Journal of Alzheimer's Disease 20 (2010) 1091–1106
DOI 10.3233/JAD-2010-091444
IOS Press

Magnesium Modulates Amyloid-β Protein Precursor Trafficking and Processing

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Handling Associate Editor: Chengxin Gong

Accepted 26 January 2010

Abstract. Alzheimer's disease (AD), the most common form of dementia, is characterized by the presence of excessive deposits of aggregated amyloid-β (Aβ), which is derived from the amyloid-β protein precursor (AβPP) following processing by β- and γ-secretase. Metal elements are implicated in the pathophysiology of AD. Magnesium affects many biochemical mechanisms vital for neuronal properties and synaptic plasticity, and magnesium levels were reported to be decreased in various tissues including brain of AD patients. However, the exact role of magnesium in the neurodegenerative process of AD remains elusive. In this study, we investigated the effects of physiological (0.8 mM, as normal control), low (0–0.4 mM), and high (1.2–4.0 mM) concentrations of extracellular magnesium ($[Mg^{2+}]_o$) on AβPP processing and Aβ secretion. Here we show the effects of varying $[Mg^{2+}]_o$ on AβPP processing is time- and dose-dependent. After 24 h treatment, high $[Mg^{2+}]_o$ increased C-terminal fragment-α (CTFα) levels and soluble α-secretase cleaved AβPP (sAβPPα) release via enhancing retention of AβPP on plasma membrane. In contrast, low $[Mg^{2+}]_o$ enhanced CTFβ accumulation and Aβ secretion, and reduced cell surface AβPP level. Varying $[Mg^{2+}]_o$ did not alter protein contents of full length AβPP. However, decreased total intracellular magnesium level by magnesium deprivation over 24 hr impaired cell viability. Normal AβPP processing could be restored when magnesium was adjusted back to physiological concentration. These data demonstrate that AβPP processing can be modulated by magnesium and at high $[Mg^{2+}]_o$, AβPP processing favors the α-secretase cleavage pathway. Our findings suggest that supplementation of magnesium has a therapeutic potential for preventing AD.

Keywords: Alzheimer's disease, amyloid-β, amyloid-β protein precursor, magnesium

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is characterized by the presence of excessive deposits of aggregated amyloid-β (Aβ). In the majority of patients with so-called sporadic late-onset AD, an age-dependent accumulation of Aβ caused by disturbed dynamic balance between anabolic and catabolic activities has been implicated [1]. Also, environmental factors such as metallic elements may play a protective or disruptive role in the pathogenesis of AD (for review, see [2,3]). Different metals may be involved in multiple aspects of the disease process, such as the regulation of amyloid-β protein precursor (AβPP) gene expression

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ISSN 1387-2877/10/$27.50

and mRNA translation, the proteolytic processing of AβPP, the aggregation and degradation of Aβ, and the formation of neurofibrillary tangles. The role of magnesium in dementia and other degenerative disorders has attracted increased attention in recent years, and magnesium levels were found to be lower in different tissues of patients with AD in clinical, experimental, and autopsy studies [4,5]. Neuronal degeneration occurs in presenilin 1 (PS1) mutant mice without extracellular Aβ deposits, suggesting it is caused by the accumulation of intracellular $A\beta_{42}$ [6]; deposits of intracellular $A\beta_{42}$ are correlated with apoptotic cell death in AD brains [7]. The Aβ is derived from AβPP through sequential cleavages by β- and γ-secretases, whose enzymatic activities are tightly controlled by subcellular localization. Thus, delineation of how the intracellular trafficking of these secretases and AβPP are regulated is important for understanding AD pathogenesis. Although AβPP trafficking has been found to be regulated by multiple factors including PS1, a major component of the γ-secretase complex, and phospholipase D1, a phospholipid-modifying enzyme; AβPP can reciprocally regulate PS1 trafficking; AβPP deficiency results in faster transport of PS1 from the trans-Golgi network to the cell surface and increased steady state levels of PS1 at the cell surface, which can be reversed by restoring AβPP levels [8]. However, it is not known whether altered magnesium level may also affect AβPP trafficking or/and processing.

In this context, the main objective of the present study is to determine whether magnesium might be implicated in AβPP processing and Aβ secretion and to understand the mechanisms involved. We cultured mouse N2a neuroblastoma cells stably expressing AβPP in varying concentrations of magnesium, analyzed the levels of total full length AβPP (flAβPP), cell surface AβPP, AβPP C-terminal fragments (CTFs), soluble AβPP (sAβPP), and secreted Aβ. We reported that compared with physiological concentration (i.e., 0.8 mM), high doses of extracellular magnesium ($[Mg^{2+}]_o$) stimulated nonamyloidogenic α-secretase-mediated AβPP processing via increasing retention of AβPP on cell surface, whereas low doses of extracellular magnesium increased amyloidogenic CTFβ and Aβ accumulation.

MATERIALS AND METHODS

Chemicals and antibodies

The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tertbutyl ester (DAPT) was purchased from Calbiochem (San Diego, CA, USA) and was dissolved in dimethylsulfoxide (Me_2SO). $MgSO_4$ and Na_2SO_4 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in H_2O to 100 mM. $A\beta_{40}$ and $A\beta_{42}$ were purchased from American Peptide (Sunnyvale, CA, USA). The following AβPP antibodies were used: 6E10 (recognizing $A\beta_{1-17}$) anti-AβPP monoclonal antibody (mAb) (Signet Laboratories, Dedham, MA, USA), 3D6 (recognizing $A\beta_{1-5}$) anti-AβPP mAb (Elan Pharmaceuticals, San Francisco, CA, USA), A8717 anti-AβPP C-terminus polyclonal antibody (pAb) (Sigma-Aldrich), A5441 anti-β-actin mAb (Sigma-Aldrich). Horseradish peroxidase conjugated anti-mouse and anti-rabbit antibodies, Protein-A agarose beads, and ECL-Plus Western blotting reagents were all purchased from Amersham Biosciences (Piscataway, NJ, USA). Complete protease inhibitor cocktail tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IL, USA). Alexa Fluor 488- or 594-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture and treatments

Mouse N2a neuroblastoma cells stably expressing both wild type presenilin 1 (PS1wt) and Swedish mutant AβPP (AβPPsw) (abbreviated as AβPPsw-N2a cells for convenience) were kindly provided by Drs. Sangram S. Sisodia and SeongHun Kim (University of Chicago), and were maintained in normal DMEM (Hyclone, South Logan, UT, USA) containing a physiological concentration of magnesium (i.e., 0.8 mM), and supplemented with 10% FBS [9]. Cells were passaged at a ratio of 1:6 when 90% confluence had been reached and discarded after 20 passages. 24 h after splitting, the conditioned DMEM medium containing 10% FBS was replaced with fresh serum-free Mg-free DMEM (Hyclone). All experiments were performed in the absence of serum in order to exclude additional Mg^{2+} from FBS. The magnesium levels in the media were adjusted with $MgSO_4$ (100 mM stock solution dissolved in H_2O; Sigma-Aldrich) to physiological (i.e., 0.8 mM, as normal control), low (0.0 and 0.4 mM), and high (1.2, 1.6, and 4.0 mM) concentrations. Cells were treated with different concentrations of extracellular magnesium ($[Mg^{2+}]_o$) for the time periods of 0, 3, 6, 12, 24, or 36 h.

Inductively coupled plasma mass spectrometry (ICP-MS) analysis of magnesium levels

At the indicated time points after treatments, cells were washed three times with phosphate-buffered

saline (PBS, pH 7.4). The cells were scraped into PBS, an aliquot was taken for protein determination using a BCA protein assay kit (Pierce, Rockford, IL, USA), and the remaining cells were collected by centrifugation at 14,000 g for 2 min. The total intracellular magnesium levels ($[Mg^{2+}]_i$) were determined in cell pellets by ICP-MS as described previously [10], and converted to fold increases in Bromophenol blue) and subjected to Western blot analysis with the method metal compared with controls.

