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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 26, pp. 17670–17680, June 30, 2006

Degradation of the Alzheimer Disease Amyloid β-Peptide by Metal-dependent Up-regulation of Metalloprotease Activity*

Received for publication, March 16, 2006, and in revised form, April 28, 2006 Published, JBC Papers in Press, April 28, 2006, DOI 10.1074/jbc.M602487200

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Biometals play an important role in Alzheimer disease, and recent reports have described the development of potential therapeutic agents based on modulation of metal bioavailability. The metal ligand clioquinol (CQ) has shown promising results in animal models and small phase clinical trials; however, the actual mode of action *in vivo* has not been determined. We now report a novel effect of CQ on amyloid β-peptide (Aβ) metabolism in cell culture. Treatment of Chinese hamster ovary cells overexpressing amyloid precursor protein with CQ and Cu^{2+} or Zn^{2+} resulted in an ~85–90% reduction of secreted Aβ-(1–40) and Aβ-(1–42) compared with untreated controls. Analogous effects were seen in amyloid precursor protein-overexpressing neuroblastoma cells. The secreted Aβ was rapidly degraded through up-regulation of matrix metalloprotease (MMP)-2 and MMP-3 after addition of CQ and Cu^{2+}. MMP activity was increased through activation of phosphoinositol 3-kinase and JNK. CQ and Cu^{2+} also promoted phosphorylation of glycogen synthase kinase-3, and this potentiated activation of JNK and loss of Aβ-(1–40). Our findings identify an alternative mechanism of action for CQ in the reduction of Aβ deposition in the brains of CQ-treated animals and potentially in Alzheimer disease patients.

Alzheimer disease (AD)[4] is characterized by progressive neuronal dysfunction, reactive gliosis, and the formation of amyloid plaques in the brain. The major constituent of AD plaques is the amyloid β-peptide (Aβ), which is cleaved from the membrane-bound amyloid precursor protein (APP) (1). Aggregated or oligomeric Aβ can induce neurotoxicity through pathways involving free radical production and increased neuronal oxidative stress (2). Among the factors capable of promoting Aβ aggregation *in vivo*, recent evidence supports a central role for biometals such as Cu^{2+} and Zn^{2+} in this process (3).

An important factor in controlling Aβ accumulation in AD patients is the activity of Aβ-degrading enzymes. Recent studies have identified several candidate proteases that may contribute to catabolism of Aβ in the brain. Neprilysin, insulin-degrading enzyme, angiotensin-converting enzyme, and matrix metalloproteases (MMPs) have all demonstrated Aβ-degrading activity *in vitro* and/or *in vivo* (4–6). Reduced activity of these or other Aβ-degrading proteases with age may play a role in promoting accumulation and deposition of Aβ in AD patients. Development of strategies to enhance clearance of Aβ may lead to novel therapeutic treatments for AD patients.

Promoting Aβ clearance may be achieved through modulating metal sequestration or metal-protein interactions. 5-Chloro-7-iodo-8-hydroxyquinoline or clioquinol (CQ), a disused antibiotic, has received considerable attention as a potential metal ligand in AD and Parkinson disease patients (7–9). Preliminary studies revealed that CQ rapidly and potently dissolved aggregates of synthetic or AD brain-derived Aβ *in vitro* (10). In subsequent animal studies, a 9-week oral treatment with CQ resulted in a 49% reduction of Aβ levels and significantly increased Cu^{2+} and Zn^{2+} levels in brains of Tg2576 mice (10). Small clinical trials of CQ have demonstrated a significant slowing of cognitive decline together with a lowering of plasma Aβ-(1–42) levels in a subset of AD patients compared with matched placebo controls (8).

The mechanism of action by CQ was suggested to be via metal sequestration, resulting in Aβ dissolution. However, CQ could also act by alternative pathways involving modulation of cellular biometal metabolism, APP expression, or Aβ processing (11). To investigate this, Chinese hamster ovary (CHO) cells overexpressing APP were treated with CQ in the presence or absence of physiological levels of biometals. When CQ was added to cells in the presence of Cu^{2+} or Zn^{2+}, the secreted levels of Aβ-(1–40) and Aβ-(1–42) were dramatically reduced. Analogous effects were seen in N2a neuroblastoma cells. Subsequent investigation revealed that this effect was associated with uptake of Cu^{2+} and Zn^{2+} and loss of Aβ through increased MMP-mediated degradation. These findings identify a novel mechanism for the therapeutic efficacy of CQ in which CQ·Cu^{2+} or CQ·Zn^{2+} complexes promote Aβ degradation.

EXPERIMENTAL PROCEDURES

Materials—CQ, bacitracin, puromycin, Me_2SO, ascorbate, LY-294, 002, wortmannin, bathophenanthroline disulfonate (BPS), *cis*-diamminedichloroplatinum (cisplatin), LiCl, SP600125, staurosporine, thior-

* This work was supported in part by the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] Supported by Ruth L. Kirschstein National Research Service Award Individual Fellowship AG05887 from NIA, National Institutes of Health.

[3] Supported by NIA Grant R01-AG12686 from the National Institutes of Health, the Alzheimer's Association, the American Health Assistance Foundation, and the Australian Research Council.

[4] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-peptide; APP, amyloid precursor protein; MMPs, matrix metalloproteases; CQ, clioquinol; CHO, Chinese hamster ovary; BPS, bathophenanthroline disulfonate; GSK, glycogen synthase kinase; MnTMPyP, Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; ICP-MS, inductively coupled plasma mass spectrometry; PI3K, phosphoinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MAPK, mitogen-activated protein kinase.

phan, and PD 98,059 were purchased from Sigma (Sydney, Australia). SB 203580, bestatin, GM 6001, phosphoramidon, glycogen synthase kinase (GSK) Inhibitor IX, MMP Inhibitor I (broad-spectrum MMP inhibitor), MMP-2 Inhibitor I, MMP-3 Inhibitor I, MMP-9 Inhibitor I, Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), and serine/cysteine protease inhibitor mixture (EDTA-free) were obtained from Merck Biosciences (Victoria, Australia). Anti-APP antibody 22C11 was obtained from Chemicon International, Inc. (Temecula, CA). Antibody 369 (APP-(656–695) epitope) was a kind gift from Dr. Sam Gandy (Thomas Jefferson University, Philadelphia, PA). Antibodies to total or phospho-specific forms of Akt, JNK, ERK1/2, p38, and GSK3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA).

Generation of APP-transfected CHO and N2a Neuroblastoma Cells—APP-CHO and APP-N2a neuroblastoma cells were generated by expressing the 695-amino acid APP cDNA in the pIRESpuro2 expression vector (Clontech). Cells were transfected using Lipofectamine 2000 and cultured in RPMI 1640 medium supplemented with 1 mM glutamine and 10% fetal bovine serum (all from Invitrogen, Mount Waverley, Victoria). Transfected cells were selected and maintained using 7.5 μg/ml puromycin (Sigma).

Exposure of Cells to CQ and Metals—APP-overexpressing cells were passaged at a ratio of 1:6 and grown in 6- or 12-well plates for 2–3 days before experiments. CQ was prepared as a 10 mM stock solution in Me_2SO and added to serum-free RPMI 1640 medium supplemented with puromycin as described above. Basal metal levels in the medium were 0.5, 1.3, and 2.1 μM for Cu^{2+}, Zn^{2+}, and Fe^{2+}, respectively, as determined by inductively coupled plasma mass spectrometry (ICP-MS). Additional metals were added (10 μM unless stated otherwise), and the medium was briefly mixed by aspiration prior to addition to cells. Control cultures were treated with vehicle (Me_2SO) alone. Inhibitors of phosphoinositol 3-kinase (PI3K) (LY-294,002 and wortmannin), JNK (SP600125), MEK1/2 (PD 98,059), p38 (SB 203580), GSK3 (GSK Inhibitor IX), and metalloproteases (GM 6001, phosphoramidon, thiorphan, bestatin, MMP-2 Inhibitor I, and MMP-9 Inhibitor I) were prepared as 10 mM stock solutions in Me_2SO and added at the indicated concentrations. Ascorbate, MnTMPyP, bacitracin, BPS, LiCl, and MMP-3 Inhibitor I were prepared as 10 mM solutions in distilled H_2O. Serine/cysteine protease inhibitor mixture (EDTA-free) was prepared as a 10× solution in distilled H_2O. Where stated, vector only-transfected or wild-type (non-APP-overexpressing) cells were exposed to synthetic human Aβ-(1–40) with or without CQ, metals, and inhibitors (see below). Cultures were incubated for up to 6 h, and conditioned media were taken for measurement of Aβ levels by enzyme-linked immunosorbent assay (ELISA). Cell viability was determined by lactate dehydrogenase release following kit instructions (Promega Corp., Annandale, New South Wales, Australia). For immunoblotting, cells were harvested into PhosphoSafe extraction buffer (Novagen) containing Protease Inhibitor Cocktail III (Calbiochem) and stored at −80 °C until used. Alternatively, cells were washed three times with phosphate-buffered saline (PBS) and harvested for analysis of metal levels by ICP-MS.

ICP-MS—Cells were treated with CQ and/or metals for 6 h unless stated otherwise and washed three times with Chelex 100-treated PBS (pH 7.4). Cells were scraped into PBS; an aliquot was taken for protein determination (protein microassay, Bio-Rad); and the remaining cells were collected by centrifugation at 14,000 rpm for 2 min in a Hermle microcentrifuge (Labnet International, Inc., Edison, NJ). Metal levels were determined in cell pellets by ICP-MS as described previously (12) and converted to ng of metal/mg of protein.

Degradation of Synthetic Aβ-(1–40)—Human Aβ-(1–40) was purchased from the W. M. Keck Laboratory (Yale University, New Haven, CT) and dissolved in Me_2SO at 1 mg/ml. The dissolved peptide was further diluted into Chelex 100-treated distilled H_2O at 100 ng/ml before addition to vector only-transfected CHO cell cultures in serum-free medium at 10 ng/ml without aging. In separate experiments, Aβ-(1–40) was also added to N2a mouse neuroblastoma, SH-SY5Y human neuroblastoma, or HeLa human epithelial cells in serum-free Opti-MEM I (Invitrogen). After 6 h (with or without addition of inhibitors and 10 μM each CQ, Cu^{2+}, or CQ and Cu^{2+}), the medium was collected, and the remaining Aβ-(1–40) levels were determined by ELISA.

Double Antibody Capture ELISA for Aβ Detection—Aβ levels were determined in culture medium using the DELFIA® double capture ELISA (PerkinElmer Life Sciences, Melbourne, Australia). 384-Well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with monoclonal antibody G210 in 15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.6) for Aβ-(1–40) detection. Plates were washed with PBS containing 0.05% Tween and blocked with 0.5% (w/v) casein. Biotinylated monoclonal antibody WO2 (Aβ-(5–8) epitope) and the culture medium or Aβ standards were added (50 μl) to each well and incubated overnight at 4 °C. Plates were washed with PBS containing 0.05% Tween, and streptavidin-labeled europium (PerkinElmer Life Sciences) was added. The plates were washed; enhancement solution (PerkinElmer Life Sciences) was added; and the plates were read in a Wallac VICTOR^2 plate reader with excitation at 340 nM and emission at 613 nM. Aβ-(1–40) and Aβ-(1–42) standards and samples were assayed in triplicate. The values obtained from the triplicate wells were used to calculate the Aβ concentration (expressed as ng/ml) based on the standard curve generated on each plate. We observed a good correlation between ELISA results and Western blot analysis of Aβ levels in CQ·Cu^{2+}-treated cultures. As the ELISA offered quantitative data on Aβ levels, we chose this as the preferred method for assessing changes to secreted Aβ levels.

