Clioquinol Mediates Copper Uptake and Counteracts Copper Efflux Activities of the Amyloid Precursor Protein of Alzheimer’s Disease*

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The key protein in Alzheimer’s disease, the amyloid precursor protein (APP), is a ubiquitously expressed copper-binding glycoprotein that gives rise to the Aβ amyloid peptide. Whereas overexpression of APP results in significantly reduced brain copper levels in three different lines of transgenic mice, knock-out animals revealed increased copper levels. A provoked rise in peripheral levels of copper reduced concentrations of soluble amyloid peptides and resulted in fewer pathogenic Aβ plaques. Contradictory evidence has been provided by the efficacy of copper chelation treatment with the drug clioquinol. Using a yeast model system, we show that adding clioquinol to the yeast culture medium drastically increased the intracellular copper concentration but there was no significant effect observed on zinc levels. This finding suggests that clioquinol can act therapeutically by changing the distribution of copper or facilitating copper uptake rather than by decreasing copper levels. The overexpression of the human APP or APLP2 extracellular domains but not the extracellular domain of APLP1 decreased intracellular copper levels. The expression of a mutant APP deficient for copper binding increased intracellular copper levels several-fold. These data uncover a novel biological function for APP and APLP2 in copper efflux and provide a new conceptual framework for the formerly diverging theories of copper supplementation and chelation in the treatment of Alzheimer’s disease.

The amyloid precursor protein (APP)† of Alzheimer’s disease (AD) is a ubiquitously expressed copper-binding glycoprotein that gives rise to the Aβ amyloid peptide (1). The APP paralogs and orthologs demonstrate a significant evolutionary change in the function of the N-terminal copper-binding domain (CuBD). In higher species, CuBD has a gain-in activity toward promoting Cu(II) reduction (2, 3).

Copper deprivation strongly down-regulates APP gene expression and reduces APP levels (4). Animal model systems revealed that APP is actively involved in balancing copper concentrations in vivo. Whereas overexpression of APP results in significantly reduced brain copper levels in three different lines of transgenic mice, APP23, Tg2576, and TgCRND8 (5–7), conversely, APP and APLP2 knock-out mouse revealed increased copper levels in cerebral cortex and liver (8). Whereas the relevance of a disturbed metal ion homeostasis in Alzheimer’s disease is presently unclear (9–11), studies in two different transgenic mouse models convincingly demonstrate that a rise in peripheral copper levels reduced soluble Aβ peptide levels (5) and the number of Aβ plaques (7). In a mammalian cell line, increasing intracellular copper levels attenuated APP processing into Aβ and stimulated secreted APP levels (5, 12). This finding demonstrates that copper-binding to APP alters the turnover of brain APP and concomitantly offers an alternative therapeutic intervention. Based on earlier observations that copper and chelators have an effect on amyloid fibril formation (13), the Cu,Zn chelator clioquinol was administered to APP-overexpressing Tg2576 mice (14). Intriguingly, the oral treatment with clioquinol (CQ), despite being a chelator for copper and zinc, significantly elevated brain copper by 19% and zinc by 13%. It also elevated the concentration of soluble brain Aβ by 50%, whereas Aβ deposition was markedly inhibited (14). These results lead to the conclusion that CQ did not induce a loss in metal ions systematically. Possibly, CQ might have redistributed copper from plaques to the cells, or alternatively, facilitated the uptake of CQ-copper complexes in the brain because CQ is hydrophobic and crosses the blood-brain-barrier (BBB). In a Phase II clinical trial, CQ inhibited cognitive decline and decreased plasma Aβ42 levels in moderate to severe AD patients (15).

Taken together, these findings jointly argue that APP and its paralogs may play a critical role in intracellular copper homeostasis and we anticipate that (1) APP or APLP2 expression is a substantial driving force for copper efflux and (2) CQ treatment enables copper passage into the cell. To test these hypotheses, the methylotrophic yeast Pichia pastoris was used to examine the effect of expression and secretion of the N-terminal domains of human APP (sAPP), APLP1 (sAPLP1), or APLP2 (sAPLP2) on intracellular concentrations of copper in yeast cells and the influence of CQ on the APP-expressing cells (16). Our studies showed that clioquinol in yeast culture me-
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Clioquinol drastically increased the intracellular copper concentration in wild-type and APP-expressing cells. These results indicated that the reported protective effects of CQ are not considered solely a chelator of zinc and/or copper. In addition, we reported that APP and APLP2 but not APLP1 are involved in copper homeostasis as copper efflux proteins.

