Intracellular copper deficiency increases amyloid-β secretion by diverse mechanisms

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INTRODUCTION

AD (Alzheimer’s disease) is characterized by three main pathological conditions in the brain: extraneuronal senile plaques, intraneuronal neurofibrillary tangles and diffuse loss of neural synapses (reviewed in [1]). The principal constituent of the senile plaque is β-amyloid (amyloid β-peptide) [2,3]. β-amyloid is derived from intracellular proteolytic cleavage of APP (amyloid precursor) [13]. APP localizes to the constitutive secretory pathway within cells and is orientated with a large N-terminal ectodomain, which is homologous with APLP (amyloid precursor-like protein) [14]. There is growing evidence for physiological interactions between APP (or its proteolytic fragments) and copper metabolism. APP and APLP2 knockout mice accumulate copper in tissues, notably in brain and liver [15,16], whereas transgenic mice overexpressing the Swedish mutant of APP or just the last 100 C-terminal residues have a decreased level of brain copper [17,18]. Copper levels modulate APP transcription [19] and may modify APP protein processing. In one report, APP processing was sensitive to extracellular copper levels, with an elevation in copper reducing the level of α-β-cleaved APP C-terminal fragments while increasing the secretion of APP ectodomain in cultured cells [20]. In addition, mutation of ATP7B (Wilson protein), which significantly increases liver and brain copper, has been shown to decrease brain and plasma Aβ levels [18].

Copper homeostasis is heavily dependent on the activities of the copper transporters ATP7A (Menkes protein) and ATP7B, which are defective in the genetic disorders of Menkes and Wilson disease respectively (reviewed in [21]). ATP7A is expressed in most tissues (except the liver), whereas ATP7B is expressed in liver hepatocytes, brain, breast and placenta. These copper ATPases translocate cytosolic copper across membranes and into secretory compartments. To remove extracellular copper, both proteins relocate from the TGN to exocytic vesicles [22,23]. Previously, ATP7A was found to generate vesicular copper that is based copper-binding site located in the N-terminal ectodomain, which is homologous with APLP (amyloid precursor-like protein) [2] [14].

Key words: Alzheimer’s disease (AD), amyloid, amyloid precursor protein (APP), copper, Menkes protein (ATP7A), neuron.
Figure 1 Schematic representation of antibody epitopes and secretase cleavage sites

Amino acid sequence alignment between the regions of APP and APLP2 recognized by the antibodies used in this study. Antibodies are shown in bold and their epitopes are indicated by horizontal brackets. APP/APLP2 regions are shown by their amino acid numbers under solid vertical lines. The main secretase cleavage sites are also shown.

released into the synaptic cleft from the post-synaptic neuron on NMDA (N-methyl-D-aspartate) stimulation [24]. Therefore APP, Aβ and ionic copper are co-localized to the glutamatergic synaptic cleft where amyloid pathology first occurs.

Information about the impact of intracellular copper on APP processing is limited. While increasing copper in cell culture [20] and in transgenic mice [18,25] is associated with decreased Aβ levels, the mechanism for this is unclear. Moreover, nothing is known about the effect of copper deficiency on Aβ production, which is important because AD brain tissue is copper deficient [26–29]. To address this, we have studied human fibroblast cell lines with genetically modified levels of the copper-efflux proteins ATP7A and ATP7B to determine how changes in the level of intracellular copper influence APP and APLP2 processing and Aβ production. We further investigated the influence of copper modulation on APP and APLP2 proteolysis in human neuroblastoma cells.

EXPERIMENTAL

Cells and reagents

Immortalized human fibroblast cell lines [GM2069 (normal), A12-H9, Me32a, C3-C1 and WND16] with different copper phenotypes were generated as described previously [30]. The details of these cell lines and their characterization are shown in Figure 2. Fibroblasts were cultured at 37°C and 5% CO₂ as a monolayer in BME (basal Eagle’s medium; Trace BioSciences, Noble Park, VIC, Australia), supplemented with 10% (v/v) FCS (fetal calf serum; Commonwealth Serum Laboratories, Broadmeadows, VIC, Australia), 2 mM L-glutamine, 12 mM NaHCO₃ and 100 mM Hepes (Thermo Electron). The fibroblast cell lines transfected with ATP7A or ATP7B were cultured in medium containing 500 μg/ml G418 to maintain transgene expression [30]. The human SY5Y neuroblastoma line was cultured at 37°C as a monolayer in RPMI 1640 medium (Trace BioScience, Noble Park, VIC, Australia); supplemented with 20% (v/v) FCS (Commonwealth Serum Laboratories), 2 mM L-glutamine, 12 mM NaHCO₃, 100 mM Hepes, 1 mM pyruvate (Thermo Electron) and 0.5 μM uridine (Sigma). The SY5Y cell line transfected with wt (wild-type) APP695 (695 residue APP) was cultured in medium supplemented with 2 μg/ml puromycin to maintain transgene expression.

Synthetic Aβ [Aβ(1–40) and Aβ(1–42)] were purchased from Merck. The goat polyclonal anti-ATP7A and anti-ATP7B antibodies (designated R17 and NC36 respectively) have been characterized previously [31,32]. The following antibodies were supplied by the Department of Pathology, University of Melbourne (Melbourne, VIC, Australia): mouse monoclonal antibody WO2 [33], mouse monoclonal anti-Aβ(1–40) antibody (G210) [33], mouse monoclonal anti-Aβ(1–42) antibody (G211) [33], rabbit polyclonal anti-APLP2NT antibody [34] (where NT is N-terminus) and mouse monoclonal antibody 22C11 [35]. Mouse monoclonal antibody 6E10, rabbit polyclonal anti-APPCT (where CT is C-terminus) and anti-APLP2CT antibodies were purchased from Merck, and mouse monoclonal anti-β-actin was purchased from Sigma. The epitopes recognized by the APP- and APLP2-specific antibodies are shown in Figure 1. All other reagents were supplied by Sigma unless specified otherwise.

