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Exposure to metal ions regulates mRNA levels of APP and BACE1 in PC12 cells: Blockage by curcumin

Raozhou Lin[a,1], Xiaohong Chen[b], Wenming Li[c], Yifan Han[c], Peiqing Liu[a], Rongbiao Pi[a,*]

[a] *Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, No. 74 Zhongshan II Road, Guangzhou 510060, PR China*
[b] *Department of Neurology, The Third Affiliated Hospital, Sun Yat-sen University, PR China*
[c] *Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, China*

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ABSTRACT

Amyloid β peptide (Aβ), generated by proteolytic cleavage of the amyloid precursor protein (APP), play a pivotal role in the pathogenesis of Alzheimer's disease (AD). The key step in the generation of Aβ is cleavage of APP by beta-site APP-cleaving enzyme 1 (BACE1). There is increasing evidence supporting an interaction between APP, Aβ and metal ions. Both APP and Aβ affect ion homeostasis. Conversely, metal ions may interact with several AD-associated pathways involved in neurofibrillary tangle formation, secretase cleavage of APP, proteolytic degradation of Aβ and the generation of reactive oxygen species. However, the underlying mechanisms remain elusive. Here we first reported the differential effects of AD-related metal ions at subtoxic concentrations on the transcription levels of APP and BACE1 in PC12 cells. Copper (Cu^{2+}, 50–100 μM) and manganese (Mn^{2+}, 50–100 μM) potently increased the expression of both APP and BACE1 in a time- and concentration-dependent pattern, while zinc (Zn^{2+}), iron (Fe^{2+}) and aluminum (Al^{3+}) did not. To uncover the mechanism(s) of the increasing expression by these ions, we observed the effects of several antioxidants and some specific inhibitors on the up-expression of APP and BACE1 by metal ions. Curcumin almost completely blocked the effects of these irons, while minocycline and sodium ferulate slightly suppressed the increased BACE1 mRNA level. Signaling pathway specific inhibitors PD98059, SB203580 and CEP11004 modestly blocked the up-transcription of APP induced by copper. These results suggest that these irons cause differential effects on the expression of APP and BACE1 in PC12 cells, and curcumin can significantly reverse their effects.

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in the current society. Its pathological features include synaptic degeneration, senile plaques and neurofibrillary tangles, which eventually lead to neuronal loss [18,19]. The major components of senile plaque are β-amyloid peptides (Aβ), including Aβ40 and Aβ42, which are derived by proteolytic cleavage of amyloid precursor protein (APP), a type I membrane spanning protein [30]. APP is considered to be functionally important because mice lacking all APP family genes exhibit postnatal growth deficit; however, it is also the source of Aβ during AD development [11]. Two β site APP-cleaving enzymes (BACE1, originally referred to as BACE; and BACE2) are involved in Aβ production [10]. BACE1 is mainly responsible for β-cleavage of APP in vivo [5], and overexpression of BACE1 increases the production of Aβ in vitro [21]. On the contrary, the down-regulation of BACE1 gene expression by the treatment of antisense oligonucleotides decreases the production of C99 (a C-terminal product of BACE1 cleavage of APP), leading to the reduction of Aβ [5]. Thus both APP and BACE1 are regarded as the pivotal components in Aβ production. Since less than 10% of total AD cases are associated with genetic factors [15], it is extremely important to investigate how environmental factors contribute to the pathogenesis of this disease.

Metal ions are the major pollutants from automobile, industries, etc., which cause the increasingly severe environmental hazard to human beings. Several studies indicate that metal ions such as copper, iron, aluminum, etc., participate in the regulation of Aβ production [1,2,16]. The concentrations of zinc and iron significantly increase in senile plaques (SP) rims and cores of AD patients. The level of copper significantly elevates in the rim of SP as well [16]. Aβ accumulation is greatly retarded by the treatment of metal chelator [7]. Redox active metal ions affect Aβ-mediated neurotoxicity through several mechanisms, including oxidative stress [12,13]. Does metal ions only act on the amyloid protein? Several studies

* Corresponding author. Tel.: +86 20 8733 3082.
E-mail address: pirb@mail.sysu.edu.cn (R. Pi).
[1] Current address: Department of Biochemistry, The University of Hong Kong, L3-58, 3/F, Laboratory Block, Faculty of Medicine, Building 21, Sassoon Road, PokFuLam, Hong Kong, China.

