

Zinc binds to and directly inhibits protein phosphatase 2A *in vitro*

Yan Xiong^{1,2}, Dan-Ju Luo², Xiu-Lian Wang², Mei Qiu², Yang Yang², Xiong Yan², Jian-Zhi Wang², Qi-Fa Ye^{1,4}, Rong Liu^{2,3}

¹Wuhan University, Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Wuhan 430071, China

²Key Laboratory of Neurological Disease, Ministry of Education, and Department of Pathophysiology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

³The Institute of Brain Research, Huazhong University of Science and Technology, Wuhan 430030, China

⁴The Third Xiangya Hospital of Central South University, Research Center of National Health Ministry on Transplantation Medicine Engineering and Technology, Changsha 410013, China

Corresponding authors: Rong Liu and Qi-Fa Ye. E-mail: rong.liu@mail.hust.edu.cn, yqf_china@163.com

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ABSTRACT

Zinc induces protein phosphatase 2A (PP2A) inactivation and tau hyperphosphorylation through PP2A (tyrosine 307) phosphorylation in cells and the brain, but whether Zn^{2+} has a direct inhibitory effect on PP2A is not clear. Here we explored the effect of Zn^{2+} on PP2A and their direct interaction *in vitro*. The results showed that Zn^{2+} mimicked the inhibitory effect of okadaic acid on protein phosphatase and prevented tau dephosphorylation in N2a cell lysates. PP2A activity assays indicated that a low concentration (10 $\mu\text{mol/L}$) of Zn^{2+} inhibited PP2A directly. Further Zn^{2+} -IDA-agarose affinity binding assays showed that Zn^{2+} bound to and inhibited PP2A_{C(51-270)} but not PP2A_{C(1-50)} or PP2A_{C(271-309)}. Taken together, Zn^{2+} inhibits PP2A directly through binding to PP2A_{C(51-270)} *in vitro*.

Keywords: zinc; protein phosphatase 2A; direct inhibition

INTRODUCTION

According to clinical surveys, Alzheimer's disease (AD) is the most common type of dementia^[1, 2]. It is characterized

by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles within the afflicted brain^[3]. The upstream factors and mechanisms leading to tau hyperphosphorylation remain largely unclarified. However, imbalance between the tau kinases involved such as glycogen synthesis kinase 3 and tau phosphatases such as protein phosphatase (PP) 2A has been proposed to play an important role in the abnormal hyperphosphorylation of tau in AD^[4-10]. Protein-serine/threonine phosphatase (PP) 2A is found in all eukaryotic cells^[11] and plays an important role in the regulation of many cellular events, including viral transformation, translation, replication, the cell cycle, metabolism, cell proliferation, and transcription^[12]. PP2A works in heteroassociate form. The PP2A holoenzyme is composed of catalytic C, regulatory/targeting B, and scaffolding/structural A subunits. Currently, the regulation of PP2A activity is not fully elucidated. Holoenzyme composition and post-translational modification have been suggested to modulate PP2A activity. Besides, some intracellular small inhibitory molecules such as I_1^{PP2A} and I_2^{PP2A} may bind to and directly inhibit PP2A^[13, 14].

Zinc is a trace metal abundant in the brain. In a previous study, we explored its role in PP2A regulation, and found that it induces PP2A inactivation through Src-dependent PP2Ac (tyrosine 307) phosphorylation

in both cells and rat brain. Meanwhile, the neuronal microtubule-associated protein tau — one of the PP2A dephosphorylating targets — is hyperphosphorylated^[15]. This work implies that Zn^{2+} may influence PP2A activity indirectly by changing the tyrosine phosphorylation-related signaling. As a metal ion, Zn^{2+} has been reported to tightly bind to PP2B^[26] and directly inhibit the activity of λ Ser/Thr phosphoprotein phosphatase^[27]; both phosphatases share sequence identity in the catalytic domain with PP2A^[20]. So is it possible that Zn^{2+} also has a direct effect on PP2A? In the present study, we performed *in vitro* studies to answer this question.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal antibody against glutathione S-transferase (GST) (1:500) was from Tiangen (Beijing, China). Rabbit polyclonal antibodies pS396 (1:1 000) and pS262 (1:1 000) against tau phosphorylated at Ser396 and Ser262, respectively, were from Signalway Antibody (Pearland, TX), pS212 (1:1 000) against tau phosphorylated at Ser212 was from Chemicon Antibody (Massachusetts, MA), and β -actin (1:1 000) was from Epitomics Antibody (Burlingame, CA). Purified PP2A was from Millipore (Billerica, MA). The protein phosphatase assay kit V2460 was from Promega (Fitchburg, WI). Immobilized iminodiacetic acid (IDA) resin was from Pierce (Rockford, IL). Cell culture media were from Gibco (San Diego, CA). ZnSO_4 and okadaic acid (OA) were from Sigma (St. Louis, MO).