Assessment of cell survival by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were seeded in a 96-well plate at a density of $1 \times 10e^4$ cells/100 μl. After being cultured in normal DMEM containing 10% FBS for 24 h, the cells were treated with varying $[Mg^{2+}]_o$ (0.0–4.0 mM) for 24 or 36 h. For MTT-reducing activity, the cells were then incubated with 0.5 mg/ml MTT (Sigma-Aldrich) at 37°C for 4 h. Media were removed, and dimethyl sulfoxide (100 μl) was added to each well to solubilize the formazan crystals generated by viable mitochondrial succinate dehydrogenase from MTT. The absorbance at 570 nm was measured using a VERSA MAX enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, USA) as the MTT reducing activity of the cells. The resultant data were expressed as the percentage of viable cells relative to controls (cells treated with 0.8 mM Mg^{2+}).

Immunoprecipitation (IP), electrophoresis, and Western blot analysis

After the indicated treatments, conditioned medium (CM) was collected, mixed with the complete protease inhibitor cocktail (Roche), and centrifuged at 3,000 g for 10 min to remove cell debris. Cells were harvested and lysed on ice in Western blot lysis buffer containing 50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors. The lysates were collected, centrifuged at 12,000 g for 5 min, and quantified for total proteins by the BCA protein assay kit.

To detect soluble α-secretase cleaved AβPP (sAβPPα), equivalent volumes of conditioned medium were directly analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), based on the protein concentration of cells in each plate. Secreted Aβ and sAβPPα were immunoprecipitated from conditioned medium using a monoclonal Aβ-specific antibody 6E10 as described previously [11], and culture supernatants were normalized for total cellular protein to correct for variations in cell number.

For analysis of flAβPP, CTFs, Aβ_{40} and Aβ_{42}, cell lysates, and immunoprecipitates were separated on 10% T 5% C Bicine/Tris, 8 M urea, SDS-PAGE or 10–18% regular SDS-PAGE system [12]. For Western blot analysis, protein was transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA), blocked for 1 h in 5% (m/v) nonfat milk in Tris-buffered saline (TBS, pH 7.5), supplemented with 0.1% Tween 20. Antibodies and their dilutions used in this study include 6E10 (1:1000) or A8717 (1:10000) for AβPP derivatives and anti-β-actin mouse mAb (1:5,000) as an internal reference control. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h at ambient temperature, the immunoblots were developed using the ECL system. Quantitative densitometric analyses were performed on digitized images of immunoblots with Quantity One software (Bio-Rad, Hercules, CA, USA). The γ-secretase inhibitor DAPT (500 nM, 8 h treatment) was employed in the experiments as a positive control for CTFα and CTFβ. Representative blots from at least three independent experiments are shown.

Quantification of secreted Aβ peptide levels by sandwich enzyme-linked immunosorbent assay (ELISA)

Following the 24 h treatment with varying $[Mg^{2+}]_o$, cells and conditioned media were harvested. Complete Protease Inhibitor cocktail was added to the medium to prevent degradation of Aβ protein and the cell debris was removed by centrifugation at 3,000 g for 10 min at 4°C. Medium were assayed for Aβ_{40} and Aβ_{42} by sandwich ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA). The Aβ concentration was normalized based on the amount of cells in each culture (as determined by protein content in the cell lysates). All measurements were performed in duplicate.

Immunofluorescence and confocal microscopy

Immunocytochemistry was performed as previously described [9,13]. The subcellular localization of AβPP was performed by immunocytochemistry using fluorescent secondary antibodies. After 24 h treatments with varying $[Mg^{2+}]_o$, cells grown on coverslips were fixed

and permeabilized with 100% methanol at −20 °C for 10 min. For surface AβPP staining, cells were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min without permeabilization [13]. Following extensive washing in PBS, cells were blocked with 3% bovine serum albumin (BSA) in PBS and then incubated with primary antibody in blocking buffer overnight at 4 °C. AβPP was detected with A8717 (1:2000) or 6E10 (1:200) antibody. CTFβ was specifically stained with mAb 3D6 (1:200) [9]. Cells were then washed thoroughly in PBS and the primary signal was detected for 1 h at 37 °C with Alexa Fluor 488 and 568 conjugated secondary antibodies (1:500). The coverslips were mounted on glass slides using Vecta shield mounting medium (Vector Laboratories, Burlingame, CA, USA), and were viewed with a Leica TCS-NT confocal microscope using a 60x objective oil immersion lens. Digital images were analyzed with Leica Q550IW software.

Biotinylation assay of cell surface AβPP

Cell surface biotinylation was performed with Cell Surface Protein Isolation Kit (Pierce) according to the protocol of the manufacturer. Briefly, after a 24 h treatment with $[Mg^{2+}]_o$ at 0.4, 0.8, and 1.2 mM, confluent cells grown in 10-cm dishes were cooled to 4 °C, washed with ice-cold PBS, and labeled with 0.25 mg/ml reducible sulfo-NHS-SS-Biotin in PBS for 30 min at 4°C. After washing and quenching of unreacted biotin, cells were lysed in lysis buffer containing complete protease inhibitors cocktail. After affinity precipitation with neutravidin beads, the biotinylated proteins were eluted with SDS-PAGE sample buffer and separated by SDS-PAGE using a 10% Tris-glycine gel. Biotinylated AβPP was specifically detected by Western blotting with 6E10 antibody and normalized based on the amount of AβPP in the respective total cell lysate. Blots were scanned, and the ratios of surface to total AβPP were determined.

Quantitative and statistical analysis

Quantitative data were expressed as mean ± SEM. Comparisons of the means among multiple groups were done by a one-way ANOVA followed by Dunnett's or Tukey-Kramer's *post hoc* tests using a statistical software package (GraphPad Prism, version 4.0; GraphPad Software, San Diego CA, USA). The asterisks indicate significant differences from controls as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS

Effects of varying $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$ and cell viability

Cells were cultured in normal DMEM containing 0.8 mM magnesium. To evaluate whether $[Mg^{2+}]_o$ regulates intracellular magnesium homeostasis and cell viability, normal DMEM with 10% FBS was replaced with magnesium-free and serum-free DMEM, and then magnesium was added to different concentrations as described in Materials and Methods. Cells were treated with varying $[Mg^{2+}]_o$ for the indicated time course. The $[Mg^{2+}]_i$ was measured by ICP-MS. As shown in Fig. 1, incubation of cells for 24 h in a magnesium free medium ($[Mg^{2+}]_o$ at 0.0 mM) resulted in a 12% ($p < 0.001$) decrease of $[Mg^{2+}]_i$ in comparison with normal control (cells treated with 0.8 mM Mg^{2+}). However, when cells were cultured in medium containing $[Mg^{2+}]_o$ of 0.4 to 4.0 mM for 24 h, no change of $[Mg^{2+}]_i$ was detected (Fig. 1A). Furthermore, no detectable differences in $[Mg^{2+}]_i$ were observed when cells were cultured for over 36 h in a medium containing 0.4 or 1.2 mM magnesium (Fig. 1B). Thus, variation of the magnesium concentration in culture media by about ± 50% of the physiological level (i.e., 0.8 mM) had no effect on the intracellular magnesium levels.

Next, we examined whether variation of extracellular Mg^{2+} have any effect on cell survival. MTT assays were performed on cells cultured for 24–36 h in medium containing different concentrations of magnesium. As shown in Fig. 1C, after culture for 24 h in magnesium free medium, cell viability reduced by approximately 21% ($p < 0.01$) compared with that of cells cultured in normal media containing 0.8 mM $[Mg^{2+}]_o$. However, when cells were cultured in media containing 0.4 to 4.0 mM Mg^{2+}, no significant change in cell viability were observed as determined by MTT assay. Furthermore, when cells were cultured for over 36 h in media containing 0.4 and 1.2 mM Mg^{2+}, no change in cell viability was observed (Fig. 1C, right panel). Collectively, all these results indicate that complete depletion of magnesium in a culture medium has a strong effect on cell viability and might be cytotoxic, while the changes of $[Mg^{2+}]_o$ in an range from 0.4 to 4.0 mM had no appreciable effect.

