Ceruloplasmin Protects Against Rotenone-Induced Oxidative Stress and Neurotoxicity

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Abstract To clarify the neuroprotective property of ceruloplasmin and the pathogenesis of aceruloplasminemia, we generated ceruloplasmin-deficient (CP−/−) mice on the C57BL/10 genetic background and further treated them with a mitochondrial complex I inhibitor, rotenone. There was no iron accumulation in the brains of CP−/− mice at least up to 60 weeks of age. Without rotenone treatment, CP−/− mice showed slight motor dysfunction compared with CP+/+ mice, but there were no detectable differences in the levels of oxidative stress markers between these two groups. A low dose of rotenone did not affect the mitochondrial complex I activity in our mice, however, it caused a significant change in motor behavior, neuropathology, or the levels of oxidative stress markers in CP−/− mice, but not in CP+/+ mice. Our data support that ceruloplasmin protects against rotenone-induced oxidative stress and neurotoxicity, probably through its antioxidant properties independently of its function of iron metabolism.

Keywords Ceruloplasmin · Iron · Rotenone · Oxidative stress · Antioxidant

Introduction

Ceruloplasmin (Cp) is proposed to have multiple biological functions, including oxidation of ferrous iron (Fe2+) and organic amines, copper transport, radical scavenging, and prooxidant activities [1–3]. In particular, the significance of Cp in iron transport through its ferroxidase activity has been well established. In the brain, Cp is mainly produced by astrocytes, especially perivascular astrocytes, and is localized on the membrane of astrocytes as a GPI-anchored form [4]. GPI-anchored Cp oxidizes Fe2+ released from perivascular astrocytes into ferric iron (Fe3+), and allows it to bind to transferrin [5–7]. Transferrin-bound iron is the main source of iron for neurons, thus, Cp facilitates iron uptake of neurons by its ferroxidase activity.

In the Cp-deficient state (aceruloplasminemia), Fe2+ in the interstitial space cannot be oxidized for uptake by transferrin. This might result in an increase of non-transferrin-bound iron (NTBI), and excessive NTBI then enters in astrocytes or other brain cells [5]. Finally, excessive cytosolic Fe2+ promotes the generation of cytotoxic hydroxyl radicals (OH•), leading to the oxidative damage to neuronal cells. Indeed, a huge amount of iron accumulation is observed in various brain regions of aceruloplasminemia patients [8–10]. There are several lines of evidence supporting that increased oxidative stress is closely associated with neuronal cell death in this disease [9–13]. Therefore, aceruloplasminemia is a good model disease to investigate the pathogenetic link between iron accumulation and neurodegeneration. To assess this issue, we generated Cp-deficient (CP−/−) mice by a targeted disruption of the ceruloplasmin gene (CP) on the BALB/c genetic background [14]. Contrary to our expectation, however, our CP−/− mice showed no iron accumulation in the brain or apparent neurological dysfunction, whereas...
they developed a large amount of iron accumulation in the liver [14]. The conversion of genetic background of CP−/− mice from BALB/c to C57BL/10 also failed to develop iron accumulation (unpublished data).

It is widely believed that iron accumulation is involved in the pathogenesis of several neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease [7, 15–17]. In Parkinson’s disease, iron accumulation promotes α-synuclein aggregation in the dopaminergic neurons of the substantia nigra [15–17]. Furthermore, excessive iron reduces the mitochondrial complex I activity in dopaminergic neurons [18]. Therefore, rotenone, a selective mitochondrial complex I inhibitor, has been used to reproduce the neuropathological findings of Parkinson’s disease [19–26]. The neurotoxic properties of rotenone are mediated via OH• generation following the inhibition of complex I activity in the substantia nigra [20]. To date, many researchers have attempted to attenuate the neurotoxic effects of rotenone using various therapeutic agents in vitro and in vivo models [27–29]. On the other hand, there are several clinical observations suggesting that a decreased oxidative activity of Cp is closely involved in the pathogenesis of Parkinson’s disease [30, 31].

Our CP−/− mice with both BALB/c and C57BL/10 genetic background showed no detectable iron accumulation. Therefore, this study aimed to investigate the neuroprotective properties of Cp independently of iron metabolism. For this aim, we treated the mice with rotenone to enhance oxidative stress and neurotoxicity.