Western Blot Analysis of Protein Expression and Phosphorylation—Cell lysates prepared in PhosphoSafe extraction buffer were mixed with SDS sample buffer (Novex) and separated on 12% Tris/glycine/SDS-polyacrylamide gels (Novex). Western blotting of Aβ in the conditioned medium was performed using 10–20% Tris/Tricine gels. Proteins were transferred to polyvinylidene difluoride membranes and blocked with milk solution in Tris-buffered saline/Tween before immunoblotting for total or phospho-specific proteins. Membranes were probed for 1 h with antiserum against Aβ (antibody WO2), C-terminal APP (antibody 369), or full-length APP (antibody 22C11) at 1:2000 dilution and with horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit secondary antibody at 1:5000 dilution. For detection of signal transduction molecules, membranes were probed with polyclonal antiserum against actin, JNK, phospho-JNK, p38, phospho-p38, ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, GSK3β, phospho-GSK3α/β, MMP-2, or MMP-6 at 1:5000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit secondary antiserum was used at 1:10,000 dilution. Blots were developed by chemiluminescence (ECL Advance, Amersham Biosciences) and imaged on a GeneGnome chemiluminescence imager (Syngene, Cambridge, UK). We found that the expression of total levels of kinases (Akt, JNK, ERK, and p38) was unaffected by metal uptake in APP-CHO cells. In contrast, actin, tubulin, and other proteins normally used for equalizing protein loading were found to be altered depending on metal levels.[5] Therefore, equal sample loading and protein transfer

[5] A. R. White, T. Du, K. M. Laughton, I. Volitakis, R. A. Sharples, M. E. Xilinas, D. E. Hoke, R. M. D. Holsinger, G. Evin, R. A. Cherny, A. F. Hill, K. J. Barnham, Q.-X. Li, A. I. Bush, and C. L. Masters, unpublished data.

TABLE 1
Metal concentrations in APP-CHO cells treated with CQ and metals for 6 h

Treatment	Cellular metal levels (p value compared with vehicle control)		
	Cu^{2+}	Zn^{2+}	Fe^{2+}
		ng/mg protein	
Vehicle	4.6 ± 0.1	182 ± 8	53 ± 5
CQ (10 μM)	20 ± 3 (0.01)	313 ± 39 (0.05)	25 ± 1
Cu^{2+} (10 μM)	24 ± 1	169 ± 10	48 ± 2
Zn^{2+} (10 μM)	6 ± 1	190 ± 16	16 ± 7
Fe^{2+} (10 μM)	4 ± 2	108 ± 7	845 ± 129 (0.001)
CQ-Cu^{2+} (10 μM)	472 ± 46 (0.005)	85 ± 14	17 ± 2
CQ-Zn^{2+} (10 μM)	29 ± 2	1838 ± 64 (0.001)	17 ± 4
CQ-Fe^{2+} (10 μM)	3 ± 2	269 ± 27	889 ± 22 (0.001)

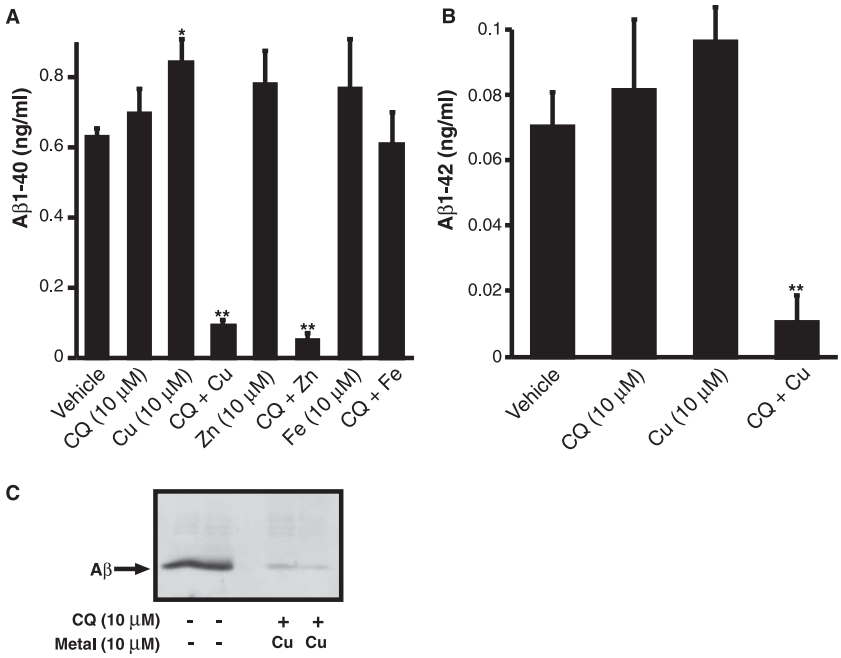

FIGURE 1. *A*, Aβ-(1–40) levels in medium from CQ-treated APP-CHO cells. Cultures were exposed to CQ (10 μM) with or without 10 μM Cu^{2+}, Zn^{2+}, or Fe^{2+} for 6 h, and Aβ-(1–40) levels were determined in culture medium by ELISA. Cu^{2+} alone induced a small but significant increase (*, $p < 0.01$) in Aβ-(1–40) secretion, whereas exposure to CQ·Cu^{2+} or CQ·Zn^{2+} significantly reduced Aβ-(1–40) levels (**, $p < 0.0001$). *B*, Aβ-(1–42) levels in medium from CQ-treated APP-CHO cells. Cultures were exposed to 10 μM CQ with or without 10 μM Cu^{2+} as described for *A*. CQ·Cu^{2+} induced a significant decrease in secreted Aβ-(1–42) levels (**, $p < 0.0001$). *Error* bars represent S.E. *C*, immunoblotting of medium from CQ·Cu^{2+}-treated APP-CHO cells. Cultures were exposed to 10 μM CQ·Cu^{2+} for 6 h, and the conditioned medium was analyzed by Western blotting using anti-Aβ antiserum (antibody WO2). Aβ levels were significantly lower in cultures treated with CQ·Cu^{2+} compared with untreated controls.

were assessed by consistency of total kinase protein levels rather than unrelated protein levels on immunoblots.

Cell Adhesion Assay—Cell adhesion to collagen type IV was determined using an InnoCyte ECM cell adhesion assay (collagen type IV; Merck Biosciences). Cells were treated with CQ, Cu^{2+}, or CQ and Cu^{2+} (with or without inhibitors) for 4 h before harvesting with a rubber policeman into the culture medium. Cells were dissociated by aspiration, replated onto collagen type IV, and cultured for an additional 2 h. The medium was discarded, and cells were washed briefly with two changes of PBS before addition of calcein acetoxymethyl ester for 1 h (37 °C). Cell adhesion was determined by fluorescence spectrophotometry on a Wallac VICTOR^2 plate reader with excitation at 490 nM and emission at 535 nm.

MMP Assays—The activity of MMPs in the conditioned medium and cell lysates was determined using an EnzoLyte MMP fluorometric assay kit (AnaSpec, Inc., San Jose, CA). Briefly, the conditioned medium or cell lysates (freshly extracted without protease inhibitors) were incubated with MMP-specific peptide substrates following the kit instructions. The substrates used were QXL520-γAbu-Pro-Cha-Abu-*S*-methyl-L-cysteine-His-Ala-Dab(5-FAM)-Ala-Lys-HN_2 (where γAbu is γ-aminobutyric acid, Cha is D-cyclohexylalanine, Dab is 2,4-diaminobutyric acid, and 5-FAM is 5-carboxyfluorescein; broad-spectrum substrate), QXL520-Pro-Leu-Ala-Leu-Trp-Ala-Arg-Lys(5-FAM)-NH_2 (MMP-1), 5-FAM-Pro-LeuAla-Nva-Dap(QXL520)-Ala-Arg-NH_2 (where Nva is norvaline and Dap is diaminopropionic acid; MMP-2), QXL520-Pro-Tyr-Ala-Tyr-Trp-Met-Arg-Lys(5-FAM)-NH_2 (MMP-3), QXL520-Pro-Leu-Gly-Met-Trp-Ser-Arg-Lys(5-FAM)-NH_2 (MMP-2/9), and QXL520-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(5-FAM)-NH_2 (MMP-8). No MMP-9-specific substrate was available. Cleavage of substrates by MMPs removed the quenching effect of QXL520 on 5-carboxyfluorescein, resulting in increased fluorescence with excitation at 490 nM and emission at 535 nm.

Statistical Analysis—All data described in graphical representations are means ± S.E. unless stated from a minimum of three separate experiments. Results were analyzed using Student's two-tailed *t* test.

RESULTS

CQ Mediates Uptake of Cu^{2+} and Zn^{2+} but Not Fe^{2+} in APP-CHO Cells—As CQ is a lipid-soluble metal ligand, we examined the effect of CQ on metal levels in APP695-transfected CHO cells (APP-CHO). Cultures were treated with CQ (10 μM) alone or in the presence of 10 μM Cu^{2+}, Zn^{2+}, or Fe^{2+} for 6 h, and cellular metal levels were assessed by ICP-MS. Basal Cu^{2+} levels were 4.6 ± 0.1 ng/mg of protein, and exposure to CQ alone increased this to 20 ± 3 ng/mg of protein ($p < 0.01$) (Table 1). Treatment with CQ and Cu^{2+} (CQ·Cu^{2+}) induced a dramatic 103-fold increase in cellular Cu^{2+} levels (472 ± 46 ng/mg of protein; $p < 0.005$) (Table 1). CQ also increased cellular Zn^{2+} levels from 182 ± 8 to 1838 ± 64 ng/mg of protein ($p < 0.001$) (Table 1). Measurement of cell survival (lactate dehydrogenase release) revealed no significant effect on cell viability after 6 h of exposure to 10 μM CQ and Cu^{2+} or Zn^{2+}. Treatment of cultures with Fe^{2+} alone (10 μM) resulted in a 16-fold

