Free Radical Research, 2002 Vol. 36 (3), pp. 303–306

Taylor & Francis
healthsciences

Glial Fibrillary Acidic Protein is Greatly Modified by Oxidative Stress in Aceruloplasminemia Brain

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Accepted by Professor B. Halliwell

(Received 15 November 2001)

Aceruloplasminemia is an autosomal recessive disorder of iron metabolism caused by mutations in the ceruloplasmin (Cp) gene. The neuropathological hallmark of this disease is intracellular iron overload, which is thought to lead to neuronal cell death through increased oxidative stress. We evaluated and characterized protein oxidation in the brain of a patient with this disease. The protein carbonyl content in the cerebral cortex of the patient was elevated compared to controls. Furthermore, peptide mass fingerprinting and partial amino acid sequencing identified glial fibrillary acidic protein (GFAP) as the major carbonylated protein in the cerebral cortex of the patient. In conjunction with the facts that Cp mainly localizes to astrocytes in the central nervous system and that astrocytes are loaded with much more iron than neurons in the cerebral cortex, our findings indicate that Cp deficiency may primarily damage astrocytes. We speculate that the dysfunction of astrocytes may be causatively related to neuronal cell loss in aceruloplasminemia.

Keywords: Aceruloplasminemia; Protein carbonyl; Oxidative stress; Glial fibrillary acidic protein; Astrocyte

INTRODUCTION

The brain is one of the major target organs in aceruloplasminemia [1,2]. The cardinal neuropathological findings are intracellular iron overload and neuronal cell loss [1,2]. The precise mechanisms by which ceruloplasmin (Cp) deficiency causes intracellular iron overload has reminded unclear, but iron overload will enhance oxidative tissue damage through an increased generation of free radicals [3]. This is supported by our previous observation that lipids peroxides increased in the basal ganglia and cerebral cortex of patients with aceruloplasminemia [4]. Free radicals attack proteins as well as lipids, and thus protein carbonyl is another marker of oxidative stress. The proposed mechanism for protein carbonylation is metal-catalyzed oxidation of lysyl, arginyl, prolyl, or threonyl residues [5]. In this study, we have identified glial fibrillary acidic protein (GFAP) as one of the most severely carbonylated proteins in the cerebral cortex in aceruloplasminemia. We speculate that glial dysfunction may be a primary event prior to neuronal cell death in this disease.

MATERIAL AND METHODS

Detection and Measurement of Protein Carbonyls

The clinicopathological details and mutation in the Cp gene of the patient (60-year-old female) were previously described [2,6]. Brain tissues (frontal cortex) were obtained at autopsy and were frozen at −80°C until dissection and analysis. The protein

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290019327

carbonyl content was measured according to the previously described methods [7], with slight modifications. For immunochemical detection of protein carbonyls on two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), brain samples were suspended in 25 mM sodium phosphate buffer (pH 7.4) containing 2 mM desferioxamine, 0.7 μg/ml pepstatin, and 20 μg/ml *p*-aminophenyl methanesulfonyl fluoride hydrochloride on ice. Homogenates were centrifuged at 16,000*g* for 15 min at 4°C. Obtained supernatants were subjected to 2, 4-dinitrophenyl hydrazine (DNPH)-derivatization as previously reported [7,8]. Briefly, the proteins were incubated in 10 mM DNPH at 15°C for 1 h. The derivatized proteins were precipitated with 10% trichloroacetic acid. The precipitated proteins were washed with 1 ml of 1:1 (vol/vol) ethanol/ethyl acetate and then with cold acetone. The precipitates were resolved in 8 M urea containing 50 mM dithiothreitol, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propenesulfonate, and 2% IPG buffer (Amersham Pharmacia Biotech), and then 200 μg of proteins were applied to Immobiline dry strip (pH 4–7) (Amersham Pharmacia Biotech). Isoelectric focusing (IEF) was done using multiphore II system (Amersham Pharmacia Biotech) as described in the manufacturer's instruction. After equilibration, IEF gel was submitted to second dimensional PAGE. For immunodetection, the proteins were transferred onto PVDF membrane and incubated with anti-DNP antibodies and then developed with ECL detection kit (Wako). For primary structure analysis, proteins on the PAGE gel were also stained with 0.25% coomassie brilliant blue (CBB).

