Journal of Cellular Biochemistry 98:912–919 (2006)

Role of Soluble Ceruloplasmin in Iron Uptake by Midbrain and Hippocampus Neurons

Ya Ke,[1,2]* Kwokping Ho,[1,2] Jinrong Du,[1,2] Li Zhu,[1,3] Youjia Xu,[1,2] Qin Wang,[1] Chen Yuen Wang,[1,2] Lianzhi Li,[1,2] Xiaohu Ge,[1] Yangzhong Chang,[1] and Zhong Ming Qian[1,2,3]*

[1]Laboratory of Brain Iron Metabolism, Department of Applied Biology & Chemical Technology, Hong Kong Polytechnic University, Hong Kong
[2]National key laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen 518060, P R China
[3]Institute for Nautical Medicine and Key Laboratory of Neuroregeneration, Nantong University, Nantong, Jiangsu 226007, P R China

Abstract Ceruloplasmin (CP) is essential for brain iron homeostasis. However, its precise function in brain iron transport has not been definitely determined. In this study, we investigated the effects of soluble CP on iron influx and efflux in primary neuronal culture from the midbrain (the substantia nigra and striatum) and the hippocampus. Our data showed that low concentrations of CP (2, 4, 8 μg/ml) can promote iron influx into iron-deficient neurons, but not the neurons with normal iron status. The same concentrations of CP had no effect on iron efflux from iron-sufficient and normal-iron neurons. Contrary to our expectation, we did not find any regional difference in the effects of CP on iron influx as well as efflux in neurons. The changes in quenching (iron influx) and also dequenching (iron efflux) of intracellular fluorescence, induced by the addition of CP with iron, in the midbrain neurons were no different from those in the hippocampus neurons. The data showed that soluble CP has a role in iron uptake by iron-deficient brain neurons under our experimental conditions. The physiological significance of the results forms the focus for future work. J. Cell. Biochem. 98: 912–919, 2006.

Key words: cerulopalsmin; iron-deficient and -sufficient neurons; iron influx and efflux; midbrain; hippocampus; brain iron homeostasis

Ceruloplasmin (CP) is an abundant serum α-2 glycoprotein. In the brain, the greatest expression of CP is found in astrocytes surrounding the brain microvasculature [Klomp and Gitlin, 1996; Klomp et al., 1996]. The glycosylphosphatidylinositol-anchored CP (GPI-CP) is the predominant form of this protein in the brain [Patel and David, 1997; Salzer et al., 1998; Patel et al., 2000]. Humans with mutations of the CP gene (aceruloplasminemia) can lead to brain iron accumulation and neurodegeneration [Harris et al., 1995; Yoshida et al., 1995, 2000; Kaplan and O'Halloran, 1996; Takahashi et al., 1996; Gitlin, 1998]. CP-knockout mice ($CP^{-/-}$) also show excessive iron in the brain with age [Patel et al., 2002; Yamamoto et al., 2002]. Dysregulation of CP expression might also be involved in a variety of neurodegenerative diseases where abnormalities in iron metabolism have been demonstrated [Qian and Wang, 1998; Gitlin, 1999; Nelson, 1999; Sayre et al., 1999; Qian and Shen, 2001; Qian et al., 2002; Ke and Qian, 2003].

Accumulated evidence showed that CP is essential for iron homeostasis in the brain.

Abbreviations used: apo-Tf, apo-transferrin; BP, bathophenanthroline disulfonate; CP, ceruloplasmin; $CP^{-/-}$, CP-knockout mice; CSF, cerebrospinal fluid; DFO, desferrioxamine; DMT1, divalent metal transporter 1; FAC, ferric ammonium citrate; FAS, ferrous ammonium sulfate; GPI-CP, glycosylphosphatidylinositol-anchored CP; Tf, transferring; TfR, transferrin receptor.

Grant sponsor: The Hong Kong Research Grants Council; Grant number: PolyU5270/01M/B-Q445; Grant sponsor: The Hong Kong Polytechnic University; Grant numbers: G-YX14, G-YD78, A-PD92, G-T616, G-T856; Grant sponsor: National Key Laboratory of Chinese Medicine and Molecular Pharmacology in Shenzhen, P R China.