Effects of varying $[Mg^{2+}]_o$ on AβPP processing

To determine the effect of extracellular magnesium on AβPP cleavage, we cultured cells in media con-

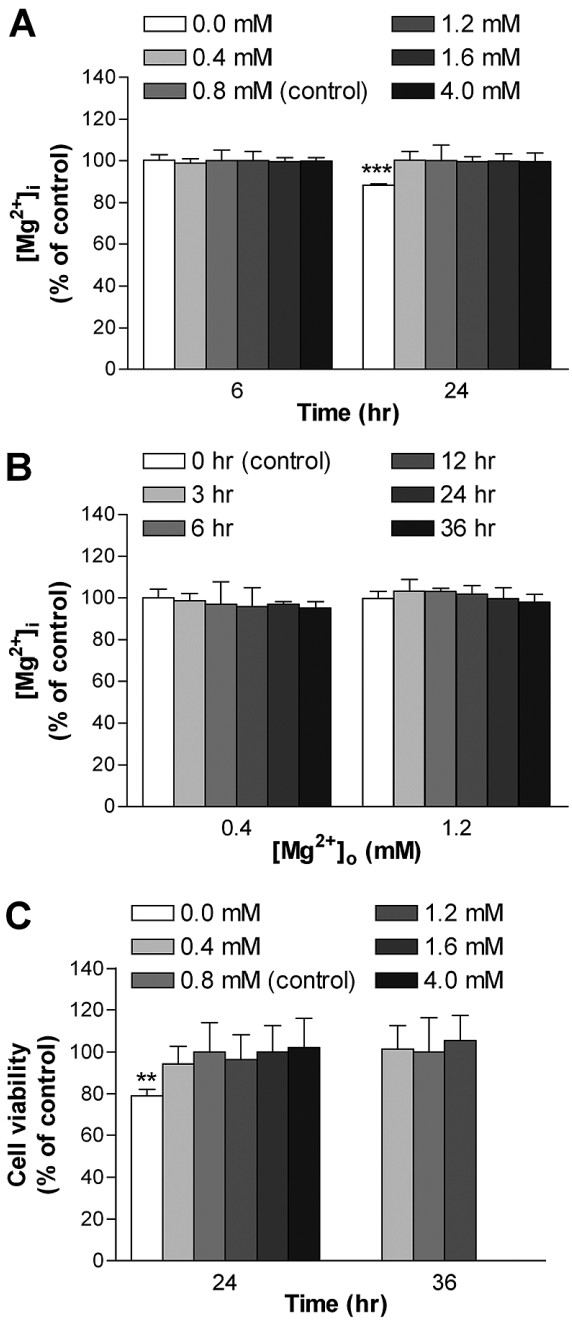

Fig. 1. Effects of varying $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$ and cell viability. A) Cells were exposed to 0.0-4.0 mM Mg^{2+}, and intracellular magnesium levels were measured at 6 and 24 h after exposure using ICP-MS. Data are represented as % change relative to control (cells treated with 0.8 mM Mg^{2+}). No change in $[Mg^{2+}]_i$ was observed after 6 h, however $[Mg^{2+}]_o$ at 0.0 mM induced significant loss of intracellular magnesium after 24 h of exposure. B) Cells were exposed to 0.4 and 1.2 mM Mg^{2+}, and intracellular magnesium levels were measured at 0, 3, 6, 12, 24, and 36 h after exposure. Data are represented as % change relative to control (time point at 0 h). No significant change was observed at each time point of exposure to $[Mg^{2+}]_o$ at 0.4 or 1.2 mM. C) Cells were exposed to 0.0–4.0 mM Mg^{2+}, and cell viability was measured at 24 and 36 h after exposure using the MTT assay. Data are represented as % change relative to control (cells treated with 0.8 mM Mg^{2+}). $[Mg^{2+}]_o$ at 0.0 mM induced a significant loss of viability after 24 h of exposure. Each bar represents the mean ± SEM from 6–10 different preparations. $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control.

