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Involvement of CTR1 and ATP7A in lead (Pb)-induced copper (Cu) accumulation in choroidal epithelial cells

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Abstract

The blood–cerebrospinal fluid barrier (BCB) plays a key role in maintaining copper (Cu) homeostasis in the brain. Cumulative evidences indicate that lead (Pb) exposure alters cerebral Cu homeostasis, which may underlie the development of neurodegenerative diseases. This study investigated the roles of Cu transporter 1 (CTR1) and ATP7A, two Cu transporters, in Pb-induced Cu accumulation in the choroidal epithelial cells. Pb exposure resulted in increased intracellular ^{64}Cu retention, accompanying with up-regulated CTR1 level. Knockdown of CTR1 using siRNA before Pb exposure diminished the Pb-induced increase of ^{64}Cu uptake. The expression level of ATP7A was down-regulated following the Pb exposure. ATP7A siRNA knockdown, or PCMB treatment, inhibited the ^{64}Cu efflux from the cells, while the following additional incubation with Pb failed to further increase the intracellular ^{64}Cu retention. Cu exposure, or intracellular Cu accumulation following the tetracycline (Tet)-induced overexpression of CTR1, did not result in significant change in ATP7A expression. Taken together, these data indicate that CTR1 and ATP7A play important roles in Cu transport in choroidal epithelial cells, and the Pb-induced intracellular Cu accumulation appears to be mediated, at least in part, via the alteration of CTR1 and ATP7A expression levels following Pb exposure.

Keywords

Blood–cerebrospinal fluid barrier; Lead; Copper; Copper transporter 1; ATP7A

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Conflict of interest

The authors declare that there is no any conflict of interest with regards to financial, personal, or their relationships with other people or organizations for the studies presented in this article.

1. Introduction

Lead (Pb) is a well-known neurotoxicant and a risk factor for neurologic disorders. It has been well documented that Pb exposure results in developmental neurotoxicity in children, including decreased intelligence quotient (IQ) (Huang et al., 2012; Tong et al., 1998), cognitive deficits (Braun et al., 2012; Boucher et al., 2012; Kuhlmann et al., 1997), poor attention span (Calderón et al., 2001; Bellinger et al., 1992), and increased aggression (Homady et al., 2002; Needleman et al., 1996). Recent studies reported that Pb exposure may also correlate with some neurodegenerative disorders, such as Parkinson's disease (Alimonti et al., 2007; Coon et al., 2006) and Alzheimer's disease (Bakulski et al., 2012; Gu et al., 2011, 2012; Basha and Reddy, 2010; White et al., 2007).

Previous studies demonstrated that Pb exposure can result in accumulation of copper (Cu) in brain tissue in experimental animals and in cultured astrocytes (Sierra et al., 1989; Qian et al., 1995, 1999, 2005). Although Cu is an essential trace element required by all living things, excess copper is detrimental to cell survival as free copper ions are readily to interact with oxygen to form toxic free radicals (Tiffany-Castiglioni et al., 2011). Cumulative evidences have implied that an imbalanced Cu homeostasis in the central nervous system (CNS) correlates with the pathogenesis of neurodegenerative disorders (Skjørringe et al., 2012; Squitti, 2012; Zheng et al., 2010; Gaggelli et al., 2006; Cuajungco et al., 2005; Rogers and Lahiri, 2004). Therefore, the accumulation of Cu ions in brain following Pb exposure may result in oxidative damage in neuronal cells and contributes to the development of neurodegenerative disorders.

Brain Cu homeostasis is regulated by two barrier systems: the barrier that restricts material fluxes between the blood and brain interstitial fluid is defined as the blood–brain barrier (BBB), while the one that restricts material traffic between blood and CSF is named the blood–cerebrospinal fluid barrier (BCB) (Zheng and Monnot, 2012). The choroid plexus, a highly vascularized tissue, constitutes the BCB. As a barrier between the blood and CSF, the choroid plexus plays an important role in regulating of essential metal ions in the CSF, and removing metabolites or unwanted materials from brain extracellular fluid to the blood (Zheng, 2001; Zheng and Zhao, 2002; Zheng et al., 2003). Several molecules mediating copper transport (CTR1, DMT1, ATOX1, ATP7A and ATP7B) that were identified in the intestinal wall by previous studies were also found to express in choroidal epithelial cells (Shi et al., 2008; Choi and Zheng, 2009; Monnot et al., 2012; Chen et al., 2012); however, the knowledge on the functions of these transport proteins in regulating Cu transport at the BCB and Pb effect on these proteins is incomplete.

Copper transporter 1 (CTR1) protein is a high-affinity Cu ion importer that is structurally and functionally conserved in yeast, plants, fruit flies, and humans and that, in all of these organisms, is localized to the plasma membrane and intracellular vesicles. Previous studies have suggested a critical role for CTR1 in dietary copper absorption in the intestinal epithelial cells (Nose et al., 2010; Nadella et al., 2007; Handy et al., 2002; Gaggelli et al., 2006). Our former works have shown that CTR1 expresses abundantly in the BBB, BCB, and brain parenchyma, and plays a major role in cellular Cu uptake (Choi and Zheng, 2009; Zheng et al., 2012). Divalent metal transporter 1 (DMT1) is a proton driven transporter that

nonselectively transports multiple divalent metals including Mn, Cu, cobalt (Co), zinc (Zn), cadmium (Cd) and lead (Pb) (Gruenheid et al., 1995; Gunshin et al., 1997). Inside the cells, Cu ions can be transported by chaperones COX17 to the mitochondria and by Cu chaperone antioxidant-1 (ATOX1) to ATPase, Cu^{2+} transporting, alpha polypeptide (ATP7A) or ATPase, Cu^{2+} transporting, beta polypeptide (ATP7B) situated in the trans-Golgi network (TGN) (Hamza et al., 2003). Our previous study demonstrated that both DMT1 and ATOX1 express in BCB cells and play a role in cellular Cu transport (Monnot et al., 2012). ATP7A and ATP7B, two Cutransporting P-type ATPase, are known to regulate cellular Cu efflux in mammals (Wang et al., 2011). When the intracellular Cu level rises, ATP7A and ATP7B translocate from the Golgi network and carry Cu to the cellular membrane for efflux from the cell (Lutsenko et al., 2007). Dysfunction in these Cu ATPases in brain barriers disrupts brain Cu homeostasis. Shutdown of ATP7A gene expression was found to result in Cu accumulation in brain capillaries of macular mutant mice (Yoshimura et al., 1995) with Cu deficiency in the brain. Former works have demonstrated that the expression of ATP7A in BCB is 3–4-fold higher than that in the BBB. In contrast, the expression of ATP7B is about 4–5-fold higher in the BBB compared to the BCB (Choi and Zheng, 2009; Zheng and Monnot, 2012; Chen et al., 2012). These data suggest that ATP7A may be a major molecule required for the regulation and maintenance of cerebral Cu homeostasis in the BCB.

As CTR1, DMT1, ATOX1 and ATP7A are abundantly expressed in the choroid plexus, we hypothesized that Pb exposure may disrupt the Cu homeostasis in the CNS by altering the expression of these Cu transporters. The purpose of this study was to investigate the mechanism whereby Pb exposure altered Cu homeostasis in the BCB by using an immortalized choroidal Z310 cell line derived from rat choroidal epithelial cells. More specifically, we determined the roles of these Cu transporters to Pb-induced Cu accumulation in the BCB.