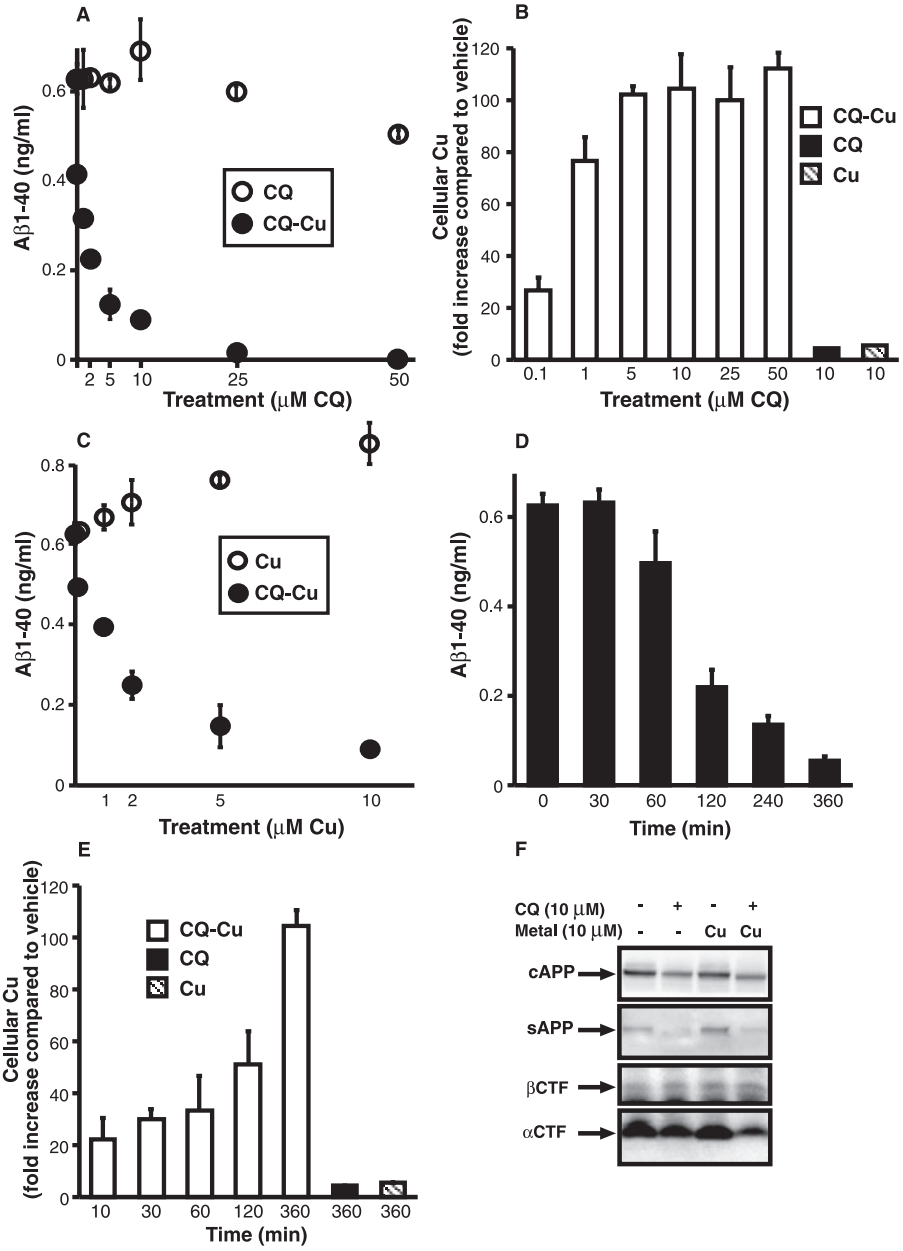

FIGURE 2. *A*, Aβ-(1–40) levels in medium from APP-CHO cells exposed to increasing concentrations of CQ. Cells were exposed to 0.1–50 μM CQ with or without 10 μM Cu^{2+} for 6 h. Aβ-(1–40) levels were determined in the culture medium by ELISA. All concentrations of CQ·Cu^{2+} tested (0.1–50 μM) induced a significant loss of secreted Aβ-(1–40) compared with CQ alone ($p < 0.001$–0.0001). *B*, cellular Cu^{2+} levels in APP-CHO cells exposed to Cu^{2+} and increasing concentrations of CQ. Cells were exposed to 10 μM Cu^{2+} and 0.1–50 μM CQ for 6 h. Cu^{2+} levels were determined in cell pellets by ICP-MS, revealing significantly increased cellular Cu^{2+} levels at all concentrations of CQ ($p < 0.001$–0.0001). The Cu^{2+} levels in vehicle-treated controls were equivalent to 1-fold Cu^{2+}. *C*, Aβ-(1–40) levels in medium from APP-CHO cells exposed to CQ and increasing concentrations of Cu^{2+}. Cells were treated with 10 μM CQ and 0.1–10 μM Cu^{2+} for 6 h. Cu^{2+} alone induced a small increase in secreted Aβ-(1–40) levels, but CQ·Cu^{2+} induced a dose-dependent decrease in secreted Aβ-(1–40) levels ($p < 0.01$–0.0001). *D*, Aβ-(1–40) levels in medium from APP-CHO cells exposed to CQ·Cu^{2+} for different time periods. Cells were treated with CQ·Cu^{2+} (10 μM) as described above, and Aβ-(1–40) levels were determined in the culture medium at time points up to 6 h after the start of treatment. Exposure to CQ·Cu^{2+} induced a time-dependent decrease in Aβ-(1–40) levels in the medium ($p < 0.05$–0.0001). *E*, cellular Cu^{2+} levels in APP-CHO cells exposed to CQ·Cu^{2+} for different time periods. Cells were exposed to CQ·Cu^{2+} (10 μM), and cellular Cu^{2+} levels were determined by ICP-MS in pellets at different time points up to 6 h after the start of treatment. CQ·Cu^{2+} induced a time-dependent increase in cellular Cu^{2+} levels ($p < 0.05$ at 120 min and $p < 0.001$ at 360 min). Relatively little change in cellular Cu^{2+} levels was induced by CQ or Cu^{2+} alone. The Cu^{2+} levels in vehicle-treated controls were equivalent to 1-fold Cu^{2+}. For all graphs, *error bars* represent S.E. *F*, APP levels in APP-CHO cells treated with CQ·Cu^{2+}. Cells were treated with 10 μM CQ with or without 10 μM Cu^{2+} for 6 h, and APP expression was determined by Western blotting in cell lysates and the conditioned medium. Equal protein loading was confirmed by immunoblotting for total JNK (not shown). CQ alone or CQ·Cu^{2+} decreased cellular APP (*cAPP*) expression and secreted APP (*sAPP*) levels in the conditioned medium. No change in APP β-C-terminal fragment (*βCTF*) C99 was observed with any treatment, whereas CQ·Cu^{2+} reduced expression of APP α-C-terminal fragment (*αCTF*) C83. Changes in APP expression did not correlate with secreted Aβ-(1–40) levels.

increase in cellular Fe^{2+} levels. However, co-treatment with CQ (10 μM) and Fe^{2+} did not further alter cellular Fe^{2+} levels. Analogous effects of CQ on cellular metal levels were also observed in vector only-transfected CHO cells. CQ·Cu^{2+} increased CHO cell Cu^{2+} levels by 94.5 ± 6-fold compared with untreated controls. Treatment with CQ·Zn^{2+} elevated Zn^{2+} levels by 10.5 ± 0.4-fold.

CQ and Cu^{2+} or Zn^{2+} Reduce Aβ Levels in Vitro—We examined whether CQ affects Aβ generation in APP-CHO cells. Treatment of APP-CHO cultures with 10 μM CQ alone for 6 h induced no significant change to Aβ-(1–40) levels in the culture medium (Fig. 1*A*). Interestingly, 10 μM Cu^{2+} alone for 6 h induced a 35% increase in Aβ-(1–40) levels ($p < 0.01$) (Fig. 1*A*).

When cultures were exposed to 10 μM CQ and 10 μM Cu^{2+}, we observed a potent reduction (~85%) of secreted Aβ-(1–40) levels ($p < 0.0001$) (Fig. 1*A*). An analogous effect was observed upon treatment with CQ and 10 μM Zn^{2+} (Fig. 1*A*). No significant changes were observed in Aβ-(1–40) levels when cells were treated with CQ plus Fe^{2+} (Fig. 1*A*). Potent inhibition of secreted Aβ-(1–42) levels also occurred with CQ·Cu^{2+}-treated cells (Fig. 1*B*). However, as Aβ-(1–42) levels in APP-CHO cells were near the detection limit of the ELISA, subsequent analysis of Aβ was restricted to Aβ-(1–40). The loss of secreted Aβ upon treatment with CQ·Cu^{2+} was confirmed by immunoblot analysis of the conditioned medium (Fig. 1*C*) and surface-enhanced laser desorption ionization mass spectrometry (data not shown).

Inhibition of Aβ Can Be Induced by Low Concentrations of CQ and Cu^{2+}—To examine the potency of CQ in inhibiting secreted Aβ levels, we treated cultures with 0.1–50 μM CQ with or without 10 μM Cu^{2+} for 6 h. Aβ-(1–40) was significantly decreased at 0.1 and 1.0 μM CQ plus Cu^{2+} (Fig. 2*A*). We also examined the effects of different concentrations of CQ on Cu^{2+} uptake in APP-CHO cells. 0.1 μM CQ induced an increase of ~25-fold in cellular Cu^{2+} levels (Fig. 2*B*). Increasing CQ concentrations resulted in further elevation of cellular Cu^{2+} levels, reaching 112-fold (at 50 μM) compared with control levels (Fig. 2*B*). The ability of low concentrations of CQ to increase cellular Cu^{2+} levels correlated with the potent reduction of secreted Aβ levels by CQ·Cu^{2+} (Fig. 2*A*). Although CQ has been reported

