Aluminum Taken Up by Transferrin-Independent Iron Uptake Affects the Iron Metabolism in Rat Cortical Cells

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We previously demonstrated that cultured human fibroblasts internalize iron *via* trans-
ferrin-independent iron uptake (Tf-IU), redox, and receptor-mediated endocytosis uptake
systems [Oshiro, S., Nakajima, H., Markello, T., Krasnewich, D., Bernardini, I., and Gahl,
W.A. (1993) *J. Biol. Chem.* 268, 21586-21591]. Of these iron transport systems, the Tf-IU
system is involved in the accumulation of transition metals in various mammalian cells. It
is also known that in experimental animals fed aluminum (Al), Al at micromolar level
selectively accumulates in the brain. In the present study, we examined the effects of Al
accumulated in the brain cells on iron transport by the Tf-IU system and iron metabolism,
using primary cultures from fetal rat cerebral cortex. Pretreatment of cells with 200 μM
Al-nitrilotriacetate upregulated the Tf-IU system for iron. Moreover, of various metals
tested, Al markedly upregulated the Tf-IU activity. To examine the influence of Al on iron
metabolism, the interaction between Al accumulated in the cells and iron-responsive
element binding protein (IRE-BP), a cellular iron regulator, was examined by Northern
blot analysis, and activity assay: Al decreased the Tf receptor mRNA level and increased
the aconitase activity of IRE-BP. The increase of aconitase activity by Al was also observed
in vitro. These results suggest that Al accumulated in cortical cells affects iron metabolism.

Key words: aluminum toxicity, iron, neuronal cells, transferrin-independent iron uptake,
transferrin receptor.

Mammalian cells take up iron *via* transferrin (Tf) recep-
tor-mediated endocytosis and the Tf-independent iron
uptake (Tf-IU) system, which is involved in the accumula-
tion of transition metals in a wide variety of cultured cells
(*1-7*). Although Tf-dependent iron uptake is regulated
through a post-transcriptional mechanism, the Tf-IU sys-
tem is upregulated by transition metals and Ca²⁺ (*3, 6*).

Aluminum (Al) is one of the most abundant element in
the earth's crust and in the environment. Though, until
recently Al has been considered harmless, Al bound to Tf
passes through the blood-brain barrier to enter neuronal
cells *via* the Tf receptor (*8*). During chronic systemic
administration to rats, Al accumulates at 100-400 μM
levels in the brain (*9*). Several recent studies have iden-
tified Al as a potential risk factor in the pathology of
Alzheimer's disease (AD), although this remains contro-
versial. Apart from its possible involvement in the neuro-

pathology of AD, Al has various neurotoxicities; for exam-
ple, it inhibits dephosphorylation of tau factor by protein
phosphatase 2A in synaptosomes and calpain-mediated
proteolysis (*10, 11*). Furthermore, its binding properties to
proteins are similar to those of heavy metals, and many
proteins are potential targets (*12*). Thus, Al is a well
established neurotoxin both *in vivo* and *in vitro*. However,
it remains unknown how Al accumulates in brain cells and
whether this metal affects iron metabolism.

This evidence led us to undertake investigations to
clarify the relation between the Tf-IU system and Al
accumulation in brain cells. The data obtained here sug-
gests that Al accumulation by Tf-IU disturbs iron metabo-
lism and can be toxic to cortical cells.

