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The Metal Chelating and Chaperoning Effects of Clioquinol: Insights from Yeast Studies

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Abstract. Clioquinol (CQ), a once popular antibiotic, was used to inhibit the growth of microorganisms. Recently, CQ and its analog PBT2 have shown encouraging effects in the animal and clinical trials for Alzheimer's disease (AD). However, the mechanism by which this class of molecules works remains controversial. In this work, we used the yeast *Saccharomyces cerevisiae* as a model to study how CQ affects molecular and cellular functions and particularly, copper, iron, and zinc homeostasis. We observed a CQ-induced inhibition of yeast growth, which could be slightly relieved by supplementation of copper or iron. Microarray results indicated that yeast cells treated with CQ sense a general deficiency in metals, despite elevated total cellular contents of copper and iron. Consistent with this, reduced activities of some metal-sensitive enzymes were observed. Intriguingly, CQ can increase the SOD1 activity, likely through Ccs1's accessibility to CQ-bound copper ions. Further studies revealed that CQ sequestrates copper and iron at the cellular membrane, likely the plasma membrane, resulting overall metal accumulation but cytosolic metal depletion. CQ's effects on metal-sensitive metalloenzymes were also verified in mammalian cell line SH-SY5Y. Together, our results revealed that CQ can regulate metal homeostasis by binding metal ions, resulting the cell sensing a state of deficiency of bio-available metal ions while simultaneously increasing available metals to SOD1 (via Ccs1) and possibly some other metalloproteins that can access CQ-bound metals. We hope this regulation of metal homeostasis may be helpful in explaining the therapeutic effects of CQ used in disease treatment.

Keywords: Alzheimer's disease, clioquinol, homeostasis metals, yeast

Supplementary data available online: http://www.j-alz.com/issues/21/vol21-4.html#supplementarydata01

INTRODUCTION

Transition metals are indispensable for life because of their ability to donate and accept electrons [1]. Increasing evidences demonstrate that these metals, especially copper [2–4], zinc [5–7], and iron [8,9] are involved in Alzheimer's disease (AD) and other neurodegenerative diseases. However, it is disputable whether transition metals are detrimental [2,6–8,10–13] or beneficial [12,14–20] in AD pathogenesis.

Clioquinol (CQ or iodochlorhydroxyquin, 5-chloro-7-iodo-8-hydroxyquinoline) is a hydrophobic chelator of copper, zinc, and iron *in vitro* [21] and was extensively used as an antibiotic for the treatment of diarrhea and skin infection in the mid-1900s. CQ was clinically withdrawn later due to possible and controversial association with subacute myelo-optic neuropathy (SMON) in Japan [22]. Interest in this drug was recently revived as it was shown that CQ specifically kills cancer cells [21,23–26] and significantly decreases amyloid-β (Aβ) level in a mouse model of AD [27]. Additionally, a pilot Phase II clinical trial revealed that CQ

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can slow down cognitive decline in AD patients [28]. Furthermore, CQ also displayed potential efficacy in the treatment of Parkinson's disease and Huntington's disease [29,30].

The possible beneficial effects of CQ or its analog PBT2 on AD is particularly attractive because there are no known effective treatments for this common neurodegenerative disease [31,32]. However, the underlying mechanisms for CQ's action are still unclear. Some evidences imply that CQ may function as a chelator of transition metals *in vivo* [30,33–35], but mounting reports indicate that CQ's effects on AD and cancers may relate to its transportation of metals into cells [15, 16,21,23,26,36,37].

It is therefore baffling how exactly CQ works as an antibiotic and combats neurodegenerative diseases and cancers. In other words, it is unclear whether the drug effects of CQ, and its analogs, are due to their ionophore or chelator roles. To elucidate this question, we used *S. cerevisiae*, which is a good model for research on metal homeostasis [1], as a platform to investigate the effect of CQ on the homeostasis of cellular transition metals. We found that CQ chelates metal ions and sequestrates them at the cellular membrane system, resulting in a general deficiency of bio-available metal ions for most metalloproteins except potent metal chaperones such as Ccs1. Similar effects of CQ on some metalloenzymes are also observed in mammalian cell line SH-SY5Y. Additionally, expression of amyloid-β protein precursor (AβPP)-reporter in SH-SY5Y can be down-regulated by CQ likely due to metal deficiency.

MATERIALS AND METHODS

Yeast strains

Yeast strains used in this work are listed in Table S1 (available online: http://www.j-alz.com/issues/21/vol21-4.html#supplementarydata01).

Plate assay

Wild-type BY4742 and Δ *ctr1* were grown overnight in liquid YPD [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose], adjusted to identical OD_{600}, and diluted 10-, and 100-fold, respectively, with sterile water. 3 μl of each diluted yeast solution was spotted onto YPD or YPG (substituting dextrose with glycerol) agar plates with or without CQ/metals.

RNA extraction and microarray analysis

Wild-type BY4742 grown in YPD to mid-log phase was 1:1 inoculated into fresh YPD, and 80 μM CQ or its vehicle control DMSO was supplemented. Experiments were performed in triplicates. 5 hours later, cells were harvested, and total RNAs were extracted with hot phenol method [38]. cRNAs were synthesized and used to hybridize Affymetrix GeneChip® Yeast Genome 2.0 Array as described in the manufacturer's protocol. More details were uploaded to Gene Expression Omnibus (GEO) database (accession number=GSE17257).

Inductively coupled plasma mass spectrometry (ICP-MS)

Yeast cells grown overnight in liquid YPD were inoculated in fresh YPD at 1:1 ratio. After chemical treatment for desired durations, cell numbers were estimated by measuring OD_{600} [39]. Cells were harvested and washed as previously described [40]. Metal contents from 15 ml yeast culture were assayed with Thermo ICP-MS XII (Thermo Fisher, USA).

Mitochondria isolation

300 ml culture of wild-type BY4742 in mid-log phase grown in YPD (1% yeast extract, 2% peptone, 0.5% dextrose) was inoculated into 1 liter of YPG supplemented with DMSO or 60 μM CQ. 5 h later, cells were harvested and washed 1X with water, 1X with 0.5 M EDTA, 1X with Milli Q water, 1X with 10% PEG1500, and then 1X with Milli Q water, 1X with buffer 1 (0.1 M Tris-HCl, 0.5 M 2-mercaptoethanol, pH 9.3), 2X with buffer 2 (0.01 M Tris-HC1, 0.5 M KC1, pH 7.0). Cells were subsequently incubated in 100 ml buffer 3 (1% snailase, 0.01 M citrate-phosphate, 1.35 M sorbitol, 1 mM EGTA, pH 5.8) for 60 min. Spheroplasts were pelleted by 900 g centrifugation at 4°C for 10 min, then washed 1X with medium 1 (1 M sorbitol, 0.5 mM EGTA, 2 mM $MgSO_4$, 1.7 mM NaCl, 10 mM potassium phosphate, 0.1% bovine serum albumin or BSA, pH 6.8), 1X with buffer 4 (0.01 M Tris-maleate, 0.75 M sorbitol, 0.4 M mannitol, 2 mM EGTA, 0.1% BSA, pH 6.8), and homogenized in 10 ml isolation buffer [5 mM HEPES, 220 mM mannitol, 70 mM sucrose, 0.1% BSA, 1% protease inhibitor cocktail (Sigma), pH 7.2] with dounce homogenizer. The homogenate was 900g centrifuged twice at 4°C for 10 min, and the supernatant was further 10,000 g centrifuged at 4°C for 10 min.