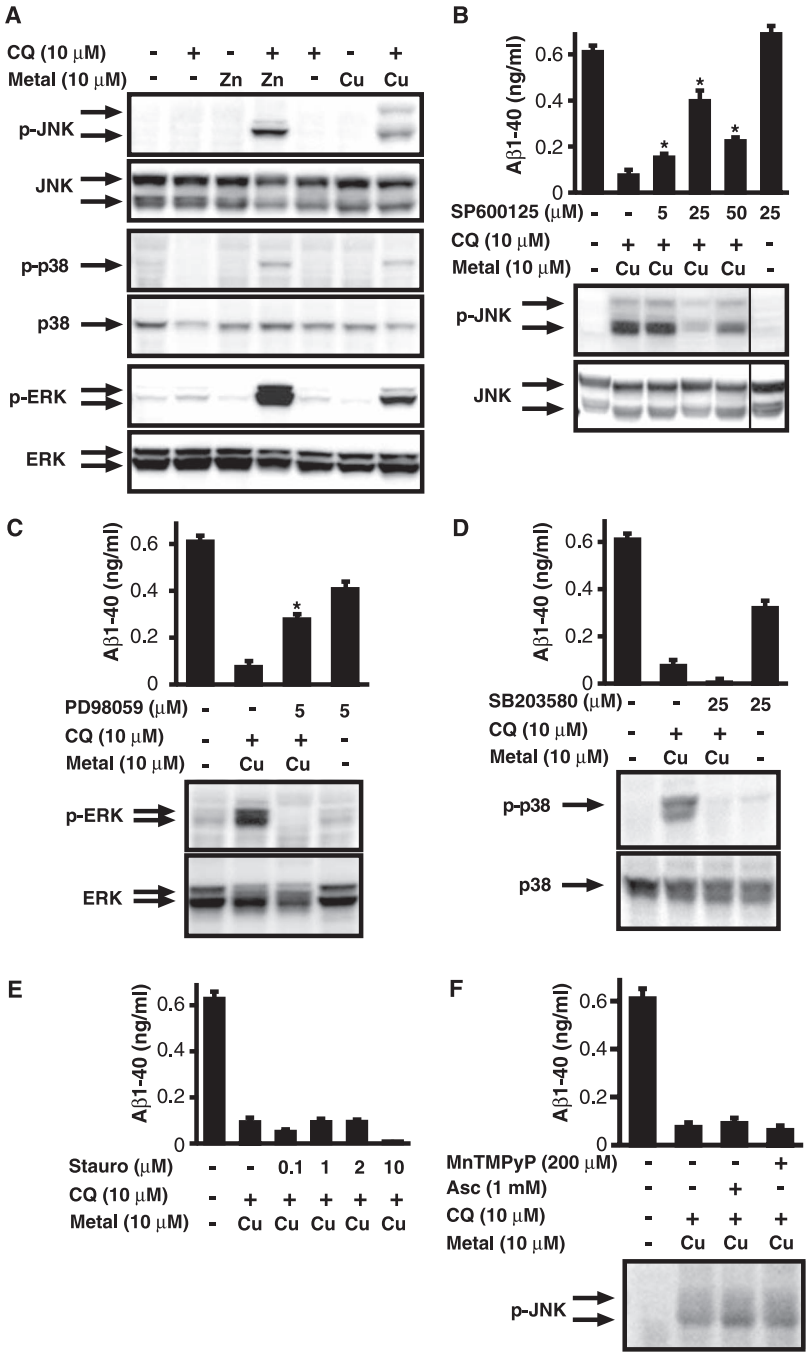

FIGURE 3. *A*, effects of CQ and metals on activation of MAPK pathways in APP-CHO cells. Cells were exposed to 10 μM CQ with or without 10 μM Cu^{2+} or Zn^{2+} for 6 h. Activation of MAPKs, including JNK, p38, and ERK1/2, was determined by Western blotting of cell lysates. CQ alone had no effect on the activity of MAPKs. In the presence of Cu^{2+} or Zn^{2+}, CQ induced activation of JNK, p38, and ERK1/2 (phospho-JNK, phospho-p38, and phospho-ERK1/2) *B*, JNK activation is associated with the loss of Aβ-(1–40) in APP-CHO cells treated with CQ·Cu^{2+}. Cells were exposed to 10 μM CQ·Cu^{2+} for 6 h in the presence or absence of the JNK inhibitor SP600125 (5–50 μM). Treatment of cells with CQ·Cu^{2+} induced activation of JNK with concurrent loss of Aβ-(1–40). Co-treatment of cells with SP600125 significantly inhibited both JNK activation and Aβ-(1–40) loss (*, $p < 0.001$). The dividing line represents removal of unrelated lanes. *C*, effect of the MEK1/2 inhibitor PD 98,059 on ERK1/2 activity and Aβ-(1–40) levels in APP-CHO cells treated with CQ·Cu^{2+}. Cells were treated with 10 μM CQ·Cu^{2+} for 6 h with or without addition of PD 98,059 (5 μM). Co-treatment with PD 98,059 abrogated ERK1/2 activation induced by CQ·Cu^{2+} and significantly inhibited Aβ-(1–40) loss (*, $p < 0.001$). *D*, the p38 inhibitor SB 203580 has no effect on the loss of Aβ-(1–40) induced by CQ·Cu^{2+}. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} as described above with or without SB 203580 (25 μM). SB 203580 prevented p38 activation by CQ·Cu^{2+}, but did not prevent Aβ-(1–40) loss induced by CQ·Cu^{2+}. SB 203580 alone reduced Aβ-(1–40) levels. *E*, staurosporine does not inhibit the loss of Aβ-(1–40) induced by CQ·Cu^{2+}. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} for 6 h in the presence or absence of the broad-spectrum protein kinase inhibitor staurosporine (*Stauro*; 0.1–10 μM). Co-treatment with staurosporine did not prevent the loss of Aβ-(1–40) by CQ·Cu^{2+}. *F*, JNK activation and Aβ-(1–40) loss by CQ·Cu^{2+} are not abrogated by antioxidants. APP-CHO cells were exposed to 10 μM CQ·Cu^{2+} for 6 h in the presence or absence of the free radical scavenger MnTMPyP (200 μM) or the antioxidant ascorbate (*Asc*; 1 mM). Co-treatment with MnTMPyP or ascorbate had no effect on JNK activation or Aβ-(1–40) loss. For all graphs, *error bars* represent S.E.

to optimally bind Cu^{2+} at a ratio of 2:1 (13), our titration studies showed no significant differences in Cu^{2+} uptake and inhibition of Aβ levels upon varying the CQ/Cu^{2+} ratios.

To determine the effects of Cu^{2+} concentration on secreted Aβ levels, cultures were exposed to 10 μM CQ with different concentrations of Cu^{2+}. 0.1 μM added Cu^{2+} significantly inhibited Aβ levels (Fig. 2*C*). Higher concentrations of added Cu^{2+} further decreased secreted Aβ levels (Fig. 2*C*). We also examined the time course of Aβ inhibition by CQ plus Cu^{2+} (10 μM each). We observed an initial decrease in Aβ levels from 30 to 60 min after addition of CQ·Cu^{2+}. A greater loss of Aβ was observed from 60 to 120 min after treatment (Fig. 2*D*). Examination of cellular metal levels revealed a 22-fold increase in Cu^{2+} after a 10-min exposure to CQ·Cu^{2+} (Fig. 2*E*). Cu^{2+} levels increased further at each time point, reaching a maximum level of 103-fold at 360 min (Fig. 2*E*).

Loss of Aβ by CQ·Cu^{2+} Does Not Correlate with Cellular APP Levels—To further understand how CQ·Cu^{2+} mediates Aβ loss, we determined whether there is a corresponding loss in APP expression. Exposure to CQ alone or to CQ·Cu^{2+} reduced both APP expression and secretion (Fig. 2*F*). However, as shown in Fig. 1*A*, only CQ·Cu^{2+} inhibited secreted Aβ levels. Interestingly, there was a reduction in the α-C-terminal 83-amino acid fragment of APP (C83) upon CQ·Cu^{2+} treatment, although no changes in APP β-C-terminal 99-amino acid fragment (C99) expression were found (Fig. 2*F*). This was consistent with our observation that the activity of BACE1 (beta-site APP-cleaving enzyme 1) in APP-CHO membrane preparations was unchanged after treatment with CQ·Cu^{2+}. Likewise, analysis of COS-7 cells transfected with a C-terminal APP construct (APP C99) (14) revealed no effect on γ-secretase cleavage of APP C99 by CQ·Cu^{2+}.[5] These findings demon-

strate that the loss of secreted Aβ upon treatment with CQ·Cu^{2+} is unlikely to result from altered APP processing.

Loss of Secreted Aβ by CQ·Cu^{2+} Is Mediated through Activation of JNK and ERK—Metal ligands can stimulate MAPK pathways (15, 16). To examine whether the effects of CQ·Cu^{2+} on Aβ occur via these pathways, we treated cultures with CQ and Cu^{2+} or Zn^{2+} (10 μM each) and measured activation of JNK, p38, and ERK1/2 in cell lysates. CQ with Cu^{2+} or Zn^{2+} induced substantial activation of JNK and ERK1/2, with moderate activation of p38 (Fig. 3*A*).

We then examined whether activation of these MAPK pathways is involved in the inhibitory action of CQ and metals on secreted Aβ levels. The JNK inhibitor SP600125 resulted in significant inhibition of JNK phosphorylation (Fig. 3*B*) and a significant elevation of Aβ-(1–40) levels compared with CQ·Cu^{2+} alone ($p < 0.001$) (Fig. 3*B*). The ERK1/2 phosphorylation inhibitor PD 98,059 (5 μM) prevented ERK activation after exposure to CQ·Cu^{2+} (Fig. 3*C*) and significantly inhibited Aβ loss ($p < 0.001$) (Fig. 3*C*). In contrast, the p38 inhibitor SB 203580 or the broad-spectrum protein kinase inhibitor staurosporine had no restorative effect on Aβ levels (Fig. 3, *D* and *E*).

JNK can be activated in response to cell stresses such as generation of reactive oxygen species or through growth factor-mediated pathways (17). Therefore, we examined whether JNK phosphorylation is mediated by generation of reactive oxygen species in the CQ·Cu^{2+}-treated cultures. APP-CHO cells were exposed to CQ·Cu^{2+} together with the reactive oxygen species scavenger MnTMPyP (200 μM) or the antioxidant ascorbate (1 mM). Treatment with these antioxidants did not inhibit JNK phosphorylation or prevent Aβ loss in CQ·Cu^{2+}-treated cultures (Fig. 3*F*). This is consistent with Zn^{2+} inducing effects analogous to those of Cu^{2+}, as Zn^{2+} is a redox-inactive metal and should not directly stimulate reactive oxygen species generation. Therefore, the results strongly suggest that activation of JNK by CQ·Cu^{2+} is not mediated through metal-induced oxidative stress.

Inhibition of Aβ by CQ·Cu^{2+} Requires Activation of the PI3K-Akt-GSK3 Pathway—Modulation of GSK3, a downstream target of PI3K and Akt activation, changes Aβ production in APP-CHO cells (18). Therefore, we examined whether the PI3K-Akt-GSK3 pathway is associated with the loss of Aβ production in CQ·Cu^{2+}-treated cells. Treatment of cells with CQ and Cu^{2+} (10 μM each) for 6 h resulted in significant activation of Akt (Fig. 4*A*). Co-treatment of cultures with the specific PI3K inhibitor LY-294,002 inhibited Akt phosphorylation induced by CQ·Cu^{2+} and significantly abrogated the decrease in secreted Aβ levels ($p < 0.0001$) (Fig. 4*A*).

Treatment of cultures with 10 μM CQ·Cu^{2+} for 6 h increased the phosphorylated forms of GSK3α/β, and this effect was blocked by LY-294,002 (Fig. 4*A*). There was also a small increase in total GSK3β levels in CQ·Cu^{2+}-treated cultures, which may partially account for the increased levels of phosphorylated GSK3.

We then investigated whether PI3K-Akt-GSK3 activation is upstream of MAPK activation. Treatment of cultures with 25 μM LY-294,002 (or 10 nM wortmannin; data not shown) inhibited phosphorylation of Akt as well as phosphorylation of both JNK and ERK1/2 (Fig. 4*B*). Conversely, treatment of cultures with inhibitors of JNK and ERK1/2 phosphorylation (SP600125 and PD 98,059 respectively) did not inhibit Akt phosphorylation (data not shown). These data demonstrate that PI3K-Akt activation is upstream of JNK and ERK activation.

Activation of the PI3K-Akt and JNK Pathways Alone Is Not Sufficient for Loss of Aβ—As inhibitors of PI3K and JNK pathways blocked the loss of Aβ by CQ·Cu^{2+}, we examined whether nonspecific up-regulation of these pathways also results in loss of Aβ in APP-CHO cells. Cultures exposed to 25–100 μM Cu^{2+} (without CQ) for 6 h revealed potent

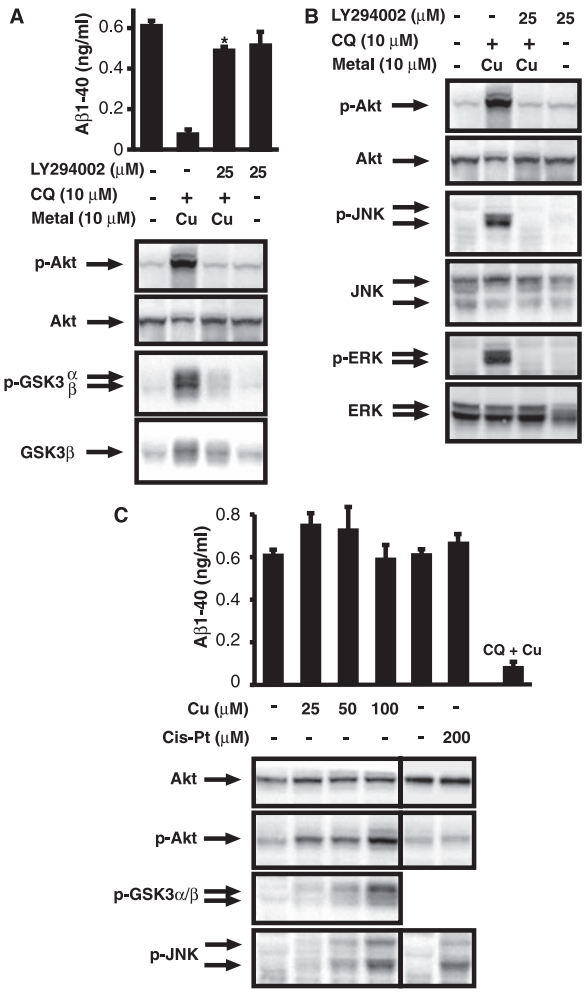

FIGURE 4. *A*, effect of PI3K inhibition on the loss of Aβ-(1–40) induced by CQ·Cu^{2+}. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} with or without the PI3K inhibitor LY-294,002 (25 μM) for 6 h. Akt and GSK3 phosphorylation was determined by Western blotting of cell lysates, and Aβ-(1–40) levels were measured in the culture medium by ELISA. Treatment of cultures with CQ·Cu^{2+} induced activation of Akt and phosphorylation of GSK3α/β. Co-treatment with LY-294,002 inhibited Akt and GSK3 phosphorylation and abrogated Aβ-(1–40) loss induced by CQ·Cu^{2+} (*, $p < 0.0001$). *B*, effect of PI3K inhibition on MAPK signaling in APP-CHO cells treated with CQ·Cu^{2+}. Cultures were exposed to 10 μM CQ·Cu^{2+} with or without 25 μM LY-294,002 for 6 h. Co-treatment with LY-294,002 prevented activation of Akt, JNK, and ERK. *C*, activation of the PI3K and MAPK pathways is not sufficient alone for the loss of Aβ. APP-CHO cells were exposed to 25–100 μM Cu^{2+} or 200 μM cisplatin (*Cis-Pt*) for 6 h. 25–100 μM Cu^{2+} induced activation of Akt, and 50–100 μM Cu^{2+} induced phosphorylation of GSK3 and JNK. However, Cu^{2+} alone did not induce Aβ loss. Cisplatin (200 μM) did not activate the PI3K pathway, but induced phosphorylation of JNK. Cisplatin did not affect Aβ levels. Aβ levels induced by 10 μM CQ·Cu^{2+} are shown for comparison. For all graphs, *error bars* represent S.E.

activation of Akt, whereas 50 and 100 μM Cu^{2+} also induced phosphorylation of GSK3 and JNK (Fig. 4*C*). Moreover, cultures treated with the apoptotic agent cisplatin (200 μM) for 6 h revealed activation of JNK but not Akt (Fig. 4*C*). However, neither of these treatments (Cu^{2+} or cisplatin) reduced secreted Aβ levels, demonstrating that the PI3K-Akt and JNK pathways are necessary, but insufficient alone, for the loss of Aβ in APP-CHO cells.

