

The Autistic Phenotype Exhibits a Remarkably Localized Modification of Brain Protein by Products of Free Radical-Induced Lipid Oxidation

¹Teresa A. Evans, ¹Sandra L. Siedlak, ²Liang Lu, ³Xiaoming Fu, ³Zeneng Wang, ⁴Woody R. McGinnis, ⁵Evelyn Fakhoury, Rudy J. Castellani⁶, ³Stanley L. Hazen³, ⁵William J. Walsh, ⁵Allen T. Lewis, ²Robert G. Salomon, ¹Mark A. Smith, ^{1,7}George Perry and ¹Xiongwei Zhu

¹Departments of ¹Pathology and ²Chemistry, Case Western Reserve University, Cleveland, OH 44106;

³Departments of ³Molecular Cardiology and of Cardiovascular Medicine, Cleveland Clinic Foundation, Cleveland, Ohio 44195; ⁴Ashland, OR 97520; ⁵Pfeiffer Treatment Center, Health Research Institute, Warrenville, IL 60555; ⁶Department of Pathology, University of Maryland, Baltimore, MD 21201;

⁷College of Sciences, University of Texas at San Antonio, San Antonio, TX 78249

Abstract: Oxidative damage has been documented in the peripheral tissues of autism patients. In this study, we sought evidence of oxidative injury in autistic brain. Carboxyethyl pyrrole (CEP) and iso[4]levuglandin (iso[4]LGE)₂-protein adducts, that are uniquely generated through peroxidation of docosahexaenoate and arachidonate-containing lipids respectively, and heme oxygenase-1 were detected immunocytochemically in cortical brain tissues and by ELISA in blood plasma. Significant immunoreactivity toward all three of these markers of oxidative damage in the white matter and often extending well into the grey matter of axons was found in every case of autism examined. This striking threadlike pattern appears to be a hallmark of the autistic brain as it was not seen in any control brain, young or aged, used as controls for the oxidative assays. Western blot and immunoprecipitation analysis confirmed neurofilament heavy chain to be a major target of CEP-modification. In contrast, in plasma from 27 autism spectrum disorder patients and 11 age-matched healthy controls we found similar levels of plasma CEP (124.5 ± 57.9 versus 110.4 ± 30.3 pmol/mL), iso[4]LGE₂ protein adducts (16.7 ± 5.8 versus 13.4 ± 3.4 nmol/mL), anti-CEP (1.2 ± 0.7 versus 1.2 ± 0.3) and anti-iso[4]LGE₂ autoantibody titre (1.3 ± 1.6 versus 1.0 ± 0.9), and no differences between the ratio of NO₂Tyr/Tyr ($7.81 \text{ E-}06 \pm 3.29 \text{ E-}06$ versus $7.87 \text{ E-}06 \pm 1.62 \text{ E-}06$). These findings provide the first direct evidence of increased oxidative stress in the autistic brain. It seems likely that oxidative injury of proteins in the brain would be associated with neurological abnormalities and provide a cellular basis at the root of autism spectrum disorders.

Keywords: autistic disorder, oxidative damage, lipid peroxidation, carboxyethylpyrrole, iso[4]levuglandin E₂, heme oxygenase

INTRODUCTION

Autism, first described in 1943 by Kanner^[1] and officially recognized in 1980^[2], is a neurodevelopmental disorder characterized by decreased levels of social interaction, lack of imaginative play or language skills and increases in repetitive activities in children, usually presented before the age of 3^[2]. Autism and closely related pervasive developmental disorders (PDDs) are referred to as autism spectrum disorders (ASD) that corresponds to what the *Diagnostic and Statistical Manual of Mental*

Disorders (DSM-IV) refers to collectively as PDD. Approximately 80% of children with autism also have some degree of mental retardation and most do not reach independence as adults^[3]. The most recent epidemiological studies of autism report rates of up to one in 150 children with ASD^[4]. The need for a better understanding of this disease is heightened by the concern that autism is increasing in frequency.

Although there is no known unique cause of autism, a number of factors have been implicated in the pathogenesis of autism, including genetic, environmental, immunological and neurological. It is

Corresponding Author: Xiongwei Zhu, Departments of ¹Pathology and ²Chemistry, Case Western Reserve University, Cleveland, OH 44106

recognized that 5-10% of autistic spectrum disorders are classified with chromosomal abnormalities, and even greater numbers may be related to one or several identified susceptibility loci^[5]. There are high concordance rates in siblings of autistic probands, as well as in monozygotic twins^[6]. Hence it has now become generally accepted that autism has a biological basis.