Culturing conditions and transfection

Culturing conditions were optimized to allow the concurrent examination of both whole-cell lysates and conditioned medium by Western–blot analysis. In a 12-well tray, each fibroblast line was seeded into separate wells (∼1.6 × 10⁵ cells) and cultured in 2 ml of medium (see above) for 24 h. The medium was then replaced with 400 μl of fresh basal medium or medium containing 2 μM DAPT and/or 10 μM cyclohexamide, or 100 μM or 200 μM CuCl₂ (see the Results section for details) and the fibroblasts were further cultured for 16 h overnight. The conditioned medium was removed and retained, and the cells were quickly washed with ice-cold PBS before the addition of 150 μl of ice-cold lysis buffer [50 mM Tris/HCl (pH 6.8), 150 mM NaCl, 0.5% (v/v) NP40 and protease inhibitor cocktail] to each well. The tray was rocked on ice at 4°C for 10 min and then the cell lysates were transferred into 1.5 ml tubes. Lysates and conditioned medium were centrifuged at 9503 g for 10 min at 4°C to remove cellular debris. These conditions were optimized so that 40–50 μg of total protein (lysate) was analysed by Western blot. SY5Y cells overexpressing wtAPP695 were cultured to ∼80% confluency in 25 cm² flasks. The medium (see above) was then replaced with 2.5 ml of fresh basal medium or medium containing treatment (2 μM DAPT, 100 μM or 200 μM CuCl₂; see the Results section for details) and the cells were further incubated for 16 h overnight. For copper chelation treatment, cells were cultured in medium supplemented with 200 μM BCS (bathocuproinedisulfonic acid) and 200 μM d-penicillamine for an initial 56 h period. Cell lysates and conditioned medium was prepared as described above for the fibroblasts; however, the cells were lysed in 500 μl of ice-cold lysis buffer.

The generation of the mammalian expression construct encoding wtAPP695 (pIRESpuro2-based) has been described...
Copper deficiency increases Aβ secretion

Previously [36], transient transfection of GM2069 and A12-H9 fibroblasts with the plasmid encoding wtAPP695 was performed using FuGENE® HD (Roche) following the manufacturer’s recommendations. Stable transfection of SY5Y cells with the plasmid encoding wtAPP695 was performed using Lipofectamine™ (Invitrogen) following the manufacturer’s instructions. The cells were recovered overnight in RPMI 1640 medium containing 20% (v/v) FCS before transfectants were isolated by treatment with 2 µg/ml puromycin for 30 days.

Western-blot analysis and densitometry

Cell lysates and conditioned media were fractionated by electrophoresis on Novex® pre-cast gels (10% or 10–20% gradient gels) using the Xcell SureLock™ mini-cell system (Invitrogen). Samples were prepared for electrophoresis by the addition of Novex® Tricine SDS sample buffer (Invitrogen) and 10% (v/v) 2-mercaptoethanol, and were heated at 90°C for 5 min. Following electrophoresis (125 V for ~90 min), proteins were transferred on to nitrocellulose (Amersham) using the XCellIII™ blot module and the Xcell SureLock™ mini-cell system (Invitrogen) according to the manufacturer’s instructions. The nitrocellulose membrane was then blocked using 5% (w/v) non-fat dried skimmed milk powder in TBST (Tris-buffered saline with Tween 20) buffer [10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.1% (v/v) Tween 20] for 1 h at room temperature (22°C). The membrane was then incubated with primary antibody diluted in TBST at 4°C overnight or for 2 h at room temperature. The following primary antibodies were used: anti-ATP7A (R17, 1:1000 dilution), anti-ATP7B (NC36, 1:1000 dilution), anti-β-actin (1:10000 dilution), WO2 (1:25 dilution), 22C11 (1:100 dilution), 6E10 (1:100 dilution), APLP2NT (1:1500 dilution), APLP2CT (1:5000 dilution) and APPCT (1:20 000 dilution) antibodies. After three washes, each for 10 min with TBST, the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (Dako; 1:10000 dilution in TBST) was applied to the membrane for 1 h at room temperature. Analysis was carried out using ECL (enhanced chemiluminescence) reagent (Amersham), c⃝2008 Biochemical Society.
following the manufacturer’s instructions and images were captured using the LAS-3000 imaging suite and analysed using Multi Gauge software (Fuji).

Densitometry was used to evaluate immunolabelled protein intensity and was expressed as a percentage comparison with the control experiment. Pixel intensities (arbitrary units) were quantified using Multi Gauge software (Fuji) and their levels were normalized relative to β-actin controls. The average of three independent experiments for each condition (as detailed in the Results section) was used for comparison. We also determined whether the correlation between the level of Aβ and pixel intensity was linear (results not shown). Incremental amounts of synthetic Aβ(1–40) were subjected to densitometry and their pixel intensity values (arbitrary units) were compared in order to determine linearity [gradient (m)]. We found that the provided amount of Aβ was below 100 pg then a linear relationship was maintained (m = 1). However, if the level exceeded 100 pg, then this linearity was lost. We therefore assumed linearity in the densitometry evaluation of the Aβ bands shown in Figure 5C, because their intensity was less than that of 100 pg Aβ(1–40). The linearity of β-actin densitometry measurements was also evaluated and a linear relationship existed when 60 µg or less of total cellular lysate was used (results not shown).

Antibody-capture ELISA for Aβ quantification

The level of Aβ in culture medium was quantified using DELFIA® double-capture ELISA as described previously [36,37]. Briefly, separate wells (of a 96-well plate) were coated with the mouse monoclonal anti-Aβ(1–40) antibody (G210, 0.4 µg/well) or anti-Aβ(1–42) antibody (G211, 0.6 µg/well) and then blocked with casein buffer [0.5 % (w/v) casein with PBST (PBS containing 0.05 % (v/v) Tween 20)] for 2 h at 37 °C. Plates were washed with PBST before biotinylated WO2 together with culture medium or the Aβ solution (PerkinElmer) was then added and the absorbance (λ = 405 nm) was measured using a microplate reader (PerkinElmer; Multiscan Spectrum, Flowtron). Enhancement of the Aβ bands in the ELISA was accomplished by using a haemocytometer and 1.5 ml of the cell suspension was centrifuged at 1000 g for 5 min at room temperature to pellet the cells, after which the supernatant was removed and the pellet was stored at −20 °C until it was analysed for copper content. Analysis of the level of copper, zinc and iron in the SY5Y overexpressing wtAPP695 cells was performed as described above; however, these cells were cultured for 56 h before the medium was replaced for 16 h overnight treatment. For copper chelation treatment, the cells were also cultured in medium supplemented with 200 µM BCS and 200 µM D-penicillamine for the initial 56 h period. Cellular metal concentration was measured using inductive-coupled plasma MS (UltraMass 700, Varian). The average of determinations for each cell line and condition was used for comparison.