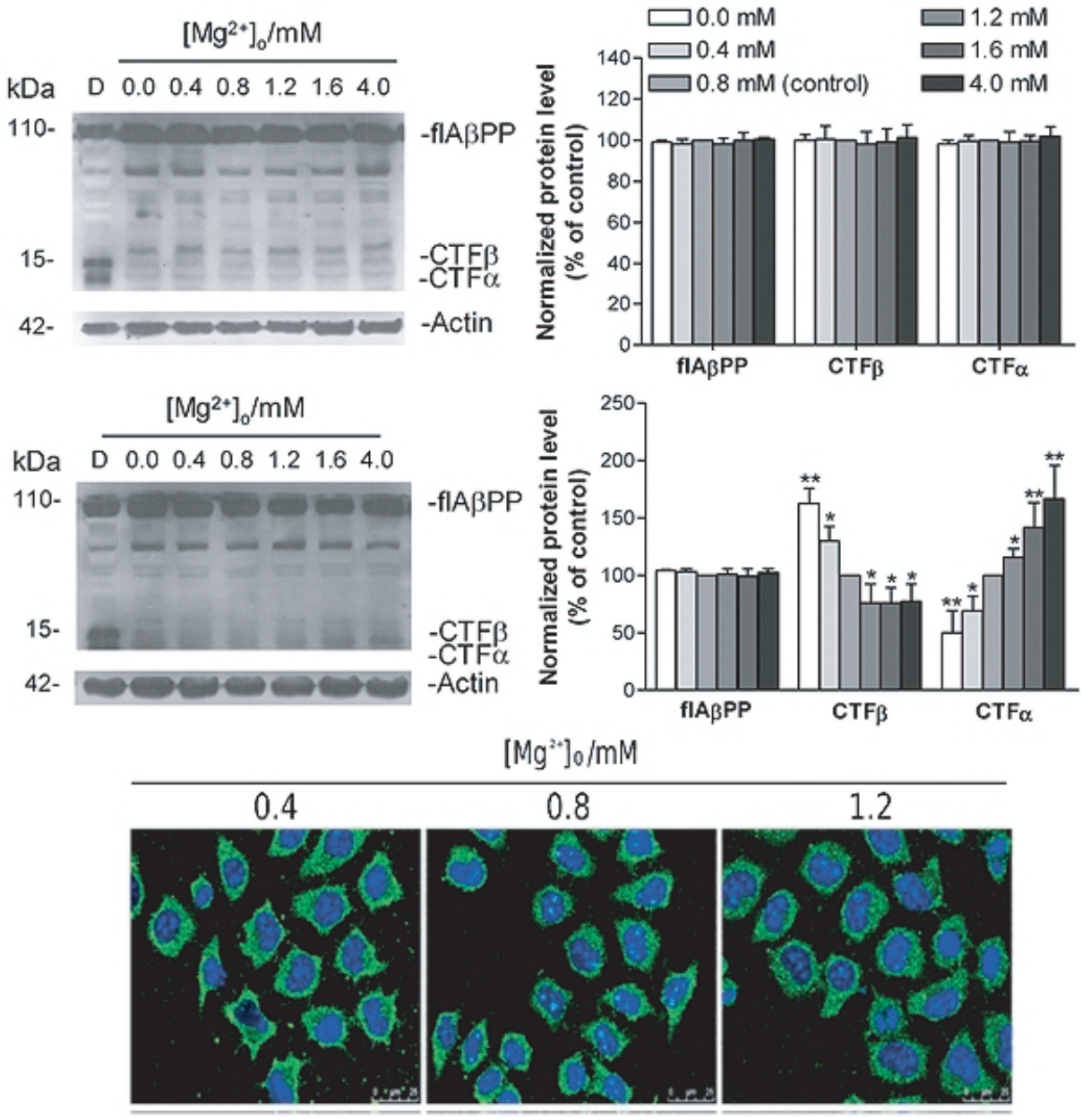

Fig. 2. Effects of varying $[Mg^{2+}]_o$ on AβPP processing. A) After treatment with varying $[Mg^{2+}]_o$ for 6 h, cell lysates were prepared, flAβPP and CTFs in cell lysates were analyzed by Western blot analysis using antibody A8717. Note that exposure to varying $[Mg^{2+}]_o$ for 6 h did not alter the levels of flAβPP, CTFβ, and CTFα normalized to β-actin. As a positive control, the γ-secretase inhibitor DAPT (500 nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (far left lane). B) After 24 h treatment, $[Mg^{2+}]_o$ induced dose-dependent increase of CTFα and reduction of CTFβ. CTFα in cell lysates were increased in high $[Mg^{2+}]_o$ whereas CTFβ were increased in low $[Mg^{2+}]_o$. Levels of flAβPP did not appear to change under any conditions. Normalized protein levels to β-actin are depicted in the corresponding diagrams. Densitometric analysis of Western blots is expressed as a percentage of the level of control (cells treated with 0.8 mM Mg^{2+}). Values are expressed as mean $\pm$ SEM from three independent experiments carried out in duplicates. *$p < 0.05$, and **$p < 0.01$ versus control. C) After treatment with low (0.4 mM), physiological (0.8 mM) and high (1.2 mM) $[Mg^{2+}]_o$ for 24 h, cells were fixed with methanol and sequentially incubated with antibody 3D6 and Alexa Fluor 488-conjugated goat anti-mouse antibody to visualize intracellular CTFβ (green) [9]. Note the profound accumulation of CTFβ within intracellular compartments in 0.4 mM Mg^{2+}-treated cells. 1.2 mM Mg^{2+} treatment showed the opposite effect, with reduced intracellular CTFβ immunoreactivities compared with the control (cells treated with 0.8 mM Mg^{2+}). Scale bars = 25 μm.

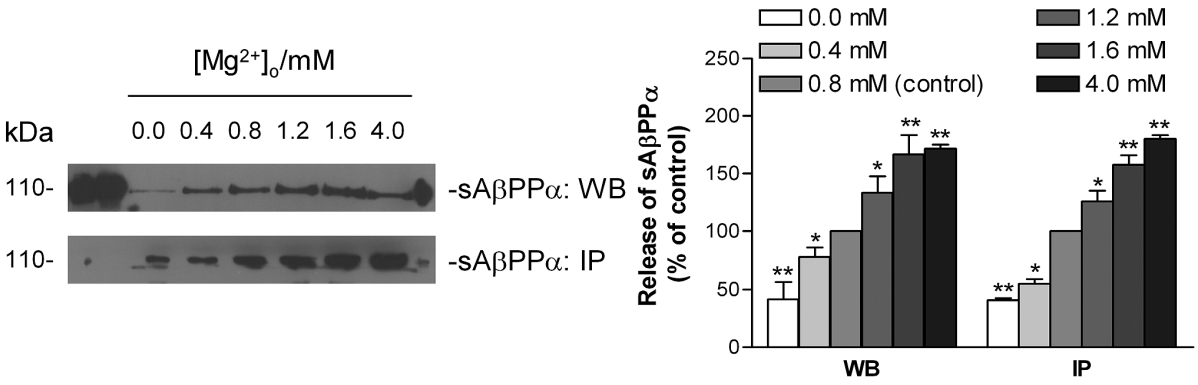

Fig. 3. Dose-response effect of $[Mg^{2+}]_o$ on sAβPPα release. Cells were incubated for 24 h with varying concentrations of magnesium (0.0–4.0 mM). Conditioned medium were collected and subjected to Western blot analysis for sAβPPα without (upper panel) or with (lower panel) immunoprecipitation of sAβPPα using antibody 6E10 first. Note that secretion of sAβPPα dose-dependently increases or decrease in response to high or low $[Mg^{2+}]_o$, respectively. The flAβPP in cell lysate was employed as positive control of sAβPPα (far left lane). Densitometric analysis of Western blots is expressed as a percentage of the level of control (cells treated with 0.8 mM Mg^{2+}). The data are the means $\pm$ SEM for three independent experiments. *, $p < 0.05$, and **, $p < 0.01$ versus control.

taining different concentrations of magnesium (0.0–4.0 mM) and for different times (0–36 h), and AβPP cleavage profiles were assayed using Western blot and immunoprecipitation analysis.

After 6 h of exposure, cells were harvested and subjected to Western blot analyses using 10% Bicine/urea SDS-PAGE as described previously [11]. As shown in Fig. 2A, immunoblotting with A8717, a pAb raised against the C-terminus of AβPP, revealed no significant difference in the level of flAβPP normalized to β-actin in cells treated with different $[Mg^{2+}]_o$ for 6 h. Likewise, changing $[Mg^{2+}]_o$ did not alter the protein level of CTFβ and CTFα. As a positive control, the γ-secretase inhibitor DAPT (500 nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (Fig. 2A, far left lane). Consistent results were obtained by using 10–18% Tris/glycine SDS-PAGE (data not shown).

Next, we assessed the effects of different doses of magnesium on AβPP processing. After 24 h of treatment, cells were harvested and subjected to Western blot analyses using A8717antibody to detect flAβPP, CTFβ, and CTFα. As shown in Fig. 2B, the different $[Mg^{2+}]_o$ caused a dose-dependent decrease in CTFβ and a concomitant increase in CTFα, indicating that low $[Mg^{2+}]_o$ and high $[Mg^{2+}]_o$ have opposite effects on α- and β-site cleavage of AβPP. Quantification of flAβPP, CTFβ, and CTFα (normalized to β-actin) revealed that a $[Mg^{2+}]_o$ at 4.0 mM resulted in a 66% increase of CTFα and 22% decrease of CTFβ ($p < 0.01$) compared with normal control (physiological $[Mg^{2+}]_o$, i.e., 0.8 mM), whereas $[Mg^{2+}]_o$ at 0.0 mM led to a 50% decrease of CTFα and 63% increase of CTFβ ($p < 0.01$). However, no significant differences in the flAβPP levels were observed in cells exposed to different concentrations of magnesium (Fig. 2B). Together, these data suggest that magnesium increases the non-amyloidogenic AβPP cleavage by α-secretase and decreases the amyloidogenic processing by β-secretase.

To further confirm these biochemical results, we also evaluated the level of CTFβ by immunofluorescence analysis following 24 h of treatment with different $[Mg^{2+}]_o$. The dose-dependent decrease in CTFβ levels was also revealed by immunocytochemistry using the monoclonal antibody 3D6 which specifically recognizes the N-terminus of Aβ and the β-secretase-generated membrane-retained stub of AβPP (i.e., CTFβ), without recognizing either the flAβPP or the CTFs generated by α-secretase [9]. As shown in Fig. 2C, after 24 h exposure, higher CTFβ levels were found in 0.4 mM Mg^{2+} treated cells whereas CTFβ levels were decreased in 1.2 mM Mg^{2+} treated cells as compared with normal control (cell treated with 0.8 mM Mg^{2+}) (Fig. 2C).

To confirm that the non-amyloidogenic α-cleavage was favored by high concentration of magnesium, we analyzed effects of different $[Mg^{2+}]_o$ on the release of sAβPPα into the conditioned medium, following 24 h incubation. sAβPPα in the medium was immunoprecipitated from the medium first, or directly detected by immunoblotting with mAb 6E10, which recognizes the first 16 residues of Aβ and therefore detects sAβPPα but not sAβPPβ. Levels of sAβPPα were normalized to corresponding total protein level in cell lysates. As shown in Fig. 3, treatment for 24 h with different $[Mg^{2+}]_o$ resulted in a significant dose-dependent in-