Experimental Procedure

Experimental Animals

The derivation of the CP−/− mice with the C57BL/10 genetic background used in this study has been already described [14, 32]. All animals were housed in a controlled-temperature environment maintained under a 12 h/12 h light/dark cycle and provided with free access to food and water. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and they were approved by the Institutional Animal Care and Use Committee of Shinshu University.

Genotyping

Tail tips were placed in a cell lysis solution (QIAGEN) supplemented with proteinase K (20 mg/ml), shaken overnight at 55°C, and then centrifuged for 5 min at 15,000×g at 4°C to remove insoluble material. The supernatant was directly subjected to PCR amplification with the Ampdirect Plus Kit (Shimadzu, Japan) for genotyping. The PCR conditions and primer sequences were described previously [14]. The genotype of the mice was reconfirmed by immunoblotting for serum Cp [14].

Rotenone Treatment

We used 13-week-old CP+/+ and CP−/− mice in this study. The mice were treated with 10 mg/kg/day rotenone or vehicle for 28 days using an osmotic pump (Alzet; DURECT Corporation, USA) subcutaneously, as previously described [32]. To see the effects of Cp under rotenone-induced neurotoxic conditions, we established the following four types of mice: (1) rotenone-untreated, wild-type mice (CP+/+/R−); (2) rotenone-untreated, Cp-deficient mice (CP−/−/R−); (3) rotenone-treated, wild-type mice (CP+/+/R+); and (4) rotenone-treated, Cp-deficient mice (CP−/−/R +).

Behavioral Analysis

In all animal groups, body weight and motor function were determined weekly during the period of treatment with rotenone or vehicle. Behavioral analysis was done by footprint assessment and accelerated rotarod test [33, 34].

Footprint assessment was performed to detect deficits in motor coordination and gait abnormalities. To obtain footprints, the hind- and forepaws of mice were coated with black and red nontoxic paint, respectively. The animals were then allowed to walk along a plastic-lined, 65 cm-long and 4 cm-wide walkway into an enclosed box with 12 cm-high walls on either side. The footprint patterns were analyzed for stride length (the average distance of forward movement between each stride) and step alternation (the average distance from the left or right front footprint/hind footprint overlap) [33].

Motor coordination, balance, and learning were tested with an accelerating rotarod (3.3 cm in diameter). The mice were allowed to run while the speed of the rotarod was gradually increased from 4 to 40 rpm. The mice were tested three times in each trial, and the mean latency to fall off the rotarod was recorded [34].

Brain tissue Preparation

After 4 weeks of treatment with rotenone or vehicle, all mice were deeply anesthetized with diethyl ether inhalation. The mice were euthanized and their whole brains were removed. One hemisphere of the removed brain was weighed and homogenized in a 10-fold volume of a high salt buffer (10 mM TrisHCl, pH 7.5, 0.5 M NaCl, 10% sucrose) supplemented with a cocktail of protease inhibitors (Complete Mini, Roche). The samples were
centrifuged at 20,000×g for 20 min, and the supernatants were stored at −80°C until analysis. The other hemisphere of the brain was fixed overnight in 10% formalin for immunohistochemical analysis.

**Measurement of Mitochondrial Complex I Enzyme Activity**

We measured the mitochondrial complex I activities in brain and liver tissue homogenates using the Complex I Enzyme Activity Microplate Assay Kit (MitoSciences, USA). The samples recovered from whole tissue homogenates (protein concentration; 3.0 mg/ml for brain and 5.5 mg/ml for liver) were used for the assay according to the manufacturer’s instructions. The activity was expressed as the change in absorbance at 450 nm per minute (mOD/min).

**Assessment of Oxidative Stress Markers**

**Western Blotting**

Brain tissues were homogenized and boiled for 5 min in a buffer containing 10% SDS, 70 mM Tris–HCl (pH 6.7), 10 mM EDTA, and 5% β-mercaptoethanol. Equal amounts of brain proteins were then separated on a graduated 12% SDS–polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane. The blot was incubated at 4°C overnight with the following primary antibodies: anti-Cp polyclonal antibody (Cp, 1:500, DAKO), anti-4-hydroxynonenal monoclonal antibody (HNE, 1:500, JaICA, Japan), and anti-Hexanoyl-Lys-adduct monoclonal antibody (HEL, 1:500, JaICA, Japan).