MATERIALS AND METHODS

Primary Cultures from Fetal Rat Cerebral Cortex—
Primary cultures were prepared from 18-day embryos
(E18) of Wistar rats by a method described previously
(*13*), which is a modification of an established method (*14*).
Cerebral cortices were dissected and rinsed twice with
Dulbecco's modified Eagle's medium (DMEM; Gibco),
followed by incubation at 37°C in Ca²⁺- and Mg²⁺-free PBS

Abbreviations: Al, aluminum; Tf, transferrin; Tf-IU, transferrin-in-
dependent iron uptake; Fe, iron; IRE-BP, iron-responsive element
binding protein; NTA, nitrilotriacetate; AD, Alzheimer's disease.

containing 0.02% papain, 0.02% bovine serum albumin, 0.02% D-cysteine HCl, and 0.5% glucose for 15 min. After the digestion, the cells were triturated using a siliconized Pasteur pipette, filtered on steril paper filter, washed twice with DMEM, and collected by centrifugation at $800 \times g$ for 5 min. The separated cells were resuspended in 5% new-born calf serum (Gibco), 5% heat-inactivated horse serum (Gibco), 90% DMEM containing 1 mM sodium pyruvate, 50 units/ml penicillin G, 0.025 mg/ml streptomycin sulfate, and 1.2 mg/ml $NaHCO_3$. The cells were plated on 35-mm poly(D-lysine)-coated dishes (Falcon) and grown in the same medium. The primary cultures were maintained at 37°C in 97% air/3% CO_2 at nearly 100% relative humidity, and used in all the experiments.

Uptake of ^{55}Fe-Citrate via the Tf-IU System—^{55}Fe-citrate (Du Pont–New England Nuclear) was used for the measurement of iron transport activity *via* the Tf-IU system according to a previous reports (5, 7). Primary cultures from fetal rat cerebral cortex were washed three times with cold sodium phosphate-buffered saline (SPB), pH 7.4, to remove Tf contained in horse serum. Moreover, the endogenous Tf in the cells was removed and depleted by incubation for 1 h in serum-free MEM. During this incubation, Tf receptors (Tf-Rc) recycle several times from the inside of the cell to the cell surface and transport endogenous Tf outside the cells. Once apo Tf is excreted to the medium, it no longer binds to the Tf-Rc. The cells were washed twice with cold SPB to remove the endogenous Tf prior to measurement of iron uptake. The cells were incubated with ^{55}Fe-citrate for appropriate times and washed three times with cold SPB. The cell pellet was solubilized in Solvable and mixed with Econoflour, then the radioactivity was measured. When the iron uptake of cultured cells was characterized under the above conditions, its properties corresponded to those of Tf-IU. This shows that there was no contamination of exogenous and endogenous Tfs in the medium.

Effect of Al or Fe Loading of the Cell Cultures on Tf-IU Activity—The cell cultures from fetal rat cerebral cortex were exposed to 20, 50, 100, and 200 μM Al-nitrilotriacetate (Al-NTA) or Fe-citrate at 37°C for 24 h. The cells were washed three times with PBS, and incubted with 1 μM ^{55}Fe-citrate in serum-free medium at 37°C for 10 min. Al-NTA was prepared as described below. The Al concentrations used were chosen based on those observed in an animal model (11, 15). After incubation, cells were washed and solubilized, and the uptake was determined. Relative uptake was expressed as the ratio of the amount of ^{55}Fe taken up by metal-loaded cortical cells to uptake by the control cells without treatment. The cell density used for all experiments was 3×10^6 cells/well.

Effect of Various Metals on the Tf-Independent Iron Transport System in Rat Cortical Cells—Rat cortical cells were washed three times and incubated in serum-free DMEM for 1 h. Thereafter, the cells were incubated with 200 μM of various transition metals for 24 h at 37°C. $AlCl_3$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $CdCl_2$, $NiCl_2$, and $FeCl_3$ were chelated with NTA. The nitrilotriacetates were made by addition of each metal to a 5-fold molar excess of the disodium salt of NTA. The Tf-IU activity was measured as described above.