EXPERIMENTAL PROCEDURES

Expression Constructs and Site-directed Mutagenesis—The APP18–350 (sAPP) construct in pPICzA (Invitrogen) was generated by PCR using the EcoRI and NotI sites in a 5'-overhang region of the forward primer (APP18–350fwd, 5'-GC GGC GGC GAA TTC TCG GAT GGA CCT ACC GAT GGT AAT G-3') and the reverse primer (APP18–350rev, 5'-TTC ATC TAA GGG GGC GTC TAG AGT AAT CTT TGG GAC ATG GCC GTC CCA ACG AC-3'), respectively. Site-directed mutagenesis of APP18–350 mutant was performed with PCR using a mismatch forward primer (APP18–350AAAfwd, 5'-AGG AGA GGA GTA TGG TTT GGG AAA CTG TTC TCT GGT CCC CCA CGA CCT CGG CCA AAG AGA C-3') and the APP18–350rev primer. The generated "megaprimer" was used with the APP18–350fwd primer on the same template. The amplified sequences were cloned into the pPICzA vector at EcoRI and NotI sites in-frame to the s-secretory factor. The vectors were linearized with NotI before transformation of P. pastoris strain SMD1168 by electroporation. Successfully transformed cells showed the metalion utilization phenotype Mut". Recombinant secreted APLP2 (sAPLP2) and APLP1 (sAPLP1) were produced in P. pastoris by using the vector pPIC9 (Invitrogen) as has been described elsewhere (17).

Expression in P. pastoris—Selection of Zeocin-resistant clones was done on YPD plates (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) agarose, 2% (v/v) methanol instead of glycerol to induce heterologous expression) containing copper and zinc at 1, 5, 10, 25, 50, 100, 200, and 400 parts/billion with Rh-103 as the internal standard for all of the isotopes of copper and zinc. The data obtained were normalized by the amount of yeast cells to compare the determined intracellular amount of metal ion per cell. Statistical analysis was performed by using the Student's t test. Error bars are shown as the mean ± S.E.

SOD1 Assay—The assay for SOD activity was purchased from Calbiochem and is based on the SOD-mediated increase in the rate of autodissociation of 5,6,6'-tetrathydro-3,9,10-trihydroxybenzo[cl]fluorene in aqueous alkaline solution, which generates a chromophore with a wavelength of maximum absorbance at 652 nm. Cell pellets were washed 1× in H2O, 1× in 0.5 mM EDTA, pH 8.0, incubated for 10 min at room temperature, and washed 1× in H2O, and cell lysates were prepared as described above, with the exception that a EDTA-frie lysine buffer was used. Lysates were diluted 1.1 in H2O, and the measurements were performed according to the manufacturer's instructions.

RESULTS

The methylo trophic yeast P. pastoris was used to examine the influence of sAPP, sAPLP2, and sAPLP1 expression on intracellular concentrations of copper. Yeast is an appropriate system, because most of what we know regarding copper transport in eukaryotes is derived from yeast studies (18–20). There is a conservation of the copper-handling pathways in yeast and mammalian cells, e.g. proteins involved in copper utilization in yeast have counterparts in mammals, and these counterparts can substitute functionally for yeast proteins. Moreover, the yeast genome does not encode a structural homolog of APP or APLPs, making it an attractive model system for analyzing the role of APP and APLPs in copper homeostasis. We have derived P. pastoris lines that secrete the ectodomain of APLP1 (sAPLP1), APLP2 (sAPLP2), and C-terminally truncated APP (sAPP) with amino acid residues 18–350. The APP18–350 construct contains the CuBD together with the N-terminal growth factor-like domain but lacks the C-terminal half, which includes the central APP domain (residues 365–570) (21, 22) and the Aβ sequence (3, 16, 29).

In agreement with previous transgenic mouse studies showing a reduction in copper brain tissue levels (5–7), the expression of sAPP decreased the intracellular copper level in yeast cells by 40% compared with mock control cells as determined by ICP-MS analysis (Fig. 1a). There was a slight but not significant increase in zinc levels between sAPP-expressing cells and control cells (Fig. 1, b and d). When the cells were grown in the presence of 1 mM copper, the concentration of copper atoms in the sAPP-expressing cells were significantly lower (Fig. 1c) (2, 5). Thus, copper efflux activity in sAPP-expressing cells was superior to mock-transfected cells (Fig. 1, a and c). To demonstrate the specificity of this effect, sAPLP2- and sAPLP1-secreting cells were analyzed. When compared with the mock control (Fig. 1a), the expression of sAPLP2 decreased the intracellular level of copper by 60% (Fig. 1e), whereas no changes were observed for the sAPLP1-expressing cells (Fig. 1f). Therefore, only sAPP and sAPLP2 have copper efflux activities. sAPLP1 might be unable to exert copper efflux activities, because it preferentially binds Cu(I) compared with intracellular Cu(I) (2), sAPLP1 lacks the His-X-His motif and has an "inert phenotype" that is neither toxic nor protective toward copper-mediated toxicity (3).