Children with autism often present with abnormal immune and digestive systems, such as inflammation of the bowel, and changes in digestive enzymes, and often suffer from bowel problems and constipation, and occasionally liver problems^[7].

The cause of these changes is not entirely known. Similarly increased NO levels in red blood cells and higher antioxidant enzyme activity as well as zinc deficiency and copper excess in plasma from autistic children are noted^[8-10]. Treatment with vitamin C, carnosine, or vitamin B6 leads to significant improvement in autistic children compared with placebo^[11-13], and this may be a result of protection against oxidative injury.

Autism is apparently a disorder of prenatal and postnatal brain development. The gross brain changes reported include changes in brain volume in young children, later returning to normal volume, using head circumference measurements. Thus, very large differences between autistic and normal children are evident at early ages, but differences are not seen in adult cases. Decreased gray matter volumes are also sometimes noted^[14]. There is substantial evidence from neuroimaging studies that dysfunctions in the cerebellum and possibly the temporal lobe and association cortex result in autistic symptoms^[15]. There is a reduction in granule and Purkinje cell density in the neocerebellum^[16] and alterations in neuronal size, density and dendritic branching in the cerebellum and limbic structures of autistic individuals^[17]. Significantly fewer neurons in the autistic amygdala overall and in its lateral nucleus are noted^[18], suggesting neuronal loss occurs in more than one brain area. The corpus callosum is smaller, and neuroimaging suggests under connectivity of brain areas^[19]. However, it is unclear what causes these abnormalities in the disease.

In view of some evidence for elevated oxidative stress in peripheral tissues from autistic patients and the sensitivity of brain to oxidative injury, we postulated that oxidative stress plays a role in the brain abnormalities in autism. In this regard, impaired mitochondrial function in autism is suggested by magnetic resonance imaging that showed decreased ATP levels in autistic brain. Furthermore, elevated levels of lactate, pyruvate, and ammonia, and lower

levels of carnitine are documented in autistic children^[20]. Indeed, mitochondrial abnormalities, a potential source of elevated oxidative stress, were reported in autistic case studies^[21].

A major cause of damage to cells results from reactive oxygen species (ROS)-induced alteration of proteins and DNA by reactive electrophilic oxidation products from polyunsaturated fatty acyls in membrane lipids. Oxidative stress and ROS have been implicated in disease states such as Alzheimer's disease, Parkinson's disease, cancer, atherosclerosis, age-related macular degeneration (AMD), and aging. Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione peroxidase (GPx). SOD, MnSOD in the mitochondria and CuZnSOD in the cytoplasm, removes superoxide anion by converting it into hydrogen peroxide (H₂O₂). Catalase and GPx reduce H₂O₂ to water. In the presence of unbound Cu, under certain conditions, SOD can promote oxidative injury owing to a Cu catalyzed, Haber-Weiss, reaction of H₂O₂ to generate •OH, a potent ROS. Statistically significant elevations in ZnCuSOD were documented in erythrocytes and in platelets of autistic individuals compared with controls^[22]. Plasma concentrations of reduced glutathione (GSH) are generally lower (4.1 ± 0.5 µmol/L) for autistics compared with healthy controls (7.6 ± 1.4 µmol/L), *p* < 0.001^[23, 24]. These abnormally low levels of plasma GSH could favor oxidative stress because GSH is a cofactor for glutathione peroxidase (GPx), an antioxidant enzyme. Furthermore, severely depressed levels of GPx (-44.4%) are found in erythrocytes of autistic individuals compared with controls^[25, 26]. As a result, the H₂O₂ formed by the action of SOD would not be efficiently removed owing to diminished levels of GPx. The imbalance is exacerbated by the low plasma GSH levels found in ASD patients, because GSH is a cosubstrate needed for the GPx-promoted reduction of H₂O₂. Lipid peroxidation products in the urine are now considered a biomarker for autism^[27].

In this study, to substantiate the role of oxidative stress in autism brain, we used antibodies against two distinct lipid-derived oxidative protein modifications. One of these, carboxyethyl pyrrole (CEP) is derived exclusively from free radical-induced oxidative cleavage of docosahexaenoates, e.g., the docosahexaenoic acid (DHA) ester of 2-lysophosphatidylcholine (PC) DHA-PC, to afford HOHA-PC that then reacts with protein to generate CEP modifications of the ε-amino groups of lysyl residues (Fig. 1)^[28].