The resultant supernatant was taken as the cytosolic fraction for the aconitase activity assay. The precipitate was resuspended with 0.2 ml isolation buffer and taken as the mitochondrial fraction for the cytochrome *c* oxidase (C*c*O) activity assay and aconitase activity assay. Protein concentrations were determined by the Bradford method.

Cytochrome coxidase activity assay

Isolated mitochondria samples (1 mg protein per ml) were incubated at 30°C in buffer containing 75 mM sucrose, 225 mM mannitol, 5 mM $MgCl_2$, 5 mM KH_2PO4, 1 mM EGTA, 5 mM Hepes-KOH (pH 7.4), 10 mM ascorbate, 200 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 2 μM antimycin A. Oxygen consumption of isolated mitochondria was measured using a Clark-type oxygen electrode in a total volume of 1 ml [41].

Aconitase activity assay

Aconitase activity was estimated by the spectrophotometric method of Racker [42]. Briefly, isolated mitochondria or cytosol samples (10 μg protein) were added to 0.5 ml reaction buffer (0.05 M phosphate buffer, pH7.4, containing 0.03 M citric acid). The increase of absorbance at 240 nm was monitored for 2 min.

Alkaline phosphatase activity assay

Yeast cultures in mid-log phase were 1:1 inoculated into fresh YPD with 80 μM CQ or 50 μM TPEN [*N, N, N', N'*-tetrakis (2-pyridylmethyl) ethylenediamine]. After 5 h treatment, cells were harvested from 20 ml culture and washed 1X with cold H_2O and 1X with ALP lysis buffer (10 mM Tris-HCl, pH7.4, 0.5 mM $MgCl_2$, 0.1% Triton X-100, 1% protease inhibitor cocktail). After centrifugation, the resultant cells were then lysed in 0.2 ml ALP lysis buffer by glass bead homogenization. The extracts were 13,200 g centrifuged at 4°C for 15 min. Then the supernatants were transferred to new Eppendorf tubes. Protein concentrations were determined by the Bradford method. Enzyme activity was measured in a 96-well plate with *p*-nitrophenyl phosphate (pNPP) as the substrate. 90 μl solution A (1.0 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8), 10 μl solution B (150 mM pNPP), and 1 μg extracted total protein were added into every reaction. After incubation at 30°C for 30 min, reactions were terminated with addition of 50 μl 1.5 M NaOH. Their absorbances at 405 nm were then determined.

Malate dehydrogenase activity assay

Yeast cells treated with DMSO or 80 μM CQ for 5 h were lysed in extraction buffer (20 mM Hepes, pH7.1, 1 mM DTT, 100 mM KCl, 1% protease inhibitor cocktail) by glass bead homogenization. After 3,000 g centrifugation at 4°C for 5 min, the supernatants were transferred to new eppendorf tubes and were 13,200 g centrifuged at 4°C for 15 min. Protein concentrations of the resultant supernatants were determined by Bradford method. To assay the malate dehydrogenase activity, about 15 μg extracted total protein samples were added into wells of a 96-well plate containing 100 μl reaction mixture (50 mM imidazole-HCl, pH7.1, 100 mM KCl, 1 mM EDTA, 5 mM $MgSO_4$, 0.2 mM NADH, 5 mM oxaloacetate). Decreases of OD_{340} in the following 5 min were measured.

SOD1 activity assay

Cells in mid-log phase were 1:1 inoculated separately into 10 ml fresh YPD supplemented with DMSO or 80 μM CQ. After 5 h treatment, cells were harvested and washed once in cold H_2O. The resultant cell pellet was resuspended in 0.5 ml lysis buffer containing 10 mM sodium phosphate (pH 7.8), 1.0 mM EDTA, 0.1% Triton X-100, 1% protease inhibitor cocktail (Sigma), and 1.0 mM phenylmethylsulfonyl fluoride. Cells were then broken in 0.2 ml lysis buffer by glass bead homogenization. The extracts were 13,200 g centrifuged at 4°C for 15 min. The supernatant was transferred to a new eppendorf tube. The protein concentrations were determined by the Bradford method. Samples were run on 10% non-denaturing polyacrylamide gels, and SOD1 activities were determined by the nitro blue tetrazolium in-gel staining assay as described elsewhere [43].

Plasma membrane and cytosol fractionation

Plasma membrane and cytosol were isolated by use of a modified version of the protocol described by Forbes et al. [44]. Yeast cells from 30 ml treated culture were washed twice with ice-cold water and once with homogenization buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl), followed by resuspension in 300 μl homogenization buffer. Cells were broken by vortexing with glass beads for 5 min. Yeast membranes were recovered by the addition of 1 ml homogenization buffer. The supernatants were twice cleared of unbroken cells and heavy organelles by centrifugation at 10,000 g for

