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Ceruloplasmin Immunoreactivity in Neurodegenerative Disorders

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Ceruloplasmin (CP) is a 132 kd cuproprotein which, together with transferrin, provides the majority of anti-oxidant capacity in serum. Increased iron deposition and lipid peroxidation in the basal ganglia of subjects with hereditary CP deficiency suggest that CP may serve as an anti-oxidant in the brain as well. The present study compared CP immunoreactivity in brain specimens from normal controls and subjects with neurodegenerative disorders (Alzheimer's disease [AD], Parkinson's disease [PD], progressive supranuclear palsy [PSP], and Huntington's disease [HD]) ($n = 5$ per group). The relative intensity of neuronal CP staining and the numbers of CP-stained neurons per 25x microscope field were determined in hippocampus (CA1, subiculum, and parahippocampal gyrus), parietal cortex, frontal cortex, substantia nigra, and caudate. CP was detected in both neurons and astrocytes in all specimens, and in senile plaques and occasional neurofibrillary tangles in AD brain. Neuronal CP staining intensity tended to increase in most AD brain regions, but was statistically significant vs controls only in the CA1 region of hippocampus ($p = .016$). Neuronal CP staining in brain specimens from other neurodegenerative disorders showed a slight but nonsignificant increase vs controls. The numbers of CP-stained neurons per field did not differ between the various neurodegenerative disorders and controls. These results suggest that a modest increase in neuronal CP content is present in the AD brain, and lesser elevations in neuronal CP occur in the other neurodegenerative disorders in this study. Though CP functions as both an acute phase protein and an anti-oxidant in peripheral tissues, whether it does so in the brain remains to be determined.

Keywords: Alzheimer's disease, ceruloplasmin, Huntington's disease, oxidative stress, Parkinson's disease, progressive supranuclear palsy

INTRODUCTION

Ceruloplasmin (CP) is a systemic acute phase protein whose hepatic synthesis and plasma concentration increase in response to a variety of conditions including inflammation, oxidative stress, pregnancy, and neoplasia.[1] CP provides the majority of anti-oxidant protection in plasma, together with transferrin (TF).[2] The ferroxidase activity of CP converts ferrous ion

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(Fe^{+2}) to ferric ion (Fe^{+3}),[3] which is bound by TF and transported into cells bearing TF receptors. Because Fe^{+2} reacts with hydrogen peroxide (H_2O_2) to produce the highly toxic hydroxyl radical (•OH), the limiting of free Fe^{+2} in tissues by CP is an important anti-oxidant protective mechanism. Additional anti-oxidant capacity of CP results from scavenging of free radicals.[4] However, under certain conditions CP may function as a pro-oxidant. When exposed to peroxynitrite, for example, CP releases copper ions[5] which promote formation of •OH. Similar findings have recently been reported following exposure of CP to H_2O_2.[6]

Oxidative stress has been reported to be present in the brain in numerous neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), progressive supranuclear palsy (PSP), Huntington's disease (HD), Down syndrome, and amyotrophic lateral sclerosis (see reviews by Refs. [7–10]). The major anti-oxidants in the brain are catalase, glutathione peroxidase, and superoxide dismutase;[11] the brain is considered to be particularly susceptible to oxidative damage because of its low levels of catalase and glutathione peroxidase.[12] Studies from our laboratory[13] and elsewhere[14] have demonstrated that CP is present within neurons. The findings of increased iron content[15] and elevated lipid peroxidation[16] in the basal ganglia from individuals with hereditary CP deficiency (aceruloplasminemia) suggest that CP may function as an anti-oxidant in the CNS. In neurodegenerative disorders, the status of CP is unclear. A previous study from our laboratory[13] detected increased CP concentrations in brain homogenates from AD, PD, and HD brain specimens, compared with normal controls. Castellani *et al.*[14] also found elevated CP levels in AD hippocampus, while Connor *et al.*[17] found decreased CP concentrations in AD superior frontal cortex. However, more recent work in our laboratory has shown that the concentrations of acute phase reactants, including CP, in brain homogenates reflect primarily the levels of these proteins within the cerebral vasculature, rather than in brain parenchyma; the concentrations of CP and other acute phase proteins in rat brain homogenates decreased by more than 80% following perfusion to remove peripheral blood proteins.[18] Therefore these previous studies of CP levels in brain homogenates do not provide information regarding cellular CP content. In order to determine the status of intracellular CP in neurodegenerative disorders, in the present study immunocytochemical staining for CP was performed on brain specimens from AD, PD, PSP, HD, and normal controls.