2. Materials and methods

2.1. Reagents

Chemicals and reagents were obtained from the following sources: lead acetate ($Pb(AC)_2$), copper chloride ($CuCl_2$), methyl thiazolyl tetrazolium (MTT), RIPA cell lysis buffer, Laemmli sample buffer and p-chloromercuribenzoate (PCMB) from Sigma Aldrich; Dulbecco's modified Eagle's medium (DMEM) from Cellgro; fetal bovine serum (FBS), antibiotic–antimycin solution, and Opti-MEM medium from Gibco; TRIzol reagent, Lipofectamine 2000, and tetracycline (Tet) from Invitrogen; reverse transcription kit from Applied Biosystems. FastStart Universal SYBR Green Master (Rox) from Roche. BCA Protein Assay Kit and ECL Western Blotting Substrate from Pierce. $^{64}CuCl_2$ (specific activity 15–30 mCi/μg) was obtained from Washington University at St. Louis. All reagents were analytical grade, HPLC grade, or the best available pharmaceutical grade.

2.2. Cell culture

Z310 choroidal epithelial cells were cultured as previously described (Zheng et al., 2012; Wang et al., 2006; Zheng and Zhao, 2002). Briefly, the cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml

of gentamycin in a humidified incubator with 95% air–5% CO_2 at 37 °C. Trypsin–EDTA was used to digest cells during subculture and Z310 cells were passaged (1:12–16) twice a week. When treated with Pb, 0, 2.5, 5 or 10 μM $Pb(AC)_2$ was added to the cells 24 h after initial seeding, and the following studies were performed.

2.3. Cell viability assay

Cell viability was measured by using MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Z310 cells (5×10^3 cells per well) were seeded into 96-well microtiter plates. Following the Pb^{2+} treatment, 20 μl MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for 4 h at 37 °C. Then, the supernatant layer was removed, and 150 μl of dimethyl sulfoxide was added into each well. The plates were shaken vigorously for 10 min to ensure complete solubilization. MTT metabolism was quantitated spectrophotometrically at 490 nm in a microplate reader.

2.4. ^{64}Cu uptake study

$^{64}CuCl_2$ (specific activity 15–30 mCi/μg) was produced at Washington University by cyclotron irradiation of an enriched ^{64}Ni target by using methods reported (McCarthy et al., 1997). Z310 cells of each group were washed twice with PBS and incubated at 37 °C for 60 min with serum-free medium containing 5 μCi/ml ^{64}Cu and 5 μM $CuCl_2$. After the incubation, cells were washed with PBS for 3 times and then were scraped into microcentrifuge tubes. The cell-retained ^{64}Cu radioactivity was determined with a gamma-counter (PerkinElmer 1480 WIZARD 3″) as well as protein concentration determined by the Bradford assay.

2.5. RNA isolation and quantitative real-time PCR

Total RNA from cells was extracted using the TRIzol reagent. The quality of RNA (A260/A280) was 1.8–2.0 for all RNA preparations. 0.5 microgram of total RNA from each sample was reverse-transcribed in cDNA at 48 °C for 1 h using Moloney murine leukemia virus reverse transcriptase. For qPCR analysis, the levels of mRNAs were quantified by using FastStart Universal SYBR Green Master (Rox) in a Mx3000p Real-Time PCR System. All amplification reactions were conducted in triplicate. Relative expression ratios were calculated by the $\Delta\Delta C_t$ method where C_t is the threshold cycle value (Pfaffl, 2001). The GAPDH gene was used for normalization. We previously checked that amplification efficiencies of both target gene and endogenous reference were approximately similar by looking at how C_t varies with template dilutions.

The following primers were used in experiments: rat CTR1 (accession No. NM 133600.1), sense, 5′-TCG GCC TCA CAC TCC CAC GA-3′, and antisense, 5′-CGA AGC AGA CCC TCT CGG GC-3′; rat DMT1 (accession No. NM 013173.1), sense, 5′-TCG CAG GCG GCA TCT TGG TC-3′, and antisense, 5′-TAC CGA GCG CCC ACA GTC CA-3′; rat ATOX1 (accession No. NM 053359.2), forward primer 5′-AGT TCT CTG TGG ACA TGA CCT G-3′, reverse primer 5′-AAG GTA GGA GAC CGC TTT TCC T-3′; rat ATP7A (accession No. NM 052803.1), sense, 5′-CCC TCA ACA GCG TCG TCA CT-3′, and antisense, 5′-GAC TAG CAG CAT CCC CAA AGG-3′; rat GAPDH (accession No. NM 017008.3),

sense, 5′-TGC CGC CTG GAG AAA CCT GC-3′, and antisense, 5′-AGC AAT GCC AGC CCC AGC AT-3′.

2.6. Cell lysis and Western blotting

Monolayer cells were washed three times with ice-cold PBS followed by incubation in RIPA cell lysis buffer (Sigma) with PMSF and Protease Cocktail on ice for 10 min. Cells were scraped into microcentrifuge tubes and then briefly sonicated, centrifuged for 15 min at 12,000 × *g* to remove the cellular debris. Postnuclear supernatants were analyzed for protein concentration using the BCA method. For Western blotting, samples were boiled in Laemmli sample buffer and electrophoresed on 12% Tris–glycine SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore), which was first blocked with 5% milk powder in TBST (0.1% Tween 20 in TBS) for 1 h and then incubated overnight at 4 °C with the corresponding primary antibodies, diluted in blocking solution (rabbit anti-CTR1, 1:300, Santa Cruz; rabbit anti-DMT1, 1:500, Alpha Diagnostic International; rabbit anti-ATOX1, 1:200, Sigma; rabbit anti-ATP7A, 1:500, Santa Cruz; mouse anti-β-actin, 1:10,000, Sigma). After incubation with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature, the blots were developed using ECL Western Blotting Substrate and exposed to film (Kodak). The integrated densities of each band on the film were quantified using the Image Lab software (Bio-Rad).

2.7. siRNA knockdown

The siRNA transfection was performed following the manual of Silencer® siRNA Starter Kit were obtained from Ambion (Applied Biosystems). Briefly, the Z310 cells were ~50% confluent in 6-well plates at transfection. The cells were incubated with either stealth small interfering RNA (siRNA) duplexes against ATP7A, or scramble siRNA Negative Control in Opti-MEM medium for 8 h, and then an equal volume of normal DMEM was added to each well. The medium was replaced with fresh DMEM 24 h after the transfection. Cells were harvested for the determination of mRNA and protein levels 48 h following the siRNA transfection. Candidate sequences for rat CTR1 knockdown were self-designed by using the Ambion website. The forward target sequence is: 5′-CCAUCCUUAUGGAGACACAtt-3′; reverse target sequence: 5′-ttGGUAGGAAUACCUCUGUGU-3′ . A predesigned silencer select sequences for knocking down ATP7A (ID:s128718) knockdown were obtained commercially from Ambion.

2.8. Statistical analysis

All data are reported as the mean ± SD from at least three independent biological samples. Difference between means was determined by one-way ANOVA followed by a least significant-difference test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Pb exposure results in intracellular Cu accumulation

Z310 cells were treated with $Pb(AC)_2$ at different concentrations (0, 1, 2.5, 5, 10, 20, 50 and 100 μmol/L) for 24 h, and then the cell viability of Z310 cells was assessed by MTT assay.

The cell viability significantly decreased following the Pb treatment at 20 μmol/L and higher concentrations (Fig. 1A). Therefore, 2.5, 5, and 10 μmol/L were chosen as the working concentrations of Pb exposure in this study.