RT-PCR (reverse transcription–PCR)

The generation of cDNA from the four fibroblast cell lines was achieved using the SuperScript™ III CellsDirect cDNA synthesis system (Invitrogen) following the manufacturer’s protocol. Oligonucleotides used to amplify APP, APLP2 and β-actin were designed for specificity and complemented the human sequence. The APP-specific oligonucleotides are capable of amplifying all APP alternatively-spliced products. The sequences of these primers are as follows: APP#1 (forward) 5′-TGTGATCTTGTTAGGTGGGAG-3′, APP#2 (reverse) 5′-CCATTCTCTCATGACCTGCTAG-3′, APLP2#1 (forward) 5′-TCCTTTTGGTTACACCTTTC-3′, APLP2#2 (reverse) 5′-TAGCTTTGAAGCTTCGTGCT-3′, β-actin#1 (forward) 5′-GGCGCAACACCCTGTACCCCT-3′ and β-actin#2 (reverse) 5′-AGGCACGTCTGACCGACTGACT-3′. The PCR reaction contained 1 µl PCR buffer, 0.2 mM of each dNTP, 2 mM MgCl₂, 0.2 µM of each primer, 2.5 units Platinum Taq DNA polymerase and 3 µl of cDNA (Invitrogen). Reactions were run on an Eppendorf Gradient Mastercycler on the following program: one cycle of 94 °C for 2 min, 38 cycles of 94 °C for 45 s, 57 °C for 60 s and 72 °C for 60 s, followed by one cycle of 72 °C for 2 min. The PCR reactions were resolved on 1.8 % (w/v) agarose gels using standard electrophoresis procedures.

RESULTS

Human fibroblasts with altered intracellular copper levels

Human fibroblast cell lines with genetically modified levels of the copper-efflux proteins ATP7A and ATP7B, and therefore different copper phenotypes (Figure 2A), were used to determine if

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Copper deficiency increases $\alpha\beta$ secretion

Figure 3 Intracellular copper levels dramatically influence APP and APLP2 metabolism

(A) Western-blot analysis of APP and APLP2 in whole-cell lysates and conditioned medium from the indicated fibroblast cell lines. Whole-cell lysates and medium were Western blotted using WO2, 22C11 and APLP2NT antibodies, with anti-$\beta$-actin antibody as a control. The percentage increases and decreases in parenthesis refer to the change in copper levels compared with the control cell line. (B) Densitometric analysis of the level of APP secreted into medium conditioned by the fibroblast cell lines. The experiment shown in (A) was repeated three times and the pixel intensity of the immunolabelled sAPP$\alpha$ and sAPP$\beta$ detected were evaluated and normalized against $\beta$-actin controls. Results are the percentage of secreted APP [total sAPP (22C11) and sAPP$\alpha$ (WO2)] compared with the control cell line (GM2069). Results were analysed using the Student's t test (*, $P < 0.01$; **, $P < 0.05$). (C) Western-blot analysis of the $\alpha$- and $\beta$-secretase-cleaved APPCT and APLP2CT in the indicated fibroblast cell lines by Western blotting with APPCT and APLP2CT antibodies, with anti-$\beta$-actin antibody used as a control. (D) Western-blot analysis of APP and APLP2 in whole-cell lysates and conditioned medium from normal fibroblast cells (GM2069) and two copper-deficient fibroblast cell lines (A12-H9 and C3-C1). Whole-cell lysates and medium were Western blotted using the, 22C11, WO2, APPCT, APLP2CT and anti-ATP7A antibodies, with anti-$\beta$-actin antibody used as a control. All Western blots shown are representative of three independent experiments. Molecular-mass-markers are indicated on the left (in kDa).

Changes in intracellular copper levels influence APP metabolism. Ordinarily ATP7A is endogenously expressed in fibroblasts (Figure 2B) and is primarily responsible for maintaining a homeostatic level of intracellular copper [21]. Fibroblasts derived from a Menkes disease patient (Me32a), which are devoid of functional ATP7A (Figure 2B) [30], accumulate $\sim 650\%$ more copper than normal fibroblasts (GM2069) (Figures 2A and 2C). In contrast, when ATP7A is overexpressed in the same Menkes patient cell line (A12-H9; Me32a cells transfected with ATP7A) (Figure 2B), the intracellular level of copper is drastically depleted, with cells containing $\sim 65\%$ less copper than normal fibroblasts (GM2069) (Figures 2A and 2C). The copper accumulation phenotype of the Menkes patient line (Me32a) can also be partially corrected by exogenous expression of ATP7B (WND16) (Figures 2A and 2C) [30]. However, this fibroblast line (WND16) still contains $\sim 100\%$ more copper than normal fibroblasts (GM2069) (Figures 2A and 2C), indicating that there may be inadequate expression of ATP7B to completely compensate for ATP7A disruption (Figure 2B). ATP7A eliminates excess intracellular copper directly across the plasma membrane [38], whereas ATP7B sequesters excess copper into exocytic vesicles [22]. This mechanistic difference becomes apparent when cells are exposed to an elevated level of copper, which stimulates the trafficking of only ATP7A to the cell periphery (Figure 2D). We utilized these cell lines to explore the influence of intracellular copper level on APP metabolism.