MATERIALS AND METHODS

Brain Specimens

Formalin-fixed, paraffin-embedded brain samples from subjects ($n = 5$/group) with AD (77–88 years old), PD (63–85 y.o.), HD (51–75 y.o.), and PSP (64–83 y.o.), as well as clinically normal individuals (51–78 y.o.) were obtained from the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA. Each brain met standard neuropathological criteria for confirming the clinical diagnosis of these disorders.[19–23] Control specimens were from non-demented individuals dying from causes other than CNS disease, and without clinically apparent neurological disorders or evidence of neurodegenerative processes at autopsy. Clinical records were reviewed to support tissue diagnoses.

Immunocytochemical Staining

Tissue sections were stained for CP using the avidin-biotin-peroxidase complex (ABC) technique, as described previously.[13] Sections were deparaffinized through graded ethanol baths, incubated with 0.3% H_2O_2 in methanol to eliminate endogenous peroxidase activity, then incubated for 30 min with phosphate-buffered saline (PBS, 0.01 M, pH 7.4) with 0.1% Triton

X-100 (Sigma Chemical Co., St. Louis, Mo) with 10% normal horse serum (NHS) (Gibco Laboratories, Life Technologies Inc., Grand Island, NY) and 1% bovine serum albumin (BSA) (Sigma). Sections were then incubated overnight at 4 °C with goat anti-human CP (Chemicon International, Inc., Temecula, CA) diluted 1:4000 in PBS-Triton-BSA. As negative controls, serial sections were incubated with a 1:4000 dilution of normal goat serum (NGS) (Gibco Laboratories). After washing, a 1:200 dilution of biotinylated horse anti-goat IgG (Vector Laboratories, Inc., Burlingame, CA) was applied for 1 h at room temperature, followed by avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 30 min at room temperature. Sections were developed with diaminobenzidene/H_2O_2 with nickel enhancement.[24] Confirmation of the specificity of CP staining, via pre-absorption of the goat anti-CP serum with human CP, was reported previously.[13]

Quantification of Neuronal CP Staining

Slides were coded and evaluated in a blinded fashion by one investigator (D.L.). The relative intensity of neuronal CP staining was scored on a scale of 0–3+, with 0 = no cell-specific staining above that observed in the absence of primary antibody, 1+ = slight cell-specific staining, 2+ = moderate cell-specific staining, and 3+ = marked cell-specific staining. The relative intensity of neuronal CP staining was estimated, and the numbers of CP-stained neurons were counted, in five 25x fields (field size 0.48 mm^2, Zeiss Standard 14 microscope) in hippocampus (CA1, subiculum, and parahippocampal gyrus), parietal cortex (Brodmann area 7), frontal cortex (Brodmann area 9), the head of the caudate nucleus, and substantia nigra pars compacta (lateral one-third). Hippocampal staining was examined in transverse sections containing the hippocampal formation, perirhinal cortex, and inferior temporal cortex. In parietal and frontal cortices, immunoreactivity was assessed in the crest of a gyrus.

Statistics

The relative intensity of CP neuronal staining per field and the numbers of CP-stained neurons per field were compared between groups by the Kruskal-Wallis nonparametric test. When significant differences were detected, values for each of the neurodegenerative disorders were compared with control values via the Mann-Whitney U-test. Statistical significance was set at $p < .05$ for all analyses.

RESULTS

Relative Intensity of Neuronal CP Staining (Table I)

Neuronal CP immunoreactivity tended to be higher in AD specimens than in other groups.

TABLE I Relative intensity of neuronal CP staining in control and neurodegenerative brain specimens

Region	CTL	AD	HD	PD	PSP
CA1	0.60 + 0.24	2.26 + 0.27[a]	1.80 + 0.44	1.69 + 0.34	1.12 ± 0.31
Parahipp.	0.90 ± 0.54	1.96 ± 0.47	1.80 ± 0.71	1.83 ± 0.27	1.36 ± 0.47
Subiculum	1.00 ± 0.41	2.32 ± 0.28	1.45 ± 0.61	1.84 ± 0.31	1.48 ± 0.52
Par. cortex	0.48 ± 0.32	2.35 ± 0.34	0.68 ± 0.43	1.80 ± 0.54	1.80 ± 0.32
Fr. cortex	0.68 ± 0.38	1.85 ± 0.36	1.04 ± 0.37	1.16 ± 0.22	1.32 ± 0.49
Sub. nigra	1.44 ± 0.39	1.76 ± 0.19	1.69 ± 0.28	2.29 ± 0.24	1.71 ± 0.33
Caudate	0.60 ± 0.30	1.60 ± 0.34	0.88 ± 0.46	1.12 ± 0.23	1.15 ± 0.49

Data are expressed as mean ± SEM.
[a] $p = .016$ vs control mean.