Northern Blot Analyses—The cultured cells were exposed to Al-NTA or Fe-NTA at the indicated concentra-tions at 37°C for 24 h, and cytoplasmic RNAs were isolated from the cultured cells according to Chomczynsky and Sacchi (16). Extracted RNAs were denatured and spotted onto a nylon membrane. The filters were then incubated with random-primer-labeled DNA probes for 24 h at 45°C. After hybridization, the filters were washed first with $2 \times$ SSC (0.15 M NaCl, 15 mM sodium citrate) for 30 min, and then twice with $0.1 \times$ SSC, 0.1% SDS for 30 min. The filters were then exposed to imaging plates (Fuji Photo Film) at room temperature for 4 h, followed by quantification with a Fuji phosphor-imager, BAS 2000. Tf-Rc mRNA was detected using a *Hind*III fragment (3.5 kb) of human Tf Rc pcD plasmid (American Type Culture Collection) (17).

Aconitase Activity—Aconitase activity was measured by coupled aconitase and isocitrate dehydrase assay (18). The reaction components were 100 mM Tris·HCl (pH 8.0), 1 mM citrate, 1 mM NADP, and isocitrate dehydrogenase (100 μg/ml). The soluble fractions from the cortical cells were prepared by lysing cells in 20 mM HEPES, pH 7.5, 2 mM dithiothretol, 5% glycerol, 40 mM KCl containing 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride. The lysates were spun at $13,000 \times g$ for 10 min. The soluble fractions thus obtained were added to the above assay components and the production of NADPH was followed by measuring the change in absorption at 340 nm.

Chemicals—Aluminum chloride, NTA, and ferric citrate were obtained from Sigma. ^{55}Fe-citrate, Solvable, and Econoflour were purchased from Du Pont–New England Nuclear. $[\alpha-^{32}P]CTP$ was from Amersham. Other chemi-cals were of analytical grade, and double-distilled water was used. Human β-actin DNA probe was purchased from Toyobo, Tokyo.

RESULTS AND DISCUSSION

Aluminum Upregulates the Transferrin-Independent Iron Uptake System in Cortical Cells—The maximum serum aluminum (Al) concentration in normal individuals is about 20 μM (19). After chronic systemic administration to animals, Al accumulates at levels of 100 to 400 μM in the brain (9, 11). Therefore, first of all, we examined the effect of Al at micromolar level on transferrin-independent iron uptake (Tf-IU) which is involved in uptake of transition metals in various cultured cells (Fig. 1). Al began to upregulate Tf-IU at the physiological concentration. At the level accumulated in the brain in the animal model, Al increased the Tf-IU 1.2- to 2.2-fold over the control cells. Fe increased the Tf-IU 1.2- to 1.4-fold under the same conditions, showing that Al had a greater effect than Fe.

These results suggest that Al accumulated at micromolar level in an animal model can upregulate the Tf-IU system and cause accumulation of Fe in cortical cells.

Al More Markedly Upregulated the Tf-IU System of Cortical Cells Than Transition Metals—To compare the upregulation of Tf-IU by Al with those by various transi-tion metals, we measured the Tf-IU after the loading and removal of various metals. As shown in Fig. 2A, Al-nitrilo-triacetic acid (NTA) more markedly upregulated the Tf-IU system than Cu-, Zn-, Mn-, Cd-, Ni-, or Fe-NTA at 200 μM and the increase was more than 2.0-fold over the Tf-IU of Fe-loaded cells. To determine the specific Tf-IU to the cortical cells, we measured the Tf-IU in the cortical cells and human skin fibroblasts as a control after the upregula-
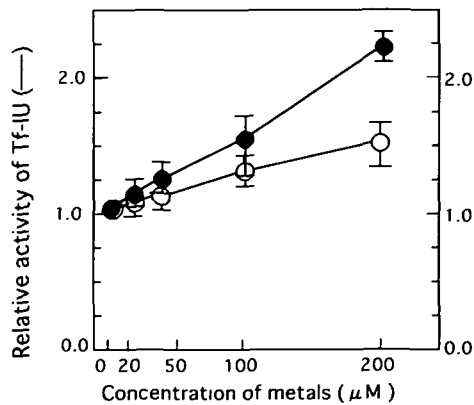
Fig. 1. **Upregulation of Tf-IU in rat cortical cells after the exposure to Al and Fe.** Primary cultures from fetal rat cerebral cortex were prepared as described in "MATERIALS AND METHODS." Cells were exposed to the indicated concentrations of Al-NTA and Fe-NTA for 24 h. Al-NTA was made as described in "MATERIALS AND METHODS." Tf-IU activities were measured after the removal of metals. Each point is the mean±SD of three separate experiments. Tf-IU of Al-loaded cortical cells, ●; Tf-IU of Fe-loaded cortical cells, ○.