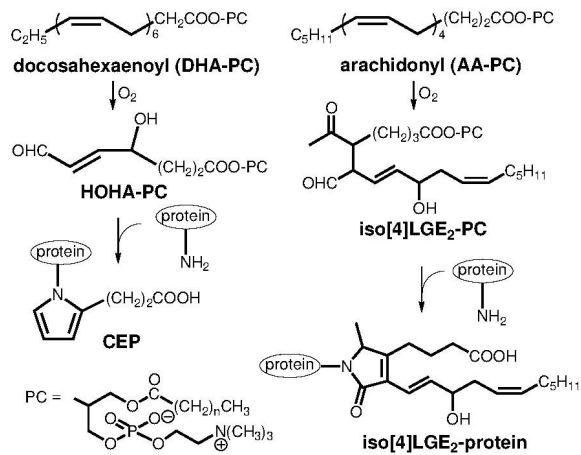


Fig. 1: Generation of CEP and iso[4]LGE₂-protein adducts.

Prominent elevations of CEP levels were noted previously in retinas of individuals with AMD. We also used antibodies against iso[4]LGE₂-protein adducts that arise exclusively through free radical-induced cyclooxygenation of arachidonates, e.g., the arachidonic acid (AA) ester AA-PC, and subsequent adduction of an intermediate iso[4]LGE₂-PC with protein (Fig. 1). Iso[4]LGE₂-protein adducts are indelible markers that provide a cumulative index for oxidative injury, such as that associated with inflammation^[29-32].

ASD seems to arise from environmental factors interacting with a genetic predisposition. It often occurs in conjunction with a family history of autoimmune diseases^[33]. As mentioned above, autistic patients often have immune abnormalities. Autoantibodies IgG, IgM, IgE and IgA against brain proteins are present in ASD patients^[34]. Inflammatory over-activation is suggested by the observation that many autistic children appear to produce excessive amounts of TNF α and other pro-inflammatory cytokines^[33]. IgG anti-brain autoantibodies were present in 27% of sera from children with ASD compared with 2% from healthy children. IgM autoantibodies were present in 36% of sera from children with ASD compared with 0% of control sera^[35]. In another study, sera from 40 healthy subjects and 40 autistic children were analyzed for the presence of IgG, IgM, and IgA antibodies against nine neuron-specific antigens and three encephalitogenic and cross-reactive proteins. Only 7.5-10% of controls had IgA, IgG or IgM antibodies against neurofilaments compared to 37.5%, 50% and 57.5% of autistic subjects^[28]. The possible operation of an immune response against altered self proteins, has not been examined. Our previous studies

demonstrated significantly elevated levels of CEP epitopes and the corresponding autoantibodies in the blood of AMD patients compared with healthy age and sex matched controls^[36]. We sought to determine whether levels of CEP epitopes and autoantibodies in the blood of ASD are also elevated as it is in blood from AMD patient.

We now report that CEP and iso[4]LGE₂-protein adducts in cortical brain tissues are a hallmark of autism, and more specifically, we demonstrated neurofilament heavy chain (NFH) to be a major protein target for CEP-modification. To our knowledge, this is the first direct evidence of increased oxidative stress in the autistic brain. We also compared levels of CEP and iso[4]LGE₂-protein adducts in plasma from patients with documented ASD with age-matched healthy controls. Anti-CEP, anti-iso[4]LGE₂-protein, autoantibodies were also measured. In addition, since recent studies proposed a possible participation of enhanced nitrate stress in AMD pathologies^[37], levels of protein bound nitrotyrosine in plasma from ASD and normal controls were compared in this study by stable isotope dilution tandem mass spectrometry. No significant elevations in the levels of any of these markers of oxidative injury were detected in the plasma of patients with ASD. However, an unanticipated strong elevation of plasma iso[4]LGE₂ immunoreactivity levels was found in ASD patients born prematurely compared with ASD patients with no birth events or other birth events.