An increase of greater than two-fold in neuronal CP staining intensity vs controls was detected in six of the seven AD brain regions examined (86%), three regions each for HD and PD (43%), and one PSP brain region (14%). These elevations achieved statistical significance only in CA1 of the hippocampus (Kruskal-Wallis p value = .044; Mann-Whitney U-test $p = .016$ between AD and controls). In parietal cortex, the between-group differences approached but did not attain statistical significance (Kruskal-Wallis $p = .053$).

Numbers of CP-Stained Neurons per Field (Table II)

Marked variation in the numbers of CP-stained neurons was present both within and between groups. There were no significant differences between neurodegenerative disorders and controls. Despite the marked neuronal dropout that occurs in some brain regions in these neurodegenerative disorders (i.e., PD substantia nigra and AD hippocampus), the numbers of CP-immunoreactive neurons in these regions did not differ from control specimens.

CP Staining Patterns

CP staining of neurons in the various brain specimens is described below. CP staining of astrocytes was also present in most specimens, mainly in white matter, though differences with regard to the intensity of astrocyte staining were not observed between neurodegenerative and control specimens.

Hippocampus (Figures 1A–D)

Faint staining (1+) of scattered pyramidal neurons was present in control CA1-3. In AD specimens, moderate (2+) staining of pyramidal neurons was observed in the hippocampal endplate (CA4), CA1-3, subiculum, and parahippocampal gyrus. Granule cells and senile plaques were stained in the dentate fascia. Immunoreactivity in both diffuse and neuritic plaques was observed in CA1 and the parahippocampal gyrus. Staining patterns in PD, HD, and PSP sections were similar to those observed in AD, but immunoreactivity was not as pronounced.

Parietal and Frontal Cortices (Figure 2)

Similar patterns of neuronal staining for CP were observed in both cortical regions. Control specimens contained occasional lightly-stained neurons. Moderate staining of pyramidal neurons was present in AD specimens, as well as staining of diffuse and neuritic plaques. CP immunoreactivity in the other neurodegenerative disorders was variable, with moderate staining of neurons in some specimens and little staining in others.

Substantia Nigra (Figure 3)

In contrast to the other regions evaluated in this study, moderate staining of neurons for CP was

TABLE II Numbers of CP-stained neurons in control and neurodegenerative brain specimens

Region	CTL	AD	HD	PD	PSP
CA1	8.00 ± 4.80	9.02 ± 3.44	7.85 ± 2.30	7.30 ± 3.26	6.48 ± 2.96
Parahipp.	9.05 ± 5.36	9.48 ± 3.34	12.35 ± 3.05	2.28 ± 0.98	12.88 ± 5.30
Subiculum	7.85 ± 2.63	12.04 ± 2.54	14.04 ± 2.24	5.05 ± 2.58	6.15 ± 2.85
Par. cortex	0.15 ± 0.15	13.95 ± 6.16	6.04 ± 2.42	1.48 ± 1.05	9.45 ± 2.97
Fr. cortex	2.35 ± 2.22	4.75 ± 0.78	4.32 ± 1.67	4.28 ± 2.21	5.00 ± 1.70
Sub. nigra	6.98 ± 2.58	10.24 ± 0.80	6.13 ± 1.17	4.04 ± 0.76	4.63 ± 1.20
Caudate	1.64 ± 1.26	5.72 ± 2.35	3.60 ± 1.46	3.68 ± 2.67	4.40 ± 3.61

Data are expressed as mean ± SEM. (No significant differences were present between values for neurodegenerative disorders and controls.)

