showed that the IRE binding activity of IRP2 was specifically increased by the aluminum in the presence of FAC. Due to the competition between the two metals, the effect of aluminum on IRP2 could not be observed any more in the presence of 300 μM FAC. We then examined the effect of aluminum on TfR or ferritin production in Neuro 2A or Ba/F3 cells. The effects of aluminum in these cell lines are similar to those observed in MEL cells (Fig. 3C–F), suggesting that aluminum suppresses the production of ferritin, and stimulates TfR biosynthesis through the induction of the IRE binding activity of IRP2, and these observations are common to many cell lines.

3.4. Iron-induced oxidative modification of IRP2 is inhibited by the presence of aluminum in vitro

Since the oxidative modification of IRP2 by iron has been shown to signal the protein for degradation via the ubiquitin-proteasome pathway [16], it was important to monitor the effect of aluminum on iron-induced oxidative modification of IRP2 in vitro. IRP2 was incubated with $FeCl_3$ and DTT in the presence or absence of $AlCl_3$, $CuSO_4$, or $ZnSO_4$, then oxidative modification of IRP2 was monitored as described before [16,18]. As shown in Fig. 4A, IRP2 was oxidized in a time-dependent fashion in the presence of $FeCl_3$ (lanes 2, 3). Aluminum could inhibit the oxidative modification over the same incubation periods, however copper and zinc could not (lanes 4–9). Neither aluminum, copper, nor zinc alone could induce oxidation of IRP2 (lanes 10–12). Coomassie staining of the membrane showed equal amounts of IRP2 were loaded (Fig. 4B). These results suggested that the inhibition of the iron-induced oxidative modification of IRP2 was specific to aluminum. To titrate the effect of aluminum on the IRP2 protein, we next incubated IRP2 with increasing concentrations of $AlCl_3$ in the presence of $FeCl_3$ (Fig. 4C), and the oxidative changes were monitored. IRP2 was oxidized in the presence of either 10 μM (lane 14) or 20 μM (lane 18) $FeCl_3$, however, these modifications were suppressed by aluminum in a dose-dependent fashion in the presence of both 10 μM (lanes 15–17) and 20 μM (lanes 19–21) of $FeCl_3$. Coomassie

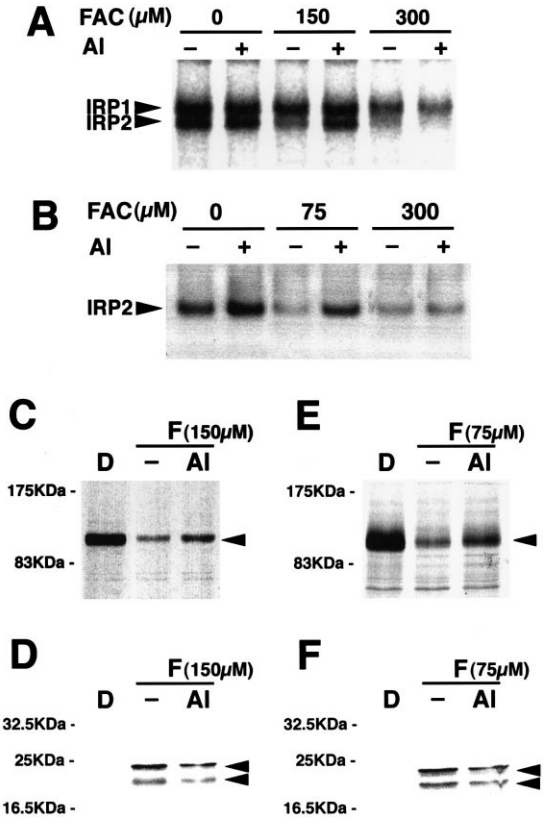

Fig. 3. Aluminum suppresses ferritin biosynthesis and increases TfR production via increased IRE binding activity of IRP2 in Neuro 2A cells and Ba/F3 cells. Neuro 2A cells (A) or Ba/F3 cells (B) were incubated with the indicated concentration of FAC in the presence (+) or absence (−) of 500 μM of $AlCl_3$ for 48 h. Lysates were assessed by gel retardation assays (A, B). Neuro 2A cells (C, D) or Ba/F3 cells (E, F) were incubated with (Al) or without (−) 500 μM $AlCl_3$ in the presence of 150 μM or 75 μM FAC (F) for 48 h, respectively. Cells that were incubated with 100 μM Df for 16 h were used as controls (D). Cells were labelled metabolically, and biosynthesis of TfR or ferritin was monitored as described in Section 2. Arrows indicate TfR (C, E) or ferritin H and L chains (D, F).