MATERIALS AND METHODS

Brain Samples: Autopsy brain samples from children with autism were obtained from the University of Maryland Brain Bank. Control cases of similarly age matched as well as older ages were also sampled. Table 1 gives the case information. Initial studies were analyzed using an array of slides prepared from formalin-fixed tissue, consisting of 5 cases of autism and 5 controls, with small core samples blocked simultaneously of cerebellum, white matter and cortical area (a gift from Dr. Charles Eberhart from Johns Hopkins University, coordinated graciously through the Autism Tissue Program). After interesting results were obtained with these samples, blocks of larger cortical (Brodmann area 39) as well as hippocampal samples were analyzed (NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland). Also from this source, frozen tissue was obtained from one case of autism, which was compared with young control cases from the Case Western Reserve University Brain Bank for protein and biochemical analysis.

Immunohistochemistry: 6 μ m sections were deparaffinized in xylene and rehydrated in graded

alcohol, the endogenous peroxidase activity eliminated by incubation in 3% hydrogen peroxide in methanol for 30 min, and finally rinsed in Tris buffered saline. Sections were blocked in 10% normal goat serum (NGS) for 30 min followed by overnight incubation with primary antibody in 1% NGS at 4°C in a humidified chamber. Staining was completed using the peroxidase-anti-peroxidase procedure with 3,3-diaminobenzidine (DAB) as chromagen, and sections were dehydrated and mounted with permount. Antisera recognizing heme oxygenase-1 (Stressgen), CEP and iso[4]LGE₂-protein adducts (available in our laboratories) and monoclonal antibodies against neurofilament protein (Sternberger Meyer Inc.) were used. Antibody specificity for CEP was confirmed by performing an adsorption experiment with its corresponding antigen. Diluted antibody was incubated overnight with 20 µg of CEP-KLH and applied to an adjacent section with antibody alone. Omission of primary antibody was also performed as a negative control (data not shown).

Table 1: Cases used in this study

<i>Autism</i>	<i>Age</i>	<i>PMI (hr)</i>	<i>Cause of Death</i>	<i>Tissue</i>
1	5.6	39	Drowning	Cortex/Array
2	7.8	14	Multi-system organ failure	Frozen Cortex/Array
3	8	23	Drowning	Hippocampus
4	8.5	24	Drowning	Hippocampus /Array
5	8.8	16	Drowning	Hippocampus
6	9	39	Drowning	Hippocampus
7	9.3	13	Drowning	Cortex/Array
8	10	24	Smoke inhalation	Cortex/Array
9	12	23	Drowning	Hippocampus
<i>Control</i>	<i>Age</i>	<i>PMI (hr)</i>	<i>Cause of Death</i>	<i>Tissue</i>
1	1.8	20	Biliary atresia	Hippocampus
2	4	7	Congenital heart disease	Hippocampus
3	5	20	Drowning	Array
4	6	18	Accident/multiple injuries	Array
5	8	36	Head trauma/hemorrhage	Array
6	9	20	Asthma	Array
7	11	19	Drowning	Array
8	11	12	Leukemia	Hippocampus
9	14	6.5	Embryonic rhabdomyosarcoma	Hippocampus
10	17	15	Gunshot	Hippocampus
11	17	16	Drowning	Hippocampus
12	19	42	Sickle cell anemia	Hippocampus
13	31	4	Cystic fibrosis	Hippocampus
14	43	N/A	Anoxic encephalopathy	Hippocampus
15	64	2	Aortic hematoma	Hippocampus
16	68	5	Breast carcinoma	Hippocampus
17	72	N/A	Alzheimer	Hippocampus
18	83	37	Alzheimer	Hippocampus
19	87	7	Alzheimer	Hippocampus

Western blot analysis and immunoprecipitation:

Frozen cortical tissue samples were collected from one case of autism (age 8 years) and 1 controls (age 17 years). Tissue was homogenized in 10 vol. of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM PMSF (lysis buffer), assayed for protein concentration using BCA (Pierce), and proteins were separated using SDS gel electrophoresis and transferred to immobilon (Millipore). After blocking in 10% dry milk in TBS-tween, anti-CEP was applied overnight, the blots rinsed in TBS-tween 4 times for 5 minutes each and then incubated in peroxidase labeled secondary antibody. Detection was performed using enhanced chemiluminescence (Millipore).