For the ^{64}Cu uptake study, Z310 cells were exposed to 2.5, 5 and 10 μmol/L $Pb(AC)_2$ for 24 h, and then were incubated with 5 μCi/ml ^{64}Cu and 5 μmol/L $CuCl_2$ in serum-free medium for another 1 h, followed by γ-counting. The results showed that the cellular ^{64}Cu retention was increased by 1.4-, 2.3- and 2.4-fold, respectively, following the treatment of Pb (Fig. 1B). To investigate the impact of Pb exposure on the cellular Cu efflux, Z310 cells were treated with 5 μCi ^{64}Cu and 5 μmol/L $CuCl_2$ in serum-free medium for 1 h, followed by 3 washes with PBS, and then the cells were incubated with fresh DMEM, or with DMEM containing 2.5, 5 and 10 μmol/L $Pb(AC)_2$. The intracellular ^{64}Cu retention was determined 24 h later. The results of γ-counting revealed that after the additional Pb treatment for 24 h, the Cu-preloaded cells retained 1.6-, 2.0-, and 2.3-fold higher levels of ^{64}Cu, respectively, when compared with their controls (Fig. 1C). These results clearly demonstrated that the Pb exposure results in Cu accumulation in the cells.

3.2. Effects of Pb exposure on the expression levels of Cu transporters

To explore whether the increased intracellular Cu level is due to alteration of Cu transporters following the Pb exposure, the expression levels of CTR1, DMT1, ATOX1, and ATP7A in Z310 cells were determined. The quantitative real time PCR (qRT-PCR) showed that following the treatment with 2.5, 5, and 10 μmol/L $Pb(AC)_2$ for 24 h, CTR1 mRNA level significantly increased by 21.8%, 43.1% and 49.2%, respectively. The mRNA levels of DMT1 and ATOX1 were not significantly changed upon the addition of Pb at all doses. In the cells treated with 5 and 10 μmol/L $Pb(AC)_2$, ATP7A mRNA level decreased by 35.8% and 44.7%, respectively (Fig. 2A). Further investigation of protein expression by Western blotting confirmed the up-regulation of CTR1 protein and down-regulation of ATP7A protein, and the protein levels of DMT1 and ATOX1 remained unchanged in the Pb-treated Z310 cells (Fig. 2B). These results above suggested that CTR1 and ATP7A may be involved in the Pb-induced intracellular Cu accumulation.

3.3. CTR1 siRNA knockdown prevented the Pb-induced intracellular Cu accumulation

To understand the roles of CTR1 and ATP7A in the Pb-induced intracellular Cu accumulation, CTR1 or ATP7A siRNA knockdown was performed before the Pb exposure. The siRNA duplexes against CTR1 caused ~59.1% reduction in the CTR1 mRNA level relative to the scramble siRNA transfected cells (negative control, NC), and Pb exposure for 24 h after CTR1 siRNA knockdown failed to up-regulate the mRNA level of CTR1 (Fig. 3A). Western blotting also confirmed the decreased expression of CTR1 on the protein level, and Pb exposure for 24 h following the CTR1 siRNA knockdown did not result in any significant increase of CTR1 protein level in these cells (Fig. 3B). Next, we investigated the impact of CTR1 knockdown on cellular Cu uptake. The data from γ-counting showed that CTR1 knockdown prevented the Pb-induced increase of cellular ^{64}Cu uptake (Fig. 3C). These data suggest that the up-regulation of CTR1 expression may contribute to the increased intracellular Cu level in Z310 cells following the Pb exposure.

3.4. Both ATP7A siRNA knockdown and inhibition resulted in elevated intracellular Cu retention

The cells treated with ATP7A siRNA showed a significant reduction of ATP7A mRNA (decreased by ~69.6%) and protein (decreased by ~75.9%) levels as compared with the scrambled siRNA controls, and the following Pb exposure for 24 h did not result in increased ATP7A expression levels in the ATP7A knockdown cells (Fig. 4A and B). In the ^{64}Cu uptake study, the cells were incubated with 5 μCi ^{64}Cu and 5 μmol/L $CuCl_2$ in serum-free medium for 1 h, followed by 3 washes with PBS, and then the cells were incubated with fresh DMEM, or with DMEM containing 5 μmol/L $Pb(AC)_2$. The results from γ-counting revealed that in the ATP7A knockdown cells, the cellular ^{64}Cu retention was approximately 4 times higher than that in the controls and the negative controls. Additional Pb treatment for 24 h did not result in further increase of the ^{64}Cu retention in the ATP7A knockdown cells (Fig. 4C).

PCMB is a specific inhibitor of ATP7A and has been used in previous work (Qian et al., 1998, 1997). PCMB treatment was carried out by incubate Z310 cells with 0.2 mM PCMB or vehicle (DMSO) for 30 min at 37 °C after 1 h incubation with ^{64}Cu, and then remove unreacted PCMB by rinsing the cells with PBS for 3 times, followed by incubation with fresh DMEM, or with DMEM containing 5 μmol/L $Pb(AC)_2$, for 24 h. The results of γ-counting showed the PCMB inhibited the efflux of ^{64}Cu, and additional Pb treatment for 24 h did not result in further increase of the cellular ^{64}Cu retention in the PCMB treated cells (Fig. 4D). These results suggested that the decreased ATP7A level or activity may account for the Pb-induced inhibition of Cu efflux in Z310 cells.

3.5. Elevated Cu level down-regulated the CTR1 protein level

As Pb exposure resulted in increased intracellular Cu level, it is possible that the elevated intracellular Cu level has a negative feedback on the CTR1 expression. To test this hypothesis, the cells were treated with 5, 25, and 50 μmol/L $CuCl_2$ for 24 h, and the levels of CTR1 protein and mRNA were determined by Western blotting and qRT-PCR. The results showed that the CTR1 protein level significantly decreased following the Cu treatment (Fig. 5A), while its mRNA level was not changed (Fig. 5B).

3.6. ATP7A level was not affected following the increased intracellular Cu level

To investigate whether the reduction of ATP7A expression is resulted from the Pb exposure or the increase of cellular Cu level, we determined the influence of the cellular Cu status on ATP7A expression. Following the exposure to 5, 25, and 50 μmol/L $CuCl_2$ for 24 h, no significant changes of the ATP7A expression were detected at mRNA or protein level (Fig. 6A and B). Furthermore, we employed a choroidal Z310 cell-based tetracycline (Tet)-inducible CTR1 expression cell line (iZCTR1) previously established in this lab (Zheng et al., 2012) to investigate the impact of intracellular Cu accumulation on ATP7A expression. The iZCTR1 cells were treated with 1 μg/ml tetracycline (Tet) for 4 h to induce the high expression of CTR1, followed by 1 h incubation with 5 μCi ^{64}Cu and 5 μmol/L $CuCl_2$ in serum-free medium, followed by 3 washes with PBS, and then the cells were submitted to γ-counting or continued to be incubated with fresh DMEM, or with DMEM containing 5 μmol/L $Pb(AC)_2$ for another 24 h, and followed by γ-counting. The results revealed that the

cellular ^{64}Cu uptake in the Tet-pretreated iZCTR1 cells was about 6.5 times higher than that in the controls (Fig. 7A). Additional Pb treatment for 24 h resulted in much higher intracellular ^{64}Cu retention compared to the cells only treated with Tet (Fig. 7B). These results suggested that Pb treatment inhibited the Cu efflux and resulted in intracellular Cu accumulation in iZCTR1 cells. The results from qRT-PCR and Western blotting demonstrated that the Tet-induced Cu accumulation in iZCTR1 cells did not significantly affect ATP7A mRNA and protein levels, while additional incubation with Pb following the intracellular Cu accumulation resulted in a significant down-regulation of ATP7A expression at both protein and mRNA levels (Fig. 7C and D).