*Correspondence to: Ya Ke, MD, PhD and Zhong Ming Qian, MD, PhD, Department of Applied Biology & Chemistry Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong.
E-mail: bcyake@polyu.edu.hk; bczmqian@polyu.edu.hk

Received 1 October 2005; Accepted 1 November 2005
DOI 10.1002/jcb.20740

However, the precise function of CP in brain iron transport via its ferroxidase activity has not been definitely determined. Relevant studies have generated controversial results [Yamamoto et al., 2002]. Classical studies suggested that CP has a role in promoting iron efflux from storage cells to plasma [Ragan et al., 1969; Roeser et al., 1970]. Some in vitro experiments [Young et al., 1997; Richardson, 1999] and studies on human aceruloplasminemia and $CP^{-/-}$ mice strongly suggested an iron-releasing function of CP [Gitlin, 1998; Harris et al., 1999]. On the other hand, other in vitro studies [Mukhopadhyay et al., 1998; Attieh et al., 1999, Qian et al., 2001; Qian and Ke, 2001; Xie et al., 2002] found that CP has a role in iron uptake rather than iron release. In this study, we investigated the effects of CP on iron influx and efflux in midbrain (substantia nigra and striatum) and hippocampal neurons. These two brain regions were chosen because they are very active areas of brain iron metabolism. The low concentrations of CP (2, 4, 8 μg/ml) were used because the level of CP in the cerebrospinal fluid (CSF) is very low, about 1–2 μg/ml [Loeffler et al., 1994]. The results revealed that soluble CP at low concentrations has a role in promoting iron uptake by iron-deficient neurons, whether they are from the midbrain or hippocampus, under our experimental conditions. The same concentrations of CP had no effect on iron uptake by neurons with normal iron status, and on iron release from iron-sufficient or normal-iron neurons of these two brain regions. No regional difference was found in the effects of CP on iron transport in the midbrain and hippocampus neurons.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals, including bathophenanthroline disulfonate (BP), desferrioxamine (DFO), ferric ammonium citrate (FAC), ferrous ammonium sulfate (FAS), trypsin, cytarabine and DNase were obtained from Sigma Chemical Co., St. Louis, MO. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Invitrogen, Carlsbad, CA. Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes, The Netherlands. Sprague–Dawley (SD) rats were supplied by the Centralized Animal Facilities of the Hong Kong Polytechnic University. Heat inactivated fetal calf serum and Ham's F12 medium were bought from Gibco BRL. DMEM was obtained from Grand Island, NY. All other solutions were freshly prepared with double distilled water on the day of use. The solutions, buffers and media used in cell culture were disterilized by passing through a 0.22 μm Millipore and autoclaved at 121°C at 15 PSI for 20 min.

Primary Neuron Cultures

The primary neuronal culture was prepared using a procedure described previously [Ho et al., 2003]. In brief, the midbrain (substantia nigra and striatum) and the hippocampus were dissected out from the brain of one- to three-day-old SD rats, minced with sterile surgical blades, incubated in 0.125% trypsin and dissociated by trituration in DNase/trypsin inhibitor solution. Dissociated cells were diluted to 4×10^5–5×10^5/ml and transferred to 50 mm culture dishes in DMEM/Ham's F12 medium (1:1, v/v, pH 7.2) containing 7% heart inactivated fetal bovine serum, 7% fetal horse serum and penicillin (100 U). Non-neuronal cell division was inhibited by exposure to cytarabine for 24 h. The cultures were maintained at 37°C in a humidified environment with 5% CO_2 in a CO_2 incubator (TC2323). After 10 days in culture (10 days in vitro, DIV), observation through a phase-contrast microscope demonstrated that the cells were predominantly neuronal cells (>98%). The cells at this stage were used in the experiment. The neuron-specific enolase (NSE) was used a marker to identify the cell type (neuron).

Preparation of Rat CP and Determination of Ferroxidase Activity

The rat CP was isolated from rat serum by a method described previously [Qian et al., 2001]. In brief, freshly isolated rat serum was diluted threefold with buffer A (25 mM phosphate buffer [PB], pH 6.8, containing 20 mM E-aminocaproic acid [ACA]). This was applied to a DEAE-sepharose column equilibrated with buffer B (100 mM PB, pH 6.8, containing 20 mM ACA), at 1 ml/min flow rate. The column was washed with about 150–200 ml buffer B (100 mM PB, pH 6.8, containing 20 mM ACA) until the A280 of the effluent decreased to 0, and then with 100 ml of 30% v/v (buffer C) and 45 % v/v (buffer E) 200 mM/100 mM PB (both pH 6.8 and with 20 mM ACA). CP was eluted with

70% v/v 200 mM/100 mM PB, pH 6.8 with 20 mM ACA (buffer E2). The blue fractions (eluent) were collected and concentrated to 2–4 ml. The concentrated CP was loaded onto a Superdex 200 column and eluted with buffer F (25 mM Na-Acetate, pH 5.6 with 100 mM NaCl and 20 mM ACA) at 0.5 ml/min flow rate. The fractions (about 4 ml) were collected and A610 and A280 of each fraction were measured. All procedures were conducted at 4°C. The ferroxidase activity of CP was tested by an in-gel ferroxidase assay and the structural integrity of the protein was determined using SDS–PAGE and Coomassie blue staining.