For immunoprecipitation, the homogenate was precleared by incubating with Protein G-agarose (Roche) at 4°C for 2 hours, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. anti-CEP was added to the supernatant and incubated at 4°C for 4 hours with end-over-end rotation, followed by the addition of Protein G-agarose and incubated overnight. Following centrifugation at 10,000 rpm for 10 minutes at 4°C, the supernatant was carefully aspirated and discarded. The pellet was washed 4 times with lysis buffer, and the sample boiled for 10 minutes prior to SDS-PAGE. For immunoblot analysis of the precipitate, 10 µl of bead slurry was used for precipitation of 500 µg of protein homogenate. The entire precipitate was loaded in one lane, and proteins were separated by SDS-PAGE followed by transfer onto Immobilon-P (Millipore, Bedford, MA). SMI32 monoclonal antibody was used to detect NFH^[38].

Human plasma: Blood samples were obtained by phlebotomists at the Institute's Pfeiffer Treatment Center. Eligibility for the study was based on a diagnosis of ASD, as defined by DSM-IV. Subjects with questionable diagnoses and patients with comorbidity for seizures, and birth anoxia were excluded. In total, 27 autistic subjects and 11 healthy control subjects were recruited for the study. Plasma (5 mL) was collected in 7 mL lavender top vacutainer tubes with EDTA. The tube was then gently inverted 5-6 times to distribute the anticoagulant. After spinning in a refrigerated centrifuge (4°C) at 3000 rpm for 15 minutes, plasma (2.0 mL) was transferred (careful to avoid the buffy coat) into a 15 mL Falcon tube. A BHT/ethanol solution was prepared by combining BHT (0.04408 g) with ethanol (10 mL). If refrigerated, the BHT solution is good for 3 weeks. Then 10 µL of BHT/ethanol solution (5 µL/mL plasma) and 20 µL of

protease inhibitor cocktail kit (10 $\mu\text{L}/\text{mL}$ plasma, Sigma, Cat. P8340) were added to the plasma and mixed by gentle inversion (do not vortex) of the tube 5-6 times. The plasma was finally aliquoted (250 μL) into 8 prelabeled screw-top microfuge tubes that were gently flushed with argon and sealed with screw caps. The samples were then quench frozen in liquid nitrogen by placing the 8 vials into a Scienceware Round Bubble Rack (Fisher, Cat. 14-792-14 Bel-Art, fishersci.com) and then immersing the bottom of the rack and the tubes into liquid nitrogen in a Nalgene polyethylene Dewar flask for 1 minute. The vials were stored in Fiberboard Storage Boxes (Fisher, Cat. 11-678-24A, fishersci.com) with Fiberboard Box Dividers (Fisher, Cat. 11-678-24C, fishersci.com) in dry ice for transport or at -80°C .

ELISA: A competitive enzyme-linked immunosorbent assay (ELISA) of plasma from ASD and healthy controls was performed as described previously for CEP and iso[4]LGE₂ immunoreactivities^[28]. For CEP immunoreactivity, CEP-BSA was used as a coating agent and CEP-HSA was used as a standard. The initial concentration of CEP-HSA was 16.7 nmol/mL. For iso[4]LGE₂ immunoreactivity, iso[4]LGE₂-BSA was used as a coating agent and iso[4]LGE₂-HSA was used as a standard. The initial concentration of iso[4]LGE₂-HSA was 810 nmol/mL. A dilution factor of 0.2 was employed for standard and samples. Eight serial dilutions for standard, five serial dilutions of samples were performed.

A direct ELISA^[39] was performed for anti-CEP and anti-iso[4]LGE₂ autoantibodies. Absorbance values were measured on a Bio Rad Microplate Reader using dual wavelength (405 nm to read the plate and 650 nm as a reference). The plates were coated with CEP-BSA or iso[4]LGE₂-BSA (100 $\mu\text{L}/\text{well}$). BSA (2%, 100 μL) was added to the corresponding blank wells. The plates were incubated for 1 h at 37°C , washed with PBS (10 mM, 300 μL) 3 times, and blocked with 1% chicken egg ovalbumin (CEO, 300 μL) for 1 h at 37°C . After washing once with 0.1% CEO plus 0.05% Tween 20 (300 μL), the plates were loaded with plasma, diluted 20 times in 0.2% CEO plus 0.05% Tween 20, and then incubated for 1 h at room temperature. The plates were then washed 3 times with 0.1% CEO plus 0.05% Tween 20 (300 μL), and incubated 1 h at room temperature with alkaline phosphatase conjugated goat anti-human IgG (Sigma-Aldrich, Milwaukee, WI, Cat. Sigma A-8542), which was diluted 1:2000 with 1% CEO plus 0.05% Tween 20 (100 μL). After washing with 0.1% CEO (3 x 300 μL), a solution of *p*-nitrophenyl phosphate in 0.2 M Tris buffer (1.0 mg/mL, 100 μL ,

Sigma-Aldrich, Milwaukee, WI, Cat. Sigma N1891) was added. The absorbance was read at 405 nm with reference at 650 nm after incubation at room temperature for 30 minutes. The titer was defined as the ratio of plasma binding to antigen (A) vs. binding to BSA blank (A₀).