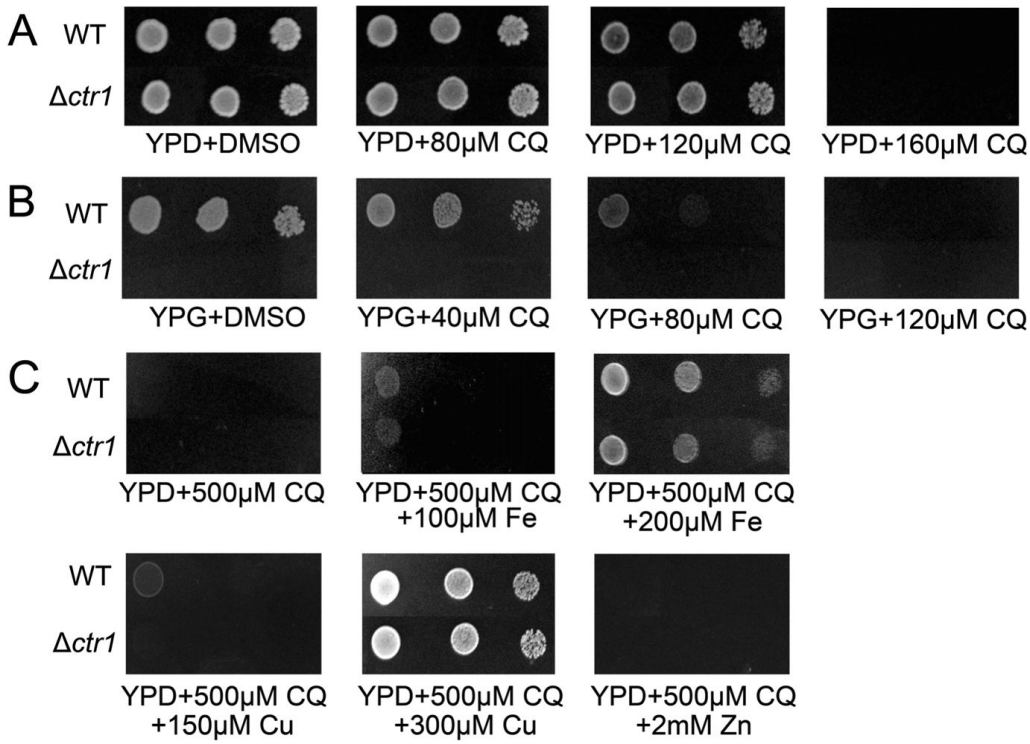

Fig. 1. CQ-induced yeast growth inhibition. A) At 160 μM, CQ can virtually prevent yeast growth on YPD media. B) About 100 μM CQ inhibits yeast on YPG. Lower amount of CQ required for inhibition on YPG suggests mitochondrial activity is more sensitive to CQ's action. Δ *ctr1* yeast lacks copper and is not able to grow on YPG. CQ could not rescue this deficiency. C) Supplemented $FeCl_2$ or $CuCl_2$, but not $ZnCl_2$, ameliorates the growth inhibition by CQ.

30 s. Membranes were collected by centrifugation at 20,000 g for 30 min. The supernatant was taken as cytosol. The final membrane pellet was solubilized in 200 μl homogenization buffer containing 1% Triton X-100. Insoluble material was removed by centrifugation at 20,000 g. Protein was quantified by use of the Bradford method.

Western blotting analysis

Protein samples were loaded and separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, followed by western blotting analysis with the specific antibodies to Ccs1 and SOD1 [45] and enhanced chemiluminescence reagents, as described in the figure legends.

Mammalian cell culture and enzymatic assays

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in humidified 5% CO_2 air. To investigate the effect of CQ, cells were treated with DMEM containing 20 μM CQ or its vehicle control DMSO (0.1%) for 24 h. Then cells were harvested and sonicated in PBS. The lysates were used for C*c*O assay as described elsewhere [46], or aconitase, alkaline phosphotase, and SOD1 activity assays as described above. The input of total protein for SOD1 assay was normalized by western blotting with the monoclonal antibody to α-tubulin (Clone DM1A, Sigma, 1:8000) according to the manufacturer's protocol.

Luciferase reporter assay

The DNA fragment containing *AβPP* promoter and *AβPP* 5'-UTR (–639 $\sim$ +153) was cloned into pGL3-basic plasmid. The AβPP-luciferase and Renilla reporter plasmids were co-transfected into SH-SY5Y cells with Lipofectamine 2000 (Invitrogen). 5 hours after transfection, cells were treated with 20 μM CQ or its vehicle control DMSO for 24 h. Dual luciferase assay (Promega) was employed to estimate the AβPP-luciferase expression.

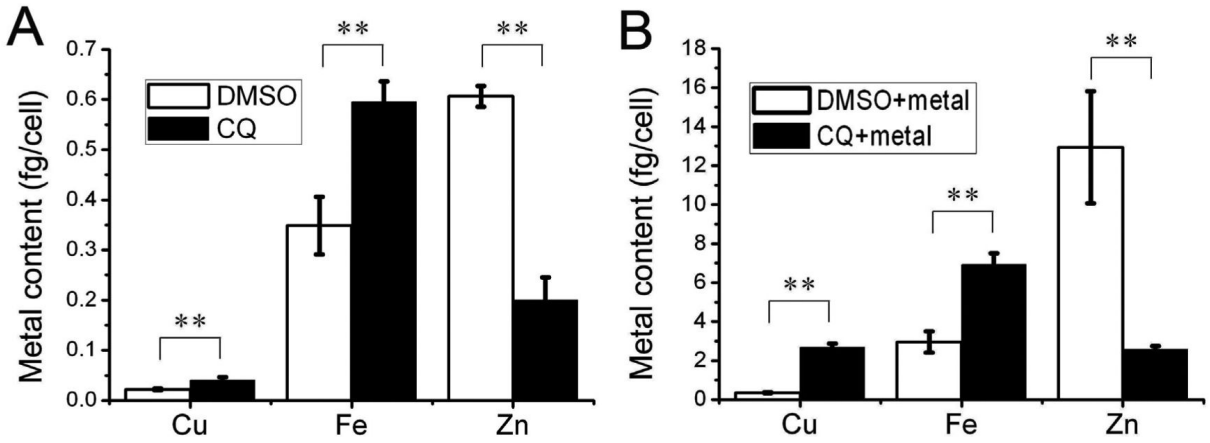

Fig. 2. Altered cellular metal contents by CQ. In the absence (A) or presence (B) of supplemented metal ions ($CuCl_2$, $FeCl_2$, and $ZnCl_2$, 10 μM each), treatment with 80 μM CQ for 5 h significantly increases cellular copper and iron contents, but decreases zinc content, in wild type BY4742 yeast. $^{**}p < 0.01$, unpaired t-test, $n = 3$.

Statistical analysis

Data are shown as means $\pm$ standard deviations (SD). P-values were calculated using the Student's t test. The integrated density values of SOD1 staining were determined by the software Image-J from NCBI.

RESULTS

CQ inhibits yeast growth

CQ is a potent antibiotic [21]. We analyzed the effect of various amounts of CQ on yeast growth. At higher concentrations, CQ can potently inhibit yeast growth (Fig. 1). On YPD plates, about 160 μM CQ was needed to completely inhibit colony formation (Fig. 1A), whereas the inhibition was noticeable at much lower level of CQ (80 μM) on nonfermentable medium (i.e., glycerol, ethanol, or lactate as the carbon source) (Fig. 1B). CQ inhibition could be slightly or marginally rescued by the addition of iron and copper, but not zinc. Higher level of copper and iron can dramatically suppress inhibition of CQ but that may be due to its neutralization action: the excess metal binds with strong affinity to CQ so that not much CQ was left to take effect. The slight but noticeable ameliorating effect of copper or iron on CQ's inhibition, together with the observation that much more potent inhibition by CQ on nonfermentable than fermentable media suggests the possibility that CQ might make yeast deficient at least in copper or iron, because yeast growth on nonfermentable media requires functional mitochondria, which is especially vulnerable to copper or iron deficiency.