Cell Iron Loading and Preparation of Iron-Deficient Cells

Iron-sufficient and iron-deficient neurons were prepared as described previously [Qian et al., 2001]. To prepare iron-sufficient neurons, the cells were pre-loaded with 50 μM FAC + 50 μM FAS for 16 h. Iron-deficient neurons were prepared by incubating the cells with 1 mM BP and 1 mM DFO for 16 h in serum-free DMEM medium at 37°C. Before this preparation, the effects of the iron chelators and iron (50 μM FAC + 50 μM FAS) on the growth of neurons were determined by MTT assay. It was found that incubation of the cells with 1 mM BP, 1 mM DFO or iron (50 μM FAC + 50 μM FAS) for 16 h did not significantly inhibit cell growth. These incubation conditions were therefore used.

Cells Calcein Loading and Iron Transport Assay

The neurons were loaded with calcein-AM according to a procedure we used previously [Ci et al., 2003]. Briefly, the neurons were incubated with 0.125 μM calcein-AM for 5 min at 37°C in HEPES-buffered, bicarbonate-free α-DMEM medium containing 20 mM HEPES, pH 7.3 (α-DMEM–HEPES). Excess calcein-AM on the cell surface was removed by three washes with Hank's balanced salt solution (HBSS, pH 7.4). Just prior to measurements, 100 μl of calcein-loaded neuron suspension (approximately 6×10^5 cells) and 100 μl HEPES were added to a 96-well plate. After initial baseline of fluorescence intensity was collected, 2 μM FAS or 30 μg/ml apo-transferrin (apo-Tf) with different concentrations of CP (0, 2, 4, 8 μg/ml) were added to the plates to determine the effects of soluble CP on iron uptake (CP + 2 μM FAS) or release (CP + 30 μg/ml apo-Tf) in the neurons. The fluorescence was measured on the Fluostar Galaxy fluorescence plate reader (BMG, Durham, NC) (λ_{ex} of 485 nm, λ_{em} of 520 nm, 37°C) equipped with excitation and emission probes directed to the bottom of the plate. The changes of calcein fluorescence were recorded every 5 min for 30 min. Data were normalized to the baseline values of fluorescence. To avoid the potential effects of intracellular transferrin (Tf) on experimental results, the C6 rat glioma cells were suspended in DMEM–HEPES medium and maintained at 37°C for a minimum of 4 h to deplete intracellular store of Tf before calcein loading of the cells and other relevant cell treatments in some experiments.

Statistical Analysis

The results were expressed as mean ± SEM of three independent experiments. Difference between mean was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $P < 0.05$ was taken to be statistically significant.

RESULTS

Effect of CP on Iron Efflux From Neurons

First, we investigated the possible role of CP in iron efflux from neurons with normal iron status. After the initial baseline of fluorescence intensity was collected, different concentrations of CP (0, 2, 4, 8 μg/ml) with apo-Tf (30 μg/ml) were added by pipetting 2 μl of CP and apo-Tf solution to a suspension of the calcein-loaded neurons with normal iron status in the plate and gently mixed. The dequenching of the cytoplasmic calcein by iron release was measured for 30 min. Although a small increase in fluorescence after the addition of CP with apo-Tf, as compared to the initial values (before adding the CP and apo-Tf), was observed in most of the time-points, there was no significant difference in the changes of the dequenching signal among the cells treated with or without CP (0, 2, 4 or 8 μg/ml) and 30 μg/ml of apo-Tf (Fig. 1A,B). Also, no difference in the changes of the dequenching signal was found between neurons of the midbrain (Fig. 1A) and hippocampus (Fig. 1B).