Copper depletion switches APP secretase cleavage from $\alpha$-cleavage to $\beta$-cleavage

Western-blot analysis and densitometry were used to investigate the expression profile of APP in the fibroblast cell lines (Figure 3). Two antibodies (WO2 and 22C11) were used to differentiate between sAPP (soluble APP ectodomain) $\alpha$ and sAPP$\beta$ (see...
Figure 1). The 22C11 antibody detects cleaved APP ectodomain generated through either the non-amyloidogenic (α-cleaved) or the amyloidogenic (β-cleaved) pathway. The WO2 antibody detects only sAPPα, as β-secretase activity cleaves upstream of its epitope. Fibroblasts with normal (GM2069) or an elevated level of intracellular copper (Me32a and WND16) produced similar levels of intracellular APP, as detected by both WO2 and 22C11 antibodies (Figure 3A). These cells also secreted an abundant amount of sAPPα into their medium, as determined by WO2 detection (Figure 3A). Medium conditioned by copper-accumulating Me32a and WND16 fibroblasts had a marked and significant increase in the level of sAPPα compared with medium conditioned by normal fibroblasts (GM2069) (∼100% and ∼48% increases respectively) (Figure 3B). These results indicate that an elevation in intracellular copper increases APP production and subsequent sAPPα secretion, although the steady-state intracellular level of APP is unchanged (Figure 3B). Copper-deficient fibroblasts (A12-H9) expressed and secreted an sAPP species that was detectable by using the 22C11 antibody, but not with the WO2 antibody (Figure 3A), and migrated at a slightly lower molecular-mass (Figure 3A). These results are consistent with profound copper-depletion switching APP proteolysis from α-secretase cleavage (sAPPα secretion) to β-secretase cleavage (sAPPβ secretion). Additionally, the copper-depleted fibroblasts (A12-H9) secreted a level of total sAPP similar to that secreted by control (GM2069) fibroblasts (Figure 3B). These results indicate that there is a basal amount of total sAPP secretion that is maintained even under copper-deficient conditions, whereas total sAPP secretion rises under conditions of copper excess. Analysis of APLP2 in these fibroblast cell lines revealed that APLP2 also increased its steady-state expression under conditions of copper deficiency (A12-H9) (Figure 3A). Full-length APLP2 was only detected in the copper-depleted (A12-H9) fibroblasts. No secreted APLP2 was detected in the medium.

To further investigate APP cleavage in these fibroblast cell lines, an anti-APLP2CT antibody was used to assay any C-terminal APP cleavage fragments (see Figure 1). Fibroblasts with normal (GM2069) or elevated (Me32a and WND16) intracellular copper levels contained a similar steady-state level of an APP C-terminal fragment (10 kDa in size) consistent with C83 (α-cleaved APP C-terminal fragment) (Figure 3C). No C-terminal fragment was detected in the copper-deficient fibroblasts (A12-H9) (Figure 3C), despite the cells abundantly secreting β-cleaved APP ectodomain (sAPPβ) (Figures 3A and 3B), suggesting that C99 (β-cleaved APP C-terminal fragment) is rapidly turned over. Use of an anti-APLP2CT antibody (raised against the last 12 amino acids of APLP2) demonstrated that only full-length APLP2 and not its C-terminal cleavage fragment(s) could be detected in the copper-deficient A12-H9 cells, but the C-terminal fragment(s) were present in the other fibroblast cell lines (Figure 3C). This suggested that APLP2 cleavage is inhibited by copper deficiency and there is consequently no C-terminal fragment generated. In summary, these results indicate that processing of both APP and APLP2 is sensitive to changes in intracellular copper concentrations.

To demonstrate that the differences in the cleavage of APP and APLP2 seen in the copper-deficient A12-H9 cells were not due to the transfection procedure itself (e.g. gene interruption by transgene integration), an independently derived clonal line (C3-C1) was also examined (Figure 3D). The C3-C1 fibroblast cell line was previously shown to have very similar characteristics to the A12-H9 fibroblast cell line [30], including the level of ATP7A overexpression and copper status. Indeed, this fibroblast line exhibited a similar APP and APLP2 Western blot profile to the A12-H9 cells (Figure 3D).

Figure 4 Copper deficiency modulates APP and APLP2 mRNA levels

RT-PCR amplification of APP and APLP2 in the indicated fibroblast cell lines. Amplification from A12-H9 cells treated with cycloheximide (CH) is also shown. The percentage increases and decreases in parenthesis refer to the change in copper levels compared with the control cell line. Note that fibroblasts transcribe the 770, 751 and 695 bp alternatively spliced APP mRNA as shown (657 bp, 599 bp and 432 bp products respectively). The sizes of the amplified products are indicated on the left in bp.

Copper depletion changes APP and APLP2 transcription/translation

It was reported previously that copper depletion in A12-H9 fibroblasts significantly down-regulates APP gene expression, as determined by Northern-blot analysis [19]. To validate this finding, we examined the level of APP mRNA in the fibroblast cell lines using RT-PCR (Figure 4). In addition, we also investigated APLP2 mRNA levels. Fibroblasts with normal (GM2069) or elevated (Me32a and WND16) intracellular copper levels contained a steady-state level of both APP and APLP2 mRNA (Figure 4). However, we could not amplify either APP or APLP2 mRNA from the copper-deficient A12-H9 cells. Since these cells produce both APP and APLP2 protein (Figure 3), the inability to detect their respective mRNA transcripts was puzzling. We hypothesized that in these copper-deficient cells, the existence of APP/APLP2 mRNA is low and transient and therefore any transcript produced is rapidly translated to protein. To demonstrate that copper-deficient A12-H9 cells do indeed produce mRNA transcripts for both APP and APLP2, the cells were treated with cycloheximide to prevent protein translation (Figure 4). Consequently, APP and APLP2 mRNA molecules were faintly detected in the cycloheximide treated A12-H9 cells (Figure 4). Because steady-state APP protein levels are not decreased in the copper-deficient A12-H9 fibroblasts and medium compared with normal fibroblasts (GM2069) (Figures 3A and 3B), our findings of low APP mRNA message in A12-H9 cells indicate that copper deficiency either accelerates the translation of APP or inhibits the degradation of APP.