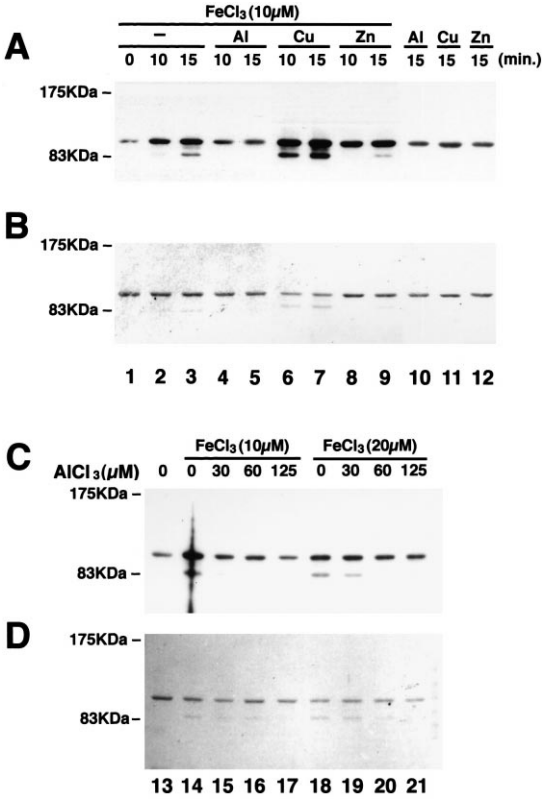

Fig. 4. Aluminum chloride inhibits the iron-induced oxidation of IRP2 in vitro. A: 4 μg of recombinant IRP2 was incubated with 10 μM $FeCl_3$ and 10 mM DTT for 10 or 15 min in the absence (lanes 2, 3) or presence of 125 μM $AlCl_3$ (lanes 4, 5), 125 μM $CuSO_4$ (lanes 6, 7), or 125 μM $ZnSO_4$ (lanes 8, 9) together with 10 mM DTT at 37°C. Or, IRP2 was incubated with 125 μM $AlCl_3$ (lane 10), 125 μM $CuSO_4$ (lane 11), or 125 μM $ZnSO_4$ (lane 12) in the presence of 10 mM DTT for 15 min at 37°C. Oxidative modifications of IRP2 were assessed by the assay for carbonyl detection together with non-treated IRP2 (lane 1) as described in Section 2. B: The same membrane was stained with Coomassie brilliant blue. C: 4 μg of recombinant IRP2 was incubated with the indicated concentrations of $AlCl_3$ and 10 mM DTT in the presence of either 10 μM (lanes 14–17) or 20 μM (lanes 18–21) $FeCl_3$ for 15 min at 37°C. Oxidative modifications of IRP2 were monitored and compared with non-treated IRP2 (lane 13). D: The same membrane was stained with Coomassie brilliant blue.

staining of the membrane revealed that equal amounts of IRP2 protein were loaded (Fig. 4D). Collectively, these results indicate that aluminum competitively inhibits the iron-catalyzed oxidative modification of IRP2.

4. Discussion

IRPs are the central regulators of iron metabolism in mammalian cells. They act by modulating the expression of the molecules involved in iron metabolism through the specific binding to IREs on the transcripts of the respective genes under conditions of iron depletion. Administration of iron to Neuro 2A cells leads to the induction of hsp70 [20], as the metal is an inducer of oxidative stress. Thus, identification of iron-independent inducers of IRPs has broad biological implications. In this report, we identified aluminum as an IRP2-specific enhancing agent.

Aluminum is known to be taken up by cells via the Tf/TfR pathway [21]. Therefore, the effect of aluminum on the accumulation of IRP2 may be due to its inhibition of transferrin receptor-mediated iron uptake, which leads to a decrease in the cellular iron concentration. However, this is not the only explanation as aluminum also induces the IRE binding activity of IRP2 in the presence of hemin, which is known to be taken up via a transferrin-independent pathway (Fig. 1E,F).

The IRE binding activity of IRP2 has been shown to be regulated by rapid degradation of the protein in the presence of iron [9]. In addition, the oxidative modification of IRP2 by iron has been shown to be the first event in the iron-dependent degradation of the protein via the ubiquitin-proteasome pathway [16]. It has been shown that the direct binding of iron to proteins leads to metal-catalyzed oxidation of amino acid residues in the vicinity of the metal binding sites in the presence of reducing agents and molecular oxygen [22]. However, the iron binding site(s) of IRP2, which appears to be critical for the oxidative modification of the protein, has not been identified. When added to cultured cells, manganese, copper, and zinc could not induce the IRE binding activity of IRP2 in cells (Fig. 1A). Neither copper nor zinc could inhibit the iron-catalyzed oxidation of IRP2 in vitro even when excess amounts of the metals are used (Fig. 4A). We showed aluminum induced IRE binding activity of IRP2 in cells (Fig. 1C–G) and inhibited oxidation of IRP2 in vitro (Fig. 4C,D). These effects of aluminum were competitive to that of iron. Taken together, these observations strongly indicate that iron binding site(s) are present in IRP2 and that the affinity of aluminum to the iron binding site of IRP2 seems to be much higher than other metals. Hanson et al. have reported that cobalt increased the amount of IRP2 protein, suggesting that cobalt also has substantial affinity for the iron binding site of IRP2 [23]. However, cobalt cannot perturb iron metabolism through IRP2 since they also showed that cobalt-induced IRP2 lacked IRE binding activity [23].

The iron-independent induction of the IRE binding activity leads to enlargement of the cytoplasmically available iron pool, which results in an increase in reactive oxygen species in cells [12]. IRP2 is preferentially expressed in the intestine and brain [9]. In the neuronal cell line, Neuro 2A, IRP2 was stabilized by aluminum. Aluminum has been shown to be the critical agent for dialysis dementia [24]. Our results suggest the possibility that the neurotoxicity induced by aluminum may be provoked by the perturbation of cellular iron regulation through stabilization of IRP2. Accumulation of reactive iron, aluminum, IRP2 has been noted in the tangles of Alzheimer lesions [25–27], but the involvement of aluminum in the pathogenesis of the disease is still controversial. Therefore, the identification of other inducing agents of the IRE binding of IRP2, and further analyses of the relationship between neurotoxicity and the accumulation of IRP2 will elucidate the roles of IRP2 in those disorders.

Acknowledgements: This work was supported by grants from the Ministry of Education and Science and the Ministry of Health and Welfare, Japanese Government.

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