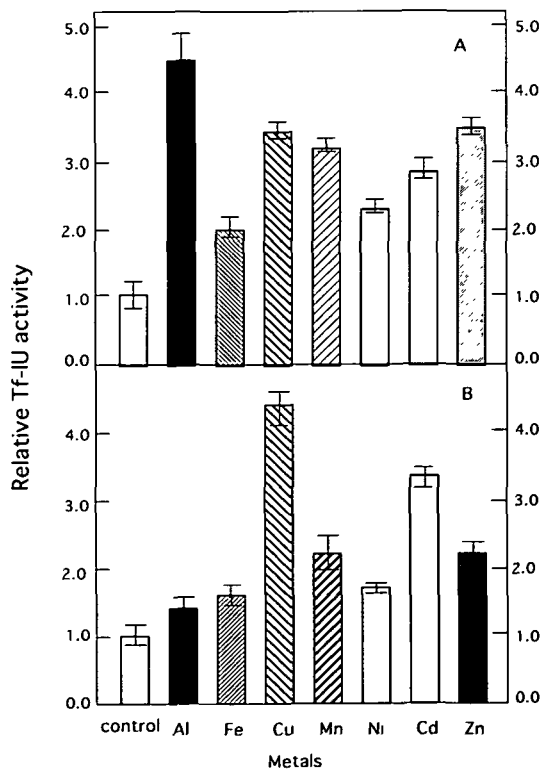Fig. 3. **Effect of Ca^{2+} on the Tf-IU in cultured cells from fetal rat cerebral cortex.** Cells were washed three times with Ca^{2+}-free Hank's balanced salt solution (HBSS) and then incubated in Ca^{2+}-free HBSS containing different concentrations of Ca^{2+}. The cells were then incubated with 1 μM ^{55}Fe-citrate for 10 min at 37°C. The Tf-IU activity represents the relative uptake with respect to the control. The cell density used was 3×10^6 cells/well. Results are means±SD of three experiments.

Fig. 2. **Effect of various metals on the Tf-independent iron uptake in rat cortical cells and fibroblasts.** Rat cortical cells (A) and human fibroblasts (B) were washed three times and incubated in serum-free MEM for 1 h. Thereafter, the cells were incubated with 200 μM Al-, Cu-, Zn-, Mn-, Cd-, Ni-, or Fe- NTA for 24 h at 37°C. The Tf-IU activity was measured as described in "MATERIALS AND METHODS." Results are means±SD of three experiments.

tion. As shown in Fig. 2B, Al did not strongly upregulate the Tf-IU in the control cells, compared with the cortical cells.
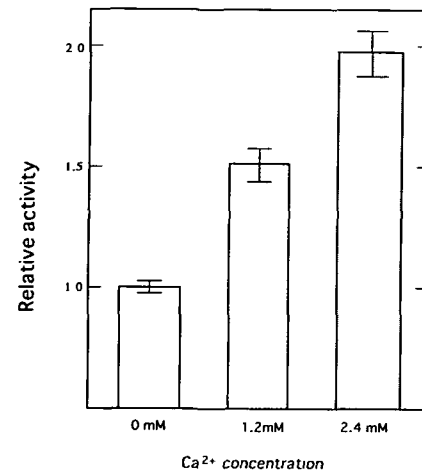
We also observed similar phenomena in HeLa cells. Thus, Al upregulated the Tf-IU system in rat cortical cells more markedly than did transition metals.