Amyloidogenic processing of APP is elevated in copper-deficient fibroblasts

An alternative approach to studying the processing of APP involves capturing the C-terminal fragments of APP by inhibiting γ-secretase activity and determining their levels through Western-blot analysis [39,40]. To capture the C-terminal fragments produced by normal (GM2069) and copper-deficient (A12-H9) fibroblasts, these cell lines were treated with the γ-secretase inhibitor DAPT [39] (Figures 5A and 5B). Inhibiting γ-secretase activity in normal fibroblasts (GM2069) had no effect on the levels of intracellular APP or secreted sAPPα.
Copper deficiency increases Aβ secretion

Figure 5 Copper deficiency increases γ-secretase cleavage of APP

(A) Western-blot analysis demonstrating that DAPT treatment prevents γ-secretase cleavage of de novo APP/APLP2 in normal fibroblasts (GM2069). GM2069 fibroblasts were cultured for 16 h in fresh basal medium (Control) or medium supplemented with 2 µM DAPT (DAPT), 10 µM cycloheximide (CH) or both 2 µM DAPT and 10 µM cycloheximide (DAPT + CH). Unconditioned medium (No cells) was also analysed. Lysates and medium were Western blotted using 22C11, APPCT and APLP2CT antibodies, with anti-β-actin antibody as a control. (B) Western-blot analysis demonstrating that DAPT treatment prevents γ-secretase cleavage of de novo APP/APLP2 in copper-deficient fibroblasts (A12-H9) was performed as in (A), but using the copper-deficient A12-H9 cell line. C99 (solid arrow) and C83 (dotted arrow) are indicated on the left. The percentage decrease in parenthesis refers to the change in copper level compared with the control cell line. (C) Western-blot analysis of DAPT-captured APP and APLP2 C-terminal fragments in the indicated fibroblast lines. The fibroblast cell lines were cultured for 16 h in medium supplemented with 2 µM DAPT and whole-cell lysates were Western blotted using APPCT and APLPCT antibodies, with anti-β-actin antibody used as a control. The percentage increases and decreases in parenthesis refer to the change in copper levels compared with the control cell line. C99 (solid arrow) and C83 (dotted arrow) are indicated on the left. (D) Densitometric analysis of the level of DAPT-captured C83 and C99 fragments in control (GM2069) and copper-deficient (A12-H9) fibroblasts. The experiment was repeated three times and the pixel intensity of the immunolabelled C83/C99 bands (detected by anti-APPCT antibody) were evaluated and normalized against β-actin controls. The level of C83 is expressed as a percentage compared with the control cell line (GM2069), whereas the level of C99 is expressed as arbitrary units, as there was no C99 detection in the control cell line (GM2069). Results were analysed using the Student’s t test (*, P = 0.031). All Western blots shown are representative of three independent experiments. Molecular-mass markers are indicated on the left (in kDa).

However, DAPT treatment markedly increased the level of C83 (Figure 5A). These results demonstrated that DAPT treatment did not perturb the production and subsequent secretion of sAPPα (and by inference did not influence α-secretase-mediated cleavage of APP), but only obstructed downstream γ-secretase processing. This is consistent with previous observations [39,40]. Inhibiting γ-secretase also increased the amount of APLP2 C-terminal fragment (Figure 5A). To establish if DAPT prevented γ-secretase-mediated cleavage of de novo APP or existing intracellular APP molecules, cycloheximide was used in conjunction with DAPT treatment to prevent new protein synthesis (Figure 5A). Cycloheximide treatment alone markedly diminished the level of both intracellular APP and secreted sAPPα (Figure 5A) and reduced the amount of C-terminal fragments captured by DAPT treatment (Figure 5A), indicating that DAPT predominantly prevents γ-secretase cleavage of de novo APP. Taken together, these experiments demonstrate that DAPT treatment captures APP C-terminal fragments without affecting APP transcription or translation.

These experiments were repeated using the copper-deficient A12-H9 fibroblast line (Figure 5B). It is important to note that A12-H9 cells secrete only sAPPβ (Figure 3) and therefore these cells are predicted to produce C99 and therefore more Aβ. Inhibiting γ-secretase (DAPT treatment) in the copper-depleted fibroblasts (A12-H9) had no effect on the levels of intracellular APP or secreted sAPPβ (Figure 5B). DAPT treatment also had no effect on the intracellular level of full-length APLP2 (Figure 5B). However, DAPT treatment captured two C-terminal fragments (Figure 5B): a predominant fragment corresponding to C83 (∼10 kDa) and a faint larger fragment corresponding to C99 (∼12 kDa). Therefore despite copper-depleted fibroblasts (A12-H9) secreting only sAPPβ (Figure 3), this did not result in the production of only C99. In fact, the majority of the C-terminal fragments produced by A12-H9 cells were still C83, with C99 molecules being an increased product (Figure 5B). The activities of α- and β-secretase are therefore not mutually exclusive, and in copper-depleted fibroblasts each APP molecule was processed by both secretases producing sAPPβ and C83.
Furthermore, the predicted sAPPα product was not detectable in the medium and therefore must be either relatively low in abundance or rapidly degraded. In addition, γ-secretase inhibition captured APLP2 C-terminal fragments (Figure 5B). A12-H9 cells, when left untreated, exhibited only full-length APLP2 and no C-terminal cleavage product below 15 kDa (Figures 3C and 3D). Therefore APLP2 is still processed in copper-deficient A12-H9 fibroblasts, although possibly more slowly, leading to an increase in full-length APLP2.

To determine if the level of APP C-terminal fragment(s) produced in the copper-deficient A12-H9 cells is comparable with that produced by the other fibroblast cell lines, each cell line was treated with DAPT and their level of APP C-termini captured directly compared (Figures 5C and 5D). Copper-deficient fibroblasts (A12-H9) treated with DAPT produced as much or more C83 and C99 compared with the other cell lines (GM2069, Me32a and WND16). This was surprising since, in contrast to the other cell lines, A12-H9 fibroblasts did not exhibit any APP C-terminal fragments (C83 or C99) under non-DAPT conditions (Figures 3C and 3D). In other words, A12-H9 cells produce more C-terminal APP fragments (C83 and C99), but they can only be detected when cells are treated with the γ-secretase inhibitor DAPT (Figures 5B–5D). Therefore the processing of the C-terminus by γ-secretase must be markedly increased in the copper-depleted A12-H9 fibroblasts compared with fibroblast cell lines with normal or elevated copper. Western-blot analysis revealed that presenilin-1 expression level was not increased in copper-deficient A12-H9 cells (results not shown).