Expression and Purification of GST-Tagged PP2Ac₍₁₋₅₀₎, PP2Ac₍₅₁₋₂₇₀₎, and PP2Ac₍₂₇₁₋₃₀₉₎

The truncated PP2Ac₍₁₋₅₀₎, PP2Ac₍₅₁₋₂₇₀₎, and PP2Ac₍₂₇₁₋₃₀₉₎ segments were expressed as thrombin-cleavage GST fusion proteins in *Escherichia coli*. The DNA sequences of PP2Ac₍₁₋₅₀₎, PP2Ac₍₅₁₋₂₇₀₎, and PP2Ac₍₂₇₁₋₃₀₉₎ were amplified by PCR with the following primers: sense 1, 5'-cgcggtatccccatggacgagaaggtgttcaccaaggagctgg-3' and antisense 1, 5'-aacgcgtcgaccacatcgaaacctctgtcagcttggtatctttgtc-3'; sense 2, 5'-cgcggtatccccagttactgtctgtggagatgtgcatgggcaatttcgatctcatgg-3' and antisense 2, 5'-aacgcgtcgaccaccacaacgataacaatagttggagcactgaaatcggttac-3'; sense 3, 5'-cgcggtatcccaaccaagctgcaatcatggaacttgacgatactctaaatac-3' and antisense 3, 5'-aacgcgtcgacccaggaag-

tagtctggggtagcagcagtaacatgtgg-3'. The products were then separately cloned into the pGEX-5X vector. The plasmids were expressed in BL21-CodonPlus-RIL cells (Stratagene, La Jolla, CA), then the cells at OD 0.8 were induced by 0.2 mmol/L isopropyl- β -thiogalactopyranoside for 3 h at 37°C. Pelleted cells were lysed in buffer containing 50 mmol/L Tris-HCl, pH 7.0, 1 mg/L lysozyme, 1.0 mmol/L PMSF, 1:200 protease inhibitor cocktail, 1% TritonX-100, 5 mg/L DNase, and 5 mg/L RNase; and GST-PP2Ac₍₁₋₅₀₎, GST-PP2Ac₍₅₁₋₂₇₀₎, and GST-PP2Ac₍₂₇₁₋₃₀₉₎ were solubilized with detergents. The fusion proteins were purified using a B-PER GST fusion protein purification kit (Thermo Scientific, Rockford, IL). Then the purified GST-PP2Ac₍₁₋₅₀₎, GST-PP2Ac₍₅₁₋₂₇₀₎, and GST-PP2Ac₍₂₇₁₋₃₀₉₎ and PP2A were incubated with or without 100 $\mu\text{mol/L}$ ZnSO_4 for 30 min at 30°C. At the end of incubation, the lysates were collected for western blotting and PP2A activity assays.

Cell Culture and Treatment

Cell culture and incubation of cell lysates with Zn^{2+} were performed as previously described^[16]. Mouse neuroblastoma N2a cells were grown to 70%–80% confluence in DMEM/Opti-MEM (1:1) supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin, washed twice, harvested in 0.2 mL of cell lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EGTA, 2 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton-X100, 0.5 mmol/L PMSF, 1:1 000 cocktail) and sonicated on ice for 30 min. Then the cell lysates were incubated with or without 100 $\mu\text{mol/L}$ ZnSO_4 for 5, 15, or 30 min at 30°C. For analysis of the time- and concentration-dependence of PP2A activity changes caused by Zn^{2+} , N2a cell lysates were incubated with 0, 10, 50, or 100 $\mu\text{mol/L}$ ZnSO_4 for 30 min, or with 10 $\mu\text{mol/L}$ ZnSO_4 for 0, 10, 30, or 60 min at 30°C. At the end of incubation, the lysates were collected for western blotting and PP2A activity assays.