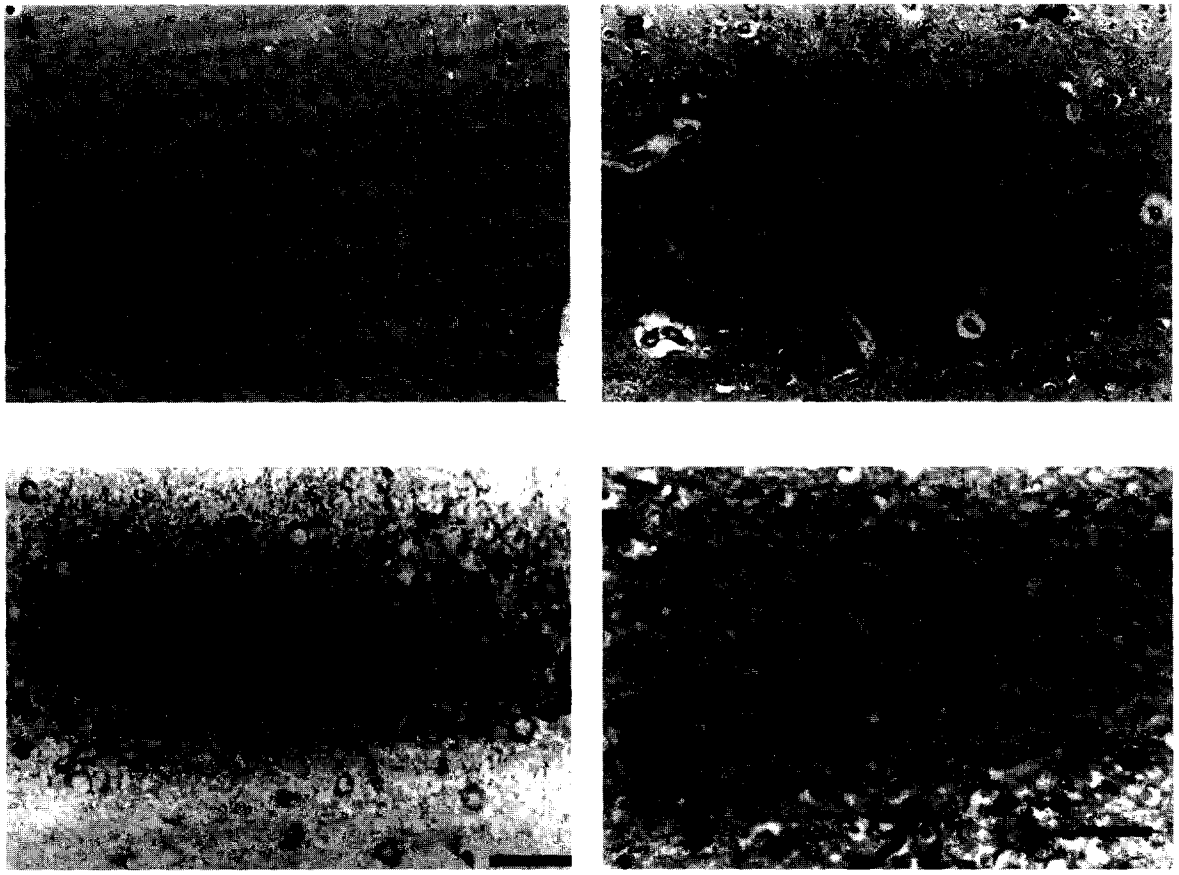

FIGURE 1 CP staining in hippocampus and perihippocampal regions. (A) Pyramidal neurons, AD subiculum (bar = 50 μm); (B) Neuritic plaque, AD temporal cortex (bar = 25 μm); (C) Granule cells, AD dentate fascia (bar = 18 μm); (D) Astrocytes in white matter, AD hippocampus (bar = 12.5 μm).

FIGURE 2 CP staining of pyramidal neurons, AD parietal cortex (bar = 25 μm).

FIGURE 3 CP staining of melanized and non-melanized neurons, AD substantia nigra (bar = 50 μm).

typically present in control as well as neurodegenerative specimens. Both melanized and non-melanized neurons were immunoreactive. No differences with regard to the neuronal CP staining pattern were found between groups, though immunoreactivity was highest in PD specimens.

Caudate

The intensity of neuronal CP staining was lower in the caudate than in the other brain regions in this study. Relatively low numbers of CP-stained neurons were present in all specimens, and no differences in staining patterns were present between groups. Immunoreactivity was observed in neuronal cell bodies, but only rarely in neuronal processes.

DISCUSSION

This study demonstrated the presence of CP within both neurons and astrocytes in brain specimens from a variety of neurodegenerative disorders, as well as normal controls. Grading of the intensity of CP neuronal staining on a scale of 0–3+ was performed to provide a basis for comparison between the various groups. CP immunoreactivity tended to increase, in comparison with controls, primarily in AD specimens, though with the exception of the AD hippocampus (CA1) these elevations did not achieve statistical significance. The relatively small numbers of specimens in each group ($n = 5$), the variability of CP staining within groups, the limitations of the 0–3+ rating scale, and the relatively conservative nature of the nonparametric statistical procedures employed may have contributed to this lack of statistical significance despite the 2- to 4-fold increases in mean neuronal CP staining intensity between AD and control specimens.