4. Discussion

Previous studies have clearly indicated that Pb exposure resulted in disturbed Cu status in the CNS (Sierra et al., 1989; Qian et al., 1995, 1999, 2005). Since BCB plays an important role in regulating the homeostasis of essential metal ions in the CSF and brain parenchyma, a thorough understanding of how Cu is regulated by BCB under Pb exposure thus becomes imperative. The results presented in this study support a significant elevation of intracellular Cu level in choroidal epithelial cells following Pb exposure. Furthermore, our observations suggest both CTR1 and ATP7A were involved in the Pb-induced cellular Cu accumulation in the BCB.

Cu is an essential trace element in all living organisms playing an important role as a catalytic cofactor for metalloenzymes (Horn and Barrientos, 2008); however, excessive intake of Cu can be pathogenic. It has been shown in some human studies that the Cu levels in the CSF are associated with some neurodegenerative diseases (Skjørringe et al., 2012; Boll et al., 2008; Hozumi et al., 2011). Previous studies have shown that Pb exposure can result in accumulation of Cu in the brain or in cultured astrocytes (Sierra et al., 1989; Qian et al., 1999, 2005), while the change of Cu status in the BCB was less studied. Our current data based on the *in vitro* choroidal Z310 model showed that 5 μM Pb treatment resulted in significant increase of intracellular Cu level, suggesting that the Cu transport in BCB can be disrupted by Pb exposure.

Previous studies in this lab and other group have suggested that the BCB may function as the regulatory site to maintain the Cu homeostasis in brain extracellular fluid and CSF by taking up Cu from the CSF *via* CTR1 enriched on the apical surface of the choroid plexus (Monnot et al., 2011; Zheng and Monnot, 2012; Kuo et al., 2006). In the current study, we found that Pb treatment resulted in a significant increase of CTR1 expression at both the mRNA and protein levels in Z310 cells. Furthermore, the siRNA knockdown of CTR1 significantly decreased the cellular Cu uptake in control cells as well as the cells treated with Pb. Hence, it became evident that CTR1 was involved in mediating the intracellular Cu overload by its induction in response to Pb exposure. Previous studies have shown that the CTR1 protein level was regulated by cellular Cu availability. Exposure of mammals to a low-copper diet results in increased CTR1 protein expression in intestinal epithelial cells without affecting its mRNA level (Nose et al., 2010; Lee et al., 2000). In addition, elevated intracellular Cu level could stimulate the rapid endocytosis and degradation of CTR1 protein from the cell membrane (Gaggelli et al., 2006). In this study, we also demonstrated that the

protein level of CTR1 in Z310 cells was down-regulated upon the exposure to Cu, while the mRNA level remained unchanged. Thus, the intracellular Cu overload following Pb exposure may in turn lead to decreased CTR1 protein level and therefore counteract the Pb-induced CTR1 induction, resulting in attenuated cellular Cu uptake. Further *in vivo* studies are necessary to investigate the combined effect of Pb exposure and the following cellular Cu overload on the CTR1 expression in BCB cells.

ATP7A was reported to regulate the release of Cu from the cells (Kim et al., 2008). The role of ATP7A in the Pb-induced intracellular Cu accumulation in BCB was also investigated. The results revealed a significant drop of ATP7A expression in Z310 cells following the Pb exposure, indicating the capacity of Cu clearance of the cells may be impaired. Our previous work demonstrated that siRNA knockdown of ATP7A resulted in Cu accumulation in choroidal cells (Monnot et al., 2012). In this study, the siRNA knockdown data confirmed the increase of intracellular Cu retention by diminishing the ATP7A expression. Additionally, knocking down ATP7A in conjunction with Pb treatment prevented the increased intracellular Cu retention seen during Pb treatment alone. Previous studies have shown that a shutdown of ATP7A gene expression led to Cu accumulation in the capillaries of the BBB (Yoshimura, 1994; Yoshimura et al., 1995). Conceivably, a decrease in ATP7A expression may result in a weaken capacity to release Cu by the choroidal epithelial cells, leading to increased intracellular Cu level. Pb was also demonstrated to inhibit the sulfhydryl-dependent Cu efflux from glial cells (Qian et al., 1997). In the current study, PCMB, a sulfhydryl-binding reagent, was found to inhibit the Cu efflux from Z310 cells, and Pb treatment following the PCMB treatment did not result in further increase of the intracellular Cu retention. Previous studies suggested that both Pb and PCMB can inhibit the Cu-transporting function of P-type ATPases by engaging sulfhydryl groups in the heavy metal binding region or the transduction site on these proteins (Qian et al., 1995, 1997). Thus, our results indicated that Pb may also bind to the sulfhydryl groups on ATP7A and thereby inhibited the Cu efflux from the cells.

As Pb exposure results in intracellular Cu accumulation, the impact of Cu on ATP7A expression was also determined. Although ATP7A expression was found to be induced by Cu exposure in tilapia and zebrafish (Chen and Chan, 2011); in mammals, it is thought to be regulated mainly at the post-translational level by alterations in the membrane transport of copper ions (Nyasae et al., 2007). In the current study, the results showed that both the mRNA and protein levels remained stable following the addition of $CuCl_2$ to the cell culture medium for 24 h. To confirm the results, a former established choroidal Z310 cells-based CTR1-inducible cell line (iZCTR1) (Zheng et al., 2012) was employed to investigate the impact of the intracellular Cu accumulation on ATP7A expression. The expression of CTR1 in iZCTR1 cells can be induced to increase drastically upon the addition of tetracycline (Tet), and thereby render the cells to absorb Cu approximately 6 times higher than that of the controls. The expression of ATP7A did not significantly changed following the intracellular Cu accumulation; however, additional incubation with Pb for 24 h resulted in significantly decreased both the ATP7A mRNA and protein levels, accompanying with much higher intracellular Cu retention, as compared to the controls. These results suggested that it is the Pb exposure, but less likely the changed intracellular Cu level, affects the expression of

ATP7A in BCB cells. It has been reported that the subcellular localization of ATP7A changes from the trans-Golgi network to the cell periphery under acute conditions of elevated Cu levels (Nyasae et al., 2007; Balamurugan and Schaffner, 2006). Thus, when the extracellular Cu levels elevated in BCB, ATP7A may undergo a subcellular relocalization as a first response to remove excess Cu ions rather than a transcriptional change. Previous study also demonstrated that in a chronic situation, dietary Cu supplement resulted in an increased mRNA level of ATP7A in rat intestine (Bauerly et al., 2004). In a study in zebrafish, an increased expression of intestinal and liver ATP7A mRNA was observed after exposure to 8 μg/L Cu for 21 days (Craig et al., 2009). In a recent study in rat intestinal epithelial cells, Cu exposure was demonstrated to increase ATP7A protein level by keeping this protein at the steady-state, without affecting the ATP7A mRNA level, inferring posttranscriptional regulation (Xie and Collins, 2012). Additionally, it was reported that the expression of Cu-ATPase was changed after exposure to excess Cu in a manner dependent on exposure route and tissue type (Minghetti et al., 2010). Further studies are needed to investigate the long-term impact of elevated Cu level on ATP7A expression and its regulatory mechanisms in BCB cells.