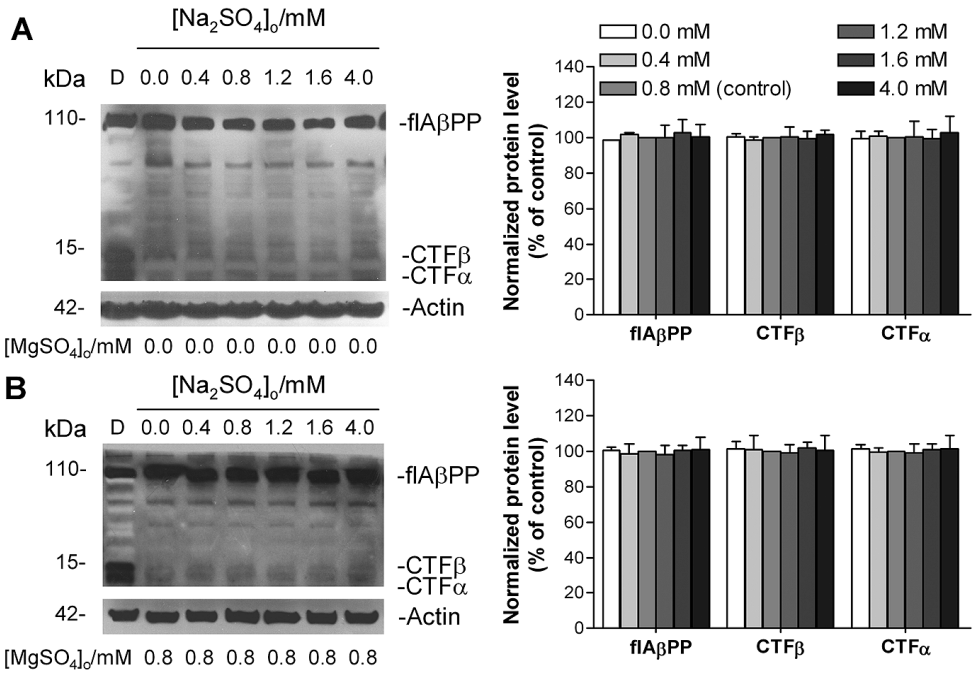

Fig. 4. Effects of varying $[Na_2SO_4]_o$ on AβPP processing after 24 h treatment. Cells were exposed to 0–4.0 mM of Na_2SO_4 for 24 h, in the absence (A) or presence (B) of 0.8 mM Mg^{2+}. Following treatment, immunoblot analysis revealed no change in normalized protein level of flAβPP and CTFs. As a positive control, the γ-secretase inhibitor DAPT (500nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (far left lane). Densitometric analysis of Western blots is expressed as a percentage of the level of control (cells treated with 0.8 mM Na_2SO_4). The data are the means $\pm$ SEM from three independent experiments.

crease in sAβPPα released into the medium. Compared with physiological $[Mg^{2+}]_o$ at 0.8 mM, high $[Mg^{2+}]_o$ (1.2, 1.6, and 4.0 mM) increases sAβPPα release, and the maximal effect was obtained at 4.0 mM, which resulted in a 80% increase ($p < 0.01$) of sAβPPα secretion. However, the levels of sAβPPα decreased upon treatment with low $[Mg^{2+}]_o$ (0.0 and 0.4 mM), and the maximal effect was obtained at 0.0 mM, which resulted in a 60% reduction of sAβPPα secretion over the control level ($p < 0.01$). The concentration-dependent effects of $[Mg^{2+}]_o$ on sAβPPα release was detected by both immunoprecipitation and direct Western blot analysis of the conditioned medium.

As mentioned in the Materials and Methods, we used concentrated $MgSO_4$ stock solution to adjust $[Mg^{2+}]_o$ to final concentration of 0–4.0 mM. Although $MgSO_4$ dose-dependently regulates AβPP processing, Na_2SO_4 had no such effect. In the presence of 0.0 or 0.8 mM of extracellular magnesium, cells exposed to different $[Na_2SO_4]_o$ (0–4.0 mM) for 24 h have relatively the same level of flAβPP, CTFβ, and CTFα (Fig. 4). This excluded the possibility that dose-dependent effect of $MgSO_4$ on AβPP processing is due to the increase in concentration of sulfate ion ($[SO_4^{2-}]_o$).

As mentioned above, high $[Mg^{2+}]_o$ can promote the α-secretase cleavage and demote the β-secretase cleavage. We also found such an effect is time dependent. Cell lysates were prepared from cells treated with $[Mg^{2+}]_o$ at 1.2 mM for different time lengths (0–36 h) and the cell lysates were subjected to Western bolt analysis for flAβPP and CTFs. As illustrated in Fig. 5, Western blot analysis clearly shows that significant increase of CTFα and decrease of CTFβ induced by 1.2 mM Mg^{2+}, occurred until time length was extended up to 24 h. Quantification of flAβPP, CTFβ, and CTFα (normalized to β-actin) revealed that $[Mg^{2+}]_o$ at 1.2 mM led to a 32% decrease of CTFβ and 33% increase of CTFα over 24 h treatment ($p < 0.05$). Most notably, CTFα level dramatically increased up to 2.3 fold after 36 h of 1.2 mM Mg^{2+} treatment ($p < 0.01$).

Effects of varying $[Mg^{2+}]_o$ on secreted Aβ levels

The high levels of amyloidogenic CTFβ indicated that β-secretase cleavage of AβPP increased in cells treated with low $[Mg^{2+}]_o$, and therefore, it is expected that Aβ levels would also be elevated in response to low $[Mg^{2+}]_o$ treatment. To investigate this pos-

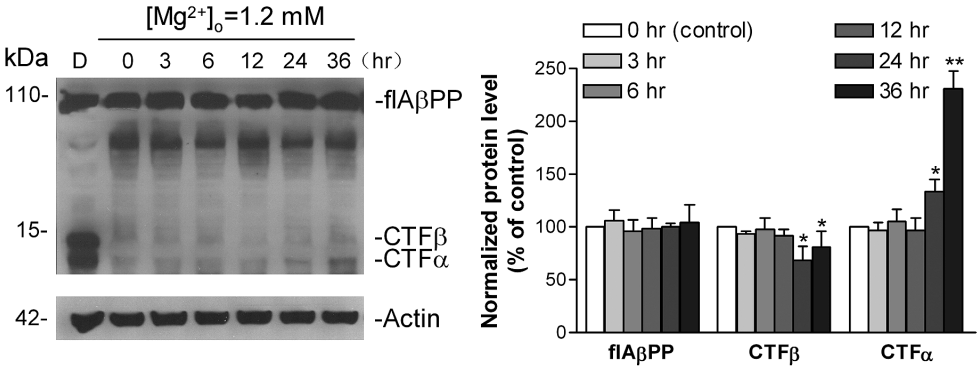

Fig. 5. Time-dependent effect of magnesium on AβPP processing. Cell lysates were prepared from cells treated with $[Mg^{2+}]_o$ at 1.2 mM for 0–36 h and subjected to Western blot analysis using antibody A8717. Note that high $[Mg^{2+}]_o$ induced significant decrease of CTFβ and increase of CTFα is notable up to 24 h after treatment. As a positive control, the γ-secretase inhibitor DAPT (500 nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (far left lane). Densitometric analysis of Western blots were expressed as a percentage of control (time point at 0 h). The data are the means $\pm$ SEM of three different experiments. *$p < 0.05$, and **$p < 0.01$ versus control.