The presence of CP within neurons was previously demonstrated in AD brain specimens in our earlier study[13] and that of Castellani *et al.*,[14] who compared CP staining between AD and control temporal lobe (including hippocampus and cortex), cerebellum, and brain stem. In the latter study, little differences in staining intensity were noted between AD and control specimens, and immunoreactivity was not quantified. This result was interpreted to suggest that neurons in the AD brain may fail to upregulate CP synthesis in response to oxidative stress, and that this lack of CP upregulation may contribute to the neurodegenerative process. In the present study, additional neurodegenerative disorders and additional brain regions were evaluated, and semi-quantitative assessment was performed on neuronal CP staining. Though we detected increased CP staining in most AD brain regions, these increases were not statistically significant with the exception of AD hippocampus. These results suggest a conclusion similar to that of Castellani *et al.*; neuronal CP content was not significantly increased in the various neurodegenerative disorders in this study. The biological significance of the increased CP staining in the AD hippocampus, and of the tendency for CP staining to increase in most of the other AD regions (and, to a lesser extent, in some regions in the other neurodegenerative disorders), is unknown. Some differences in staining patterns were noted between the present study and that of Castellani *et al.*; we detected staining of astrocytes (particularly in white matter, in both neurodegenerative and control specimens) as well as granule cells in the dentate gyrus (mainly in neurodegenerative specimens), while Castellani *et al.* reported no CP staining in these cell types. A glycosylphosphatidylinositol (GPI)-anchored form of CP has been reported in astrocytes,[25,26] and it may be this form of CP that was detected with our staining protocol. Klomp and Gitlin[27] previously detected glial cell-specific CP gene expression in an *in situ* hybridization study of the human brain, providing further evidence for the presence of CP within astrocytes. The differing staining patterns between the present study

and that of Castellani *et al.* may have been due to differences in fixation procedures and/or in the anti-CP sera used for immunocytochemistry. We used formalin fixation and Chemicon's goat anti-CP serum (1:4000 dilution), whereas the other study employed methacarn fixation and Accurate Chemical Company's rabbit anti-CP (1:250 dilution).

The conditions which resulted in the elevation of neuronal CP in the AD hippocampus are unknown. Oxidative stress and inflammation are likely candidates. Both conditions are present in AD,[28,29] and increased anti-oxidant activity in the AD brain has been suggested to be a compensatory response to oxidative stress.[30] Both conditions also promote the systemic acute phase response, but whether CP acts as an acute phase reactant in the CNS is unknown. The present results suggest that CP undergoes a less marked acute phase-type response in the CNS than in the periphery.

The mechanism by which neurons acquire CP is unknown. As mentioned above, *in situ* hybridization studies have detected CP mRNA within astrocytes, but not neurons, in normal brain.[27,31] Cell-surface receptors for CP have been described in the brain and other organs,[32] and cellular internalization of CP has been reported.[33] Neuronal CP may therefore reflect CP synthesis by these cells and/or receptor-mediated uptake of CP from the extracellular space. Extracellular CP might also increase in the brain secondary to blood–brain barrier damage; if so, this could allow for increased receptor-mediated uptake of CP into neurons.

Lastly, despite our finding of, at most, modest increases in neuronal CP immunoreactivity in these neurodegenerative disorders (with the possible exception of AD hippocampus), the significance of CP in the brain remains unclear. Though CP is clearly an anti-oxidant in peripheral blood, it may not necessarily function as such in the CNS. Accumulation of iron in the basal ganglia of subjects with aceruloplasminemia[15] indicates an important role for CP in brain iron metabolism; because iron promotes the production of oxyradicals, CP might well be a neuronal anti-oxidant. However, the studies cited above of the pro-oxidant effects of CP following its exposure to peroxynitrite[5] and H_2O_2[6] leave open the possibility that intraneuronal CP may in some cases contribute to, rather than defend against, oxidative stress. CP might function as a pro-oxidant in the substantia nigra, for example, where H_2O_2 is generated as a byproduct of dopamine metabolism.[7] Whether the lack of a more robust increase in neuronal CP in these disorders allows for greater oxidative damage depends upon whether CP is ultimately found to be an anti-oxidant or a pro-oxidant in the brain.

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