In conclusion, the present results indicate that Pb exposure may lead to Cu accumulation in choroidal Z310 cells in two different ways: one associated with cellular Cu uptake and the other with its clearance. Exposure to Pb up-regulates the expression of CTR1, which take up more Cu into the cells. On the other hand, Pb down-regulates the expression of ATP7A, as well as inhibiting the Cu-transporting function by binding to the sulfhydryl groups on this Cu-ATPase, causing a reduction of Cu efflux from the cells. These data suggest that the choroidal epithelial cells absorb more Cu into the cytosol but fail to clear the Cu completely through the barrier following the Pb exposure, and may thereby resulting in disturbed Cu homeostasis in the brain.

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Abbreviations

CNS	central nervous system
BCB	blood–cerebrospinal fluid barrier
CSF	cerebrospinal fluid
CTR1	Cu transporter 1
DMT1	divalent metal transporter 1
ATP7A	ATPase, Cu^{2+} transporting, alpha polypeptide
ATP7B	ATPase, Cu^{2+} transporting, beta polypeptide

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HIGHLIGHTS

- Pb exposure resulted in Cu accumulation in choroidal Z310 cells.
- Pb up-regulated CTR1 expression, while down-regulated ATP7A level in Z310 cells.
- CTR1 knockdown blocked the Pb-induced increase of cellular Cu uptake.
- ATP7A knockdown led to cellular Cu overload similar to that of the Pb-treated cells.
- CTR1, but not ATP7A protein level, was regulated by the cellular Cu status.

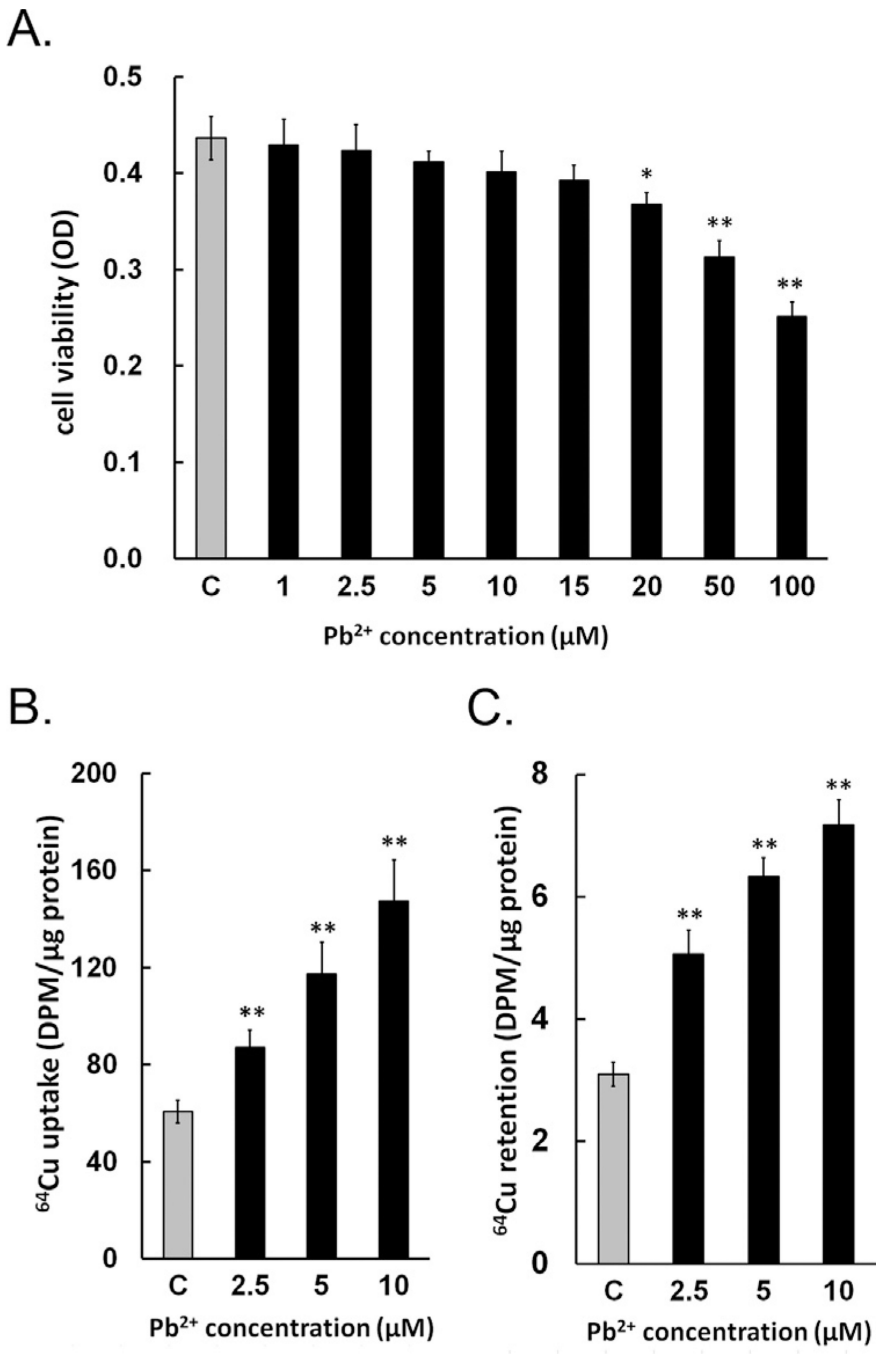

Fig. 1.

Pb exposure result in Cu accumulation in Z310 cells. (A) Cell viability following the Pb exposure, determined by MTT assay. Z310 cells were treated with 0, 1, 2.5, 5, 10, 15, 20, 50, 100 μM $Pb(AC)_2$ for 24 h, and then the cell viability was assessed by MTT assay. Data represent mean ± SD, $n = 8$. *$p < 0.05$ *vs*. controls; **$p < 0.01$ *vs*. control. (B) Increased Cu uptake in Z310 cells following Pb exposure *in vitro*. The cells were incubated with DMEM containing Pb for 24 h, followed by the incubation with 5 mCi/ml ^{64}Cu and then were submitted to γ-counting. Data represent mean ± SD, $n = 6$. **$p < 0.01$ *vs*. control. (C)

Increased Cu retention in Z310 cells following Pb exposure *in vitro*. The cells were incubated with 5 μCi ^{64}Cu for 1 h, followed by incubation with DMEM, or with DMEM containing Pb for 24 h, and then were submitted to γ-counting. Data represent mean ± SD, $n = 6$. $^{**}p < 0.01$ *vs*. control.