To establish that the CuBD of sAPP is responsible for this phenotype, we mutated the copper-binding residues H147A, H149A, and H151A (24–26) in APP (Fig. 2, a and b) and assayed sAPPH147A/H149A/H151A for its influence on intra-
cellular copper levels. *P. pastoris* cells overexpressing sAPPH147A/H149A/H151A repeatedly demonstrated that neither copper supplementation nor the presence of clioquinol significantly changed the expression levels of wild-type or mutant sAPP as shown by the integrated density values measured (Fig. 2, a and b).

Surprisingly, under standard conditions, the copper levels of the sAPPH147A/H149A/H151A cells showed a 5-fold increase compared with control cells. In the presence of 1 mM copper, intracellular copper levels are increased 16-fold because of the expression of the mutant construct (Fig. 3, a and b). These results indicate that the CuBD of sAPP is responsible for the copper efflux activity of APP. Elevating the copper concentrations in the growth medium enhanced the cytoplasmic copper concentrations in the sAPPH147A/H149A/H151A cells and partially enhanced the secretion of sAPP (Fig. 2b) as revealed by an integrated density value determined by densitometry. This finding suggests that the expression of sAPPH147/H149A/H151A may interfere with copper acquisition, sorting, or distribution systems in yeast cells (27–29).

To determine whether the reduced copper level in sAPP-, sAPLP2-, or sAPLP1-expressing cells lowers the bioavailability of copper, we measured the activity of cellular ySOD1 in yeast cells grown either in normal or in copper-supplemented medium by using a quantitative SOD assay (30). There was a 38% decrease of ySOD1 activity in sAPP-expressing cells (Fig. 4a) and a 21% decrease of ySOD1 activity in sAPLP2-expressing cells (Fig. 4b) that were not observed for sAPLP1 (Fig. 4c) compared with mock-transfected cells. The overexpression of mutant sAPPH147/H149A/H151A resulted in a 5-fold increase in ySOD1 (31) activity that correlated with the altered intracellular copper levels (Fig. 4d). Thus, the expression of sAPP or...
sAPLP2 limited the intracellular copper availability for apo-
ySOD1 but not sAPLP1, whereas mutant sAPP H147A/H149A/
H151A inhibited copper efflux and increased the intracellular
accessible pool of copper to apo-
ySOD1.

To test the hypothesis that CQ can facilitate the uptake of
copper through the formation of a CQ-copper complex, cells
were treated either alone with CQ or with equimolar additions
of CQ and vitamin B12 in the medium. There was a significant
attenuation of cell growth either in the presence of CQ or CQ
and vitamin B12. The cytotoxic effects of CQ could be blocked by
adding an equimolar amount of copper or cobalt. This could not
be achieved with other divalent metal ions tested, such as
Mn(II) or Zn(II) (data not shown). Accordingly, unconjugated
CQ was responsible for the growth inhibitory effect, which
might be reversed by inclusion of the cobalt-vitamin B12
complexes. In the 1970s, it was proposed that CQ leads to
subacute myelo-optic neuropathy and that subacute myelo-
optic neuropathy was caused by a vitamin B12 deficiency due to
a chelation of Co(II) from vitamin B12 by CQ (29).

There was facilitated uptake of copper into yeast cells grown
in medium supplemented with 1 mM CQ plus 1 mM copper. A
35-fold increase of intracellular copper levels was found in
mock control cells (Fig. 5a), treated with CQ-copper compared
with cells treated with copper alone. In the presence of 1 mM
vitamin B12, 1 mM CQ, and 1 mM copper, the elevation of
intracellular copper was further increased to 60-fold (Fig. 5a).
This effect was specific for copper levels, because the copper-CQ
treatment did not change zinc levels (Fig. 5b). In the absence of
CQ, neither combination of the compounds tested had a
strong effect on intracellular copper concentrations (Fig. 5c).
Interestingly, medium supplementation with vitamin B12 in-
duced a significant increase of zinc levels in mock-transfected
cells (Fig. 5d), an effect that was reverted by the expression of
sAPP, indicating that sAPP is involved in the adjustment of the
cellular zinc level. This finding suggests that the ho-
meostatic defect caused by CQ was rather due to a facilitated
uptake of copper than to a redistribution as suggested for the
elevated copper and zinc levels in CQ-treated APP2576 mice
(14).