Ca^{2+} Increases the Tf-IU Activity—Smith and Smith reported that fibroblasts contain a cell surface receptor for transition metals, which stimulates the release of inositol phosphate, resulting in an increase in intracellular Ca^{2+} concentration (20). Therefore, we tested the effect of Ca^{2+} on Tf-IU in cortical cells. At physiological concentrations in cerebrospinal fluid and plasma (1.2 and 2.4 mM) (21), Ca^{2+} resulted in 2- and 3-fold increases above control cells without Ca^{2+} (Fig. 3) suggesting that Ca^{2+} also upregulates the Tf-IU and causes accumulation of Fe in the cortical cells under physiological conditions. In human fibroblasts, extracellular Ca^{2+} resulted in an increase of intracellular Ca^{2+} at physiological and supraphysiological concentrations (3). Our observations on the effect of Ca^{2+} also suggest that the effect of iron on the Tf-IU system might result from an iron-induced change in the intracellular Ca^{2+}. Thus, Al, transition metals and Ca^{2+} upregulated the Tf-IU in the cortical cells.

Al Increases Tf-IU in Neuronal Cells by Increase in V_{max} without a Change of K_m—We showed that Al- and transition metal-loading increased the Tf-IU activity in the cortical cells (Fig. 2). Next, we examined the effects of Al- and Fe-loading on the kinetics of ^{55}Fe uptake to clarify the mechanism by which Al upregulates the Tf-IU system in cortical cells (Fig. 4). From the kinetic data in Al- and Fe-loaded cells, the apparent V_{max} for iron uptake increased from 12 pmol/mg cell protein/min (control cells) to 20 and 17 pmol/mg cell protein/min, respectively. However, the apparent K_m values for the uptake by Fe- and Al-loaded cells were approximately 37 μM, nearly the same as the value of control cells, showing that the increase in Tf-IU was due to an increase in V_{max} without a change of K_m. These results strongly suggest that Al is transported by the same or a similar mechanism (*e.g.* cryptic transporters) as
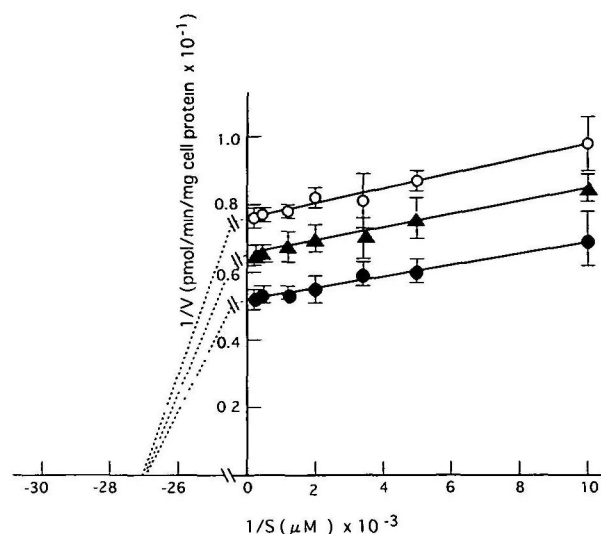
Fig. 4. **Effect of Al- or Fe-loading on the uptake of ^{55}Fe in rat cortical cells.** The cells were exposed to the specified concentrations of Al-NTA or Fe-NTA for 24 h at 37°C. After the removal of metals the cells were incubated with 1 μM ^{55}Fe-citrate for 10 min at 37°C, and washed with cold PBS. The solubilized cells were used as samples for determination of cell protein and radioactivity. Results are means±SD of four experiments. ●, Al-NTA; ▲, Fe-NTA; ○, control.