After three washes, the membrane was incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse or rabbit IgG secondary antibody (1:300, DAKO), streptavidin-HRP conjugate (1:300, GIBCO BRL), and reacted with diaminobenzidine (0.3 mg/ml, SIGMA) in 50 mM Tris buffer (pH 7.4) with 0.001% H2O2. Finally, the sections were counterstained with hematoxylin.

**Immunohistochemistry**

The paraffin-embedded brain tissues were cut at 10 μm. The sections were deparaffinized and hydrated through graded alcohol. These sections were first microwaved in 0.1 M sodium citrate, pH 6.5. They were then incubated in 3% aqueous hydrogen peroxide (H2O2) to remove endogenous peroxidase activity, and in phosphate buffered saline containing 10% goat serum for 30 min. Subsequently, the sections were incubated overnight at 4°C with primary antibodies: HNE (1:100) and HEL (1:50). The sections were then incubated with anti-mouse or rabbit IgG secondary antibody (1:300, DAKO), streptavidin-HRP conjugate (1:300, GIBCO BRL), and reacted with diaminobenzidine (0.3 mg/ml, SIGMA) in 50 mM Tris buffer (pH 7.4) with 0.001% H2O2, finally, the sections were counterstained with hematoxylin.

**Statistical Analysis**

All values are presented as mean ± standard error (SEM). Statistical analysis of data was performed using two-way analysis of variance (ANOVA) or repeated-measures ANOVA, followed by post hoc Student’s t-test to determine the significance of differences between two groups. The analysis was performed using software Excel Toukei 2010 (Social Survey Research Information, Tokyo, Japan). Statistical significance was attributed as *P* < 0.05.

**Results**

**Effects of Rotenone Treatment on Motor Function**

All mice completed the protocol without any troubles in general health. There were no significant differences in body weight between the four experimental groups (data not shown). By western blotting, rotenone treatment did not change the level of Cp expression in C57Bl/6 mice (Fig. 1a). There were no significant differences in mitochondrial complex I enzyme activity of brain (Fig. 1b) and liver tissue homogenates (Fig. 1c) among the four experimental groups.

To capture the endogenous IgG with Dynabeads Protein G, the same amount (5 μg) of brain proteins was added to 50 μl of beads and incubated with rotation overnight. Then the tube was placed onto a magnet to separate the beads from solution, and the supernatant was used for analysis.

The carbonyl contents of brain tissues were assayed with the Protein Carbonyl Enzyme Immuno-assay Kit (Biocell PC Test, BioCell Crop, New Zealand) according to the manufacturer’s instructions. Absorbance was measured at 450 nm and expressed as nmol carbonyl/mg protein.

The paraffin-embedded brain tissues were cut at 10 μm. The sections were deparaffinized and hydrated through graded alcohol. These sections were first microwaved in 0.1 M sodium citrate, pH 6.5. They were then incubated in 3% aqueous hydrogen peroxide (H2O2) to remove endogenous peroxidase activity, and in phosphate buffered saline containing 10% goat serum for 30 min. Subsequently, the sections were incubated overnight at 4°C with primary antibodies: HNE (1:100) and HEL (1:50). The sections were then incubated with anti-mouse or rabbit IgG secondary antibody (1:300, DAKO), streptavidin-HRP conjugate (1:300, GIBCO BRL), and reacted with diaminobenzidine (0.3 mg/ml, SIGMA) in 50 mM Tris buffer (pH 7.4) with 0.001% H2O2. Finally, the sections were counterstained with hematoxylin.
In the footprint test, \( CP^{-/-}/R^+ \) mice tended to develop gait abnormalities, such as a shorter stride length and an irregular placement of the fore- and hind-limbs (Fig. 2a). Stride length significantly decreased in \( CP^{-/-}/R^- \) mice (0 w: 64.0 \( \pm \) 4.0 mm vs. 4 w: 54.4 \( \pm \) 3.3 mm, \( P < 0.05 \), \( n = 8 \)) and \( CP^{-/-}/R^+ \) mice (0 w: 63.5 \( \pm \) 1.3 mm vs. 4 w: 53.6 \( \pm \) 2.3 mm, \( P < 0.05 \), \( n = 6 \)) during the experiment, while there was no obvious change in \( Ch^{-/-} \) mice, even with rotenone treatment. There was no difference in stride length between \( CP^{-/-}/R^- \) and \( CP^{-/-}/R^+ \) mice. Step alternation of \( CP^{-/-} \) mice appeared more irregular than in \( CP^{+/+} \) mice before treatment, but this was not statistically significant. Step alternation gradually worsened with rotenone treatment in \( CP^{+/+} \) mice. In \( CP^{+/+} \) groups, step alternation tended to get worse with rotenone treatment, but the difference was not significant between \( CP^{+/+}/R^- \) and \( CP^{+/+}/R^+ \) mice after 4 weeks. At the end of rotenone treatment, step alternation was more conspicuous in \( CP^{-/-}/R^- \) mice than \( CP^{+/+}/R^- \) or \( CP^{+/+}/R^+ \) mice (Fig. 2c). The time spent on the rotarod was not different before rotenone treatment between the four mouse groups, and increased over time in all groups. The fall latency of \( CP^{+/+}/R^- \) mice drastically increased at 4 weeks (0 w: 10.8 \( \pm \) 2.7 s vs. 4 w: 41.2 \( \pm \) 4.0 s, \( P < 0.05 \), \( n = 5 \)). However, after 4 weeks, \( CP^{-/-}/R^- \) mice and \( CP^{-/-}/R^+ \) mice showed shorter fall latencies than \( CP^{+/+}/R^- \) mice (Fig. 2d). There was no significant difference in the fall latency between the \( CP^{-/-}/R^- \) and \( CP^{-/-}/R^+ \) mice.