Identification of Carbonylated Proteins by Peptide Mass Fingerprinting and Partial Amino Acid Sequencing

Protein identification was performed by mass spectrometry combined with sequence database searches [9,10]. The "in-gel" digestion was done as previously described [11], with slight modifications. In brief, protein spots of interest were cut out from CBB-stained 2-D PAGE gel. After reduction and carboxymethylation, the gel pieces were incubated with 0.01 μg/μl trypsin (Roche Diagnostics) in 50 mM NH_4HCO_3 buffer (pH 8.8) at 37°C overnight. Peptide fragments were extracted in 50% acetonitrile/5% formic acid and then concentrated with rotary evaporator. After desalting with Zip Tip (Millipore), the peptides were dissolved in 70% acetonitrile containing 10 mg/ml α-cyano-4-hydroxycinnamic acid and 0.1% trifluoroacetic acid. The resulting tryptic peptide mixtures were directly analyzed by matrix-assisted laser desorption ionization reflector time-of-flight mass spectrometry (MALDI/TOF-MS). Protein identification by MS data was accomplished using PeptideSearch software to query comprehensive sequence database on internet (MS-Fit, URL;http//prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). Other gel pieces were incubated with endoprotease Lys-C. Obtained peptide fragments were isolated on reversed-phase HPLC. One of the major fragments was subjected to automatic peptide sequencer.

Immunochemical Detection of GFAP on SDS (1-D) PAGE

Tissues were homogenized in 2% SDS, 50 mM Tris (pH 7.6), 2 mM EDTA, 2-mercaptethanol, and cocktail proteinase inhibitor (Complete™, Boehringer Mannheim) and then centrifuged at 16,000*g* for 15 min to separate supernatants (soluble fraction) and pellets (insoluble fraction). The pellets were resuspended in a volume of SDS buffer equivalent to the volume of the soluble fraction. Proteins were separated on a 5–15% polyacrylamide gradient slab gel and transferred to PVDF membrane. Ten micrograms of protein in 10 μl of sample buffer were applied to each lane of the gel. Following blocking with 5% skim-milk, the membrane was incubated with 10 μg/ml of goat anti-GFAP antibody (anti-N or anti-C terminal specific antibody, Santa Cruz Biotechnology, Incorporation) for 16 h at 4°C. After extensive washing, the membrane was incubated with peroxidase-labeled donkey anti-goat IgG antibody and developed with the ECL detection kit. Protein concentrations were determined with the Bradford method throughout this study.

RESULTS

The protein carbonyl content was elevated in the brain of the patient (1.20 nmol/mg protein) compared to controls (0.91 ± 0.15 nmol/mg protein, $n = 6$). Indirect western blot analysis using the anti-DNP antibody on 1-D PAGE revealed that many protein bands stained more noticeably in the patient than those in controls (data not shown). Immunoblotting in 2-D PAGE shown intense and broad signals around molecular weight 40 kDa in acidic pI region (arrow head in Fig. 1A and B). This protein spot was identified as GFAP by peptide mass fingerprinting and partial amino acid sequencing (Fig. 1C and D). Furthermore, immunoblotting using either N-terminal or C-terminal specific anti-GFAP antibodies could not detect significant signal in buffer soluble fractions (data not shown). Thus, the fragments in buffer soluble fraction might be corresponding to a middle portion of GFAP. In buffer insoluble fractions, however, both antibodies detected several bands with small molecular weight in addition to the intact 50 kDa band of GFAP in both