GSK3 Phosphorylation Promotes Activation of JNK in Cultures Treated with CQ·Cu^{2+}—Our data suggested that phosphorylation of GSK3 in CQ·Cu^{2+}-treated cells may modulate downstream JNK activation. To examine this, we treated cultures with CQ·Cu^{2+} in the presence of LiCl (an inducer of GSK3 phosphorylation). In the presence of CQ·Cu^{2+}, 5 mM LiCl increased phosphorylated GSK3 levels compared with CQ·Cu^{2+} alone (Fig. 5*A*). LiCl had no effect on phospho-Akt levels, demonstrating that the effect was not mediated through increased PI3K

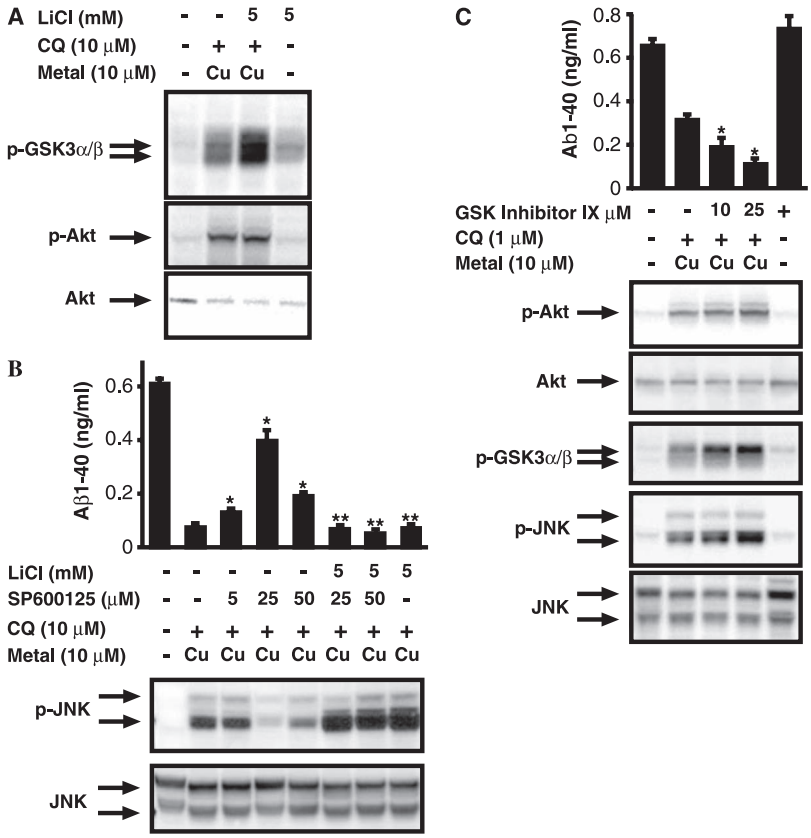

FIGURE 5. *A*, effect of LiCl on GSK3 phosphorylation in APP-CHO cells exposed to CQ·Cu^{2+}. Cells were treated with 10 μM CQ·Cu^{2+} with and without 5 mM LiCl for 6 h. Phosphorylation of GSK3α/β was determined by Western blotting of cell lysates. LiCl alone induced a low level of GSK3 phosphorylation, but, together with CQ·Cu^{2+}, greatly increased GSK3 phosphorylation without effect on Akt phosphorylation. *B*, effect of LiCl on Aβ-(1–40) in cultures treated with CQ·Cu^{2+}. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} with or without the JNK inhibitor SP600125 (5, 25, or 50 μM) and/or LiCl (5 mM) for 6 h. CQ·Cu^{2+} induced JNK phosphorylation, and this was inhibited by co-treatment with SP600125. The loss of Aβ-(1–40) induced by CQ·Cu^{2+} was also inhibited by SP600125. Co-treatment with 5 mM LiCl substantially increased JNK activation even in the presence of the JNK inhibitor SP600125. Co-treatment with LiCl also restored the loss of Aβ-(1–40) induced by CQ·Cu^{2+}, overcoming the inhibitory action of SP600125 (*, $p < 0.001$ compared with control cultures, **, $p < 0.001$ compared with cultures treated with CQ·Cu^{2+} and SP600125). LiCl alone had no significant effect on Aβ-(1–40) levels (not shown). *C*, effect of GSK Inhibitor IX on GSK3, JNK, and Aβ levels in APP-CHO cells. Cultures were treated with 10 μM CQ·Cu^{2+} for 6 h with or without 10 or 25 μM GSK Inhibitor IX. GSK Inhibitor IX increased GSK3 and JNK phosphorylation induced by CQ·Cu^{2+}. GSK Inhibitor IX also potentiated the loss of Aβ-(1–40) (*Ab1–40*) induced by CQ·Cu^{2+} (*, $p < 0.001$). For all graphs, *error bars* represent S.E.

and Akt activities (Fig. 5*A*). LiCl potentiated JNK phosphorylation in cultures treated with CQ·Cu^{2+} (Fig. 5*B*). This potentiation was sufficient to overcome the inhibitory action of 25 or 50 μM SP600125 on JNK phosphorylation (Fig. 5*B*). Interestingly, potentiation of JNK phosphorylation by LiCl also overcame the ability of SP600125 to prevent Aβ loss in the medium (Fig. 5*B*). To confirm the potentiating effect of GSK3 phosphorylation on JNK activation, we treated cultures with 1 μM CQ and 10 μM Cu^{2+} in the presence of GSK Inhibitor IX (10 or 25 μM). This increased phosphorylation of GSK3 and JNK compared with CQ·Cu^{2+} alone (Fig. 5*C*). The increased GSK3 and JNK phosphorylation correlated with a down-regulation of Aβ levels in the culture medium (Fig. 5*C*). These results provide strong evidence that increased phosphorylation of GSK3 in CQ·Cu^{2+}-treated cultures promotes activation of JNK and leads to loss of secreted Aβ.

CQ·Cu^{2+} Induces Metalloprotease-dependent Loss of Aβ—Exposure of APP-CHO cells to CQ·Cu^{2+} for 6 h resulted in morphological changes consistent with altered cell adhesion (detachment of cells), but without an obvious role for cytotoxicity or oxidative stress (Fig. 3*F*). To examine this, we measured adhesion of cells to a collagen type IV matrix after treatment with 10 μM CQ and Cu^{2+}. As shown in Fig. 6*A*, CQ·Cu^{2+} inhibited APP-CHO cell adhesion to collagen type IV by ~50%. The loss of adhesion could be prevented by treatment with SP600125 or LY-294,002 (Fig. 6*A*). As loss of cell adhesion is commonly associated with activation of metalloproteases (19), we treated cells with broad-spectrum metalloprotease inhibitors. GM 6001 (10 μM), BPS (500 μM), and MMP Inhibitor I (20 μM) significantly inhibited CQ·Cu^{2+}-mediated loss of cell adhesion to collagen type IV (Fig. 6*A*).

To determine whether metalloproteases mediate Aβ loss, APP-CHO cells were treated with a range of metalloprotease inhibitors, and Aβ levels were measured after exposure to CQ·Cu^{2+}. All metalloprotease inhibitors tested except thiorphan (neprilysin inhibitor) significantly inhibited the decrease in secreted Aβ levels induced by CQ·Cu^{2+} (Fig. 6*B*). To confirm that the loss of secreted Aβ was mediated through increased metalloprotease-mediated degradation rather than altered APP processing, vector only-transfected CHO cell cultures were exposed to 10 ng/ml synthetic human Aβ-(1–40) for 6 h with or without CQ·Cu^{2+}. Measurement of Aβ-(1–40) levels in the conditioned medium revealed 0.89 ± 0.11 ng/ml remaining in the control medium after 6 h, indicating substantial clearance by cell uptake and/or degradation (Fig. 6*C*). Exposure of cultures to 10 μM CQ or 10 μM Cu^{2+} increased the levels of synthetic Aβ-(1–40) remaining in the medium after 6 h (Fig. 6*C*). However, treatment of cultures with CQ·Cu^{2+} significantly decreased Aβ-(1–40) levels by 56% compared with controls and by 77% compared with CQ alone (Fig. 6*C*). Interestingly, this effect was prevented by co-treatment of cultures with LY-294,002 (25 μM) or inhibitors of metalloproteases (Fig. 6*C*). The results clearly support a role for PI3K-mediated metalloprotease degradation of Aβ as the primary cause of Aβ loss in cultures treated with CQ·Cu^{2+}.

CQ·Cu^{2+} Induces Up-regulation of MMP-2 and MMP-3 through Activation of the PI3K and JNK Pathways—The efficacy of GM 6001 and MMP Inhibitor I against loss of Aβ and cell adhesion strongly supported a role for up-regulation of MMPs in CQ·Cu^{2+}-treated cultures. Therefore, we measured the activity of MMPs in cells treated with CQ·Cu^{2+} using MMP-specific fluorescent substrates. MMP assays of cell lysates or the conditioned medium after treatment with CQ·Cu^{2+} for 6 h revealed a significant elevation of the specific activities of MMP-2 and MMP-3 (Fig. 7*A*). No significant changes were observed in the activities of MMP-1, MMP-8, and MMP-9. Western blot analysis of cell lysates with antisera to MMP-2 and MMP-9 confirmed the results from the fluorescent substrate assay. Both latent and activated forms of MMP-2 were up-regulated in cultures exposed to CQ·Cu^{2+}, whereas MMP-9 revealed only a minimum change (Fig. 7*A*, *inset*).