Western Blotting

The total protein content in cell lysates was measured using a BCA kit (Rockford, IL), and then normalized to 5 $\mu\text{g}/\mu\text{L}$. The protein samples (30 μg) were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies, followed by anti-rabbit or anti-mouse IgG conjugated to IRDyeTM (800CW; Licor Biosciences,

Lincoln, NE) for 1 h at 25°C, and visualized using the Odyssey Infrared Imaging System (LicorBiosciences).

Protein Phosphatase Activity Assay

The activity of PP2A in cell lysates and purified truncated PP2Ac was assayed using the V2460 Phosphatase Kit (Fitchburg, WI) according to the manufacturer's protocol. Briefly, endogenous phosphate was removed using the spin columns provided, then the N2a cell lysates were normalized for protein content, and 5 µg protein samples in triplicate were incubated with a synthesized phosphopeptide (RRA (pT)VA)^[17] for 30 min at 33°C. Then an equal volume of the Molybdate Dye/Additive mixture was added to all wells to stop the reaction. Absorbance at 600 nm or 630 nm was measured with a VersaMax plate reader (Molecular Devices). The PP2A activity was evaluated by the release of phosphate per microgram protein per minute (pmol/µg/min).

Zn²⁺-IDA-Agarose Affinity Binding Assay

The Zn²⁺-iminodiacetic acid (IDA)-agarose affinity binding assay was based on previous descriptions^[18]. First, IDA column was warmed to room temperature, then 50 µL IDA equilibrated in Buffer A (10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 0.5% TritonX-100) was incubated for 30 min with freshly-prepared 10 mmol/L ZnSO₄ at 25°C. The fully Zn²⁺-charged IDA-agarose was thoroughly washed twice with Buffer A to remove the uncharged Zn²⁺. Then 50 µL purified GST-PP2Ac₍₁₋₅₀₎, GST-PP2Ac₍₅₁₋₂₇₀₎, or GST-PP2Ac₍₂₇₁₋₃₀₉₎ protein was added separately to the pelleted Zn²⁺-IDA-agarose at room temperature for 30 min. The effluent (designated F) was collected, then the agarose resin was washed 6 times with 50 µL Buffer A, and the wash solution (W1...6) was collected for analysis. Then the bound proteins were eluted from the Zn²⁺-IDA-agarose 2 or 3 times with 500 µL 100 mmol/L EDTA (E1, E2, or E3). In addition, one group was treated with the same procedure, except for the lack of ZnSO₄. All the samples were boiled with sample loading buffer for western blotting using antibody against GST.

Statistical Analysis

Data are presented as mean ± SD and were analyzed with SPSS10.0 software (SPSS Inc., Chicago, IL). Student's *t* test and one-way analysis of variance (ANOVA) followed by the LSD *post hoc* test were used to determine group differences. *P* < 0.05 was considered statistically significant.

RESULTS

Zinc Mimics the Inhibitory Effect of Okadaic Acid on PP2A *in vitro*

To explore whether Zn²⁺ has a direct inhibitory effect on PP2A, we first incubated N2a cell lysates with Zn²⁺ in the absence of exogenous ATP, to exclude the influence of protein kinases on the phosphorylation state of proteins. The results showed that with control incubation, tau was dephosphorylated at Thr212, Ser262, and Ser396 by protein phosphatases, while OA, a potent protein phosphatase inhibitor, prevented the dephosphorylation of tau at 10 nmol/L, a concentration that only inhibits PP2A^[19]. Zn²⁺ at 100 µmol/L had an effect similar to OA on tau phosphorylation at all the sites (Fig. 1), suggesting that Zn²⁺ may also directly inhibit PP2A.