Protein hydrolysis: Plasma samples were delipidated and desalted using two sequential extractions with a single phase mixture of H₂O/methanol/H₂O-saturated diethyl ether (1:3:8 v/v/v). Oxidized tyrosine standards, ¹³C₆-ortho-tyrosine (o-Tyr), ¹³C₆-meta-tyrosine (m-Tyr), ¹³C₆-nitro-tyrosine (Nitro-Tyr), ¹³C₆-chloro-tyrosine (Cl-Tyr), ¹³C₆-bromo-tyrosine (Br-Tyr) (2 pmol each) and universal labeled tyrosine, ¹³C₉-¹⁵N-tyrosine (2 nmol) were added to protein pellets. Samples were hydrolyzed in degassed 4M methane sulfonic acid (500 μL) supplemented with 1% phenol for 24 h at 110°C under argon atmosphere. Amino acid hydrolysates were resuspended in 0.1% trifluoroacetic acid (2 mL) and applied to mini solid-phase C18 extraction columns (Supelclean LC-C18 SPE mini-column, 3 mL; Supelco, Inc., Bellefonte, PA) pre-equilibrated with 0.1% trifluoroacetic acid. Following sequential washes with trifluoroacetic acid (2 mL, 0.1%), oxidized tyrosines and tyrosine were eluted with 2 mL of 30% methanol in 0.1% trifluoroacetic acid, dried under vacuum, and then analyzed by mass spectrometry as described below. Synthetic [¹³C₆]-labeled 3-nitrotyrosine was used as internal standard for quantification of natural abundance 3-nitrotyrosine. Simultaneously, a universal labeled precursor amino acid, [¹³C₉, ¹⁵N₁]tyrosine, was added, permitting potential intrapreparative formation of nitro[¹³C₉, ¹⁵N₁]tyrosine to be routinely monitored and shown to be negligible (i.e., \ll 5% of the level of the natural abundance product observed). Results are normalized to the content of the precursor amino acid tyrosine (i.e., nitrotyrosine/tyrosine, micromoles/moles), which was monitored within the same injection.

Mass spectrometric analysis of nitrotyrosine:

Protein-bound nitrotyrosine in plasma from ASD and healthy controls was quantified by stable isotope dilution LC/MS/MS as described^[40]. Nitrotyrosine in lysates were analyzed by HPLC with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) using stable isotope dilution methodology on a triple quadrupole mass spectrometer (API 365; Applied Biosystems, Foster City, CA) with Ionics EP 10+ upgrade (Ionics, Concord, Ontario, CA) interfaced to a Cohesive Technologies (Franklin, MA) Aria LX Series HPLC multiplexing system. Samples