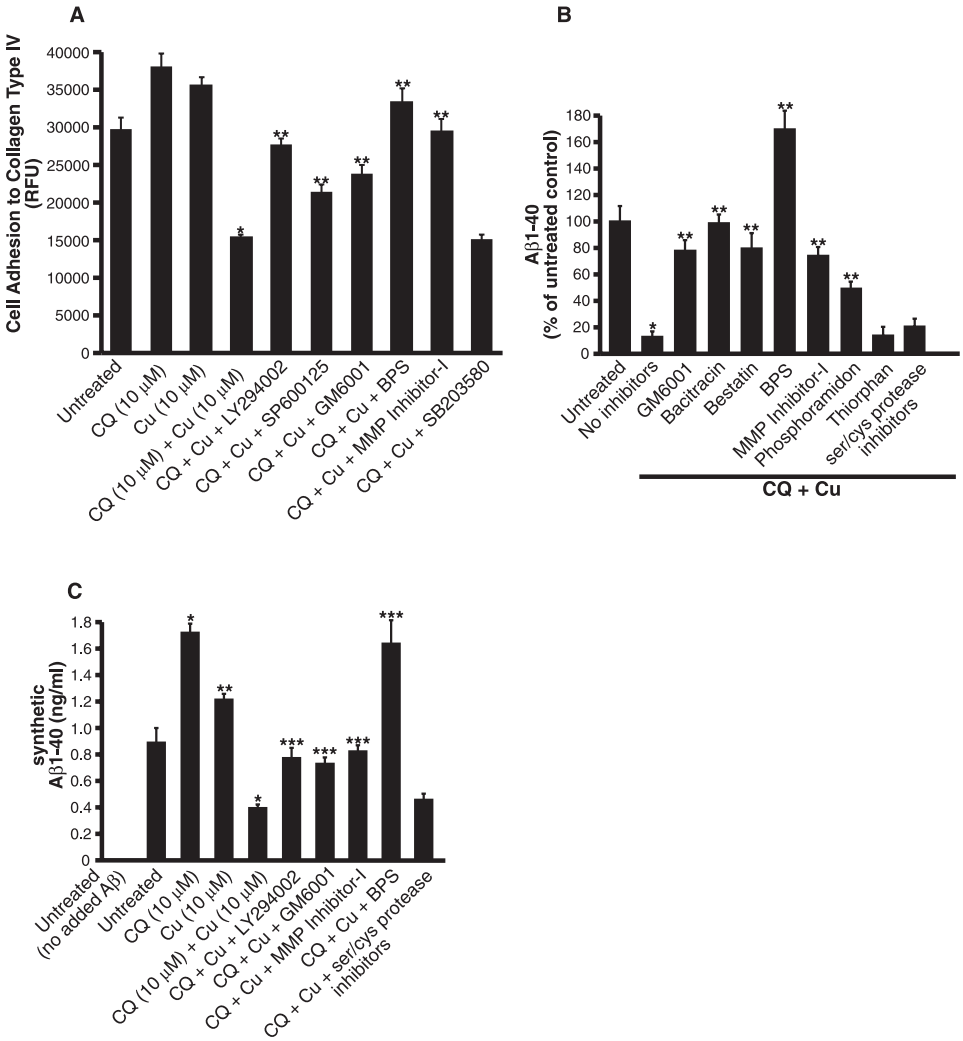

FIGURE 6. *A*, cell adhesion to a collagen type IV matrix. APP-CHO cells were exposed to CQ (10 μM), Cu^{2+} (10 μM), or CQ·Cu^{2+} with or without inhibitors, and adhesion to collagen type IV was determined by uptake of calcein acetoxymethyl ester by attached cells. Cells treated with CQ·Cu^{2+} revealed significantly lower adhesion to collagen type IV (*, $p < 0.0001$). The loss of adhesion was significantly inhibited (**, $p < 0.001$–0.0001) by co-treatment with LY-294,002 (25 μM), SP600125 (25 μM), GM 6001 (10 μM), BPS (500 μM), or MMP Inhibitor I (20 μM), but not by SB 203580 (25 μM). *RFU*, relative fluorescence units. *B*, Aβ loss is inhibited by metalloprotease inhibitors. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} for 6 h with or without 10 μM GM 6001, 10 μM bacitracin, 10 μM bestatin, 500 μM BPS, 20 μM MMP Inhibitor I, 50 μM phosphoramidon, 25 μM thiorphan, or 1× serine/cysteine protease inhibitor mixture. Aβ-(1–40) levels were measured in the culture medium by ELISA. CQ·Cu^{2+} induced a significant loss of Aβ-(1–40) (*, $p < 0.0001$). Co-incubation with metalloprotease inhibitors significantly inhibited the loss of Aβ-(1–40) induced by CQ·Cu^{2+} (**, $p < 0.001$ compared with CQ·Cu^{2+} alone). *C*, effect of CQ·Cu^{2+} on the loss of synthetic Aβ-(1–40). Vector only-transfected CHO cells were exposed to CQ (10 μM), Cu^{2+} (10 μM), or CQ·Cu^{2+} for 6 h with or without LY-294,002 (25 μM), GM 6001 (10 μM), MMP Inhibitor I (20 μM), BPS (500 μM), or 1× serine/cysteine protease inhibitor mixture. Cultures were co-incubated with 10 ng/ml synthetic human Aβ-(1–40) for 6 h, and the medium was assessed for remaining Aβ-(1–40) by ELISA. Treatment of cultures with CQ or Cu^{2+} alone resulted in significantly increased levels of Aβ-(1–40) remaining in the conditioned medium after 6 h (*, $p < 0.0001$; **, $p < 0.01$). Treatment with CQ·Cu^{2+} resulted in significantly decreased Aβ-(1–40) levels compared with controls. The loss of Aβ-(1–40) could be inhibited by addition of LY-294,002 or metalloprotease inhibitors (***, $p < 0.001$–0.0001 compared with CQ and Cu^{2+} alone). For all graphs, *error bars* represent S.E.

To further confirm activation of MMP-2 and MMP-3 by CQ·Cu^{2+}, cultures were treated with selective MMP inhibitors. Incubation of cultures with MMP-2 Inhibitor I prevented activation of MMP-2 by CQ·Cu^{2+}, but had no significant effect on MMP-3 activity (Fig. 7*B*). Likewise, MMP-3 Inhibitor I blocked activation of MMP-3, but did not affect MMP-2 activation by CQ·Cu^{2+} (Fig. 7*B*). An MMP-9 inhibitor had no effect on either MMP-2 or MMP-3 activation by CQ·Cu^{2+} (Fig. 7*B*). Moreover, we observed that LY-294,002 and SP600125 blocked activation of MMP-2 and MMP-3 (Fig. 7*B*).

Interestingly, the inhibitors of MMP-2 and MMP-3 significantly abrogated the loss of Aβ-(1–40) caused by CQ·Cu^{2+} (Fig. 7*C*). These effects were consistent with a previous report that both MMP-2 and MMP-3 can cleave Aβ at several sites (10). In fact, surface-enhanced laser desorption ionization analysis of medium from our control cultures revealed Aβ cleavage products consistent with MMP-2-mediated degradation.[5] Unfortunately, few Aβ fragments of any size were observed in CQ·Cu^{2+}-treated cultures. This suggested that further degradation of the Aβ cleavage fragments may be occurring in these cultures, possibly through activation of aminopeptidases. This was supported by inhibition of Aβ loss by co-treatment with bestatin (aminopeptidase inhibitor) (Fig. 6*B*).

Finally, we examined alternative cell types for their ability to degrade Aβ when exposed to CQ·Cu^{2+}. Treatment of APP-overexpressing N2a murine neuroblastoma cells with 10 μM CQ and 10 μM Cu^{2+} for 6 h reduced Aβ levels from ~1.1 to 0.4 ng/ml ($p < 0.001$), whereas CQ or Cu^{2+} alone had no significant effect (Fig. 8*A*). In addition, non-transfected N2a, SH-SY5Y human neuroblastoma, and HeLa human epithelial cells were treated with 10 μM CQ and Cu^{2+} together with 10 ng/ml synthetic human Aβ-(1–40). Measurement of Aβ levels in the conditioned medium after 6 h revealed significantly reduced synthetic Aβ-(1–40) levels in all cell types after treatment with CQ·Cu^{2+}, and this could be prevented by co-treatment with GM 6001 (Fig. 8*B*). These results demonstrate that CQ·Cu^{2+} can modulate secreted Aβ levels via metalloproteases in different cell types, including neuroblastoma cells.

DISCUSSION

In this study, we have shown for the first time that the lipid-soluble metal ligand CQ modulates secreted Aβ levels *in vitro*. Whereas treatment of APP-expressing cells with CQ alone had little effect on Aβ levels in the culture medium, treatment with CQ complexed to Cu^{2+} or Zn^{2+} dramatically decreased extracellular Aβ levels. We have shown that this effect is closely related to the ability of CQ to mediate substantial increases in cellular Cu^{2+} or Zn^{2+} levels, resulting in selective up-regulation of MMP activity.

Interestingly, we found that even low concentrations of CQ or Cu^{2+} (0.1–1 μM each) could induce a significant loss of Aβ after only 6 h. The potency with which CQ·Cu^{2+} inhibited Aβ underscores the potential physiological relevance of our findings. A recent study reported human plasma levels of CQ at ~13–25 μM during small

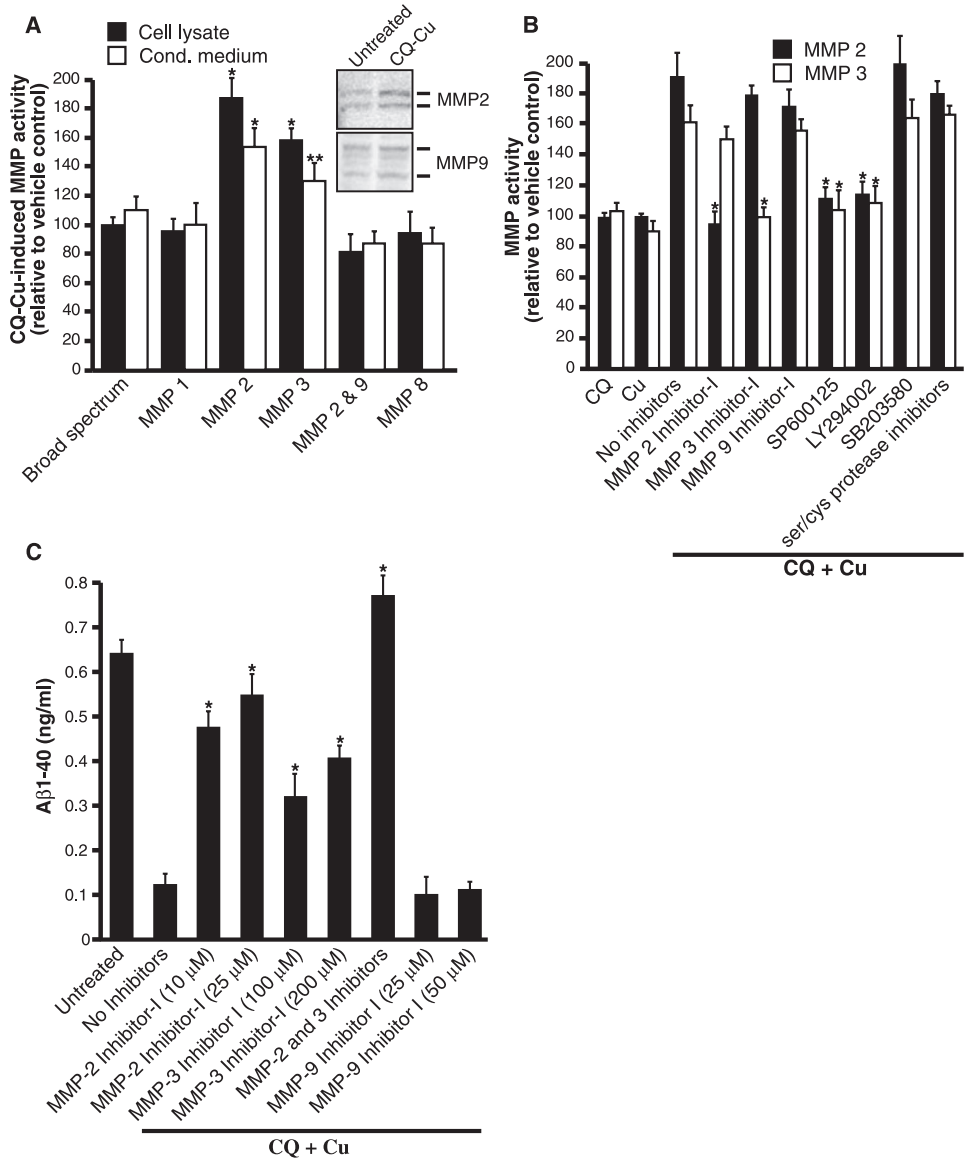

FIGURE 7. *A*, MMP activity induced by CQ·Cu^{2+}. APP-CHO cells were treated with 10 μM CQ·Cu^{2+} for 6 h, and MMP activity was assayed in cell lysates and the conditioned (*Cond.*) medium. MMP-1 and MMP-8 activities were not significantly altered in cells exposed to CQ·Cu^{2+}. MMP-2 and MMP-3 activities were significantly elevated by CQ·Cu^{2+} (*, $p < 0.001$; **, $p < 0.05$). No significant effects were observed using a broad-spectrum MMP substrate or a substrate recognized by both MMP-2 and MMP-9. *Inset*, Western blot analysis of cell lysates using antisera to MMP-2 and MMP-9. Western blotting confirmed that latent (*upper band*) and active (*lower band*) forms of MMP-2 were up-regulated in cultures treated with CQ·Cu^{2+} compared with controls. No change in latent MMP-9 (*upper band*) was observed in CQ·Cu^{2+}-treated cultures, although a slight increase in active MMP-9 (*lower band*) was seen. *B*, effect of inhibitors on MMP activity induced by CQ·Cu^{2+}. APP-CHO cells were exposed to CQ·Cu^{2+} (10 μM) for 6 h with or without MMP-2 inhibitor I (25 μM), MMP-9 inhibitor I (25 μM), LY-294,002 (25 μM), SB 203580 (25 μM), GM 6001 (10 μM), MMP-3 inhibitor I (200 μM), or 1× serine/cysteine protease inhibitor mixture. CQ·Cu^{2+} significantly activated both MMP-2 and MMP-3. MMP-2 activity induced by CQ·Cu^{2+} was significantly inhibited by co-treatment with MMP-2 Inhibitor I, GM 6001, or LY-294,002. MMP-3 activity was significantly inhibited by MMP-3 inhibitor I, GM 6001, or LY-294,002 (*, $p < 0.001$–0.0001). *C*, effect of MMP inhibitors on the loss of Aβ-(1–40) in CQ·Cu^{2+}-treated cultures. APP-CHO cells were exposed to 10 μM CQ·Cu^{2+} for 6 h with or without MMP-2 inhibitor I (10 and 25 μM), MMP-3 inhibitor I (used at 100 and 200 μM, as this is a large, peptide-based inhibitor, not a small, lipid-soluble molecule), MMP-2 and MMP-3 inhibitors (25 and 200 μM), or MMP-9 Inhibitor I (25 and 50 μM), and Aβ-(1–40) levels were measured in the culture medium by ELISA. MMP-2 inhibitor I and MMP-3 inhibitor I but not MMP-9 inhibitor I prevented the loss of Aβ-(1–40) induced by CQ·Cu^{2+} (*, $p <$ 0.0001). For all graphs, *error bars* represent S.E.