Zinc Inhibits PP2A Directly *in vitro*

To further confirm the direct inhibitory effect of Zn²⁺ on PP2A, we incubated N2a cell lysates with Zn²⁺ at 10, 50 or 100 µmol/L without addition of ATP, and assessed the PP2A activity. The results showed that Zn²⁺ inhibited PP2A at all three concentrations, but the higher concentration showed a weaker inhibitory effect (Fig. 2A). We then incubated the lysates with 10 µmol/L Zn²⁺ for 10, 30, or 60 min and found that it time-dependently inhibited PP2A activity (Fig. 2B). These data indicate that Zn²⁺ at a low concentration directly inhibits PP2A.

Zinc-induced PP2A Inactivation Occurs on the PP2Ac₍₅₁₋₂₇₀₎ Segment

Next, we explored the possible mechanism of direct inhibition of PP2A by Zn²⁺. We found that incubation of full-length PP2A with 100 µmol/L ZnSO₄ resulted in significant inhibition of PP2A (Fig. 3B), consistent with the above findings in N2a cell lysates. Further, among the three GST-tagged PP2Ac truncates, PP2Ac₍₅₁₋₂₇₀₎ had the highest phosphatase activity (Fig. 3A), consistent with previous speculation that the catalytic activity of PP2A is located on this segment^[20]. In addition, Zn²⁺ significantly inhibited the PP2Ac₍₅₁₋₂₇₀₎ activity (Fig. 3B), with no effect on the other two PP2A truncates, suggesting that the interaction site of Zn²⁺ with PP2A is located on the PP2Ac₍₅₁₋₂₇₀₎ segment.

Zinc Binds Directly to PP2Ac₍₅₁₋₂₇₀₎

The Zn²⁺-IDA-agarose affinity binding assay showed that

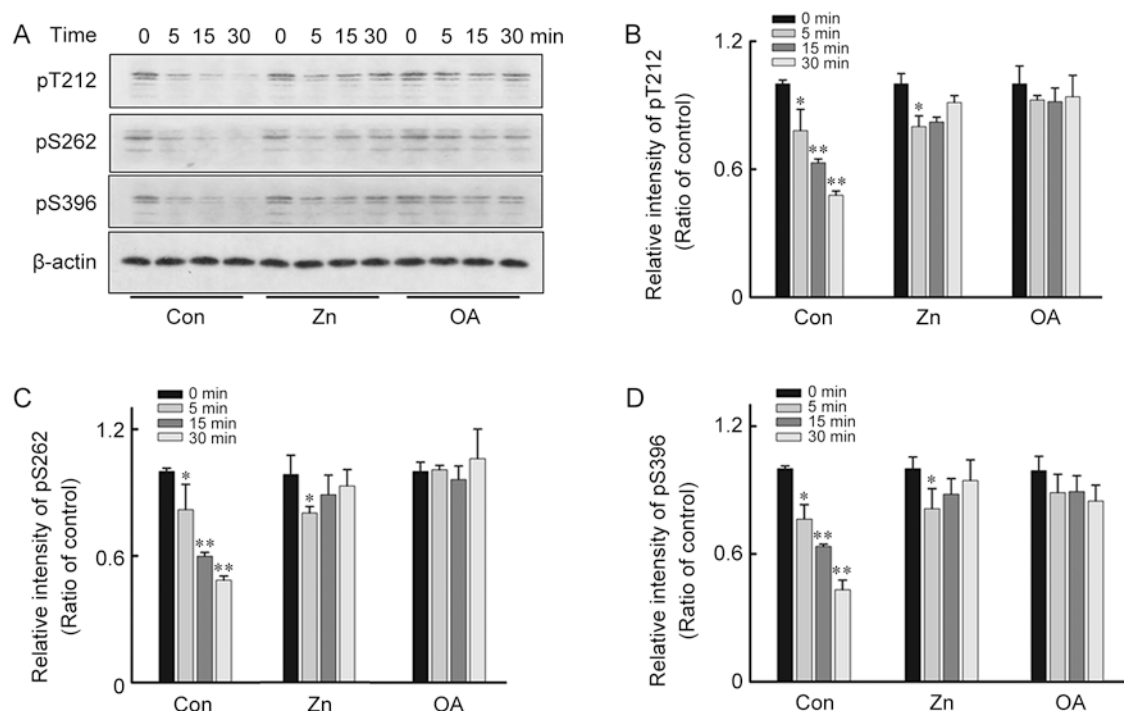


Fig. 1. Zinc mimics the inhibitory effect of okadaic acid (OA) on PP2A *in vitro*. (A) Western blots of the phosphorylation levels of tau at Thr212, Ser262, and Ser396 in N2a cell lysates incubated with or without 100 $\mu\text{mol/L}$ ZnSO_4 or 10 nmol/L OA for 0, 5, 15, or 30 min. (B–D) Quantitative analysis normalized against β -actin. All data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs 0 min group.