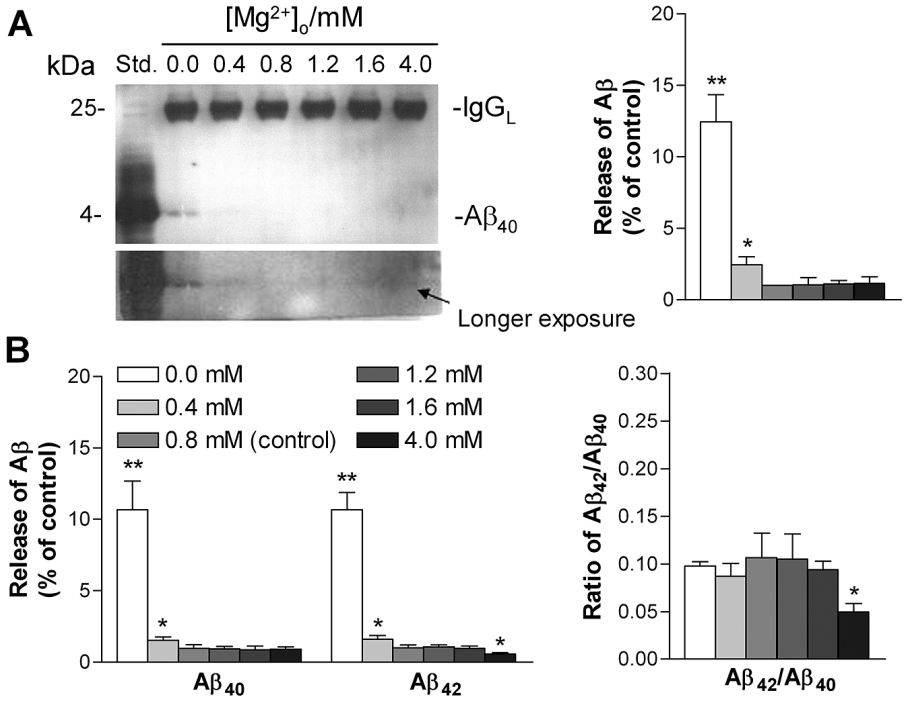

Fig. 6. Effects of varying $[Mg^{2+}]_o$ on Aβ secretion after 24 h exposure. Cells were cultured in the presence of indicated $[Mg^{2+}]_o$ for 24 h. A) Secreted Aβ was immunoprecipitated from conditioned medium with antibody 6E10, and subjected by Western blot analysis on 10% bicine-urea gel. Aβ levels were densitometrically analyzed and depicted in diagrams as a percentage of control (cells treated with 0.8 mM Mg^{2+}). Note that the amount of Aβ_{42} peptides in medium was beyond the detection limit, and Aβ_{40} level were significantly increased at low $[Mg^{2+}]_o$. To confirm bands corresponding to Aβ_{40}, synthetic Aβ_{40} was employed as positive control (far left lane). B) Secreted Aβ_{40} and Aβ_{42} in medium were also determined by ELISA. Levels of Aβ are represented as % change relative to control (cells treated with 0.8 mM Mg^{2+}). Note that $[Mg^{2+}]_o$ at low doses increased both Aβ_{40} and Aβ_{42} levels (normalized to total protein amount in respective cell lysate) as compared with control treatment, whereas at high doses only 4.0 mM Mg^{2+} induced a significant reduction of Aβ_{42}. 4.0 mM Mg^{2+} induced a significant reduction of Aβ_{42}/Aβ_{40} ratio from 0.1 to 0.05 compared with other conditions. IgG_L is the light chain of immunoglobulins. The data are the means $\pm$ SEM from 3–6 independent experiments. *$p < 0.05$, and **$p < 0.01$ versus control.

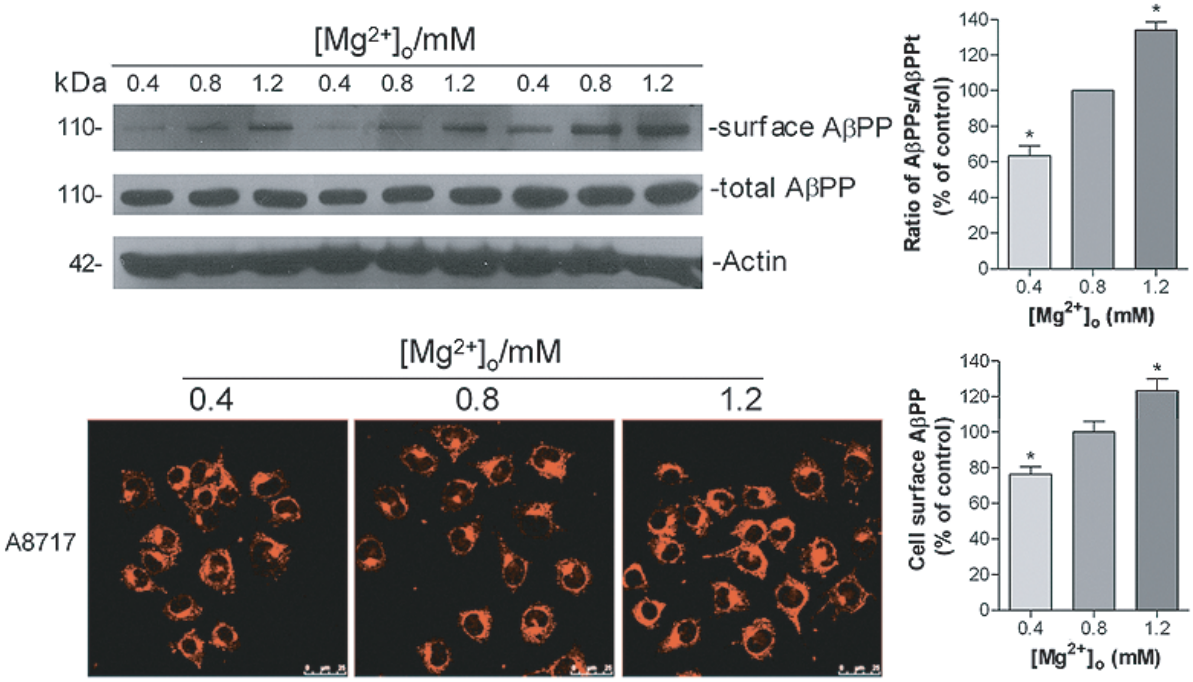

Fig. 7. Effects of varying $[Mg^{2+}]_o$ on the presence of AβPP at the plasma membrane after 24 h treatment. Cells were exposed to low (0.4 mM), physiological (0.8 mM), and high (1.2 mM) $[Mg^{2+}]_o$ for 24 h. A) Following treatment, surface proteins were labeled with sulfo-NHS-SS-Biotin solution, isolated using neutravidin beads, and immunoblotted with antibody 6E10. Cell surface level of AβPP was normalized to total flAβPP levels in the complete cell lysates. Top, compared with physiological concentration, $[Mg^{2+}]_o$ at 0.4 and 1.2 mM, decreased and increased surface levels of AβPP, respectively. Middle, levels of full-length AβPP were unaffected by varying $[Mg^{2+}]_o$. The cell surface AβPP (AβPPs) to total AβPP (AβPPt) ratio was calculated and is expressed as a percentage of the level of control (cells treated with 0.8 mM Mg^{2+}). The data are the means $\pm$ SEM from three independent experiments. B) Non-permeabilized cells were immunolabeled with antibody A8717 (Alexa594; red), and the immunofluorescent intensity of cell surface AβPP were quantified ($n = 121$–155 cells under each treatment condition). Data are represented as % change relative to control (cells treated with 0.8 mM Mg^{2+}). Note that $[Mg^{2+}]_o$ at 1.2 mM increased, whereas $[Mg^{2+}]_o$ at 0.4 mM reduced cell surface AβPP immunofluorescent staining compared with the control. Values are expressed as mean $\pm$ SEM from three independent experiments. *, $p < 0.05$ versus control. Scale bars = 25 μm.

sibility, we treated cells with different $[Mg^{2+}]_o$ for 24 h. The conditioned medium was collected and the protein concentration in cell lysates was determined. Normalized volumes of medium, relative to protein concentration in cell lysates, were subjected to immunoprecipitation/Western blot analysis and specific ELISA. As shown in Fig. 6A, immunoblotting with antibody 6E10 following immunoprecipitation revealed that $[Mg^{2+}]_o$ at low dose resulted in a 1.4-fold (0.4 mM) and 11.5-fold (0.0 mM) increase of Aβ_{40} levels as quantified by densitometric analysis of the Western blot results. In contrast, high $[Mg^{2+}]_o$ did not induce significant changes in the release of Aβ_{40}. Aβ_{1-42} peptides, which migrate faster than Aβ_{1-40} in Bicine/urea gels [11], were below the level of detection limit in the immunoprecipitation/Western blot analysis for the doses and times of magnesium treatment tested. To confirm bands corresponding to Aβ_{40}, synthetic Aβ_{40} was employed as markers. Similar increase in the level of Aβ after treatment with low $[Mg^{2+}]_o$ were evidenced by ELISA analyses (Fig. 6B). Specifically, after 24 h treatment, compared with normal control, $[Mg^{2+}]_o$ at 0.0 mM resulted in a 9.7-fold increase of Aβ_{40} and Aβ_{42} ($p < 0.01$) compared to control, and $[Mg^{2+}]_o$ at 0.4 mM markedly increased the level of secreted Aβ_{40} and Aβ_{42} by 55% and 62% ($p < 0.05$), respectively, whereas high $[Mg^{2+}]_o$ at 1.2 and 1.6 mM did not alter levels of secreted Aβ. Interestingly, 4.0 mM Mg^{2+} did not change Aβ_{40}level, but reduced secretion of Aβ_{42} by 42% ($p < 0.05$). Aβ is secreted in several different forms, with Aβ_{40} as the predominant species and Aβ_{42} as a minor but more amyloidogenic form. ELISA analysis also revealed that 4.0 mM Mg^{2+} induced a significant reduction of Aβ_{42}/Aβ_{40} ratio to 0.05, whereas cells treated with 0.0–1.6 mM Mg^{2+} secreted Aβ_{42} and Aβ_{40} at a relatively similar ratio (approximately 0.1). Collectively, these data indicate that low concentration of magnesium might promote Aβ secretion.