As shown with DAPT treatment, a small percentage of the C-terminal fragments generated by A12-H9 cells were amyloid-harbouring C99 fragments (Figures 5B and 5C). We hypothesized that when A12-H9 cells are not treated with DAPT, these C99 fragments would be cleaved by γ-secretase, liberating the Aβ peptide, and therefore the copper-deficient fibroblasts (A12-H9) should exhibit increased production of Aβ. The Aβ secreted endogenously by A12-H9 cells was below detection limits (results not shown). Therefore we overexpressed wtAPP695 in normal (GM2069) and copper-deficient (A12-H9) fibroblasts to test whether copper deficiency caused an increase in the production of Aβ (Figure 6A). In normal fibroblasts (GM2069), wtAPP695 overexpression resulted in the abundant secretion of sAPPα, which was detected by WO2 (Figure 6A). Unlike non-transfected cells that exclusively secreted sAPPβ (Figure 3), A12-H9 fibroblasts overexpressing wtAPP695 secreted abundant sAPPα (Figure 6A). This suggests that wtAPP695 expression exceeded the capacity of the A12-H9 fibroblasts to process APP via the β-cleavage pathway (sAPPβ) (Figures 3A and 3B) and, as a consequence, much of the APP was processed by α-secretase (Figure 6A). Nevertheless, overexpression of APP permitted sufficient production of Aβ for it to be detected by WO2 (Figure 6A) and 6E10 (Figure 6B) antibodies. When transfected with wtAPP695, the copper-deficient fibroblasts (A12-H9) clearly generated more Aβ than normal fibroblasts (GM2069) (Figures 6A and 6B). The level of wtAPP695 expressed in both fibroblast cell lines was equivalent, indicating that a difference in expression level was not responsible for the elevated Aβ production seen in copper-deficient fibroblasts. Therefore copper depletion caused by ATP7A overexpression increases β- and γ-secretase activities in human fibroblasts.

Copper depletion in human neuronal cells increases Aβ secretion

To ascertain if the level of intracellular copper influences APP metabolism in neuronal cells, we investigated a human SY5Y neuroblastoma line overexpressing wtAPP695 (SY5Y-wtAPP695). We found that we were unable to transfect these cells with ATP7A; therefore, in order to deplete these cells of copper they were cultured in medium supplemented with BCS (200 µM) and D-penicillamine (200 µM), which chelate Cu²⁺ and Cu⁺ respectively. This approach reduced intracellular copper by ∼27 % (Figure 7A). Alternatively, to elevate the level of intracellular copper, the culture medium was supplemented with 200 µM CuCl₂. This relatively high concentration of CuCl₂ was necessary to counteract the inherent copper-sequestering nature of serum [20% (v/v) FCS] and increased intracellular copper by ∼600 % (Figure 7A). The copper-modulating treatments had no significant effect on the level of intracellular zinc, but treatment with copper chelators elevated intracellular iron levels by ∼29 % (Figure 7A), which is consistent with the inhibition of ceruloplasmin activity [41].

Figure 6 Copper deficiency in fibroblasts increases the generation of Aβ

(A) Western-blot analysis of normal (GM2069) and copper-deficient (A12-H9) fibroblasts transfected with wtAPP695, with transfection with vector only as a control. Unconditioned medium was also analysed as a control. Whole-cell lysates and medium were Western blotted using 22C11 and WO2 antibodies, with anti-β-actin antibody as a control. The percentage decrease in parenthesis refers to the change in copper level compared with the control cell line. (B) Western-blot analysis confirming Aβ secretion from copper-deficient fibroblasts overexpressing wtAPP695. GM2069 fibroblasts were used as a control cell line. Synthetic Aβ1–40 and Aβ1–42 standards (100 pg) were run concurrently with whole-cell lysate and medium samples. Samples were Western blotted using 22C11, WO2 and 6E10 antibodies, with anti-β-actin antibody used as a control. All Western blots shown are representative of three independent experiments. Molecular-mass-markers are indicated on the left (in kDa).
Copper deficiency increases Aβ secretion from human neuronal cells

(A) Copper, zinc and iron content of SY5YwtAPP695 cells was measured using inductive-coupled plasma MS. The neuronal cells were cultured in basal medium (Control), medium supplemented with either 200 µM BCS and thio-pancuronium (BCS+PEN) or with 200 µM CuCl₂. Results are normalized means ± S.D. (n = 3) and are shown as ng of metal per 1 × 10⁶ cells. Results were analysed using the Student’s t test (*, P = 0.039; **, P = 0.018). (B) Western-blot analysis demonstrating that copper supplementation or chelation does not directly affect the detection of secreted Aβ. Conditioned medium from SY5YwtAPP695 cells was Western blotted using the 6E10 antibody. Molecular-mass-markers are indicated on the left (in kDa). (C) Western-blot analysis demonstrating that copper deficiency increases the level of Aβ secreted from human neuronal cells. SY5YwtAPP695 cells were cultured in basal medium (Control), 200 µM BCS and thio-pancuronium (BCS/PEN), 100 µM CuCl₂ or 200 µM CuCl₂. Whole-cell lysates and medium were Western blotted using 22C11, W02 and 6E10 antibodies, with anti-β-actin antibody as a control. Unconditioned medium (no cells) and synthetic Aβ₁₋₄₀ standard were also analysed. Molecular-mass-markers are indicated on the left (in kDa). (D) Western-blot analysis demonstrating that modulating medium copper levels does not alter APP processing in human neurons. SY5YwtAPP695 cells were cultured in 200 µM BCS and thio-pancuronium (BCS/PEN) or 200 µM CuCl₂ after treatment with 2 µM DAPT. Anti-β-actin antibody was used as a control. Molecular-mass-markers are indicated on the left (in kDa). (E) Copper deficiency significantly increases the level of Aβ secreted from human neuronal cells as determined by densitometry. The experiment shown in (C) was repeated three times and the pixel intensity of the immunolabelled Aβ bands were evaluated and normalized against β-actin controls. Results are the level of secreted Aβ as a percentage compared with the control sample. Results were analysed by the Student’s t test (*, P = 0.028). (F) ELISA-based quantification confirmed that copper deficiency increases the level of Aβ secreted from human SY5YwtAPP695 neuronal cells. The concentration of Aβ₁₋₄₀ secreted into the medium from SY5YwtAPP695 cells cultured in 200 µM BCS and thio-pancuronium (BCS/PEN), 100 µM CuCl₂ or 200 µM CuCl₂ was measured by ELISA. Background Aβ measurements in non-conditioned medium were also made (No cells). Results are normalized means ± S.D. (n = 3) and were analysed using the Student’s t test (*, P = 0.006).
Copper promotes $\alpha\beta$ cross-linking, whereas metal chelators have been shown to dissolve $\alpha\beta$ aggregates both in vitro and in vivo [5,9]. Therefore it was possible that copper supplementation or chelation could cause secreted $\alpha\beta$ to oligomerize or dissolve into monomers respectively. To determine if this was the case, medium conditioned by SY5YwtAPP695 cells was divided evenly and incubated in equal volumes with either the chelators (BCS and D-penicillamine), PBS (control) or 200 $\mu$M CuCl$_2$ (Figure 7B). An equivalent level of $\alpha\beta$ was observed in all three conditions, indicating that these treatments do not directly affect the detection of monomeric $\alpha\beta$ in this time frame. This is probably because of agents in the medium that scavenge H$_2$O$_2$ generated by $\alpha\beta$ [8,12]. Therefore modulation of copper in our experiments does not alter the amount of monomeric $\alpha\beta$ detected in the cell-free medium.