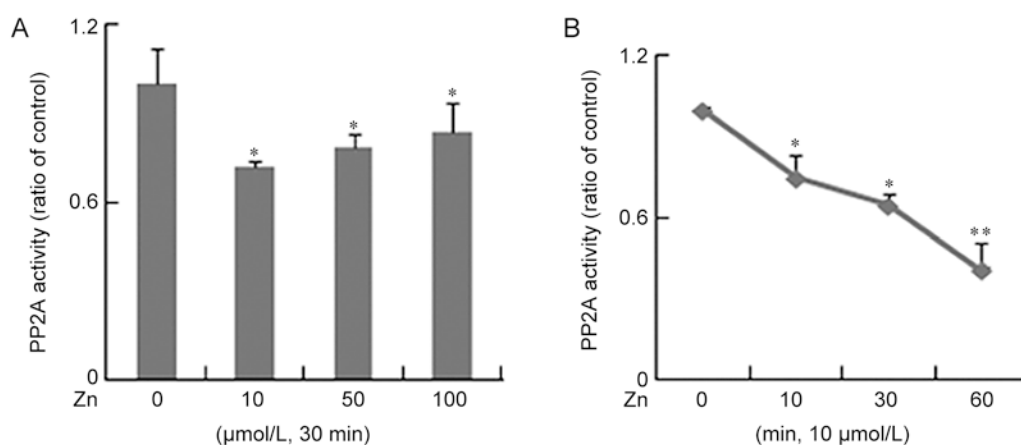


Fig. 2. Zinc inhibits PP2A in a time-dependent manner *in vitro*. (A–B) N2a cell lysates were incubated with 0, 10, 50, or 100 $\mu\text{mol/L}$ ZnSO_4 for 30 min (A) or with 10 $\mu\text{mol/L}$ ZnSO_4 for 0, 10, 30, or 60 min (B) without ATP; PP2A activity was measured using a protein phosphatase activity assay kit. All data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs control group.

the recombinant GST-tagged $\text{PP2Ac}_{(51-270)}$ bound to Zn^{2+} -charged IDA-agarose (Fig. 4B) but not to uncharged IDA-agarose (Fig. 4A, C). The bound GST-tagged $\text{PP2Ac}_{(51-270)}$ protein was eluted by the metal-chelating agent EDTA

(100 mmol/L). No Zn^{2+} binding was detected for the other two PP2A segments. These data indicate that Zn^{2+} binds to the $\text{PP2Ac}_{(51-270)}$ segment directly and then inhibits PP2A.