Second, we investigated the effect of CP on iron release from the iron-sufficient cells. The iron-sufficient neurons were preloaded with 50 μM FAC + 50 μM FAS for 16 h at 37°C and then loaded with 0.125 μM calcein-AM for

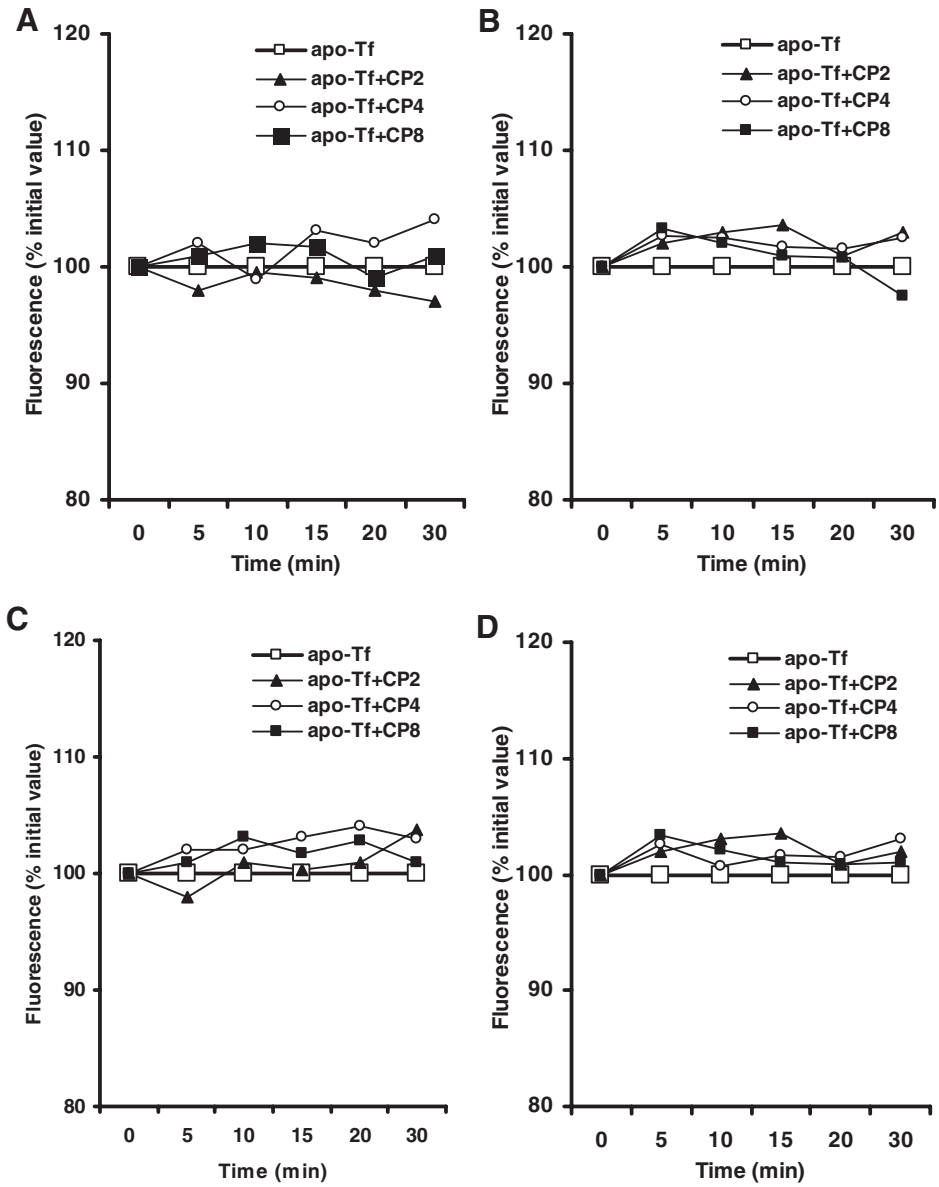

Fig. 1. Effects of ceruloplasmin on iron efflux from cultured neurons. Neurons with normal iron status (**A**: midbrain and **B**: hippocampus) and iron-sufficient neurons (**C**: midbrain and **D**: hippocampus) were pre-loaded with 0.125 μM calcein-AM as described in Materials and Methods. After the initial baseline of fluorescence (the steady-state value) was collected, CP (0, 2, 4, 8 μg/ml) with apo-transferrin (apo-Tf) 30 μg/ml was then added. The changes in fluorescence induced by iron release were measured using a BMG Fluostar Galaxy fluorescence plate reader (λ_{ex} of 485 nm, λ_{em} of 520 nm, 37°C) for 30 min. The data were mean ± SEM (% initial value) of three independent experiments.