CQ causes a general metal deficiency despite increase of total cellular copper and iron

Although CQ is thought to be a chelator with moderate affinities for metals [K_{A1}(Zn) $= 10^{7.0}$, K_{A1} (Cu) $= 10^{8.9}$, K_{A1} (Ca) $= 10^{4.9}$, K_{A1} (Mg) $= 10^{5.0}$] [27], it has also been reported CQ can increase cellular metal contents, possibly due to its lipophilic nature. Indeed, after treating wild-type BY4742 with 80 μM CQ in the absence or presence of additional metal ions ($CuCl_2$, $FeCl_2$, and $ZnCl_2$, 10 μM each) for 5 h, CQ significantly increased cellular copper and iron contents in either the absence of (Fig. 2A) or presence of (Fig. 2B) supplemented metal ions, consistent with reports by others [16,27,40]. Simultaneously and surprisingly, we observed a significant decrease of zinc content by CQ (Fig. 2A, B).

The intriguing metal increase as measured and the deficiency data suggested by growth analysis prompted us to investigate whether the metal accumulated is biologically available. To this end, we tested CQ's effect on Δ *ctr1* yeast. Copper-transporting deficient yeast Δ*ctr1* cells are unable to grow on nonfermentable media YPG because of copper deficiency [47,48]. Since studies in yeast as well as mammalian cells have shown CQ facilitates copper uptake [27,37,40], we postulated that this might rescue respiration in Δ *ctr1* if indeed CQ could increase effective intracellular copper level. However, CQ had no effect on the rescue no matter how much CQ was used (Fig. 1B), implying CQ may not poison the cell with metal excess, instead, may deplete metal availability.

To verify the above analysis, and to investigate the systematic effect of CQ on yeast cells, we then utilized

Table 1
Transporters (or their regulators) of transition metal ions are up-regulated in response to CQ treatment according to microarray results. Gene descriptions are from http://www.yeastgenome.org. The fold changes were calculated by ratio of signals in CQ-treated samples to that in DMSO-treated controls, and presented as the averages of three experiments. All genes listed in this table are significantly changed according to our microarray results. $p < 0.01$, unpaired t-test, $n = 3$ (3 samples versus 3 controls)

Gene	Gene description	Fold change
CTR1	High-affinity copper transporter of the plasma membrane, mediates nearly all copper uptake under low copper conditions; transcriptionally induced at low copper levels and degraded at high copper levels	5.8
CTR2	Putative low-affinity copper transporter of the vacuolar membrane; mutation confers resistance to toxic copper concentrations, while overexpression confers resistance to copper starvation	2.9
FRE1	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels	4.3
FRE2	Ferric reductase and cupric reductase, reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low iron levels but not by low copper levels	2.4
FRE3	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels	2.4
FRE5	Putative ferric reductase with similarity to Fre2; expression induced by low iron levels; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies	7.1
FRE7	Putative ferric reductase with similarity to Fre2; expression induced by low copper levels	14.3
FTR1	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3; expression is regulated by iron	3.7
FET3	Ferro-O_2-oxidoreductase required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity, belongs to class of integral membrane multicopper oxidases	4.4
ENB1	Endosomal ferric enterobactin transporter, expressed under conditions of iron deprivation; member of the major facilitator superfamily; expression is regulated by Aft1 and affected by chloroquine treatment	3.0
ARN1	Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates; responsible for uptake of iron bound to ferrirubin, ferrirhodin, and related siderophores	6.7
VEL1	Protein of unknown function; highly induced in zinc-depleted conditions and has increased expression in*NAP1* deletion mutants	36.0
ZAP1	Zinc-regulated transcription factor, binds to zinc-responsive promoter elements to induce transcription of certain genes in the presence of zinc; regulates its own transcription; contains seven zinc-finger domains	6.9
ZPS1	Putative GPI-anchored protein; transcription is induced under low-zinc conditions, as mediated by the Zap1 transcription factor, and at alkaline pH	22.5
ZRT1	High-affinity zinc transporter of the plasma membrane, responsible for the majority of zinc uptake; transcription is induced under low-zinc conditions by the Zap1 transcription factor	2.2
ZRT3	Vacuolar membrane zinc transporter, transports zinc from storage in the vacuole to the cytoplasm when needed; transcription is induced under conditions of zinc deficiency	2.8
PCA1	Cadmium transporting P-type ATPase; may also have a role in copper and iron homeostasis; stabilized by Cd binding, which prevents ubiquitination; S288C and other lab strains contain a G970R mutation which eliminates Cd transport function	3.2
SMF1	Divalent metal ion transporter with a broad specificity for divalent and trivalent metals; post-translationally regulated by levels of metal ions; member of the Nramp family of metal transport proteins	2.0
SMF3	Putative divalent metal ion transporter involved in iron homeostasis; transcriptionally regulated by metal ions; member of the Nramp family of metal transport proteins	2.0

chip analysis to examine the effect of CQ on the expression of an array of metal genes at the genomic level. Affymetrix GeneChip® Yeast Genome 2.0 Array was hybridized with cRNA samples from CQ-treated yeast. The microarray data are available online at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17257. Of the 2,729 genes with positive signals, 448 (16.42%) were reduced $> 50\%$, and 600 (21.99%) were increased $>$2-fold, after treatment with 80 μM CQ for 5 h. Amazingly, CQ dramatically increased the transcription of a number of genes encoding transport components for transition metals, including copper, iron, zinc, and cadmium, and the key regulators that control them (Table 1). Most of these genes have been reported with enhanced transcription under metal deficiency states. These upregulated genes include: *CTR1* and *CTR2* for copper transportation; *FRE3*, *FTR1*, *FET3*, *ENB1*, and *ARN1* for iron transportation; *FRE1* and *FRE2* for transportation of both copper and iron; *ZRT1*,*ZRT3*, and*ZAP1* for zinc transportation or its reg-

ulation; *PCA1* for cadmium transportation; *SMF1* and *SMF3* for nonspecific transportation of divalent and trivalent metals; and other genes, such as *FRE5*, *FRE7*, *VEL1*, *ZAP1*, and*ZPS1*, which have previously been reported to be upregulated under metal deficient state. Alteration of some representatives of these genes was later verified by reverse transcription PCR (Fig. S1).

In conclusion, the microarray results show that after CQ treatment, cells sense a general deficiency of transition metal ions, such as copper, iron, and zinc, and consequently activate a battery of metal transport genes in attempt to combat this defective state.