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showed that some metal ions (copper, iron and calcium, and so on) modulated the expression of APP were reported in vitro or in vivo [2–4,6,17,22,24]. But, few report was not found to uncover effects of heavy ions on the expression of BACE1. Because metal-regulatory elements are located in both 5′-untranslated region of APP and BACE1 transcripts, which implies the role of metals on the expressions of APP and BACE1 [24,26], whether these ions have same effects on the expressions of APP and BACE1? Some mechanisms were also found to modulate the expression of APP mRNA [3,31]. However, direct effects of metal ions and their mechanisms on mRNA levels of APP and BACE1 are not fully uncovered. In this study, we would investigate the effects of metal ions on the transcription of APP and BACE1 and the mechanisms by pharmacological strategy in PC12 cells.

PC12 cells were cultured in a Dulbecco's modified Eagle medium (DMEM; Invitrogen), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 5% heat-inactivated horse serum (HS, Hyclone), 0.1% streptomycin (HyClone, Logan, UT, USA) and 0.1% penicillin. Cells were incubated in the culture medium at 37 °C in a humidified incubator gassed with 95% air and 5% CO_2, and passaged every 2 days in 10% FBS DMEM after digested with 2.5% trypsin. Exposure to metal ions was carried out when the confluence approached to 80%. In brief, PC12 cells were washed twice with PBS (0.1 M, pH 7.4) and then incubated in DMEM containing 5% serum (in which the ratio of FBS to HS is 2:1) with or without metal ions for different durations indicated in figures. The metal salts employed in present study are $CuCl_2{\cdot}2H_2O$, $ZnCl_2$, $MnSO_4$, $FeSO_4{\cdot}7H_2O$, $AlCl_3{\cdot}6H_2O$ and $PbCl_2$ and all of them, purchased from Sigma or Chinese company, are analytical grade. The metal was prepared freshly at the day of incubation. All metal salt were solved in millipore water at 1000× stock solution of appropriate final concentration. After diluting into the appropriate concentration in DMEM containing 5% serum, the pH of DMEM was adjusted if necessary and controlled around 7.2 to ensure the ion form of metals in the medium. Medium containing a lower concentration of serum was used to inhibit the proliferation of cells.

Cell viability was measured by a spectrophotometric method using the tetrazolium salt (3-4,5-dimethylxthiazol-2-yl-2,5-diphenyltetrazolium bromide, MTT) (Shanghai, Sangon). PC12 cells were cultured in 96-well plates and exposed to metal ions as the methods as described above. Then the cells were incubated at 37 °C for 3 h with MTT (0.5 mg/ml) and lysed in dimethyl sulfoxide (DMSO) after discarding the supernatants. The optical density was measured at 570 nm with spectrometer and all data were expressed as percentage of control.