Detection of Oxidative Stress Markers

Western blotting showed that the signals indicative of HNE and HEL were enhanced in the brains of \( CP^{-/-}/R^+ \) mice compared with mice from the other three groups (Fig. 3a, b). Consistent with the western blotting data, ELISA analyses showed a significant increase in HNE and protein carbonyls in \( CP^{-/-}/R^+ \) mice (Fig. 3c, d). Rotenone treatment did not change the levels of HNE or carbonyl proteins in \( CP^{+/+} \) mice.

Neuropathological and Immunohistochemical Findings

Histopathological findings were grossly normal in the four experimental groups. Iron staining detected no iron accumulation in the brains of \( CP^{-/-}/R^+ \) mice before and after rotenone treatment (data not shown). The immunoreactivity for HNE and HEL in the cytoplasms of the striatal neurons was more conspicuous in \( CP^{-/-}/R^+ \) mice than \( CP^{+/+}/R^- \) mice or \( CP^{-/-}/R^- \) mice (Fig. 4). Even without rotenone treatment, the immunoreactivity was apparent in \( CP^{-/-}/R^- \) mice, but not in \( CP^{+/+}/R^- \) mice (Fig. 4).

Discussion

We have generated \( CP^{-/-} \) mice in both the BALB/c and C57BL/10 genetic background to investigate the pathogenesis of aceruloplasminemia, but neither of the mice showed iron accumulation in the brain at least up to 40 or 60 weeks of age [14, 32]. In this study, therefore, we used a selective mitochondrial complex I inhibitor, rotenone, to enhance oxidative stress and to see whether Cp has a protective effect against oxidative neuronal damage. Rotenone decreases the viability of TH-immunoreactive
Fig. 2 Analysis of motor function. a Representative footprints after the 4-week administration of 10 mg/kg rotenone or vehicle. The forepaws and hindpaws were colored with red and black ink, respectively. The dotted lines indicate stride length. The irregular distance between the fore- and hindpaws in a $CP^{-/-}/R+$ mouse is indicated by the bidirectional arrow. The quantification of the stride length (b) and step alternation (c) of footprint patterns in $CP^{+/+}/R-$, $CP^{-/-}/R-$, $CP^{+/+}/R+$, and $CP^{-/-}/R+$ mice are shown ($n = 7–10$). d Fall latency in the rotarod test (4–40 rpm) ($n = 4–5$). * $P < 0.05$ in comparison with control ($CP^{+/+}/R-$).
dopaminergic neurons in vivo and in vitro, and is frequently used to investigate the pathogenesis of Parkinson’s disease [19–26]. According to the reported dosage of rotenone in mice (2.5–100.0 mg/kg/day for 4–9 weeks) [21, 23–26], we used rotenone at a comparatively low-dosage (10 mg/kg, 4 weeks). Thus, all the mice completed the protocol without any problems in their general health and the mean body weight was not significantly different between the four groups examined.