CQ decreases the activities of some metal-dependent enzymes

A number of cellular enzymes are sensitive to metal availability. We thus further tested the effect of CQ on the activities of some metal-sensitive enzymes. C*c*O is the mitochondrial respiratory chain complex IV, and its activity in cells is down-regulated by copper deficiency [49–51]. We found that C*c*O activity was reduced to about 50% in CQ-treated yeast (Fig. 3A). *ACO1* encodes an iron-dependent enzyme, mitochondrial aconitase, which can be used to indicate the cellular iron loading state [52]. Results showed that after CQ treatment, mitochondrial aconitase activity was reduced to about 30% of the control (Fig. 3B). Activity of cytosolic aconitase was also decreased to about 25% of its control. Consistent with this finding, it was reported that CQ makes mice and *C. elegans* deficient in iron and results in decreased activity of mitochondrial aconitase [53]. Pho8 is an alkaline phosphatase (ALP) whose activity is zinc dependent [54], and catalyzes the hydrolysis of p-nitrophenyl phosphate (pNPP) *in vitro*. Our results (Fig. 3C) showed that like TPEN, which is a specific zinc chelator, CQ decreased activity of alkaline phosphatase in yeast by 60%. On the contrary and as a control, the activity of malate dehydrogenase [55], which presumably should not be affected by metal ions, was not significantly altered by the CQ treatment (Fig. 3D).

To explore whether the reduction of enzymatic activities was due to reduced protein levels or changes of metal-dependent specific activities, we examined the protein levels of Aco1 and Pho8 with their respective GFP-tagged, gene knock-in strains (Fig. S2). Our analysis with *ACO1-GFP* knock-in strain demonstrated that the Aco1-GFP protein level was not significantly altered by CQ (Fig. S2B), indicating the decreased Aco1 activity was because of less specific activity after CQ

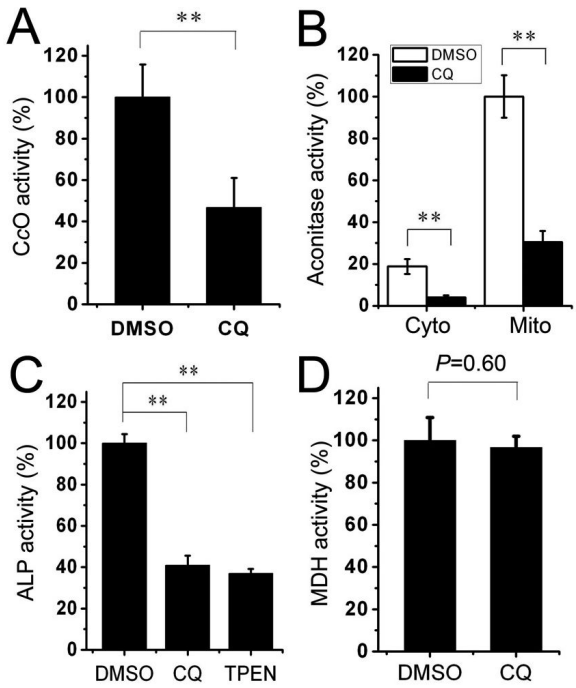

Fig. 3. Decreased activities of some metal-sensitive enzymes after CQ treatment of yeast. A) Treatment with 60 μM CQ for 5 h significantly inhibited cytochrome *c* oxidase (C*c*O), a copper-dependent enzyme, in wild-type BY4742 yeast. B) Treatment with 60 μM CQ for 5 h dramatically depressed activities of both cytosolic (cyto) and mitochondrial (mito) aconitases, which are dependent on iron ions, in wild type BY4742. C) Treatment with 80 μM CQ for 5 h exhibited significant inhibition of alkaline phosphatase (ALP) activity, which is zinc dependent, similar to the inhibition shown by TPEN (a zinc specific chelator), in wild type BY4742. D) Activity of malate dehydrogenase (MDH), which is not metal dependent, is not affected by 80 μM CQ treatment for 5 h. $*p < 0.05$ and $**p < 0.01$, unpaired t-test, $n = 3$.

treatment. However, the Pho8-GFP protein level was decreased by CQ (Fig. S2A). Because in our microarray results, *PHO8* was not affected by CQ at the transcription level, and it was reported that both Pho8 activity and protein level are zinc-dependent [54], decreased ALP activity thus could at least partially arise from the reduced protein level. In summary, in the presence of CQ, reduced metal-dependent enzymatic activities may be explained by the reduction of specific enzymatic activity or lowered enzyme stability or a combination of both, as a result of metal insufficiency.

CQ increases bio-available copper ion for Ccs1

Superoxide dismutase 1 (SOD1) is another copper-dependent enzyme in diverse organisms [43]. Ccs1, the copper chaperone for SOD1, introduces a disulfide bond and a copper ion into SOD1 to activate it [56].

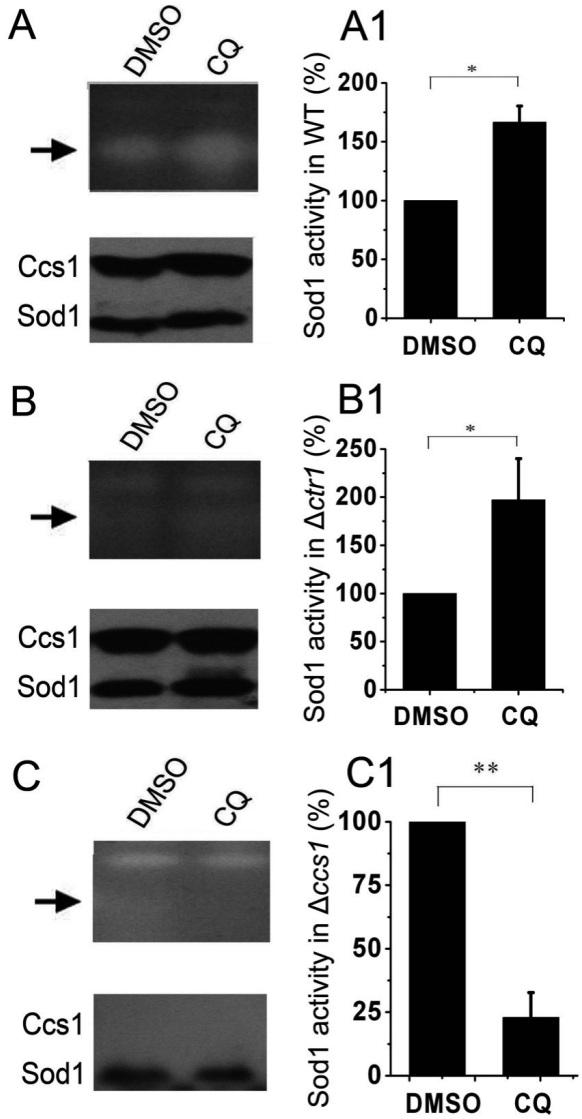

Fig. 4. CQ-induced elevation of SOD1 activity via Ccs1. A, B, and C are representative stains of SOD1 assays (upper panels: the arrow indicates the SOD1 activity bands) and western blots of Ccs1 and SOD1, probed with their respective antibodies (lower panels). In wild type BY4742 (A; 10 μg total protein/lane) or Δ *ctr1* (B; 30 μg total protein/lane), 80 μM CQ significantly elevates SOD1 activity; on the contrary, in Δ*ccs1* (C; 50 μg total protein/lane), 80 μM CQ significantly decreases SOD1 activity. A1, B1, and C1 are average results of SOD1 activity normalized with the total protein in WT, Δ *ctr1*, and Δ *ccs1*, respectively. $*p < 0.05$, paired t-test, $n = 3$ independent replicates.