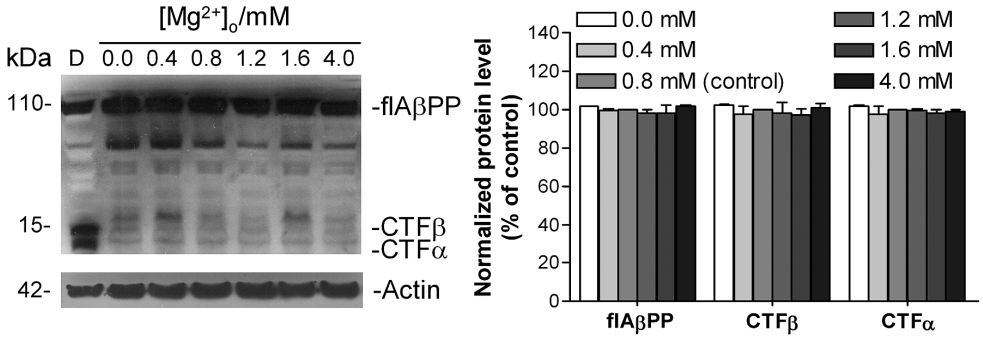

Fig. 8. Restoration of normal AβPP processing by additional 24 h culture in medium containing physiological concentration of magnesium. After 24 h exposure of varying $[Mg^{2+}]_o$, old medium was replaced with fresh medium containing physiological $[Mg^{2+}]_o$ at 0.8 mM and cells were cultured for another 24 h. Cell lysates were prepared and immunoblotted with antibody A8717. A representative of three experiments is shown, with bands corresponding to flAβPP, CTFβ, and CTFα labeled. Note that normal AβPP processing is restored by additional 24 h exposure to physiological $[Mg^{2+}]_o$ at 0.8 mM following 24 h of treatment of varying $[Mg^{2+}]_o$. As a positive control, the γ-secretase inhibitor DAPT (500 nM, for 8 h treatment) was employed to induce the accumulation of CTFβ and CTFα (far left lane). Densitometric analysis of Western blots is expressed as a percentage of the level of control (cells treated with 0.8 mM Mg^{2+}). The data are the means $\pm$ SEM from three independent experiments.

Effects of varying $[Mg^{2+}]_o$ on the presence of AβPP on the plasma membrane

Since AβPP processing depends on its exposure to the different secretases present in various subcellular compartments, we tested whether magnesium regulates AβPP processing via affecting its distribution. It has been demonstrated that non-amyloidogenic processing occurs mainly at the cell surface, where α-secretases are present [14], and BACE1 predominantly localizes to the late Golgi/TGN and endosomes, consistent with amyloidogenic cleavage of wild-type AβPP during endocytic/recycling steps [15]. To assess whether different $[Mg^{2+}]_o$ alters the presence of AβPP on the plasma membrane, we compared the surface AβPP levels of cells treated with physiological (i.e., 0.8 mM), low (0.4 mM), and high (1.2 mM) $[Mg^{2+}]_o$ for 24 h using a biotinylation assay. After treatment, cell surface proteins were biotin labeled and isolated from cell lysates with neutravidin beads. The resulting protein samples were immuno-blotted for AβPP with mAb 6E10. As shown in Fig. 7A, compared with physiological $[Mg^{2+}]_o$, cell surface level of AβPP was decreased at low $[Mg^{2+}]_o$ of 0.4 mM, and increased at high $[Mg^{2+}]_o$ of 1.2 mM, whereas total cell-associated AβPP levels remained unchanged (Fig. 7A, middle). Quantification showed that surface protein levels of AβPP were significantly decreased to 36.5% of control by 0.4 mM $[Mg^{2+}]_o$, whereas increased to 34% of control at 1.2 mM ($p < 0.05$).

We confirmed this result with another assay of surface protein levels [13]. Confocal microscopy of the nonpermeabilized cells with AβPP antibody A8717 or 6E10, again revealed magnesium dose-dependently increased the immunostaining of cell surface AβPP. Quantification of A8717 stained cells ($n = 121$–155) demonstrated that low $[Mg^{2+}]_o$ at 0.4 mM significantly decreased surface levels of AβPP by 24%, whereas high $[Mg^{2+}]_o$ at1.2 mM treated cells showed a significant 23% increase in cell surface AβPP over control cells treated with physiological $[Mg^{2+}]_o$ (i.e., 0.8 mM) ($p < 0.05$) (Fig. 7B). 6E10 immunostaining revealed similar result (21% and 25%, respectively) (data not shown). Consistent with the Western blot data, the amount of total AβPP expression, assessed by immunofluorescence, was not altered by various $[Mg^{2+}]_o$ (Fig. 2B). Taken together, these results suggest that high dose of magnesium induced a partial redistribution of AβPP to the cell surface, where it could undergo enhanced α-secretase cleavage.

Restoration of normal AβPP processing by additional 24 h culture in medium containing physiological concentration of magnesium

Next, we examined the duration of the effects of various $[Mg^{2+}]_o$ on AβPP processing. Following 24 h exposure to varying $[Mg^{2+}]_o$, cells were cultured in fresh medium containing 0.8 mM Mg^{2+} for another 24 h. Cell lysates were prepared and immunoblotted with pAb A8717. As shown in Fig. 8, no significant difference of normalized protein level of flAβPP, CTFβ, and CTFα were observed. This data indicate that the dose dependent effects of various $[Mg^{2+}]_o$ on AβPP

processing are transient, and normal AβPP processing is restored by an additional 24 hr exposure to physiological $[Mg^{2+}]_o$ (i.e., 0.8 mM).

DISCUSSION

Increasing evidence suggests that metals play a pivotal role in the pathophysiology of neurodegenerative disorders (for review, see [2,3]). Heavy metals (e.g., lead, mercury and cadmium) are neurotoxic and associated with intellectual impairment [16]. Recent studies have implicated lead exposure in the subsequent elevation of AβPP and Aβ in animals [17] as well as in the aggregation of synthetic $A\beta_{1-40}$ *in vitro* [18]. In the case of aluminum, another "toxic" metal, its relevance to AD is ascribed to the involvement in the formation of paired helical filaments (PHF), the aggregation and toxicity of Aβ and the generation of oxidative species (for review, see [19]). Transition metals (e.g., copper, zinc, and iron, which are essential in cell biology) can induce Aβ precipitation [20,21], and are found concentrated in and around the amyloid plaques in the AD brain [22]. Disturbed homeostasis of these biometals in the AD brain (decreased copper levels, and increased concentrations of iron, zinc, and manganese [23,24]) have been reported. An imbalance of zinc and copper has been shown to significantly alter the normal course of AβPP processing and Aβ generation in relevant animal models [25–30]. The levels of magnesium are found decreased in AD brains [4,5]. Moreover, Lemke found that serum magnesium levels were lower in AD patients than those of controls [31]. A causal relationship between low magnesium in hippocampal neurons and impairment of learning ability was also demonstrated in aged rats [32]. Interestingly, treatment of dementia patients with nutritional magnesium support efficiently improved memory and other symptoms [33]. However, therapeutic administration of magnesium is still controversial for the treatment of AD, and high doses of magnesium may have potential detrimental side effects [34–37]. In the present study, we show for the first time, that magnesium modulates AβPP processing in a time- and dose-dependent manner. After treatment for 24 h, $[Mg^{2+}]_o$ at high doses increased CTFα level and sAβPPα release and promoted retention of AβPP on plasma membranes. In contrast, $[Mg^{2+}]_o$ at low doses enhanced amyloidogenic fragments (CTFβ) accumulation and Aβ secretion, and reduced cell surface AβPP level. Complete deprivation of extracellular magnesium ($[Mg^{2+}]_o$ at 0.0 mM) was associated with a corresponding decrease in $[Mg^{2+}]_i$. However, $[Mg^{2+}]_i$ was not significantly affected by $[Mg^{2+}]_o$ at 0.4–4.0 mM for 24 h. Moreover, prolonged incubation (36 h) with 50% deficiency/supplementation of magnesium (0.4 and 1.2 mM, respectively, compared with the physiological $[Mg^{2+}]_o$, i.e., 0.8 mM) did not change $[Mg^{2+}]_i$. This finding is similar to those in other reports showing that several human cell lines maintained a constant intracellular magnesium level even when cultured in a medium with very high or very low extracellular magnesium [38,39]. In accordance with previous reports, which have shown the deleterious effects of extracellular magnesium deficiency on the survival of cultured human and rat hepatocytes [40–42], we found that concomitant with a reduced $[Mg^{2+}]_i$, cell viability was significantly impaired in cultures with $[Mg^{2+}]_o$ at 0.0 mM. As we measured only whole-cell magnesium content, our results cannot exclude alterations in distribution of magnesium among cytosolic, nuclear, endoplasmic reticulum, and mitochondrial compartments [39], or change in the level of free magnesium which might influence cellular phenotype and response [43,44].