Rotenone did not cause a significant change in motor behavior, neuropathology, or the levels of oxidative stress markers in the presence of Cp in this study. On the other hand, it brought about an obvious motor dysfunction and increased levels of oxidative stress markers in the absence of Cp. At the end of the 4-week rotenone treatment, $CP^{-/-}/R^+$ mice showed a slow and irregular gait with small steps, and they fell from the rotarod more easily than $CP^{-/-}/R^-$ mice. The levels of oxidative stress markers were increased in $CP^{-/-}/R^+$ mice when compared with $CP^{+/+}/R^-$ or $CP^{-/-}/R^-$ mice. In addition, we previously reported that immunoreactivity for acrolein and ubiquitin was enhanced in the brains of $CP^{-/-}/R^+$ mice [32]. Mitochondria are easily exposed to oxidative damage because they utilize large amounts of oxygen and iron, and contain numerous redox enzymes [36, 37]. In fact, there is a marked reduction in the activity of the respiratory chain complexes I and IV in the brains of aceruloplasminemia patients [11]. Thus, from the viewpoint of enhanced oxidative stress in the brain, our $CP^{-/-}$ mice treated with a low dose of rotenone will be a more faithful model for aceruloplasminemia than conventional $CP^{-/-}$ mice.

But, rotenone treatment in our mice did not affect the mitochondrial complex I activities, probably because of the low dosage. Under this condition, however, $CP^{-/-}$ mice, not $CP^{+/+}$ mice, showed a significant increase of oxidative stress markers in the brain and motor dysfunction. Rotenone is believed to cause neuron death by an inhibition of the mitochondrial complex I activities, but Choi et al. have suggested that neuronal cell death induced by rotenone is independent of mitochondrial complex I inhibition [38]. The present study may support that rotenone-induced neurotoxicity is mediated through alternative pathways other than mitochondrial complex I inhibition, and that such pathways are enhanced by Cp deficiency.

Our $CP^{-/-}$ mice with or without rotenone treatment showed no iron accumulation in the brain as far as we observed up to 60 weeks of age. The question is how Cp prevents neuronal cells from rotenone-induced...
neurotoxicity under the circumstances without iron accumulation. Cp is considered to oxidize non-iron substrates such as catecholamines and aromatic amines [1, 2, 39]. For example, Cp oxidizes the neurotoxin, 6-hydroxydopamine, which is an intermediate in the formation of dopamine and melanins. 6-hydroxydopamine undergoes spontaneous autooxidation to yield several reactive oxygen species including H₂O₂ and OH•. Unlike spontaneous autooxidation, 6-hydroxydopamine oxidation by Cp does not result in the production of H₂O₂ and OH• [2, 39]. Thus, one possible interpretation is that enhanced oxidative stress in our CP⁻/⁻/R+ mice could be due to a loss of Cp-mediated oxidation of non-iron substrates. Moreover, Atanasiu et al. have shown that Cp is capable to scavenge peroxyl, superoxide radicals, and OH• even after heat inactivation of its oxidase activity [40]. These data strongly indicate that Cp, besides its preventive antioxidant properties, has chain-breaking antioxidant property [40]. This is another possibility to explain that CP⁻/⁻/R+ mice showed motor dysfunction and increased oxidative stress markers when compared with CP⁺/+/R- mice. The antioxidant properties of Cp other than its ferroxidase activity may also explain

Fig. 4 Immunohistochemistry for oxidative stress markers. Immunohistochemical staining using anti-HNE (a–d) and anti-HEL (e–h) antibodies in the striatum is shown. CP⁺/+/R− (a, e), CP⁻/⁻/R− (b, f), CP⁺/+/R+ (c, g), and CP⁻/⁻/R+ mice (d, h). Scale bars = 20 μm.
why our CP⁻⁻/⁻ mice showed motor dysfunction when compared with CP⁺⁺/⁺⁻ mice. Even, if it is the case, we speculate that an increase of oxidative stress markers in our CP⁻⁻/⁻ mice was too subtle, or locally restricted to be detected by our assay methods using whole brain homogenates.

In summary, our CP⁻⁻ mouse model treated with rotenone indicates that Cp plays an important role in the neuroprotection against oxidative stress, even without apparent misregulation of iron homeostasis. To further elucidate the pathogenesis of aceruloplasminemia, it will be needed to clarify how the alternative functions of Cp, other than ferroxidase activity, is involved in this disease.

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