We next investigated the impact of copper deficiency and copper accumulation on the production and secretion of $\alpha\beta$ from neuronal cells (Figure 7C). Modulating intracellular copper in SY5Y cells transfected with wtAPP695 had no observable effect on the steady-state level of intracellular APP or on the level of sAPP secretion, as shown by detection with 22C11 and WO2 antibodies (Figure 7C). Nevertheless, copper deficiency did noticeably increase the level of secreted $\alpha\beta$ in comparison with the other conditions tested. This was established by Western blotting with both WO2 and 6E10 antibodies (Figure 7C). These results demonstrate that, as with fibroblasts, copper deficiency increases neuronal $\alpha\beta$ secretion.

To determine if modulating intracellular copper had an impact on the processing of APP, the SY5Y wtAPP695 cells were treated with either 200 $\mu$M CuCl$_2$ or copper-chelating agents (BCS and D-penicillamine) and then with the $\gamma$-secretase inhibitor DAPT to capture the C-terminal fragments (Figure 7D). Western-blot analysis demonstrated that there is no difference in the amount of C83 or C99 produced by the cells under any of the copper conditions tested (Figure 7D). Incubating the neuronal cells in medium containing the copper chelators for 5 or 7 days and/or inducing differentiation by culturing with 20 $\mu$M retinoic acid had no effect on C83/C99 production (results not shown). We tested whether this lack of response to copper modulation may have been the result of overexpression of APP by assaying untransfected parental SY5Y cells for C-terminal fragments. Neither CuCl$_2$ nor copper chelation affected the levels of the APP C-terminal fragments in non-transfected SY5Y cells (results not shown). These results suggest that modulating copper has no effect on APP processing in neuronal SY5Y cells, but does increase the level of $\alpha\beta$ secreted. In order for this to occur, there is most likely some variation in the degradation of $\alpha\beta$, with copper deficiency decreasing $\alpha\beta$ breakdown as reported previously [36].

Densitometry was used to measure and compare the levels of $\alpha\beta$ secreted by the SY5YwtAPP695 cells under each copper condition (based on detection with the 6E10 antibody) (Figure 7E). Copper deficiency resulted in $\sim$30% more $\alpha\beta$ in the SY5YwtAPP695 conditioned medium, whereas elevated intracellular copper had no significant effect (Figure 7E). To confirm this observation and to determine which $\alpha\beta$ species is being secreted, the levels of both $\alpha\beta$(1–40) and $\beta$(1–42) in the medium were further quantified by ELISA (Figure 7F) [36,37]. Verifying the densitometry results, ELISA quantification demonstrated that copper deficiency caused SY5YwtAPP695 cells to secrete an elevated concentration of $\alpha\beta$(1–40) ($\sim$36% increase) (Figure 7F). The level of $\beta$(1–42) in the conditioned medium was below detection limits. These results demonstrate that copper deficiency in neuronal SY5Y cells increases $\alpha\beta$(1–40) secretion.

**DISCUSSION**

In the AD brain, copper is sequestered in senile plaques by direct co-ordination to $\alpha\beta$ peptides [5–8], but there is a net decrease in tissue copper levels in neocortical tissue [26–28], reflected by diminished activities of copper-dependent enzymes such as cytochrome c oxidase [42–44] and SOD1 (superoxide dismutase 1) [45,46]. Therefore copper collects outside of the cortical cells, which are themselves copper deficient. We found that copper deficiency increased the level of $\alpha\beta$ secretion from both human fibroblast and neuroblastoma cells. These observations have implications for AD progression, as elevated $\alpha\beta$ production and subsequent aggregation (plaque formation) could further perpetuate localized neural copper deficiency by seizing available copper. This in turn, according to our results, could increase $\alpha\beta$ secretion and generate a vicious cycle.

In fibroblasts, an elevation in intracellular copper levels increased the production and subsequent secretion of $\alpha$-cleaved APP (sAPP$\alpha$), whereas copper-deficient cells secreted increased $\beta$-cleaved APP (sAPP$\beta$) (Figures 3A and 3B). These results are consistent with previous observations using CHO (Chinese-hamster ovary) cells, where excess copper increased the secretion of APP ectodomain and correspondingly reduced the level of $\alpha\beta$ production [20]. We have confirmed the previous findings of Bellingham et al. [19], who showed that sAPP$\alpha$ cannot be detected by Western blot in the copper-deficient A12-H9 cells (Figure 3A). However, we have extended these findings by showing that these fibroblasts actually secrete sAPP$\beta$ instead (Figures 3A and 3B). Since these cells still produce some C83 (Figures 5B and 5C), it is possible that sAPP$\alpha$ is rapidly cleaved or cleared under these conditions, making it undetectable. Indeed, sAPP$\alpha$ could be detected when A12-H9 cells were transfected with APP (Figures 6A and 6B). This demonstrates that APP molecules can be cleaved by both $\alpha$- and $\beta$-secretase pathways concurrently and therefore the two activities are not mutually exclusive as previously considered [47,48]. Importantly, the level of sAPP$\beta$ production does not therefore directly correlate with the level of $\alpha\beta$ production.