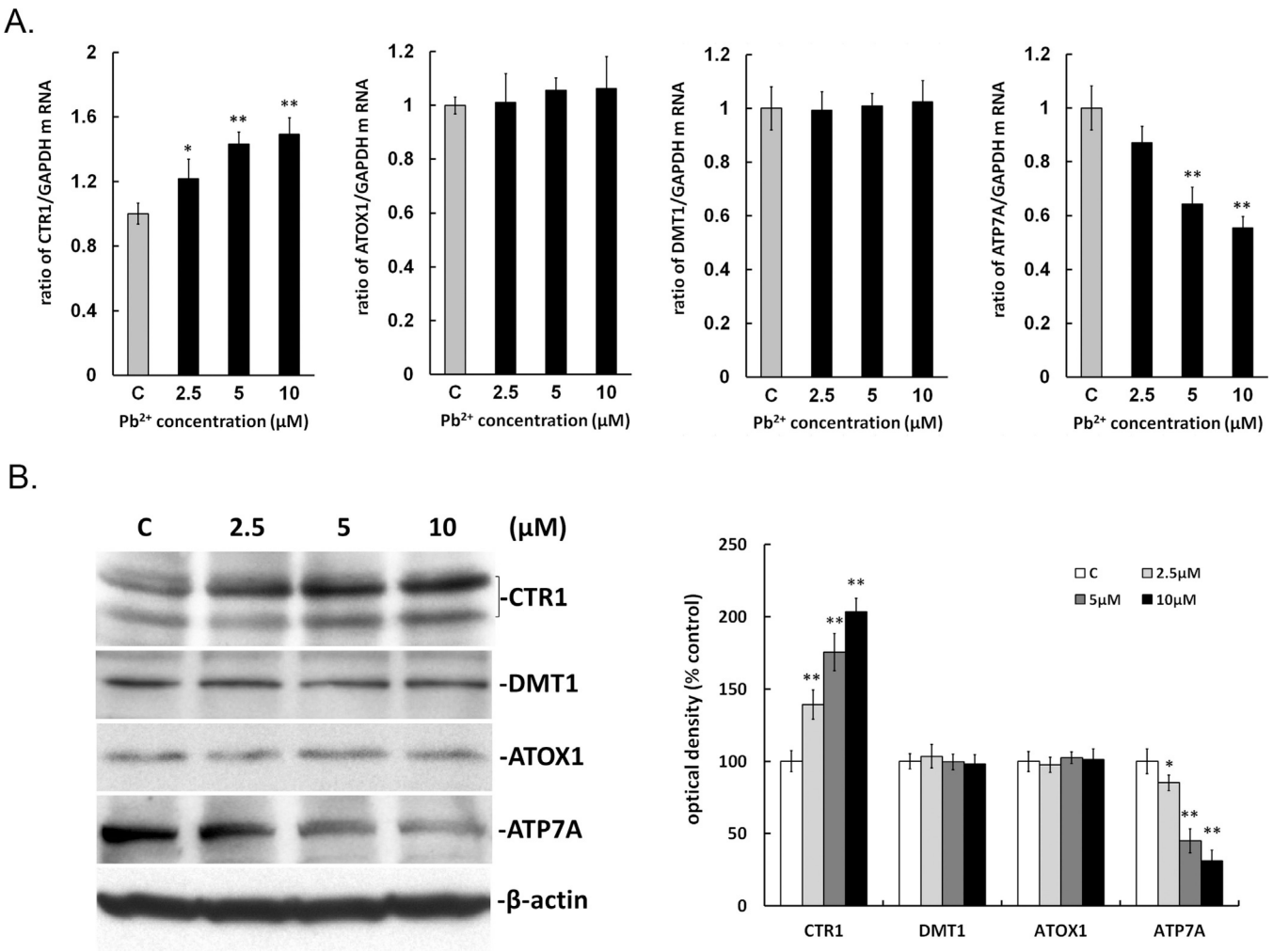

Fig. 2.

Pb exposure affected the expression levels of CTR1 and ATP7A. (A) The mRNA levels of CTR1, DMT1, ATOX1, and ATP7A following Pb (2.5, 5, 10 μM) exposure for 24 h, determined by qRT-PCR. The relative mRNA levels of the gene of interest (GOI) and GAPDH were quantified by real-time RT-PCR and expressed as the ratio of GOI/GAPDH. Data represent mean ± SD, $n = 6$. *$p < 0.05$ *vs*. controls; **$p < 0.01$ *vs*. control. (B) The protein levels of CTR1, DMT1, ATOX1, and ATP7A following Pb (2.5, 5, 10 μM) exposure for 24 h, determined by Western blotting. The bar graph represents the optical density of the target protein band normalized by the optical density of the corresponding β-actin band. Data represent mean ± SD, $n = 3$. *$p < 0.05$ *vs*. controls; **$p < 0.01$ *vs*. control.

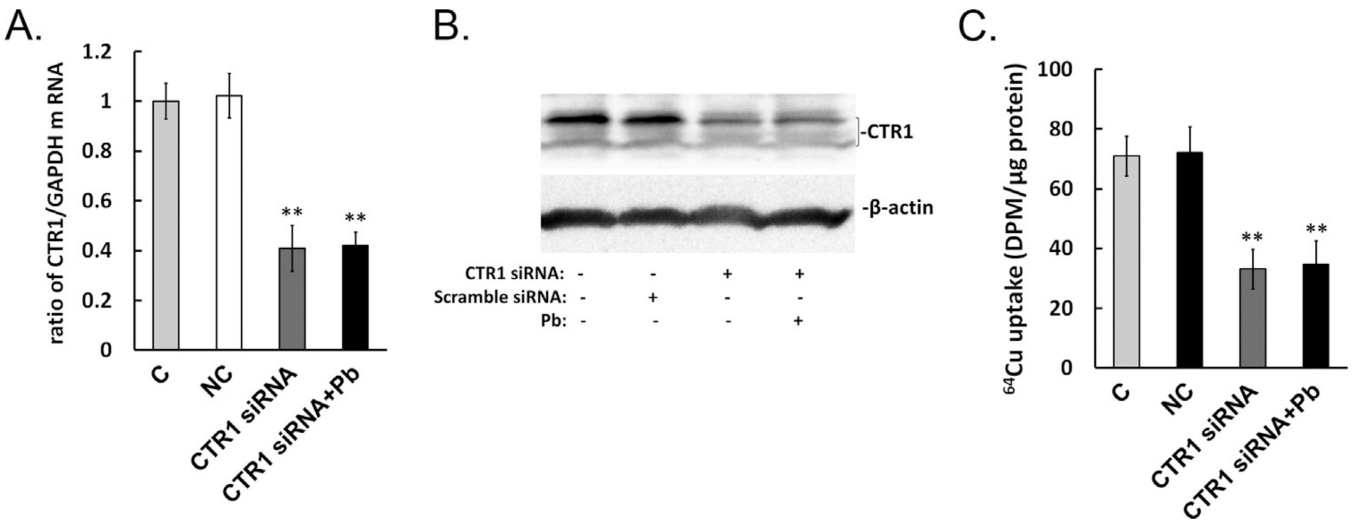

Fig. 3.

The impact CTR1 siRNA knockdown on the Pb-induced cellular Cu accumulation. (A) CTR1 siRNA knockdown decreased the CTR1 mRNA level. Data represent mean ± SD, n = 6; ** $p < 0.01$ *vs*. NC (negative control or scramble siRNA). (B) CTR1 protein level decreased following the siRNA treatment, determined by Western blotting. (C) CTR1 siRNA knockdown decreased cellular ^{64}Cu uptake. Data represent mean ± SD, $n = 6$; **$p < 0.01$ *vs*. NC.