Here we show the effects of different $[Mg^{2+}]_o$ on AβPP processing is time- and dose-dependent. $[Mg^{2+}]_o$ at high doses increased CTFα level and sAβPPα release. In contrast, $[Mg^{2+}]_o$ at low doses enhanced amyloidogenic fragments (CTFβ) accumulation and Aβ secretion. The mechanism of how varying magnesium concentrations led to shifts between α- and β-secretase cleavage of AβPP might be partially explained by the evidence that $[Mg^{2+}]_o$ at high doses promoted retention of AβPP on plasma membrane, whereas $[Mg^{2+}]_o$ at low doses reduced cell surface AβPP level. All AβPP family members are predominantly cleaved in the late secretory pathway, including the plasma membrane and endosomes [45]. Further, different secretase activities show distinct subcellular localization, namely α-secretase at the plasma membrane [46,47] and β/γ-secretases within endocytic compartments [48–52]. Because targeting of AβPP to distinct subcellular compartments determines processing into amyloidogenic or non-amyloidogenic products, much attention has focused on factors that regulate AβPP trafficking. Interestingly, several adaptor proteins are known to influence AβPP transport and processing. For example, F-spondin, a secreted factor that binds to the extracellular domain of AβPP [53], has been shown to increase levels of cell surface AβPP, promote β-cleavage of AβPP, and decrease β-cleavage of AβPP [54]. Similarly, the extracellular matrix protein Reelin caused increased surface AβPP and a preference

for α-cleavage over β-cleavage [55]. These findings suggest that trafficking and proteolysis of AβPP are regulated together. As described in this work, AβPP processing was clearly affected by varying $[Mg^{2+}]_o$. Compared with $[Mg^{2+}]_o$ at physiological concentration (i.e., 0.8 mM), high doses (1.2, 1.6, and 4.0 mM) enhanced α-secretase cleavage, whereas low doses (0, 0.4 mM) caused an increase in CTFβ accumulation and Aβ secretion. We also found $[Mg^{2+}]_o$ regulates cell surface levels of AβPP in a dose-dependent manner, with increased retention of AβPP on the plasma membrane for α-secretase cleavage under high doses. Thus a function of $[Mg^{2+}]_o$ in AβPP transport from/to the cell surface might be a possible explanation for its modulation of AβPP processing. In the light of Mg^{2+} as antagonist of the NMDA receptor, our finding is corroborated by the previous report that chronic NMDA receptor activation decreased α-secretase-mediated AβPP processing and increased Aβ production in cultured cortical neurons [56]. Furthermore, several lines of evidence suggest that AβPP metabolism and Aβ levels are closely correlated with neural activity in animals [57–59] and humans [60]. It has been demonstrated that decreasing neuronal activity by high $[Mg^{2+}]_o$ (10 mM $MgCl_2$) resulted in significant reduction of Aβ secretion, which may involve a change in AβPP processing [61]. However, the precise functional mechanism of how magnesium regulates AβPP transport and whether magnesium interacts with α- and β-sectetase or regulates enzyme activity or their subcellular localization remains undetermined but will be part of our future analysis.

Induced sAβPPα secretion might have additional advantages, for various studies have strongly established that secreted sAβPPα possesses potent neurotrophic and neuroprotective activities against excitotoxic and oxidative insults [62,63], p53-mediated apoptosis [64], the proapoptotic action of mutant PS1 by activating the transcription factor NF-κB [65]. Moreover, sAβPPα stimulates neurite outgrowth [66], regulates synaptogenesis [67], has trophic effects on cerebral neurons in culture [68], stabilizes neuronal calcium homeostasis, and protects hippocampal and cortical neurons against the toxic effects of glutamate and Aβ peptides [69]. It also has been shown that intracerebroventricular administration of secreted forms of sAβPPα to amnestic mice has potent memory-enhancing effects and blocks learning deficits induced by scopolamine [70]. Therefore, it is more than possible that high concentration of magnesium may exert protective effects against AD, by their action on AβPP processing.

In contrast to sAβPPα, which responded to increasing $[Mg^{2+}]_o$ in a dose-dependent manner, we observed an increase of secreted Aβ only upon low $[Mg^{2+}]_o$ (0.0 and 0.4 mM) compared with physiological $[Mg^{2+}]_o$ (i.e., 0.8 mM), whereas high $[Mg^{2+}]_o$ (1.2, 1.6 and 4.0 mM) could not significantly lower total extracellular Aβ level. Previous reports showed reduced Aβ secretion under conditions of enhanced release of sAβPPα secretion [71–77]. Several studies, however, have demonstrated a dissociation between sAβPPα release and Aβ generation both *in vitro* or *in vivo* [78–82], suggesting that there might be a more complex regulatory mechanism of these two processing events of AβPP. For instance, constitutive activation of PKC in guinea pig brains increased sAβPPα secretion without any effect on secreted Aβ [82], suggesting that the α- and β-secretase pathways may be differentially controlled. In our experimental conditions, we detected a significant reduction of the ratio of $Aβ_{42}/Aβ_{40}$ upon $[Mg^{2+}]_o$ at 4.0 mM, whereas no change under other conditions ($[Mg^{2+}]_o$at 0.0–1.6 mM). This is consistent with the notion that $Aβ_{40}$ and $Aβ_{42}$ may be differentially regulated. In neurons, $Aβ_{1-42}$ and $Aβ_{1-40}$ are produced in the endoplasmic reticulum and in the trans-Golgi network, respectively [83]. However, because we examined the effects of magnesium only on the pathologically high production of Aβ, the modulation of the physiological Aβ production by magnesium needs to be established in future studies.

In conclusion, in the present study we show that high $[Mg^{2+}]_o$ promotes the non-amyloidogenic AβPP cleavage by α-secretase and demote the amyloidogenic processing by β-secretase via modulation of AβPP retention on cell surface, whereas low $[Mg^{2+}]_o$ has opposite effects on AβPP processing. Given the prevalence of magnesium inadequacy in the general population [84], magnesium supplementation could constitute a potential novel pharmacological target for the treatment of AD via its action on AβPP processing.

ACKNOWLEDGMENTS

We thank Dr. Sangram S. Sisodia (University of Chicago, Chicago) for providing Mouse N2a neuroblastoma cells stably expressing PS1wt and AβPPsw.

This work was supported by the National Natural Science Foundation of China (NSFC; Grants No. 30570533, No. 30670414 and No. 30973145) and the National High Technology Research and Development

Program of China (973 Program No. 2006CB500705 and 863 Program No. 0060102A4031).

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=310).

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