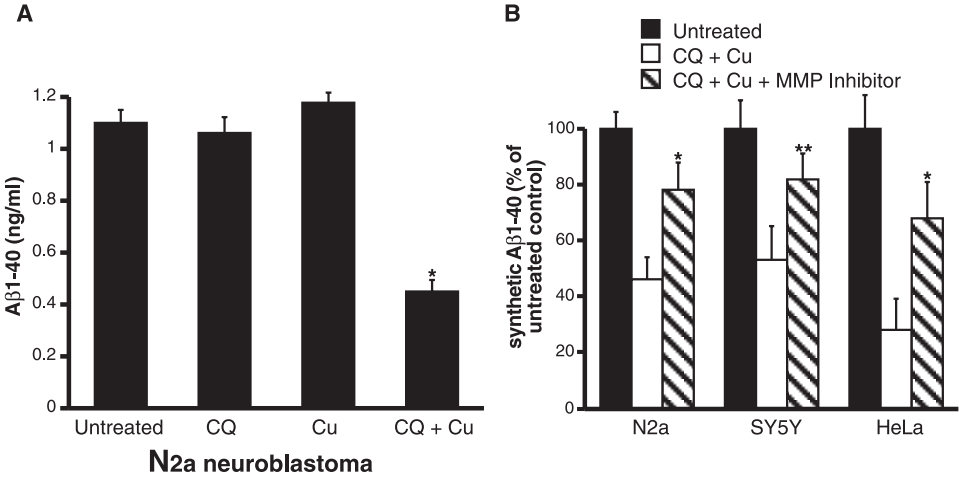

FIGURE 8. *A*, effect of CQ·Cu^{2+} on Aβ levels in APP-overexpressing N2a neuroblastoma cells. APP-N2a cells were treated with CQ or Cu^{2+} (10 μM each) or CQ·Cu^{2+} for 6 h. Aβ-(1–40) levels were determined in the conditioned medium by ELISA. Treatment with CQ·Cu^{2+} significantly reduced Aβ-(1–40) levels in APP-N2a cells (*, $p < 0.001$). *B*, loss of synthetic Aβ-(1–40) in different cell lines after treatment with CQ·Cu^{2+}. N2a, SH-SY5Y, or HeLa cells were exposed to 10 μM CQ·Cu^{2+} for 6 h with 10 ng/ml synthetic Aβ-(1–40). Treatment with CQ·Cu^{2+} significantly decreased synthetic Aβ-(1–40) levels in the conditioned medium of all cell types tested. Addition of the broad-spectrum MMP inhibitor GM 6001 (10 μM) significantly reduced the loss of Aβ-(1–40) (*, $p < 0.001$; **, $p <$ 0.01). For all graphs, *error bars* represent S.E.

phase clinical trials (8). CQ levels in the brain may reach 20% of serum levels, which equates to 2.6–5 μM (20). These concentrations were well within the range of CQ levels found to inhibit Aβ in our cultures if sufficient Cu^{2+} or Zn^{2+} was available. Cu^{2+} levels can range from 1.7 μM in the extracellular space of the brain to 250 μM in the synaptic cleft, whereas Zn^{2+} is also highly abundant in the brain, with synaptic levels reaching 300 μM (21). Further investigation is required to determine whether CQ can transport other metals (*i.e.*

nickel or cobalt) into cells and, if so, whether similar effects on APP metabolism are induced.

Although CQ is neurotoxic *in vitro* at low concentrations (22), cell lines are relatively more resistant to CQ than are primary neurons, and we found no evidence of increased cell death after 6 h of exposure to CQ and metals. Moreover, AD patients treated with 250 or 750 mg of CQ/day have not revealed complications that would indicate severe neurotoxicity (8). Similarly, mice treated with intraperitoneal injections of 28 mg/kg CQ also failed to show evidence of cytotoxicity (23). These findings suggest that the actions and toxicity of CQ *in vivo* are likely to be complex and dependent on the availability of "free" metals, antioxidants levels, and cellular resistance.

The mechanism of action by CQ·Cu^{2+} is via activation of the PI3K-Akt pathway and subsequent phosphorylation of JNK and ERK1/2. Although it is common to view PI3K-Akt and JNK/p38 as opposing pathways leading to cell survival and apoptosis, respectively (24), JNK activation can also be potentiated through PI3K activation (25, 26), as we have demonstrated here. Activation of both PI3K-Akt and JNK pathways has been reported in AD brain tissue, although the downstream consequences of this activity are not clear (27, 28).

PI3K is normally activated in response to cell stresses or growth factors, and metals can activate PI3K in some cell culture models (29). Particularly intriguing was our finding that activation of Akt and JNK by treatment with high Cu^{2+} levels alone (without CQ) or cisplatin had no effect on secreted Aβ levels, demonstrating that, although up-regulation of these pathways is required, by themselves, they are not able to decrease secreted Aβ levels. It is possible that, after exposure to CQ and metals, elevation of Cu^{2+} (or Zn^{2+}) levels in certain subcellular compartments results in specific modulation of multiple metal-dependent pathways, including PI3K activation (Fig. 9). This is consistent with reports that Zn^{2+} can activate gene expression by a PI3K- and JNK-dependent process (30). Alternatively, elevated metal levels could promote release of growth factors or other ligands that, in turn, activate PI3K, MAPK, and additional pathways. Interestingly, stimulation of MAPK pathways by metal-mediated growth factor release has been reported in lung epithelial cells (16, 31).

A common downstream signaling pathway controlled by PI3K activation involves phosphorylation of Akt and subsequent inhibition of GSK3 through phosphorylation (32). Treatment of cultures with LY-294,002 blocked phosphorylation of both Akt and GSK3α/β by CQ·Cu^{2+}. Inhibition (phosphorylation) of GSK3 can result in abrogation of Aβ production in APP-transfected cells (18), consistent with our findings. However, we found that phosphorylation of GSK3α/β correlated closely with increased JNK phosphorylation. Using inhibitors of GSK3 (LiCl and GSK Inhibitor IX), we demonstrated that increased phosphorylation of GSK3 potentiated JNK activation and subsequent Aβ loss. The mechanism behind this is not clear. As phosphorylation of GSK3 leads to its inactivation, the data suggest that activated GSK3 may inhibit or reduce JNK activation by certain stimuli. Similar effects have been reported previously, where a loss of GSK3 activity potentiated JNK activation by growth factors but not by cell stress (17, 33). This is consistent with our data suggesting that MAPK pathways are activated by CQ·Cu^{2+} via non-oxidative mechanisms. Down-regulation of GSK3 activity by CQ·Cu^{2+} could also affect tau phosphorylation, and this should be investigated in appropriate neuronal cell models.

Activation of cell signaling pathways by CQ·Cu^{2+} culminated in up-regulation of MMP activity and degradation of extracellular Aβ. Fig. 9 shows that the order of events are activation of PI3K-Akt, followed by phosphorylation of GSK3 as well as JNK and ERK. Inhibition of these

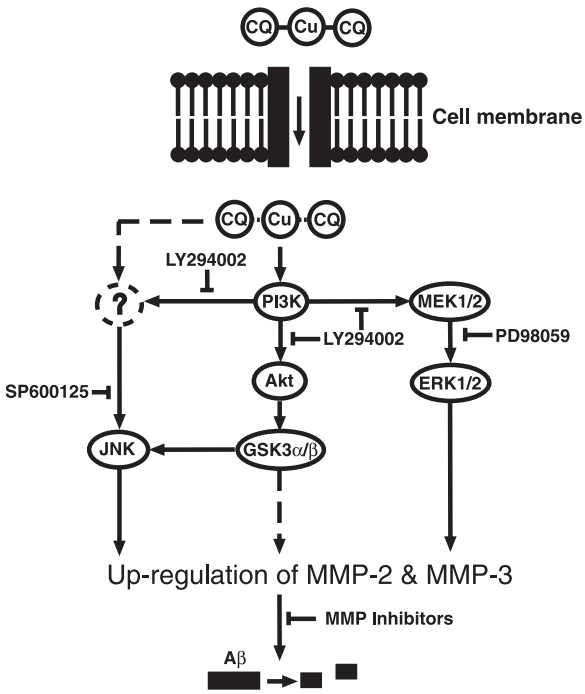

FIGURE 9. **Schematic of a proposed mechanism showing how CQ·Cu^{2+} mediates reduction of Aβ levels in APP-CHO cell cultures.** *Solid arrows* represent established pathways. *Dashed arrows* represent proposed pathways. CQ·Cu^{2+} complexes enter the cell by an unknown process. Cu^{2+} induces PI3K and additional cofactors (*?*) required for JNK activation. PI3K also activates Akt via phosphorylation, which, in turn, mediates phosphorylation of GSK3. PI3K activates MEK1/2 (not shown), resulting in phosphorylation of ERK1/2. Upon phosphorylation, GSK3 potentiates activation of JNK, which either alone or in concert with GSK3 or other signal factors, up-regulates the activities of MMP-2 and MMP-3. This induces an increase in degradation of extracellular or membrane-associated Aβ by these metalloproteases. Sites of inhibitor action are also shown.

kinases (Akt, JNK, and ERK) blocked activation of MMPs, so they are upstream of MMP activation. Moreover, inhibitors of the kinases and MMPs blocked the loss of Aβ, demonstrating that Aβ loss is downstream of these events. MMP activation is often associated with pathological changes to the cellular microenvironment in the brain, including tumor cell tissue invasion and migration and breakdown of blood-brain barrier permeability during cerebral ischemia. However, MMP activation can also have beneficial functions in the brain, including angiogenesis following ischemia and during axon guidance.

Up-regulation of MMP-2 and MMP-3 by Cu^{2+} or Zn^{2+} has been reported previously (16, 34), although excess Zn^{2+} can also inhibit MMP activity (35). In this study, we found that either Cu^{2+} or Zn^{2+} complexed to CQ induced activation of JNK, ERK, and p38 and loss of secreted Aβ. However, we investigated only CQ·Cu^{2+} complexes in detail, and it remains to be determined whether CQ·Zn^{2+} complexes mediate activation of the same MMPs.