10 min. The addition of CP (2, 4, 8 μg/ml) with apo-Tf resulted in a slight increase in the dequenching fluorescence in iron-sufficient cells at almost all the time-points (Fig. 1C,D), however, no significant difference in cellular fluorescence intensity was observed among the cells treated with or without CP (0, 2, 4 or 8 μg/ml) and apo-Tf. Also, fluorescence intensity in the midbrain neurons did not differ significantly from that in the hippocampus neurons. These findings showed that soluble CP at lower concentrations (2, 4 or 8 μg/ml) had no effect on iron release from normal iron status cells and iron-sufficient cells in vitro under our experimental conditions.

Effects of CP on Iron (Fe II) Uptake by Neurons

To determine the effects of CP on iron uptake by neurons of the two brain regions, 2 μM FAS was added to a suspension of the calcein-loaded neurons with normal iron status in the presence or absence of different amounts of CP (0, 2, 4, 8 μg/ml) at 37°C after the initial baseline of

fluorescence was collected. No significant difference was found between the total quenching in the control (no CP) group and the experimental groups (treated with 2, 4, 8 μg/ml of CP) (Fig. 2A,B). Also, no significant difference was found between the effects of CP on iron uptake by the two brain regions' neurons (Fig. 2A: midbrain and B: hippocampus). The results implied that CP at these low concentrations had no effect on iron (Fe II) uptake by neurons with normal iron status.

We also investigated the effects of CP on iron uptake by iron-deficient neurons of the two brain regions. Iron-deficient cells were pre-