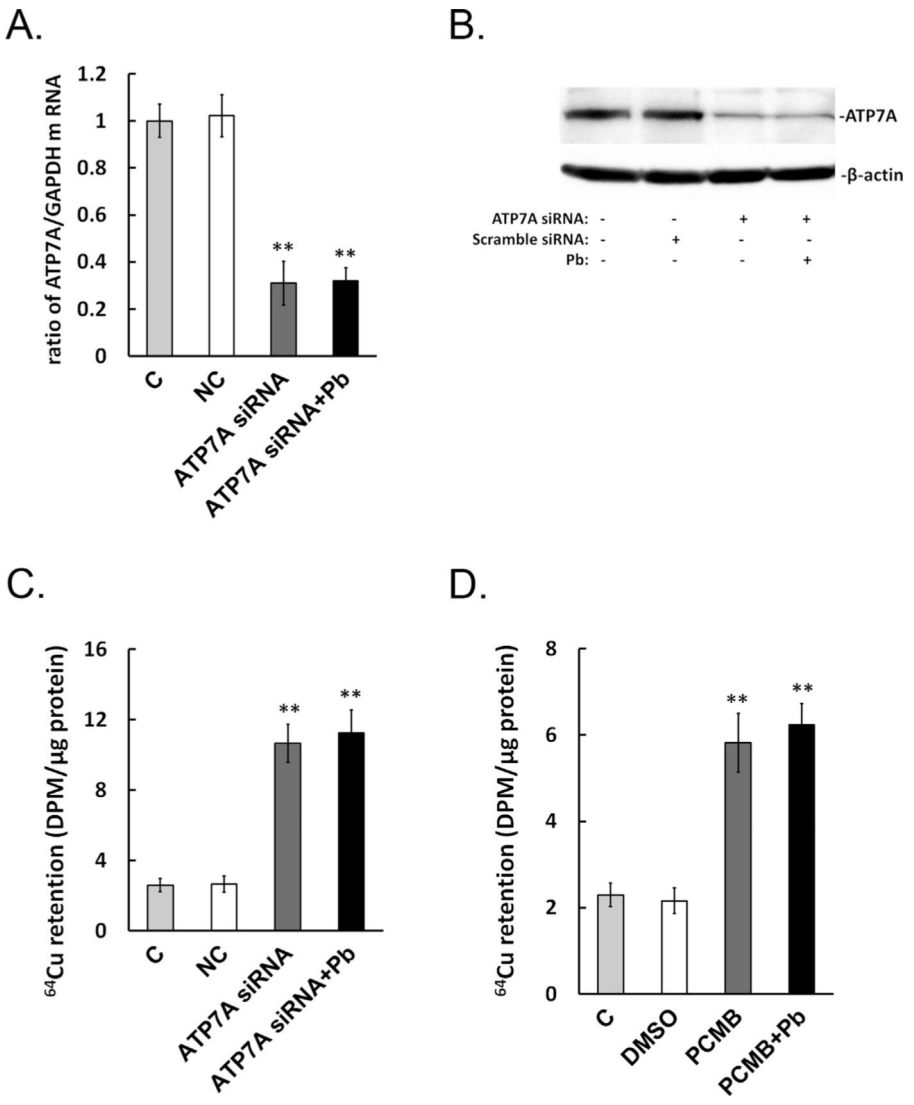

Fig. 4.

The impacts ATP7A siRNA knockdown or inhibition on the Pb-induced cellular Cu accumulation. (A) ATP7A siRNA knockdown decreased the ATP7A mRNA level. Data represent mean ± SD, $n = 6$; $^{**}p < 0.01$ *vs*. NC. (B) ATP7A protein level decreased following the siRNA treatment, determined by Western blotting. (C) ATP7A siRNA knockdown increased intracellular ^{64}Cu retention. Data represent mean ± SD, $n = 6$; $^{**}p < 0.01$ *vs*. NC. (D) PCMB increased intracellular ^{64}Cu retention. Data represent mean ± SD, $n = 6$; $^{**}p < 0.01$ *vs*. NC.

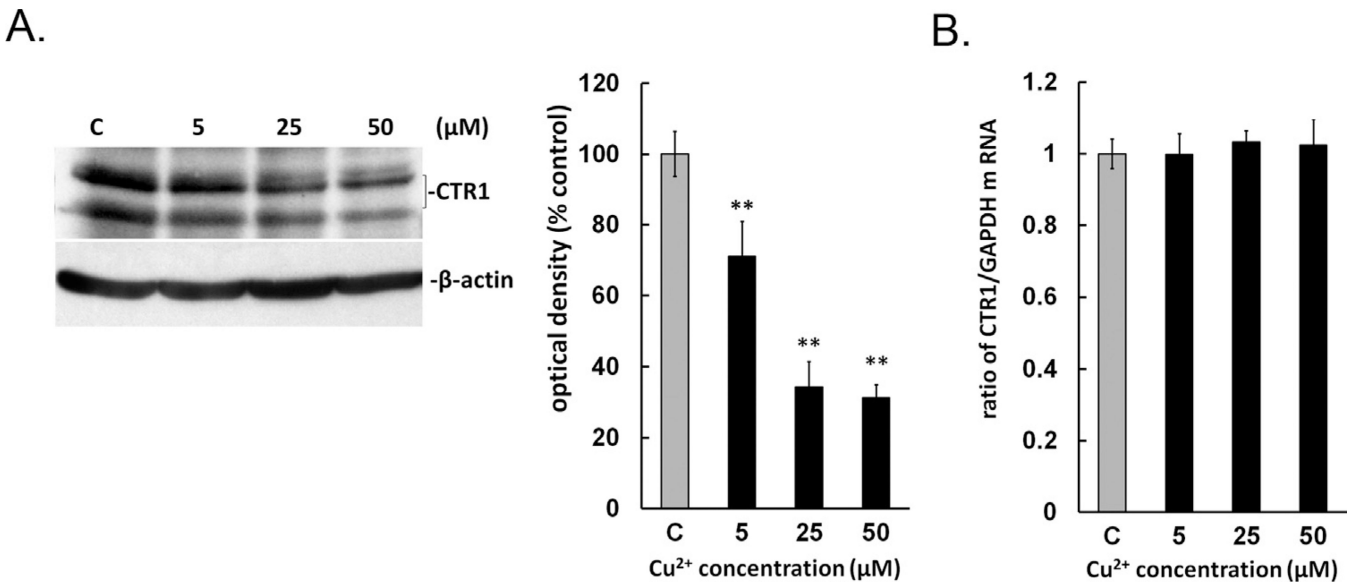

Fig. 5.

Cu exposure decreased CTR1 protein level. (A) CTR1 protein level decreased following $CuCl_2$ (5, 25, 50 μM) exposure for 24 h, determined by Western blotting. The bar graph represents the optical density of CTR1 band normalized by the optical density of the corresponding β-actin band. Data represent mean ± SD, $n = 3$. $^{**}p < 0.01$ *vs*. control. (B) The expression of CTR1 mRNA remained unchanged following the $CuCl_2$ exposure. The relative mRNA levels of CTR1 and GAPDH were quantified by real-time RT-PCR and expressed as the ratio of CTR1/GAPDH. Data represent mean ± SD, $n = 6$; $^{**}p < 0.01$ *vs.* controls.