The role of CQ in copper uptake was further evaluated in
yeast strains expressing sAPP. If sAPP expression could sub-
stantially contribute to the transport of intracellular copper
out of the cell, APP secretion should attenuate the effect of
CQ on the intracellular copper concentration. Indeed, the
intracellular copper in sAPP-expressing cells was limited to a
25-fold increase compared with a 35-fold increase in vector-
transfected cells in the presence of 1 mM CQ-copper (Fig. 5a).
With additional vitamin B12 in the medium, a drastic in-
crease of 50-fold in sAPP-expressing cells and of 60-fold in
vector-transfected cells was observed (Fig. 5a). This attenu-
ated an increase of copper levels in sAPP-expressing cells
confirmed the previously observed efflux activities, which
arose from sAPP expression itself rather than from adventi-
tious reactions.
DISCUSSION

The in vitro results indicate that sAPP or sAPLP2 secretion can promote copper efflux. The inability of the copper-binding mutant sAPP H147A/H149A/H151A to promote copper efflux indicates that the copper export requires the direct binding of copper to the CuBD of sAPP. The elevation of copper from submicromolar concentrations to 1 mM in the medium raised ySOD1 activity in control cells 2-fold, and the total number of copper atoms per cell increased by 20-fold. Whereas ySOD activity was impaired by sAPP and sAPLP2 but not by APLP1 expression or copper depletion, the expression of the sAPP H147A/H149A/H151A mutant increased ySOD activity and the total number of copper atoms per cell. In the presence of 1 mM copper, the intracellular copper level further increased by 65-fold, although the ySOD1 activity was only slightly increased (Figs. 1 and 4). Because the low availability of copper within the cell is a key limitation to ySOD1 activity (31), copper must have been readily accessible to apo-ySOD1 in mutant sAPP H147A/H149A/H151A-secreting cells under normal growth conditions but not in control cells. When the overexpression of full-length APP in three different lines, Tg2576, APP23, and TgCRND8 mice, resulted in significantly reduced brain copper levels before the appearance of pathology (5–7), the deleterious effects were explained by an interference in copper homeostasis and intracellular copper trafficking. The results presented here provide a potential answer to this conundrum and support a direct role for secreted APP in cellular efflux of copper. Based on the structural homology to copper chaperones that also possess surface Cu(I) sites, the APP CuBD may function as a neuronal metallotransporter and/or metallochaperone (26). The structure of this domain and the nature and orientation of His-147 and His-151, which are involved in copper coordination, define a novel copper-binding site, which is surface-exposed (26). The release of the APP ectodomain from the membrane would allow this secreted form to excrete...
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Fig. 5. In vivo analysis of copper uptake in the presence of CQ into vector and sAPP-transfected cells. In the presence of CQ (1 mM) and additional copper in the growth medium (1 mM), the total intracellular copper concentrations are observed to increase. This effect is alleviated through the expression of sAPP, which promotes copper efflux.

The data obtained with sAPP and a C-terminally truncated sAPLP2 indicate that copper binding to the CuBD of APP (25) and to Aβ (32) is an unlinked phenomenon.

It was postulated that subacute myelooptic neuropathy was caused by a vitamin B₁₂ deficiency due to the chelation of Co(II) by CQ (29). Our data support the hypothesis that CQ can cause damage to the nervous system by a direct interaction with vitamin B₁₂ but only in the absence of the supplemented divalent metal ions copper or cobalt. Surprisingly, vitamin B₁₂ specifically increased zinc levels in mock-transfected cells. Early observations indicated that low vitamin B₁₂ levels and zinc deficiency are linked in the cerebrospinal fluid and that vitamin B₁₂ transport to the cerebrospinal fluid was suggested to depend on the presence of zinc (33, 34). A similar mechanism may exist in P. pastoris, thus supporting the suitability of this model to study metal ion transport by APP.

Despite being a chelator, CQ did not induce a loss in metal ion levels systematically but rather inhibited plaque formation and concomitantly increased soluble brain copper and zinc levels (14). CQ facilitated the uptake of copper as CQ-copper complexes, similar to copper-nitritriacetic acid-treated mice (35). In mammals CQ-copper complexes could form in the intestinal tract and cross the BBB to enter the brain and thus explain why soluble copper and zinc levels were increased by 15% upon CQ treatment in mice brain (14). Therefore, CQ-copper complexes could selectively and markedly elevate copper levels in the brain of individuals with AD and counterbalance the changes in copper levels observed in AD, most probably mediated through the APP export function. On the background that aged APP transgenic mice treated with copper lived longer than untreated mice (5), these issues may be important in understanding the integration of copper homeostasis in AD and with other physiological processes such as aging.

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