Fig. 5. **Northern blot analysis of Tf-Rc mRNA in Al- or Fe-loaded cortical cells.** Fetal rat cortical cells were exposed to Al-NTA or Fe-NTA at 0–200 μM at 37°C for 24 h, then cytoplasmic RNAs were isolated from cultured cells according to Chomczynsky and Sacchi (*16*). Northern blot analysis was decribed in "MATERIALS AND METHODS." (A) Northern blot hybridization in rat cortical cells treated with 0, 50, 100, and 200 μM Al-NTA (a, b, c, and d). (B) Northern blot hybridization in the cells treated with 0, 50, 100, and 200 μM Fe-NTA (e, f, g, and h). (C) shows the relative amount of Tf-Rc mRNA in (A). (D) shows the relative amount of Tf-Rc mRNA in (B). The relative amounts of (C) and (D) are normalized with respect to the amount of β-actin mRNA. Results are means±SD of three experiments.

is observed in human skin fibroblasts (*3*).

Al Taken Up by the Tf-IU System Suppresses the Expression of Tf Receptor mRNA in Cortical Cells—We showed that Al- and Fe-loading increase Tf-IU in the cortical cells (Fig. 1). However, it is unclear whether Al accumulated in the cells affects the cellular iron metabolism. Therefore, we examined the effect of Al on the function of iron-responsive element binding protein (IRE-BP), an iron central regulator. The cellular Fe level is regulated by the interaction between IRE-BP and IRE located in the 3'-untranslated region of Tf receptor (Tf-Rc). When the cellular Fe level decreases, the stability of the Tf receptor (Tf-Rc) mRNA increases. Conversely, when the cellular Fe concentration increases, the longevity of Tf-Rc mRNA is reduced and the mRNA level is decreased (*22, 23*). Thus, Tf-Rc mRNA level is post-transcriptionally controlled by Fe. In order to examine the modulation of the Tf-Rc mRNA level by Al taken up, the relative amount of Tf-Rc mRNA of the Al-loaded cells was measured by slot blot analysis using human Tf-Rc cDNA as a probe, compared with that of Fe-loaded cells. Increasing concentration of Al decreased the Tf-Rc mRNA level (Fig. 5A), as in the case of Fe (Fig. 5B). To examine the expression of Tf-Rc on the cell surface after exposure to Al or Fe, we measured the Tf-binding activity of the cells at 4°C. The preincubation of cells with 200 μM AlCl$_3$ and Fe-citrate reduced Tf-binding by 75.5 or 55.6% of the control. These results suggest that Al is taken up by Tf-IU and suppresses Tf-Rc mRNA expression to reduce Tf-Rc numbers as iron does, and that Al affects iron homeostasis.

Al Accumulated in the Cells Increases the Aconitase Activity—IRE-BP was recently cloned and shown to have 30% identity with mitochondrial aconitase (*24, 25*). This similarity has led to the proposal of a model for a mechanism by which IRE-BP might act as an iron sensor (*26*). IRE-BP contains an Fe-S cluster and, in its enzymatically
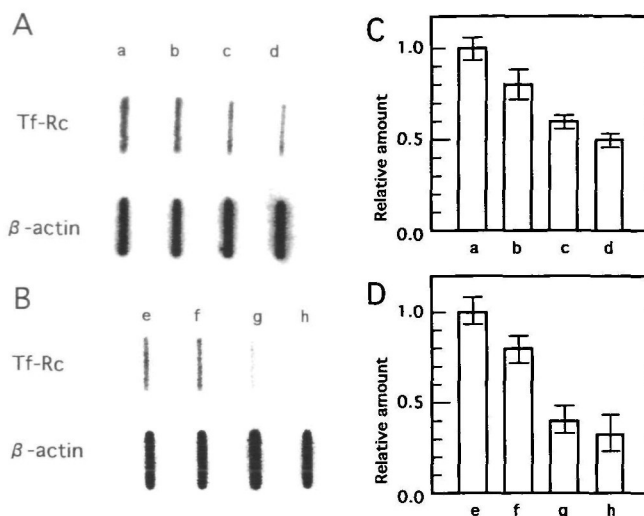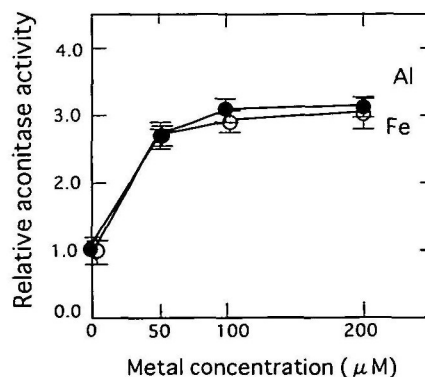