In the absence of *Ccs1*, only a small part of SOD1 molecules can acquire copper ions in either yeast or mammals [57–59]. SOD1 assay in our study showed that CQ significantly elevated SOD1 activities in wild type (Fig. 4A, A1) and Δ *ctr1* (Fig. 4B, B1) yeast cells. Meanwhile, CQ did not significantly affect protein levels of SOD1 and its copper chaperon Ccs1 according to the western blotting analysis. This suggests that CQ can increase bio-available copper to SOD1 to increase its specific activity. To test whether this copper delivery occurs directly between SOD1 and CQ or mediated by Ccs1, we tested the effect of CQ on SOD1 in Δ *ccs1* yeast. In Δ *ccs1*, CQ was no longer able to increase SOD1 activity and on the contrary significantly inhibited it (Fig. 4C, C1), indicating CQ-mediated copper delivery to SOD1 is dependent on Ccs1. In essence, CQ decreases the availability of cellular copper ions to some metalloproteins, but increases bio-available copper for Ccs1.

CQ-induced metal alteration is not mediated by normal cellular transport apparatus and is CQ dose dependent

Cells have an array of metal transporters responsible for moving metals into the cells. To examine whether CQ-induced alteration of metal content is mediated by metal uptake genes, we tested the effect of CQ on copper homeostasis in strains lacking essential genes encoding or regulating components of copper transport system. We tested Δ *ctr1*, Δ *mac1*, and Δ *fre1* Δ *fre2* yeast, which should all display copper uptake defect. In these cells, CQ treatment could still elevate total cellular copper content (Fig. 5A). Intriguingly, CQ induced an even more dramatic increase of copper content in these mutants than in their wild-type counterpart. The absolute copper levels, and magnitudes of change, were both higher in yeast mutants for copper transporters or regulators than those in the wild-type control. This shows that the elevated copper content in yeast treated with CQ is not mediated through normal copper transport apparatus, such as the high affinity copper transporter Ctr1. We also tested the effect of CQ on zinc in zinc transporter mutant strains, ZHY3 (Δ *zrt1* Δ *zrt2*) [60,61]. CQ reduced overall zinc level in wild type cells, and could further reduce zinc level in ZHY3 yeast (Fig. 5B), confirming that CQ-facilitated zinc alteration does not rely on normal zinc transporters either.

We then explored how CQ dosage may affect cellular copper accumulation. We treated yeast with different concentrations of CQ in the presence of 200 μM $CuCl_2$. The result indicates that the total cellular copper contents heavily depend on the dosages of CQ (Fig. 5C). In fact, the correlation is almost perfectly linear within the examined dosage range.

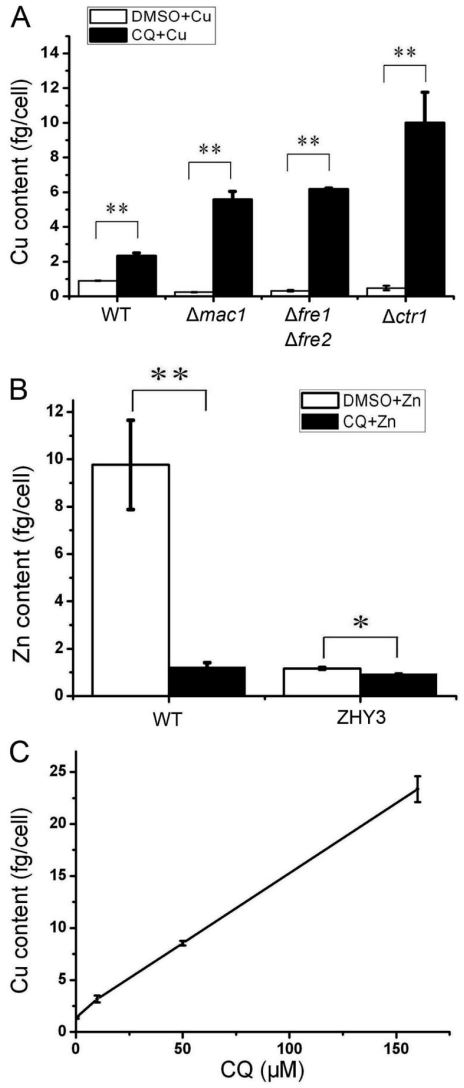

Fig. 5. Transporter apparatus independence and CQ dose dependence of CQ-induced metal alteration. A) In the presence of supplemented 10 μM $CuCl_2$, treatment with 80 μM CQ for 5 h increases copper content in wild type BY4742, Δ*ctr1*, Δ *mac1* and Δ *fre1* Δ *fre2*. B) In the presence of supplemented 10 μM $ZnCl_2$, treatment with 80 μM CQ for 5 h decreases zinc content dramatically in wild type DY1457 and mildly in ZHY3 (double deletion of zinc transporters *ZRT1* and *ZRT2*, DY1457 background). C) In the presence of supplemented 200 μM $CuCl_2$, treatment with 0, 10, 50, or 160 μM CQ for 5 h increases copper contents in BY4742 in correlation with the CQ doses. $*p < 0.05$ and $**p < 0.01$, unpaired t-test, $n = 3$.