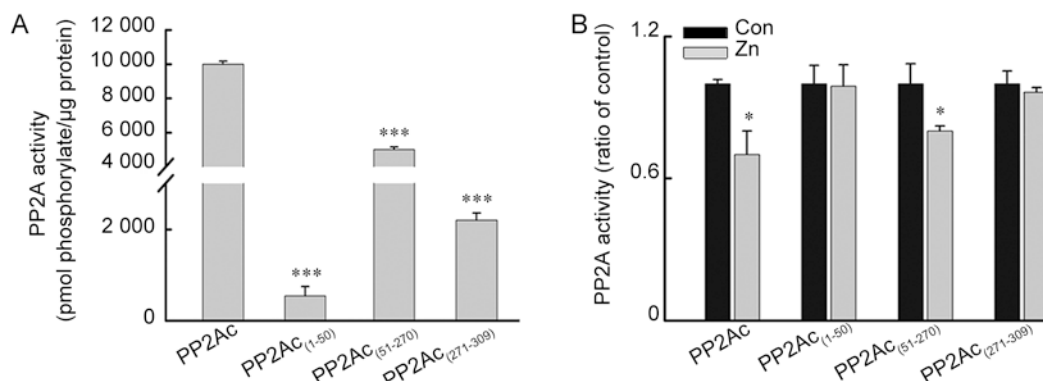


Fig. 3. Recombinant truncated PP2Ac₍₅₁₋₂₇₀₎ possesses biological activity and is inhibited by zinc. (A) Activity of full-length PP2A and purified truncated PP2Ac₁₋₅₀, 51-270, 271-309 measured by protein phosphatase activity assay. ****P* < 0.001 vs PP2Ac. (B) Full-length PP2A and purified recombinant PP2Ac₁₋₅₀, 51-270, 271-309 were separately incubated with 100 μmol/L ZnSO₄ for 30 min, and the activity was measured by protein phosphatase activity assay. **P* < 0.05 vs untreated control.

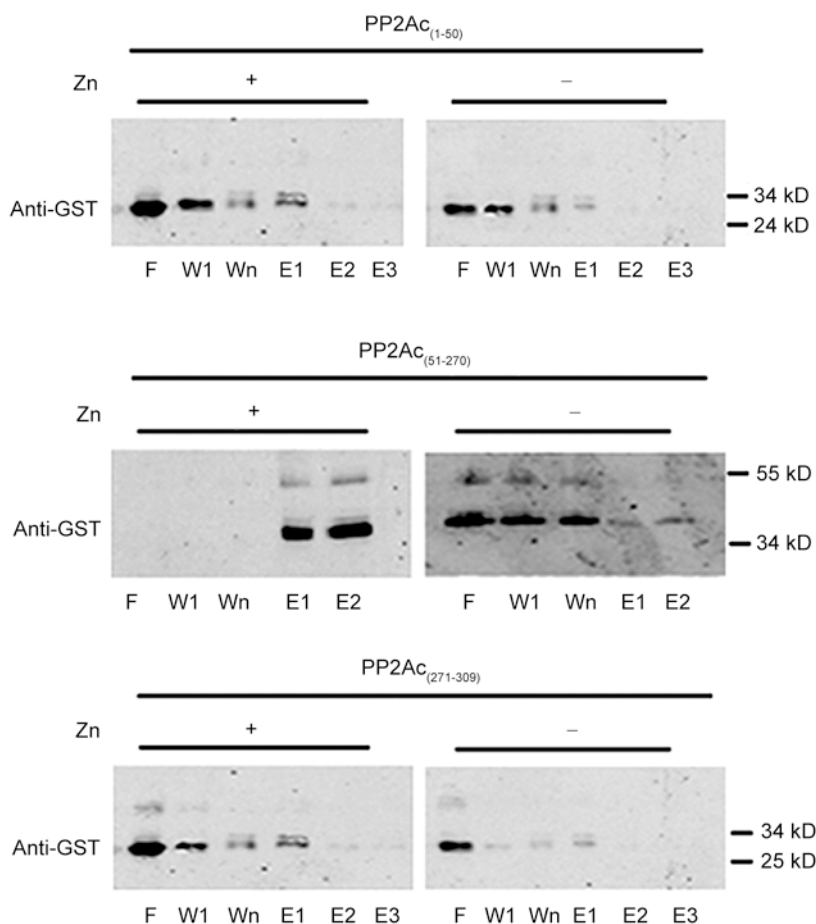


Fig. 4. Zinc binds directly to PP2Ac₍₅₁₋₂₇₀₎. Recombinant truncated GST-PP2Ac₁₋₅₀, 51-270, 271-309 segments were loaded into IDA-agarose charged with (+) or without (-) Zn²⁺, then all the fragments were collected separately and underwent Western blotting for GST. Only the elutes of GST-PP2Ac₅₁₋₂₇₀ segment loaded into Zn²⁺-charged DA-agarose show anti-GST bands. F, the effluent; W1 and Wn, wash solutions; E1, E2, and E3, the 1st, 2nd, and 3rd elutes of bound proteins.