Fig. 6. **Effect of Al and Fe on aconitase activity in rat cortical cells after the exposure to Al and Fe.** Cells were exposed to Al-NTA and Fe-NTA at indicated concentrations at 37°C for 24 h. Aconitase activities were measured as described in "MATERIALS AND METHODS" after the removal of metals. Each point is the mean±SD of three separate experiments. Tf-IU of Al-loaded cortical cells, ●; Tf-IU of Fe-loaded cortical cells, ○.

active form, contains a [4Fe-4S] cluster. However, when iron is depleted, it readily loses one iron to become a [3Fe-4S] cluster. In this state, IRE-BP has little enzymatic activity. The enzymatic activity can be readily restored by reloading the [3Fe-4S] cluster with iron. Therefore, we investigated whether IRE-BP acquires aconitase activity by binding to Al. After Al-loading, the aconitase activity increased with increasing concentrations of Al, as is the case for Fe (Fig. 6). To test whether Al directly binds with
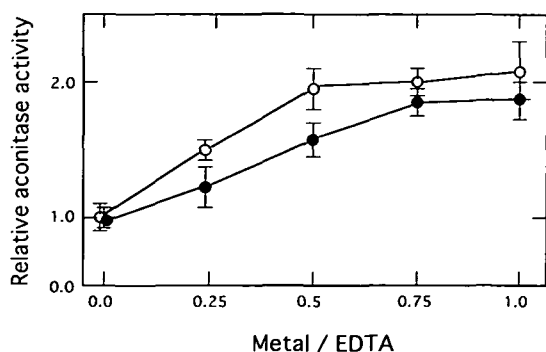
Metal / EDTA

Fig. 7. Effect of Al and Fe on aconitase activity in IRE-BP partially purified from rat cerebral cortex. IRE-BP partially purified from rat cerebral cortex was anaerobically activated in 80 mM Hepes (pH 7.5) containing 5 mM dithiothreitol, 100 μM EDTA, and various concentrations of Al-NTA and Fe-NTA at room temperature for 30 min. Aconitase activities were measured as described in "MATERIALS AND METHODS." Each point is the mean$\pm$SD of three separate experiments. Aconitase activity activated by Al-NTA, ●; aconitase activity activated by Fe-NTA, ○.

apo-aconitase, we partially purified cytosol aconitase (IRE-BP) from rat cerebral cortex by a reported method (27). When the IRE-BP was exposed to Al, the increase of activity was dependent on Al concentration (Fig. 7). Moreover, we demonstrated by band shift assay using iron-responsive element that the increasing concentration of Al decreased the amount of IRE-BP bound to IRE (S. Oshiro, unpublished information). These results suggest that Al accumulated in the cells increases aconitase activity by binding to IRE-BP.

Taken together, our data suggest that Al accumulates in the cortical cells *via* the Tf-IU system, which is upregulated by Al at the physiological concentration. Thus, Al affects the iron metabolism in the cells. These results may help to explain the increased accumulation of Al both in the animal model and in AD brain. The mechanism by which the Tf-IU system is upregulated by Al is under investigation.

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