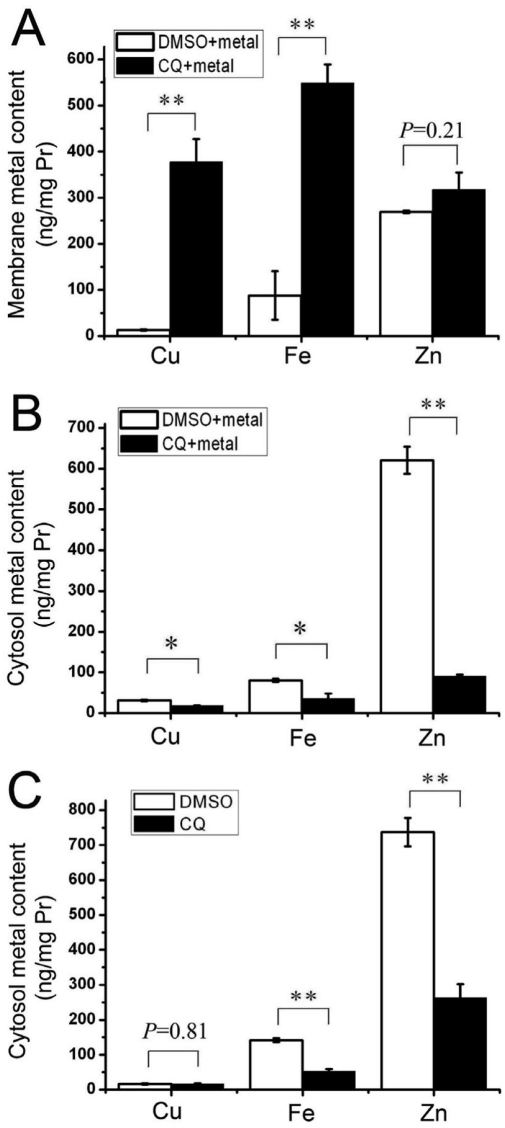

Fig. 6. Metal sequestration at the membrane by CQ. In the presence of supplemented metal ions ($CuCl_2$, $FeCl_2$, and $ZnCl_2$, 10 μM each), treatment with 80 μM CQ for 5 h significantly increases copper and iron contents, and slightly increases zinc content, in the yeast plasma membrane fraction (A), while decreases these three metals in the cytosol fraction (B). Treatment of yeast cells cultured in metal-rich ($CuCl_2$, $FeCl_2$, and $ZnCl_2$, 10 μM each) YPD media with 80 μM CQ for 30 min can significantly decrease iron and zinc, but not copper ions in cytosol (C). Metal contents were normalized with the total proteins in the samples. $*p < 0.05$ and $**p < 0.01$, unpaired t-test, $n = 3$.

CQ sequestrates copper and iron likely at the plasma membrane

Since CQ is a lipophlic chemical, we wondered whether the increased metals by CQ were located at the plasma membrane or in the cytosol. We fractioned plasma membrane and cytosol parts and subjected them to ICP-MS analysis. The ICP-MS result showed that CQ-induced metal accumulation most occurs in the isolated membrane fraction. CQ-treated yeast had dramatically higher copper and iron levels but slightly higher zinc level in the plasma membrane fraction (Fig. 6A), and

had lower levels of these three metals in the cytosol (Fig. 6B), than the vehicle control. Due to the concern of the purity of our membrane fraction, we repeated the experiment with yeast strains containing tagged proteins residing at different intracellular locations, to facilitate the monitoring of possible other membrane or cytosol contamination. We obtained similar results as above; however, the plasma membrane we isolated could be associated with a residual level of vacuolar membrane components but not the cytosol or mitochondrial marker, as revealed by the weak signal of the vacuolar protein marker after long exposure (Fig. S3). This revealed that the plasma membrane we obtained was essentially free of cytosol, but could contain a very low level of some other membrane fractions detectable under scrutinization. Taken together, our results suggest that CQ-induced metal accumulation most occurs in the membrane fraction, likely in the plasma membrane in particular.

Reduction of metals in the cytosol could arise from depletion of extracellular available metals during growth/metabolism or chelation of intracellular metals followed by their sequestration or exportation. To examine if CQ can deprive preexistent metal ions from cytosol, we cultured yeast in metal-rich media ($CuCl_2$, $FeCl_2$, and $ZnCl_2$, 10 μM each) overnight, then harvested and washed the cells. The resultant yeast cells were incubated in distilled water containing 80 μM CQ or 0.1% DMSO for 30 min. In this duration, OD_{600} monitoring showed that the cell number did not significantly increase. ICP-MS assay indicated that the cytosolic fractions contained much decreased levels of iron and zinc, while that of copper remained relatively constant (Fig. 6C). This suggests that CQ can directly deprive iron and zinc from cytosol. For copper ions, however, CQ cannot deprive them likely due to their monovalent state in the cytosol.

CQ displays similar effects on the activities of metal-dependent enzymes in mammalian cells and down-regulates expression of AβPP-luciferase

To check if CQ can similarly alter the bio-availability of transition metals in mammalian cells, we treated SH-SY5Y with 20 μM CQ, a concentration conferred no significant toxicity to SH-SY5Y cells (Fig. S4), and assayed the activities of metal-dependent enzymes mentioned above. Consistently, CQ significantly decreased activities of CcO (Fig. 7A), aconitase (Fig. 7B), and alkaline phosphatase in SH-SY5Y cells (Fig. 7C), whereas elevated SOD1 activity (Fig. 7D, D1).

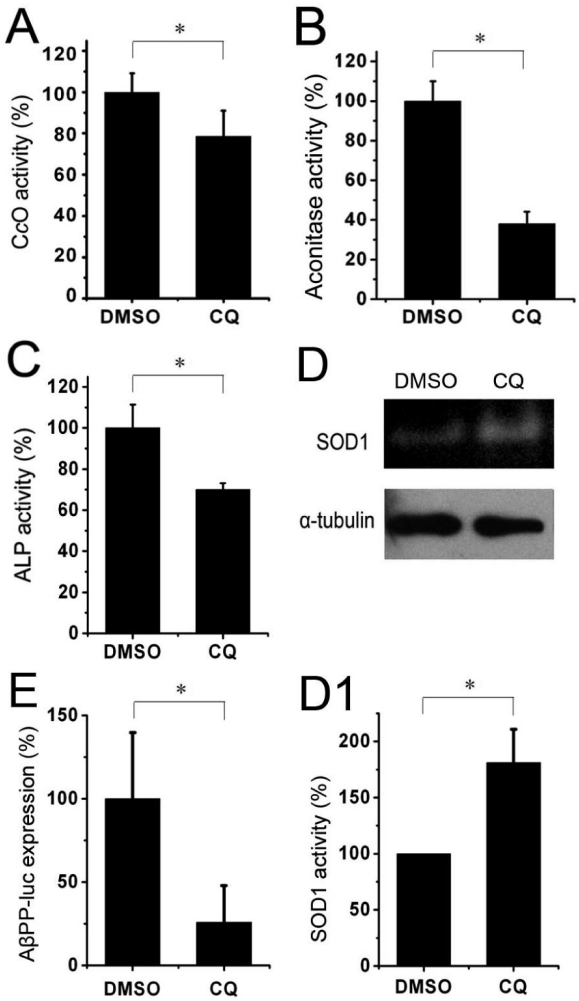

Fig. 7. Altered activities of metal dependent enzymes and expression of AβPP-luciferase reporter in human neuroblastoma SH-SY5Y cells. Cytochrome c oxidase (CcO, A), aconitase (B), and alkaline phosphotase (ALP, C) activities are decreased by treatment of cells with 20 μM CQ for 24 h (unpaired Student's t-test, $n = 3$). D) Representative gel staining of SOD1 in CQ- or DMSO-treated SH-SY5Y cell; SOD1 activity is enhanced by CQ treatment; the inputs of total protein are normalized by western blot for α-tubulin. D1 represents the result of quantified relative SOD1 activities from D (the ratio of SOD1 signal to α-tubulin signal is set as 100 percent; paired t-test, $n = 3$ independent replicates). E) CQ treatment can decrease the expression of transfected AβPP-luciferase reporter (AβPP-luc, unpaired t-test, $n = 3$) $^*p < 0.05$.