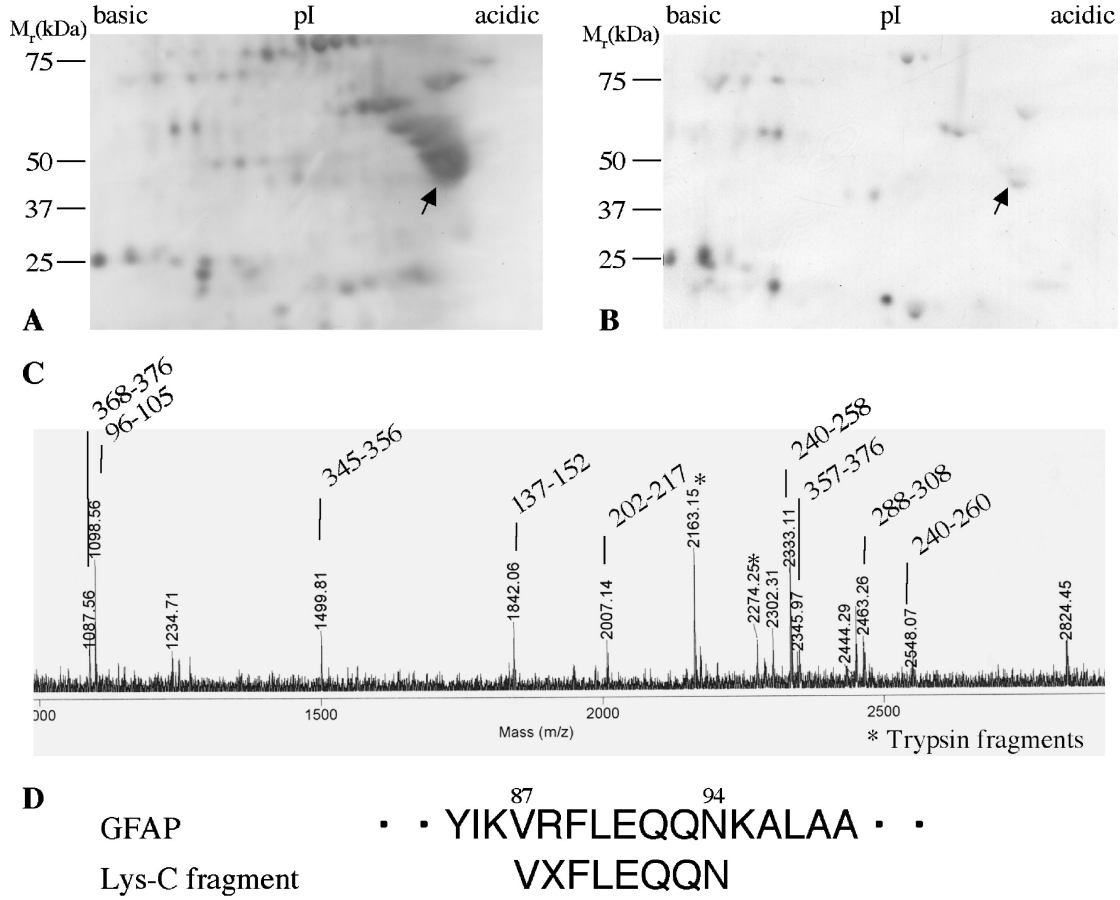

FIGURE 1 (A and B) Immunoblot of DNPH-labeled carbonylated proteins in 2-D PAGE from the patient (A) and a control (68-year-old female) (B). The arrows indicate the major carbonylated proteins around 40 kDa. (C) MALDI/TOF peptide-mass map obtained from the target protein spot in the homogenate of the cerebral cortex. Ion signals with measured masses are indicated. These signals match within 130 ppm the estimated masses of the protonated tryptic peptides of GFAP. (D) Partial amino acid sequence of the target protein. The sequence of one of the major fragments, the Lys-C fragment, is shown.

the patient and controls. The intact 50 kDa band of GFAP appeared to be reduced in the patient, while the set of smaller bands increased compared to controls. This might reflection enhanced fragmentation of GFAP by oxidative modification in the patient.

DISCUSSION

In this study, we used the cerebral cortex from the patient instead of the basal ganglia, where brain tissues were extensively destroyed [2]. In the cerebral cortex, iron overload was recognizable almost exclusively in astrocytes, but neuronal cell loss was much less prominent than the basal ganglia [2]. For this reason, these pathological changes are considered to reflect the early phase of the disease and, therefore, the cerebral cortex may be suitable to see the pathophysiological process preceding neuronal cell loss. Ceruloplasmin mainly localizes to astrocytes in the central nervous system [12] and is directly anchored to the surface of the astrocytes via a glycosylphosphatidylinositol anchor [13]. Lack of membrane-bound Cp cause intracellular iron overload in astrocytes by unknown mechanisms. Because of the Fenton reaction that generates hydroxyl radical depends on the presence of free ferrous iron, and hydroxyl radical is highly reactive and very short-lived, its concentration is likely to increase locally around astrocytes. In this situation, astrocyte components might be prone to be the targets of free radicals. GFAP is an intermediate filament specific to mature astrocytes, and maintains the structure and formation of astrocytes. Since astrocytes have protective and supportive effects on neurons, oxidative damage to GFAP may lead to dysfunction of astrocytes, which, in turn, causes neuronal cell death. One of the mechanisms of neuronal cell death that has been suggested is a decrease of glial cell derived neurotrophic factor.

In conclusion, GFAP is strongly modified by oxidative stress in the cerebral cortex of the aceruloplasminemia brain. This means that astrocytes, the major cell type of Cp production in the brain, may be primarily damaged by oxidative stress

in this disease. We speculate that the dysfunction of astrocytes may be causatively related to neuronal cell death in aceruloplasminemia.

Acknowledgements

We thank Prof. Sataro Goto for providing anti-DNP antibody. This work was supported by a Grant-in-Aid for Scientific Research (C) 12670599 from the Ministry of Education, Science, Sports, and Culture of Japan.

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