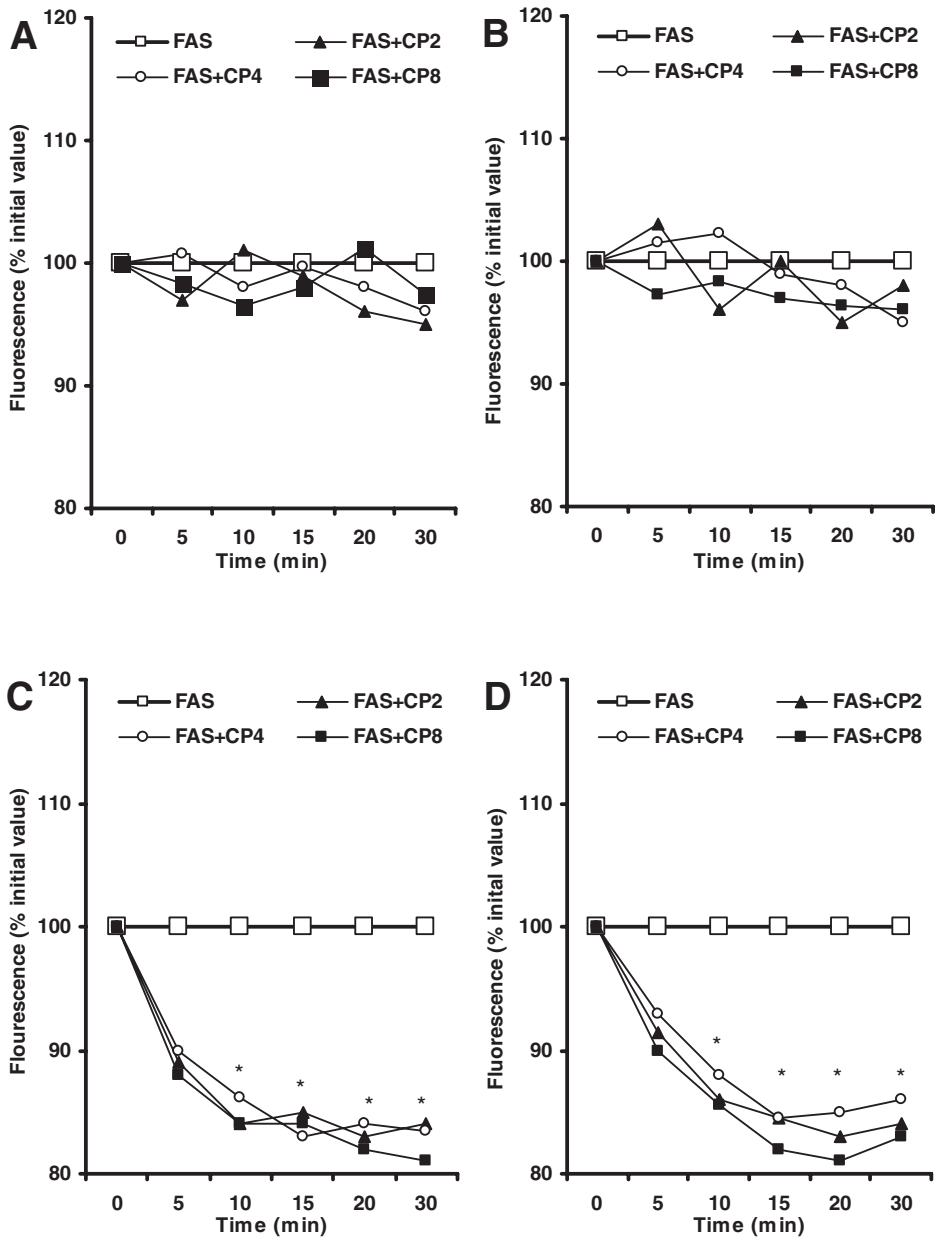

Fig. 2. Effects of ceruloplasmin on iron uptake by cultured neurons. Neurons with normal iron status (**A**: midbrain and **B**: hippocampus) and iron-deficient neurons (**C**: midbrain and **D**: hippocampus) were pre-loaded with 0.125 μM calcein-AM as described in Materials and Methods. After the initial baseline of fluorescence was collected, 2 μM FAS with varying amounts of CP (0, 2, 4, 8 μg/ml) was added to a suspension of the calcein-loaded neurons. The quenching of calcein fluorescence by iron was measured using a BMG Fluostar Galaxy fluorescence plate reader (λ_{ex} of 485 nm, λ_{em} of 520 nm, 37°C) for 30 min. The data were mean ± SEM (% initial value) of three independent experiments. $^*P < 0.05$, $^*P < 0.01$, versus the control (the steady-state values of fluorescence before addition of FAS and/or CP).

pared by incubating the cells with 1 mM BP and 1 mM DFO for 16 h in serum-free DMEM medium at 37°C. A significant quenching of fluorescence was observed after the addition of 2 μM FAS with 2, 4 or 8 μg/ml of CP at 10, 15, 20 and 30 min (all $P < 0.05$ vs. the control) (Fig. 2C,D). The results showed that soluble CP at these concentrations has a role in iron uptake by iron-deficient neurons. However, contrary to our expectation, no regional difference was found in the effects of CP on iron uptake by iron-deficient neurons. The changes in quenching of intracellular fluorescence induced by the addition of iron with CP in midbrain neurons were no difference from those in hippocampus neurons. Also, there was no significant difference in iron uptake among the iron-deficient neurons treated with 2, 4 and 8 μg/ml of CP.

DISCUSSION

To our knowledge, this study is the first investigation on the effects of soluble CP on iron transport in primary cultured neurons of the midbrain (the substantia nigra and striatum) and the hippocampus. The data in this study demonstrated that CP at low concentrations (2, 4 or 8 μg/ml CP) has a role in promoting iron uptake by the neurons of these two brain regions when they are in an iron-deficient status under our experimental conditions. The same concentrations of soluble CP had no effect on iron uptake by the normal-iron neurons nor on iron release from iron-sufficient neurons and neurons with normal iron status. The results are similar to those obtained from some other in vitro studies using different types of cells [Mukhopadhyay et al., 1998; Attieh et al., 1999; Qian et al., 2001; Xie et al., 2002]. Whether these findings imply a physiological role for soluble CP in iron uptake by neurons in the brain needs to be investigated further.

In the brain, the predominant form of CP is GPI-CP [Patel and David, 1997; Salzer et al., 1998; Patel et al., 2000]. It is mainly found in astrocytes surrounding the brain microvasculature [Klomp and Gitlin, 1996; Klomp et al., 1996], not in neurons. The level of soluble CP in the CSF is very low [Loeffler et al., 1994]. A recent study [Jeong and David, 2003] using astrocytes purified from the central nervous system of CP-null mice showed that GPI-CP is essential for iron release and is not involved in regulating iron uptake. They also demonstrated that soluble CP (1 and 300 μg/ml) increased iron release (by 9% and 50% respectively). Their data strongly suggested an iron-releasing function of CP and provided important insights into understanding the function of CP in iron transport in brain cells.