Importantly, MMP-2 and MMP-3 levels can be increased through stimulation of the PI3K-Akt and MAPK (JNK and ERK) pathways (36, 37), which is consistent with our findings in CQ·Cu^{2+}-treated cells. Nonspecific Akt and JNK phosphorylation was unable to induce the decrease in secreted Aβ levels, indicating that CQ-delivered Cu^{2+} has a more complex effect on cell signaling pathways, resulting in up-regulation of MMPs. For example, there are a number of soluble inducers of MMP-2 and MMP-3, including transforming growth factor-β and epidermal growth factor, that could be released upon exposure to CQ and metals.

Aβ can be degraded *in vitro* and *in vivo* by several proteases, including metalloproteases, neprilysin, insulin-degrading enzyme, and MMPs (4, 6, 38). These metalloproteases may have important roles in clearance of Aβ in the brain, whereas reduced activity in AD patients could promote amyloid

deposition. As thiorphan (neprilysin inhibitor) had no effect on Aβ levels in CQ·Cu^{2+}-treated cultures, increased neprilysin activity is unlikely to be involved in Aβ loss. Although the inhibition of Aβ loss by bacitracin is consistent with insulin-degrading enzyme activity toward Aβ, immunoblot analysis of the culture medium revealed no increase in insulin-degrading enzyme levels after CQ·Cu^{2+} treatment (data not shown).

It has been shown previously that Aβ-(1–40) and Aβ-(1–42) can be degraded by MMP-2 ($Lys^{16} \downarrow Leu^{17}$, $Leu^{34} \downarrow Met^{35}$, and $Met^{35} \downarrow Val^{36}$) and MMP-3 ($Glu^{3} \downarrow Phe^{4}$) (4, 39). MMP-6 is also known to degrade Aβ at $Lys^{16} \downarrow Leu^{17}$, $Ala^{30} \downarrow Ile^{31}$, $Leu^{34} \downarrow Met^{35}$, and $Gly^{37} \downarrow Gly^{38}$ (4, 39). Our study supports the MMP-mediated degradation of Aβ, as both MMP-2 and MMP-3 were up-regulated in response to CQ·Cu^{2+} treatment, and inhibition of these metalloproteases prevented the loss of secreted Aβ. Whether other MMPs (40) and proteases (*i.e.* aminopeptidases) are also involved in the loss of Aβ induced by CQ·Cu^{2+} is not known. Further investigation will be necessary to fully characterize the Aβ cleavage products in CQ·Cu^{2+}-treated cultures and to identify whether MMP-2- and MMP-3-mediated cleavage is a rate-limiting step in the rapid clearance of secreted Aβ.

Whether CQ enhances degradation of Aβ *in vivo* is not known. MMP expression and distribution are altered in AD brains, and up-regulation of MMP activity occurs in response to Aβ exposure *in vitro* (41). Although this may result from inflammatory processes, it could also be an attempt to increase Aβ degradation. Several recent studies have shown that increases in central nervous system Cu^{2+} levels result in lower Aβ levels and reduced plaque deposition (42, 43). Moreover, Cherny *et al.* (10) demonstrated that APP transgenic mice treated with CQ have elevated central nervous system Cu^{2+} and Zn^{2+} levels together with reduced Aβ deposition. These reports are consistent with our findings here that elevated Cu^{2+} or Zn^{2+} levels can reduce Aβ levels by increasing Aβ degradation. Interestingly, small phase clinical trials of CQ demonstrated lower plasma Aβ-(1–42) levels with elevated plasma Zn^{2+} levels in treated patients (8). This could reflect increased peripheral degradation of Aβ through elevated Zn^{2+} levels. If so, this would raise the possibility of targeting peripheral Aβ with metal ligands as a means of reducing the total Aβ load.

In summary, our studies indicate a potentially important therapeutic role for induction of MMP activation by metal ligands and subsequent Aβ degradation. If CQ also mediates clearance of Aβ *in vivo* through activation of Aβ-degrading MMPs, these findings will have important implications for the future direction of AD therapeutics based on modulation of metal bioavailability.

Acknowledgments—We thank Dr. Roberto Cappai for critically reading the manuscript and Dr. Ian Trounce for the APP-N2a cells.

REFERENCES

1. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4245–4249
2. Barnham, K. J., Masters, C. L., and Bush, A. I. (2004) *Nat. Rev. Drug Discov.* **3,** 205–214
3. Bush, A. I. (2003) *Trends Neurosci.* **26,** 207–214
4. Roher, A. E., Kasunic, T. C., Woods, A. S., Ball, M. J., and Fridman, R. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1755–1761
5. Howell, S., Nalbantoglu, J., and Crine, P. (1995) *Peptides* (*N.Y.*) **16,** 647–652
6. Backstrom, J. R., Lim, G. P., Cullen, M. J., and Tokes, Z. A. (1996) *J. Neurosci.* **16,** 7910–7919
7. Kaur, D., Yantiri, F., Rajagopalan, S., Kumar, J., Mo, J. Q., Boonplueang, R., Viswanath, V., Jacobs, R., Yang, L., Beal, M. F., DiMonte, D., Volitakis, I., Ellerby, L., Cherny, R. A., Bush, A. I., and Andersen, J. K. (2003) *Neuron* **37,** 899–909
8. Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiers, L., Cherny, R., Li, Q.-X., Tammer, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R. E., and Masters, C. L. (2003) *Arch. Neurol.* **60,** 1685–1691
9. Ritchie, C. W., Bush, A. I., and Masters, C. L. (2004) *Expert Opin. Investig. Drugs* **13,** 1585–1592
10. Cherny, R. A., Atwood, C. S., Xilinas, M. E., Gray, D. N., Jones, W. D., McLean, C. A., Barnham, K. J., Volitakis, I., Fraser, F. W., Kim, Y., Huang, X., Goldstein, L. E., Moir, R. D., Lim, J. T., Beyreuther, K., Zheng, H., Tanzi, R. E., Masters, C. L., and Bush, A. I. (2001) *Neuron* **30,** 665–676
11. Treiber, C., Simons, A., Strauss, M., Hafner, M., Cappai, R., Bayer, T. A., and Multhaup, G. (2004) *J. Biol. Chem.* **279,** 51958–51964
12. White, A. R., and Cappai, R. (2003) *J. Neurosci. Res.* **71,** 889–897
13. Di Vaira, M., Bazzicalupi, C., Orioli, P., Messori, L., Bruni, B., and Zatta, P. (2004) *Inorg. Chem.* **43,** 3795–3797
14. Hoke, D. E., Tan, J. L., Ilaya, N. T., Culvenor, J. G., Smith, S. J., White, A. R., Masters, C. L., and Evin, G. M. (2005) *FEBS J.* **272,** 5544–5557
15. Chung, K. C., Park, J. H., Kim, C. H., Lee, H. W., Sato, N., Uchiyama, Y., and Ahn, Y. S. (2000) *J. Neurosci. Res.* **59,** 117–125
16. Wu, W., Samet, J. M., Silbajoris, R., Dailey, L. A., Sheppard, D., Bromberg, P. A., and Graves, L. M. (2004) *Am. J. Respir. Cell Mol. Biol.* **30,** 540–547
17. Liu, S., Yu, S., Hasegawa, Y., Lapushin, R., Xu, H. J., Woodgett, J. R., Mills, G. B., and Fang, X. (2004) *J. Biol. Chem.* **279,** 51075–51081
18. Phiel, C. J., Wilson, C. A., Lee, V. M., and Klein, P. S. (2003) *Nature* **423,** 435–439
19. Hofmann, U. B., Houben, R., Brocker, E. B., and Becker, J. C. (2005) *Biochimie* (*Paris*) **87,** 307–314
20. Matsuki, Y., Ito, T., Fukuhara, K., Abe, M., Othaki, T., and Nambara, T. (1987) *Arch. Toxicol.* **59,** 374–378
21. Frederickson, C. J., Koh, J. Y., and Bush, A. I. (2005) *Nat. Rev. Neurosci.* **6,** 449–462
22. Benvenisti-Zarom, L., Chen, J., and Regan, R. F. (2005) *Neuropharmacology* **49,** 687–694
23. Ding, W. Q., Liu, B., Vaught, J. L., Yamauchi, H., and Lind, S. E. (2005) *Cancer Res.* **65,** 3389–3395
24. Shimoke, K., Yamagishi, S., Yamada, M., Ikeuchi, T., and Hatanaka, H. (1999) *Dev. Brain Res.* **112,** 245–253
25. Li, J., Chen, H., Tang, M. S., Shi, X., Amin, S., Desai, D., Costa, M., and Huang, C. (2004) *J. Cell Biol.* **165,** 77–86
26. Miyata, Y., Sato, T., Yano, M., and Ito, A. (2004) *Mol. Cancer Ther.* **3,** 839–847
27. Zhu, X., Raina, A. K., Rottkamp, C. A., Aliev, G., Perry, G., Boux, H., and Smith, M. A. (2001) *J. Neurochem.* **76,** 435–441
28. Pei, J. J., Khatoon, S., An, W. L., Nordlinder, M., Tanaka, T., Braak, H., Tsujio, I., Takeda, M., Alafuzoff, I., Winblad, B., Cowburn, R. F., Grundke-Iqbal, I., and Iqbal, K. (2003) *Acta Neuropathol.* **105,** 381–392
29. Ostrakhovitch, E. A., Lordnejad, M. R., Schliess, F., Sies, H., and Klotz, L. O. (2002) *Arch. Biochem. Biophys.* **397,** 232–239
30. LaRochelle, O., Gagne, V., Charron, J., Soh, J. W., and Seguin, C. (2001) *J. Biol. Chem.* **276,** 41879–41888
31. Wu, W., Graves, L. M., Jaspers, I., Devlin, R. B., Reed, W., and Samet, J. M. (1999) *Am. J. Physiol.* **277,** L924–L931
32. Liang, J., and Slingerland, J. M. (2003) *Cell Cycle* **2,** 339–345
33. Takada, Y., Fang, X., Jamaluddin, M. S., Boyd, D. D., and Aggarwal, B. B. (2004) *J. Biol. Chem.* **279,** 39541–39554
34. Adamson, I. Y., Vincent, R., and Bakowska, J. (2003) *Exp. Lung Res.* **29,** 375–388
35. de Souza, A. P., Gerlach, R. F., and Line, S. R. (2000) *Dent. Mater.* **16,** 103–108
36. Kubiatowski, T., Jang, T., Lachyankar, M. B., Salmonsen, R., Nabi, R. R., Quesenberry, P. J., Litofsky, N. S., Ross, A. H., and Recht, L. D. (2001) *J. Neurosurg.* **95,** 480–488
37. Matsumoto, K., Minamitani, T., Orba, Y., Sato, M., Sawa, H., and Ariga, H. (2004) *Exp. Cell Res.* **297,** 404–414
38. Leissring, M. A., Farris, W., Chang, A. Y., Walsh, D. M., Wu, X., Sun, X., Frosch, M. P., and Selkoe, D. J. (2003) *Neuron* **40,** 1087–1093
39. Rapala-Kozik, M., Kozik, A., and Travis, J. (1998) *J. Pept. Res.* **52,** 315–320
40. Stix, B., Kahne, T., Sletten, K., Raynes, J., Roessner, A., and Rocken, C. (2001) *Am. J. Pathol.* **159,** 561–570
41. Deb, S., Zhang, J. W., and Gottschall, P. E. (1999) *J. Neurosci. Res.* **55,** 44–53
42. Bayer, T. A., Schafer, S., Simons, A., Kemmling, A., Kamer, T., Tepest, R., Eckert, A., Schussel, K., Eikenberg, O., Sturchler-Pierrat, C., Abramowski, D., Staufenbiel, M., and Multhaup, G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14187–14192
43. Phinney, A. L., Drisaldi, B., Schmidt, S. D., Lugowski, S., Coronado, V., Liang, Y., Horne, P., Yang, J., Sekoulidis, J., Coomaraswamy, J., Chishti, M. A., Cox, D. W., Mathews, P. M., Nixon, R. A., Carlson, G. A., St George-Hyslop, P., and Westaway, D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14193–14198