DISCUSSION

PP2A is one of the most important serine/threonine protein phosphatases, and is ubiquitous in eukaryotic cells. It plays prominent roles in regulating the cell cycle, signal transduction pathways, morphology, and development^[21]. Downregulation of PP2A has been reported in the AD brain^[4,5,7], while PP2A suppression is necessary for immortalized cells to complete transformation in carcinogenesis^[22]. The PP2A holoenzyme is composed of scaffolding/structural A, regulatory/targeting B, and catalytic C subunits; different B subunits influence its activity by determining the substrate specificity^[23]. The activity of the catalytic subunit is affected by many factors. *In vivo*, it is regulated by PP2A inhibitory proteins such as I₁^{PP2A} and I₂^{PP2A}^[13, 14]. *In vitro*, the PP2A activity is dramatically inhibited after tyrosine 307 phosphorylation^[24, 25]. Xiong *et al.* reported that Zn²⁺ inhibits PP2A activity and induces tauopathy through activating Src-dependent PP2A (tyrosine 307) phosphorylation^[15], indicating that Zn²⁺ may inhibit PP2A indirectly through a tyrosine phosphorylation-related signaling pathway.

In this study, we investigated the direct effect of Zn²⁺ on PP2A *in vitro*. We first investigated a possible direct inhibitory effect by comparing the dephosphorylation level of tau in Zn²⁺- and OA-incubated N2a cell lysates. Tau is a favored substrate of PP2A^[10, 28]. *In vitro*, tau is gradually dephosphorylated by many serine/threonine protein phosphatases, of which PP2A contributes the major part^[7]. OA is a potent inhibitor of protein phosphatase, and only inhibits PP2A when used at 10 nmol/L *in vitro*^[19]. In our study, OA prevented the dephosphorylation of tau at Thr212, Ser262, and Ser396 *in vitro*; all are favored dephosphorylation sites of PP2A, especially Thr212 and Ser262. In addition, Zn²⁺ had an effect on tau dephosphorylation similar to OA, suggesting that Zn²⁺ inhibits PP2A *in vitro*. In our experimental system, exogenous ATP was not added to the cell lysates during incubation, so the effect of tau kinases on tau phosphorylation could be excluded.

Then we assessed the PP2A activity in Zn²⁺-incubated cell lysates without ATP. The results showed that Zn²⁺ inhibited PP2A at concentrations as low as 10 μmol/L, and the inhibitory effect was time-dependent within 1 h. As new phosphorylation (ATP needed, including tyrosine

phosphorylation) was blocked, we interpreted the PP2A inhibition as a direct effect of Zn²⁺. To confirm this hypothesis, we further incubated purified full-length PP2Ac with Zn²⁺ directly, and found that it reduced PP2A activity by ~30%. To further reveal the underlying mechanism, we cut PP2Ac into several segments and then tested their interactions with Zn²⁺ using a Zn²⁺-IDA-agarose affinity binding assay. The crystal structure of the PP2A catalytic subunit is not known, however, based on the three-dimensional structure of the PP-1 catalytic subunit, which has a catalytic domain highly homologous with PP2A, the PP2Ac₍₅₁₋₂₇₀₎ segment is thought to cover the catalytic active site and metal coordination center^[20]. Furthermore, Wozniak *et al.* reported that the motif of amino-acids 50–270 of PP2Ac contains active site with high affinity with divalent cations^[29]. So we tested the interaction of Zn²⁺ with PP2Ac₍₁₋₅₀₎, PP2Ac₍₅₁₋₂₇₀₎, and PP2Ac₍₂₇₁₋₃₀₉₎ separately. We found that Zn²⁺ bound with the PP2Ac₍₅₁₋₂₇₀₎ segment, which showed high catalytic activity in the PP2A activity assay, and Zn²⁺ directly inhibited its activity. These results indicate that Zn²⁺ inhibits PP2A directly by binding with PP2Ac₍₅₁₋₂₇₀₎.

In conclusion, Zn²⁺ binds to and inhibits PP2A directly *in vitro*, and this effect occurs on PP2Ac₍₅₁₋₂₇₀₎. Further studies are needed to reveal the detailed mechanism of the interaction.

ACKNOWLEDGEMENTS

This work was supported by Natural Science Foundation of Hubei Province, China (2012FFA044, 2013CFB258) and the National Natural Science Foundation of China (81271403, 81471304, 81261120570).

Received date: 2014-07-07; Accepted date: 2014-10-14

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