Our results also demonstrate that copper deficiency in fibroblasts increases the cleavage of APP C-terminal fragments by $\gamma$-secretase. APP C-terminal fragments were undetectable in copper-depleted A12-H9 fibroblasts, but were abundant in the other fibroblast cell lines (Figures 3C and 3D). However, inhibition of $\gamma$-secretase (with DAPT) revealed that A12-H9 fibroblasts actually produced an elevated level of C83 (Figure 5A). Therefore the C-terminus of APP must be processed more rapidly under conditions of copper deficiency. Our results show that copper deficiency may also increase the activity of BACE1 ($\beta$-site amyloid precursor protein-cleaving enzyme 1). The intracellular signalling mechanisms involved are not yet clear, but a Cu$^{2+}$ binding site has been identified on the cytoplasmic C-terminus of BACE1 [49], which mediates an interaction with the copper chaperone for SOD1. This raised the hypothesis that copper levels can influence BACE1 activity or trafficking.

Another important observation is the differential modulation of APP and APLP2 processing by copper. Both APP and APLP2 mRNA levels dropped markedly under copper-deficient conditions (Figure 4), consistent with copper-dependent transcriptional regulation for both genes. Nonetheless, rapid translation of both proteins was still evident when $\gamma$-secretase processing was inhibited (Figure 5C). Despite both proteins being cleaved by BACE1 and $\gamma$-secretase [50,51], our current results show that under copper-deficient conditions, APP processing shifts from $\alpha$-cleavage to $\beta$-cleavage, whereas APLP2 processing is generally attenuated and consequently there is more.
steady-state full-length protein (Figures 3A, 3C and 3D). There may also be an increase in β-cleavage fragments of APLP2 (Figure 5C). The purpose of this differential processing of APP compared with APLP2 under copper-deficient conditions is not clear, but it is interesting to note that a recent report described a significant decrease in the ratio of cerebrospinal fluid sAPPβ to soluble APLP2 in mild cognitive impairment and AD [52]. This finding is consistent with the effect of cellular copper deficiency that we have observed in vitro. Furthermore, with APP and APLP2 both contributing to [16] and being affected by copper homeostasis, it will be interesting to explore the contribution of both proteins and their processing to the pathophysiology of Menkes and Wilson diseases.

Contrary to results obtained using fibroblasts, changes in APP processing were not observed in copper-deficient neuroblastoma cells (Figures 7C and 7D). There are several possible explanations for this difference. The processing of APP may be cell-type dependent or depend on which spliced variant of APP is predominantly expressed (as APP770 is the prevalent form in fibroblasts; Figure 4). The lowering of intracellular copper by chelation in the neuroblastoma cells (∼27% decrease) may have been insufficient to match the response seen in the more profoundly copper-deficient fibroblasts (∼65% decrease). Lowering intracellular copper levels in fibroblasts by chelation also had no observable effect on APP processing (results not shown). Again, the decrease in copper that could be achieved in fibroblasts by chelation was only ∼20% (results not shown).

Finally, there may be a specific requirement for the copper deficiency to be induced by ATP7A. Despite the level of intracellular copper in neuroblastoma cells having no obvious effect on secretase cleavage of APP (Figures 7C and 7D), copper deficiency did raise the level of Aβ secretion by at least 30% (Figures 7E and 7F). This response was also observed using human M17 neuroblastoma cells transfected with wtAPP695 (results not shown). These results suggest that, in neuroblastoma cells, copper deficiency down-regulates the degradation of Aβ and subsequently the concentration of secreted Aβ increases.

Aβ can be degraded in vitro and in vivo by numerous metalloproteinases, including nephrilysin, matrix-metalloproteinases and insulin-degrading enzyme (reviewed in [1]). Recently, White et al. [36] demonstrated using CHO cells that clioquinol liganded to Cu²⁺ selectively up-regulated matrix-metalloprotease activity and consequently increased the degradation of Aβ by elevating intracellular copper (clioquinol alone had no effect). However, these authors found that increasing copper alone (CuCl₂, without clioquinol) in the medium induced a 35% increase in Aβ(1–40) secretion [36]. These results appear to conflict with results obtained by Borchardt et al. [20] using the same cell type (CHO), and also with our results using human neuroblastoma cells (Figure 7). This inconsistency might be explained by the presence of a serum factor or a copper carrier (such as clioquinol) modifying the behaviour of copper. Indeed, results from White et al. [36] indicate that clioquinol-liganding of Cu²⁺ induced opposite effects (decreased Aβ) in cell culture compared with the effects of free Cu²⁺ (increased Aβ). In serum, copper will complex to several ligands including α-fetoprotein (fetal albumin) and amino acids. The copper studies conducted by White et al. [36] were carried out in serum-free conditions unlike Borchardt et al. [20] or our present study. Nevertheless, results obtained in APP transgenic mice on the effect of elevated brain copper on Aβ levels in vivo support the conclusion that elevated copper decreases Aβ levels [18,25]. TgCRND8 AD model mice (expressing human APP containing both Swedish and Indiana mutations) crossed with the toxic milk (txj) mouse, a model for Wilson disease that accumulates copper in the brain and liver, have reduced Aβ plaques and diminished plasma Aβ levels [18]. Similarly, APP23 AD model mice (expressing human APP containing the Swedish mutation) on three months of dietary copper supplementation had significantly reduced Aβ production [25]. Notably, Tg2576 mice (expressing APP containing the Swedish mutation) treated with clioquinol have reduced brain Aβ burden, but levels of brain copper rise with this treatment [9]. In contrast, the addition of copper to the drinking water of cholesterol-fed rabbits induced immunoreactive Aβ accumulation in the neocortex and neurological impairment [53]. Aβ–copper complexes recruit chelator to generate neurotoxic chemical species [54], so under particular circumstances, such as in a high-cholesterol environment, a reaction of Aβ with copper ions may foster neurotoxicity and pathology. We have recently found that Aβ and copper are enriched in the cholesterol-rich lipid raft compartment of neuronal cells even under conditions of copper deficiency (Y. Hung and A.I. Bush, unpublished work). This may explain the mechanism leading to the co-mingling of copper and Aβ in copper-deficient tissue.

In summary, our results are consistent with brain copper deficiency elevating Aβ concentrations. Therefore the ionophoric properties of molecules like clioquinol that can redistribute copper from Aβ aggregates to neighbouring copper-deficient cells may be essential to their potential therapeutic benefit.

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