In CP-null mice or aceruloplasminemia patients, however, the lack of CP starts from the early stage of life, while iron accumulation in the brain does not occur until about 16 months in mice [Patel et al., 2002; Yamamoto et al., 2002] and 45 years in humans [Harris et al., 1995; Yoshida et al., 1995, 2000; Kaplan and O'Halloran, 1996; Takahashi et al., 1996; Gitlin, 1998]. The fact implies that brain iron balance depends not only on CP but also on other factors. Besides CP, many proteins such as transferrin receptor (TfR), divalent metal transporter 1 [DMT1] and ferroportin 1/hephaestin may also play key roles in brain iron homeostasis [Qian et al., 2002]. The lack of CP might lead to compensative changes in the expression of some of these iron metabolism proteins. It might be why the brain can maintain normal iron status for about 16 months in CP-null mice or 45 years in the patients with aceruloplasminemia although no CP involves in these cases at all. Our recent study provided the evidence for the existence of significant changes in the expression of DMT1(+IRE) and ferroportin 1 proteins in the brain of $CP^{-/-}$ mice at 40 weeks of age [Ke et al.; unpublished data]. It is not known whether such changes in the expression of iron metabolism proteins occur in astrocytes purified from the brain of CP-null mice. However, it is highly likely that the astrocytes of CP-null mice might be different from those of wild-type animals not only in the absence of CP but also in the expression of other iron metabolism proteins or relevant factors. The results of iron influx and efflux studies on astrocytes of CP-null mice might be associated with the more complicated factors.

In studies in vitro, the experimental conditions can be better controlled. The use of in vitro models might provide more confidence that there is a direct relationship between CP and iron transport. Although the in vitro studies have been criticized as being done 'under non-physiological culture conditions', the data obtained are valuable for our understanding of the role of CP in brain iron transport. These findings should not be simply ignored. In fact, most of the published data showing evidence of

iron influx [Mukhopadhyay et al., 1998; Attieh et al., 1999; Qian et al., 2001; Xie et al., 2002] or iron efflux function [Young et al., 1997; Richardson, 1999] of CP were done in vitro. However, published results in vitro on the function of CP in cellular iron transport are controversial. The cause is unknown although it might be partly due to the difference in experimental conditions [Yamamoto et al., 2002], including the cell types used, the time of iron loading, the incubation temperatures and/or others. These factors might not be the major points. Further studies are absolutely needed. At the present, it is too early to make any final conclusions on the function of CP in brain iron transport. It might be reasonable, however, to propose that the physiological function of CP depends on its activity of ferroxidase, based on the data presented here along with other research works. The ferroxidase activity of CP might play a role not only in iron efflux but also in iron influx in brain cells, including neurons and astrocytes.

ACKNOWLEDGMENTS

The studies in this laboratory were supported by Competitive Earmarked Grants of The Hong Kong Research Grants Council (PolyU5270/01M/B-Q445), Research Grants of The Hong Kong Polytechnic University (G-YX14, G-YD78, A-PD92, G-T616, and G-T856) and National Key Laboratory of Chinese Medicine and Molecular Pharmacology in Shenzhen, P R China.

REFERENCES

Attieh ZK, Mukhopadhyay CK, Seshadri V, Tripoulas NA, Fox PL. 1999. Ceruloplasmin ferroxidase activity stimulates cellular iron uptake by a trivalent cation-specific transport mechanism. J Biol Chem 274:1116–1123.

Ci W, Li W, Ke Y, Qian ZM, Shen X. 2003. Intracellular Ca(2+) regulates the cellular iron uptake in K562 cells. Cell Calcium 33(4):257–266.

Gitlin JD. 1998. Aceruloplasminemia. Pediatr Res 44:271–276.

Gitlin JD. 1999. The sky blue protein. J Lab Clin Med 134: 431–432.

Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RTA, Gitlin JD. 1995. Aceruloplasminemia: Molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci USA 92:2529–2543.

Harris ZL, Durley AP, Tsz KM, Gitlin JD. 1999. Targeted gene reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci USA 96:10812–10817.

Ho KP, Li L, Zhao L, Qian ZM. 2003. Genistein protects primary cortical neurons from iron-induced lipid peroxidation. Mol Cell Biochem 247:219–222.

Jeong SY, David S. 2003. GPI-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. J Biol Chem 278(29):27144–27148.

Kaplan J, O'Halloran TV. 1996. Iron metabolism in eukaryotes: Mars and venus at it again. Science 271: 1510–1512.

Ke Y, Qian ZM. 2003. Iron misregulation in the brain: A primary cause of neurodegenerative disorders. Lancet Neurol 2:246–253.