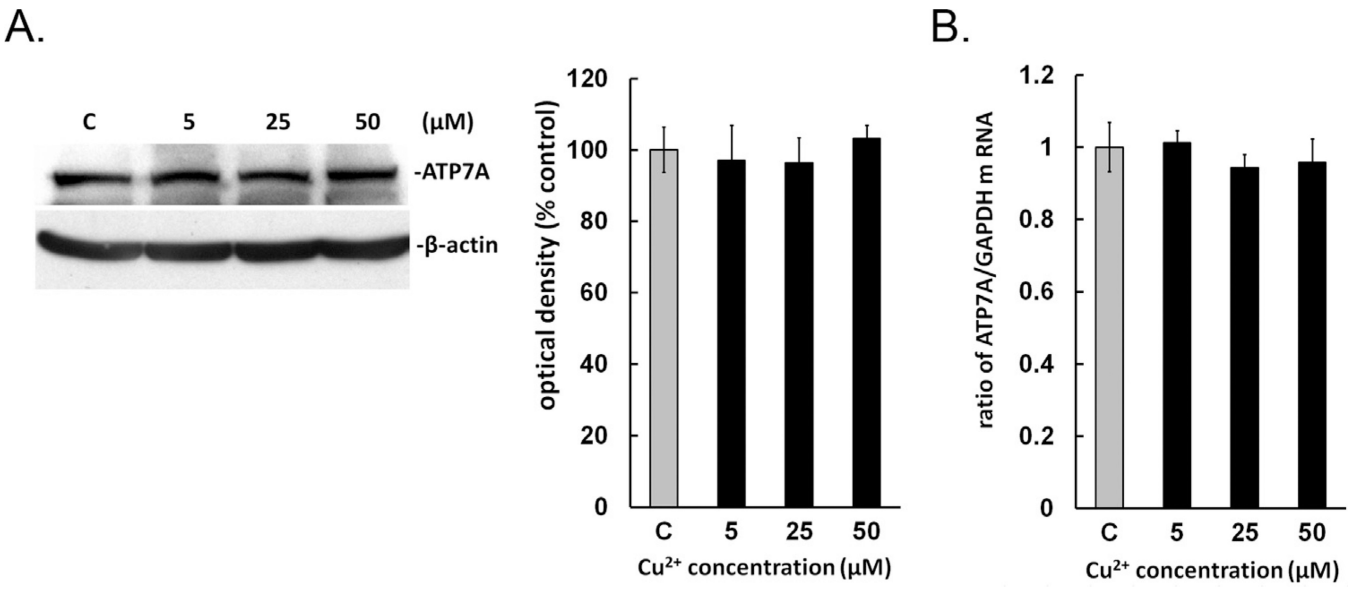

Fig. 6.

The expression level of ATP7A was not changed following Cu exposure. (A) ATP7A protein level remained unchanged following $CuCl_2$ (5, 25, 50 μM) exposure for 24 h, determined by Western blotting. The bar graph represents the optical density of ATP7A band normalized by the optical density of the corresponding β-actin band. Data represent mean ± SD, $n = 3$. (B) The expression of ATP7A mRNA remained unchanged following the $CuCl_2$ exposure. The relative mRNA levels of ATP7A and GAPDH were quantified by real-time RT-PCR and expressed as the ratio of ATP7A/GAPDH. Data represent mean ± SD, $n = 6$.

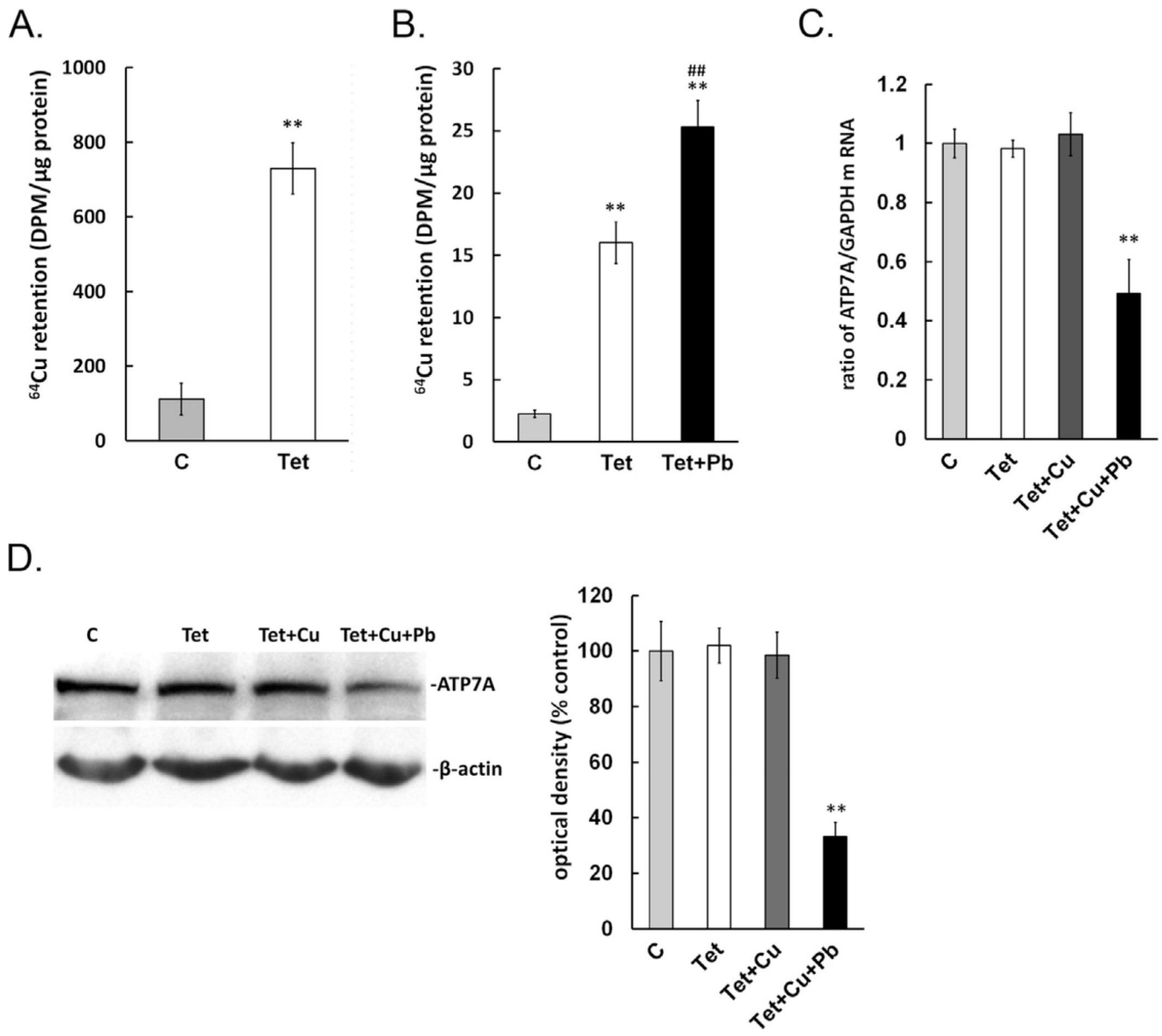

Fig. 7.

The expression level of ATP7A was not changed following intracellular Cu accumulation. (A) Increased ^{64}Cu uptake in iZCTR1 cells following the incubation with Tet, determined by γ-counting. Data represent mean ± SD, $n = 6$. $^{**}p < 0.01$ *vs*. control. (B) Pb exposure inhibited the Cu efflux from iZCTR1 cells following the intracellular Cu accumulation, determined by γ-counting. Data represent mean ± SD, $n = 6$. ** $p < 0.01$ *vs*. control; $^{\#\#}p < 0.01$ *vs*. Tet pretreated iZCTR1 cells. (C) The ATP7A mRNA level remained unchanged following the intracellular Cu accumulation, while additional treatment by 5 μM $Pb(AC)_2$ resulted in decreased ATP7A mRNA level. The relative mRNA levels of ATP7A and GAPDH were quantified by real-time RT-PCR and expressed as the ratio of ATP7A/GAPDH. Data represent mean ± SD, $n = 6$, $^{**}p < 0.01$ *vs*. control. (D) ATP7A protein level remained unchanged following intracellular Cu accumulation, while additional treatment by

5 μM $Pb(AC)_2$ resulted in decreased ATP7A protein level, determined by Western blotting. The bar graph represents the optical density of ATP7A band normalized by the optical density of the corresponding β-actin band. Data represent mean ± SD, $n = 3$, $^{**}p < 0.01$ *vs*. control.