Reverse transcriptional-polymerase chain reaction (RT-PCR) was employed to examine the transcription levels of APP and BACE1. Total RNA was extracted from PC12 cells from treatment and control groups with TRIZOL (Invitrogen) by following the manufacturer's instructions. RNA concentrations were calculated on the basis of OD values and the 260/280 values (≥1.6) of RNA samples were accepted for further assay. Total RNA was converted to cDNA by reverse transcriptase using Avian Myeloblastosis Virus (AMV) Reverse Transcriptase XL (Takara) according to the manufacturer's instructions. Briefly, 10 μl reverse transcript system mixture including 2 μg RNA, 0.5 μl random 9 mer primers, 1 μl dNTP (10 mM), 2 μl AMV RT buffer, 0.25 μl RNAse inhibitor (Takara), 0.25 μl AMV reverse transcriptase XL, and appropriate RNase-free water were added into 0.2 ml thin PCR tubes. The samples were incubated at 30 °C for 10 min and then 42 °C for 50 min. The reverse transcriptase was inactivated by heating at 99 °C for 5 min. 1 μl cDNA of each sample was amplified in PCR mixture, which contained 1.6 μl 10 mM dNTP mixture, 1 U Hot-Start Taq polymerase (TaKaRa) together with 0.2 μl 10 mM of each primer in total 20 μl volume. Primers were designed for each mRNA according to the rat APP sequence (GenBank Accession No. NM019288), the rat BACE1 sequence (GenBank Accession No. AF190727), and the rat beta-actin sequence (GenBank Accession No. NM03644). Sequence of each primer was as follows: APP (forward), 5′-GGACGACTCCGATGTCTGGT-3′; APP (reverse), 5′-ACATCA AAGTACCAGCGGGAG-3′; BACE1 (forward), 5′-GCAGACCCACATTCCGAACA-3′; BACE1 (reverse), 5′-GCCACTGTCCACGATGCTCTT-3′; Beta-actin (forward), 5′-AGCC ATGTACGTA GCCATCC-3′; Beta-actin (reverse), 5′-CTCTCAGCTGTG GTGGTGAA-3′. The reaction conditions consist of a denaturing step at 95 °C for 45 s, an annealling step at 55–61 °C for 30 s for different primers and an extension step at 72 °C for 40 s. The results were obtained in 25–32 cycles of amplification. The PCR products were separated by electrophoresis on 2.0% agarose gels and visualized by using GoldVew DNA staining solution (Beijing SBS). Grey densities of bands on scanned gels were quantitated and analyzed using the Image Quant 5.0 software. The relative gray density value of control groups were 1.0.

Data were expressed as means ± standard errors of means (S.E.M.s) by analysis of variance (ANOVA) with Student's *t*-test between treatment and control groups. Differences were accepted as significant at $p < 0.05$ or less.

Fig. 1 showed the time- and concentration-dependent effects of copper and manganese on the viability of PC12 cells as well as on the transcription levels of APP and BACE1. Treatments with copper and manganese at concentrations of 50 and 100 μM for 24 h had no significant effects on the viability of PC12 cells, respectively, while the viability slightly decreased when the cells were treated with 200 μM of manganese for 24 h. The viability of PC12 cells treated with 200 μM copper for 24 h decreased significantly ($P < 0.05$) (Fig. 1A). Thus, the ion concentrations which had no significant toxicity were defined as the subtoxic range in this study, and the transcription levels of APP and BACE1 were investigated in PC12 cells exposed to the subtoxic ranges of different metal ions. Both APP and BACE1's transcription levels increased significantly by copper and manganese. These up-regulations were repressed following the increase of their concentrations (Fig. 1B). 100 μM copper or manganese have minor effects on the viability of PC12 cells treated for 12 and 24 h, respectively, while the viability decreased significantly when the cells were treated for 48 h (Fig. 1C). The transcription levels of APP and BACE1 were increased when PC12 cells were treated with copper or manganese for 12 and 24 h (Fig. 1D). The effects of the other metal ions, including iron, zinc, aluminum and lead, on the expression of APP and BACE1 were also investigated by using subtoxic concentration, respectively (determining by MTT assay, data not shown). But the up-regulated effects on APP were only observed in the cells treated with lead (Fig. 1E), similar to the previous report [3]. Aluminum slightly reduced the APP transcription and increased the BACE1 transcription, which was not consistent with previous report [22]. The reason might be the different cell line used. No effects were observed in the cells treated with zinc and iron.

Increasing evidence has indicated that metals used in this research are closely implicated in the pathogenesis of AD, especially copper [9,25,27]. Therefore, we wondered the mechanisms of metal ions on the expression of APP and BACE1 by using copper as the representative ion. Copper can induce oxidative stress in diverse systems. To uncover the mechanisms of the copper-mediated increases of APP and BACE1 transcription levels, PC12 were treated with either diverse antioxidants (minocycline, sodium ferulate and curcumin) [20,23,29], or signaling pathway inhibitors (MEK-1 inhibitor PD98059; p38MAPK inhibitor SB203580, and JNK inhibitor CEP11004) for 30 min before exposed to copper (Fig. 2). All of these chemicals had no effect on the viability of PC12 in the treatment duration at their concentrations used here. Curcumin almost blocked the copper-induced up-regulation of APP and