Aβ is toxic to the cell and proposed to be the main initiating cause of neurodegeneration in AD. It's reported that CQ can significantly decrease serum level of Aβ in AD patients [28]. There are also evidences suggesting that expression of AβPP is regulated by copper and iron [4,62,63]. Because CQ induces alteration of metal homeostasis in cells, we tested whether CQ could

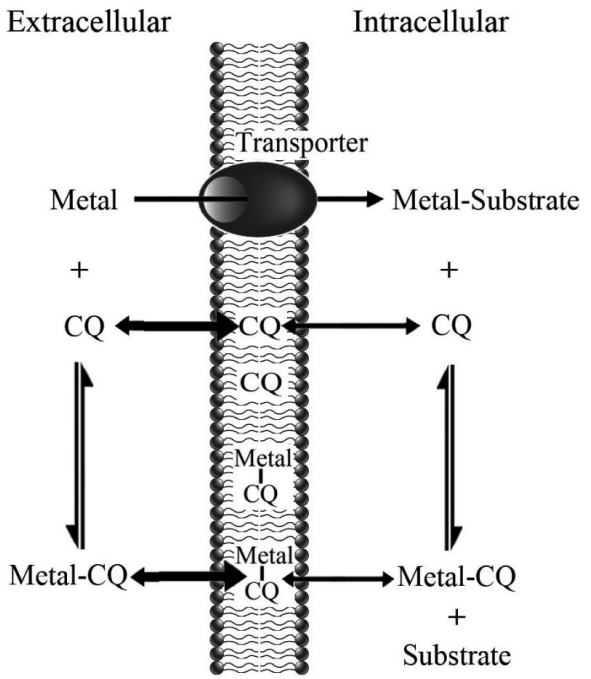

Fig. 8. Schematic of a proposed mechanism showing how CQ regulates metal homeostasis. Free metal ions are taken up by membrane transport systems. CQ binds extracellular divalent metal ions [Cu(II), Fe(II) and Zn(II)], and likely remove a part of cytosolic Fe(II) and Zn(II), which normally bind to a set of intracellular metal-binding "substrates". A large portion of metal-CQ complexes and free CQ molecules are hypothesized to be concentrated at the membrane (likely the plasma membrane in particular) due to their lipophilicity. See text for more.

down-regulate AβPP expression. AβPP-luciferase and Renilla reporter plasmids were co-transfected into SH-SY5Y cells. Relative expression of AβPP-luciferase was shown to be significantly down-regulated by 20 μM CQ (Fig. 7E). We speculate that this decrease of AβPP may also contribute to the therapeutic effect of CQ on AD.

DISCUSSION

CQ was used in the 1950s–1970s as an oral anti-parasitic agent for the treatment and prevention of intestinal amebiasis, but withdrawn from the market due to reports of neurotoxicity in Japanese patients. The mechanism of its action as an antimicrobial and its possible beneficial effect on neurodegenerative disease has been a mystery. The focus of the disputation, among other things, is on whether CQ functions as a chelator or an ionophore in cells.

Here we reported that CQ has a dual role in altering the distribution of metals in cells: it chelates metals and sequestrates them at the cellular membrane, and consequently leads to a general deficiency of transition metal ions for most metalloproteins while enhancing the bio-availability of copper to Ccs1. Although yeast cells are not neurons, the CQ-metal interaction knowledge thus gained may help to elucidate the mechanisms of CQ's effect on AD pathology. On one hand, the exacerbated extracellular accumulation of metals such as in glutamatergic synapse, likely relating to the age-related fatigue of metal uptake and restoration [64–68], can be remediated by the CQ chelating activity, reducing the external metal-dependent Aβ toxicity [69]; the chelating effect may also down-regulate expression of AβPP or relieve Aβ and tau intracellular aggregation pathology via decreasing free intracellular metal ions [2,6–8, 13,70–72]. On the other hand, the chaperoning effect of CQ may benefit the neurons through antagonizing the intracellular deficiency of copper and zinc in patients; for example, CQ can protect them from the ROS damaging effects via elevating bio-available copper to Ccs1. The accessibility of CQ-bound copper to Ccs1 is likely due to its higher affinity to copper [57]. Besides SOD1 and Ccs1, it is not clear at this stage what else metalloproteins can also access to the CQ-bound metals. The selective targeting or chaperoning of metals to some proteins may additionally explain the synergistic toxicity of CQ and copper in CQ antitumor studies, as reported previously [21,23,26,36] and also confirmed in our hands (Fig. S4). It is therefore possible that some of the previous seemingly conflicting reports may arise from the different showing of the two faces of CQ actions. Both aspects may contribute to CQ's therapeutic effects.

The fact that no obvious alteration of hematological readout or metal-dependent enzymes was reported in previous studies in the mice and clinical trial [27,28] might be because that the applied CQ dosages cannot lead to severe changes which can be readily observed above the background fluctuation in the animal/human population. These changes supposedly can be exacerbated with higher drug dosages and in addition may be more conveniently detected, in cell culture studies.

Although CQ can bind zinc and decrease cytosolic zinc level, zinc ions do not seem to significantly accumulate at the cellular membrane (Fig. 6A). This intriguing observation explains why CQ did not induce overall cellular zinc accumulation as mentioned above. It is likely that CQ-zinc complex does not stick tightly to plasma membrane. Indeed, we observed that without wash yeast cells did associate with increased amount of cellular zinc as compared to the control, and in wa-

ter phase, the solubility of CQ-Zn is relatively higher than that of CQ-Cu (data not shown). It is possible that several washes in our experiments could easily disassociate the accumulated cellular zinc, resulting in the observed reduction of total zinc (Fig. 2A, B).

We propose a model to explain how CQ regulates homeostasis of metal ions, as shown in Fig. 8. CQ is a natural metal chelator and can bind extracellular and intracellular divalent metal ions. A large fraction of CQ-induced copper and iron accumulation arises from metal sequestration in the membrane, likely in the plasma membrane in particular. This fraction of metals is not generally available to most intracellular enzymes (exceptions include SOD1 via Ccs1, and possibly some unknown others), resulting cellular metal deficiency. In essence, CQ makes cell deficient in metals through two pathways: on the one hand, CQ chelates Cu(II), Fe(II), and Zn(II) ions outside the cell and reduces the free metal level available to transmembrane transporters; on the other, CQ can deprive Fe(II) and Zn(II) ions from cytosol.

It is clear that CQ facilitated metal transport is not aided by normal transport apparatus. Nevertheless, it is not fully clear why CQ-facilitated copper accumulation is on the contrary exacerbated in copper transporter mutants. We cannot offer a satisfying explanation to this. Certainly more CQ-Cu is trapped in these mutant cells. One possibility is that the nature of the plasma membrane or the state of the cytosol may be altered in these mutant cells so that more CQ-Cu is associated with them

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