Klomp LW, Gitlin JD. 1996. Expression of the ceruloplasmin gene in the human retina and brain: Implications for a pathogenic model in aceruloplasminemia. Hum Mol Genet 5:1989–1996.

Klomp LW, Farhangrazi ZS, Dugan LL, Gitlin JD. 1996. Ceruloplasmin gene expression in the murine central nervous system. J Clin Invest 98:207–215.

Loeffler DA, DeMaggio AJ, Juneau PL, Brickman CM, Mashour GA, Finkelman JH, Pomara N, LeWitt PA. 1994. Ceruloplasmin is increased in cerebrospinal fluid in Alzheimer's disease but not Parkinson's disease. Alzheimer Dis Assoc Disord 8:190–197.

Mukhopadhyay CK, Attieh ZK, Fox PL. 1998. Role of ceruloplasmin in cellular iron uptake. Science 279:714–717.

Nelson N. 1999. Metal ion transporters and homeostasis. EMBO J 18:4361–4371.

Patel BN, David S. 1997. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. J Biol Chem 272:20185–20190.

Patel BN, Dunn RJ, David S. 2000. Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. J Biol Chem 275:4305–4310.

Patel BN, Dunn RJ, Jeong SY, Zhu Q, Julien JP, David S. 2002. Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. J Neurosci 22:6578–6586.

Qian ZM, Ke Y. 2001. Rethinking the role of ceruloplasmin in brain iron metabolism. Brain Res Rev 35:287–294.

Qian ZM, Shen X. 2001. Brain iron transport and neurodegeneration. Trends Mol Med 7:103–108.

Qian ZM, Wang Q. 1998. Expression of iron transport proteins and excessive iron accumulation in the brain in neurodegenerative disorders. Brain Res Rev 27:257–267.

Qian ZM, Tsoi YK, Ke Y, Wong MS. 2001. Ceruloplasmin promotes iron uptake rather than release in BT325 cells. Exp Brain Res 140:369–374.

Qian ZM, Li H, Sun H, Ho KP. 2002. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54:561–587.

Ragan HA, Nacht S, Lee GR, Bishop CR, Cartwright GE. 1969. Effect of ceruloplasmin on plasma iron in copper-deficient swine. Am J Physiol 217:1320–1323.

Richardson DR. 1999. Role of ceruloplasmin and ascorbate in cellular iron release. J Lab Clin Med 134:454–465.

Roeser HP, Lee GR, Nacht S, Cartwright GE. 1970. The role of ceruloplasmin in iron metabolism. J Clin Invest 49:2408–2417.

Salzer JL, Lovejoy L, Linder MC, Rosen C. 1998. Ran-2, a glial lineage marker, is a GPI-anchored form of ceruloplasmin. J Neurosci Res 54:147–157.

Sayre LM, Perry G, Smith MA. 1999. Redox metals and neurodegeneratiove disease. Curr Opin Chem Biol 3:220–225.

Takahashi Y, Miyajima H, Shirabe S, Nagataki S, Suenaga A, Gitlin JD. 1996. Characterization of a nonsense

mutation in the ceruloplasmin gene resulting in diabetes and neurodegenerative diseases. Human Mol Genet 5:81–84.

Xie JX, Tsoi YK, Chang YZ, Ke Y, Qian ZM. 2002. Effects of ferroxidase activity and species on ceruloplasmin mediated iron uptake by BT325 cells. Brain Res Mol Brain Res 99:12–16.

Yamamoto K, Yoshida K, Miyagoe Y, Ishikawa A, Hanaoka K, Nomoto S, Kaneko K, Ikeda S, Takeda S. 2002. Quantitative evaluation of expression of iron-metabolism genes in ceruloplasmin-deficient mice. Biochim Biophys Acta (Molecular Basis of Disease) 1588(3):195–202.

Yoshida K, Furihata K, Takeda S, Nakamura A, Yamamoto K, Morita H, Hiyamuta S, Ikeda S, Shimizu N, Yanagisawa N. 1995. A mutation of the ceruloplasmin gene is associated with systemic hemosiderosis in humans. Nature Genet 9:267–272.

Yoshida K, Kaneko K, Miyajima H, Tokuda T, Nakamura A, Kato M, Ikeda S. 2000. Increased lipid peroxidation in the brains of aceruloplasminemia patients. J Neurol Sci 175(2):91–95.

Young SP, Fahmy M, Golding S. 1997. Ceruloplasmin, transferrin and apotransferrin facilitate iron release from human liver cells. FEBS Lett 411:93–96.