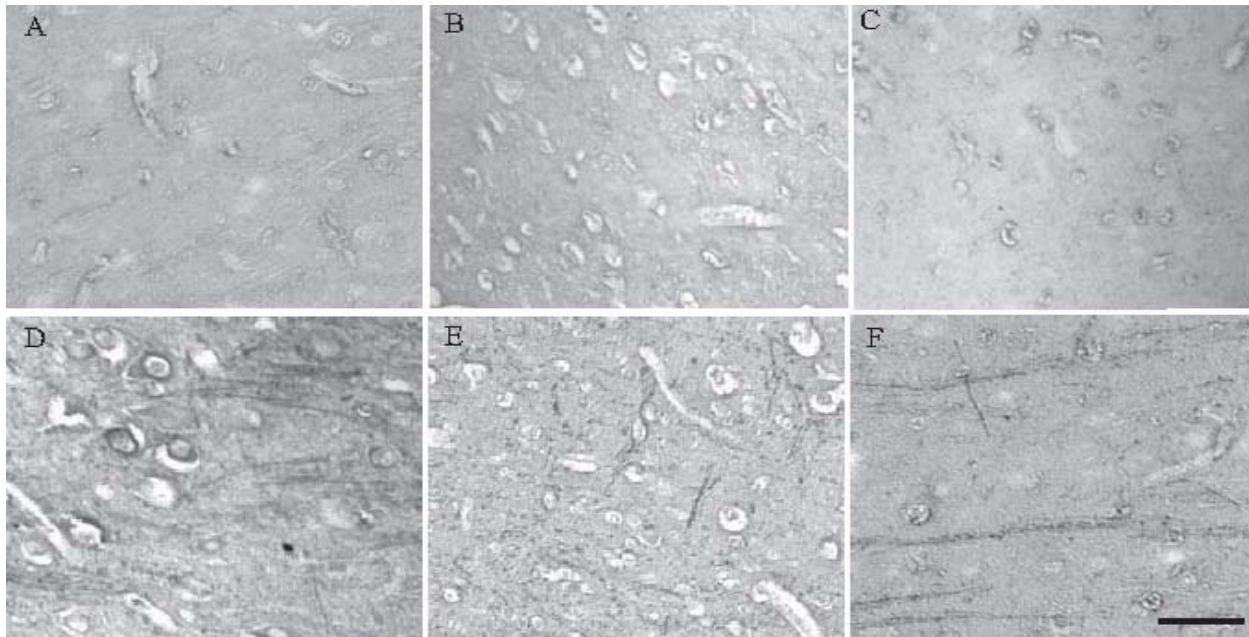


Fig. 2: CEP-modified protein is readily detected in the brains of all autism cases while no specific labeling is seen in controls. Representative cases of autism aged 9, 7, and 5 years (D, E, F) and controls aged 5, 6, and 11 years (A, B, C) are shown. In some autism cases, CEP is also present in neuronal cell bodies (D). Scale bar= 50 μ m.

were suspended in equilibration solvent (H_2O with 0.1% formic acid) and injected onto an Ultrasphere C18 column (Phenomenex, 5 μ m, 2.0 \times 150 mm). L-Tyrosine and its oxidation products were eluted at a flow rate of 0.2 mL/min using a linear gradient generated against 0.1% formic acid in acetonitrile pH 2.5, as the second mobile phase. Analytes were monitored in positive ion mode with full scan product ion MS/MS at unit resolution. Response was optimized with a spray voltage setting of 5 kV. The heated capillary voltage was set at 10 V and the temperature to 350°C. Nitrogen was used both as sheath and auxiliary gas, at a flow rate of 12 and 8 psi, respectively. The precursor ion isolation width was 1.0 and 3.0 for nitrotyrosine and tyrosine, respectively. The analyte abundance was evaluated by measuring the chromatographic peak areas of selected product ions extracted from the full scan total ion chromatogram, according to the corresponding ion trap product ion spectra. The ions monitored for each analyte were: 3-nitro[$^{12}C_6$]tyrosine (mass-to-charge-ratio (m/z) 227 \rightarrow 181); 3-nitro[$^{13}C_6$]tyrosine (m/z 233 \rightarrow 187); 3-nitro[$^{13}C_9$ $^{15}N_1$]tyrosine (m/z 237 \rightarrow 190).

The maximum ion injection time was 100 ms; a scan rate was used that permitted a minimum sampling rate

of at least 9 points/chromatographic peak. For all analyses, results were normalized to the content of the precursor amino acid L-tyrosine, which was monitored within the same injection of each oxidized amino acid.

Statistical analyses: ASD and normal controls were compared using CEP, iso[4]LGE₂ protein adducts, anti-CEP, anti-iso[4]LGE₂ antibodies and nitroTyr/Tyr ratio. P-values were calculated by independent t-test and analysis of variance (ANOVA) using Microsoft Excel 2003 for Windows. The significance of group comparisons was corrected for the effect of repeated measures. Correlations between measured parameters were ascertained using pair-wise comparisons by linear regression and were performed using JMP 5.12 (SAS Institute, Cary NC). Data were expressed as mean \pm SD. Statistical significance was defined as p less than 0.05.

RESULTS

The tissue array samples were very useful in obtaining preliminary data. Prominent staining by anti-CEP antibody was observed in all 5 cases of autism, and not in any of the control specimens. Fig. 2 shows representative cortical staining patterns from 3 of the

autism cases aged 9, 7, and 5 years (Fig. 2D, E, F) and 3 control cases aged 5, 6 and 11 years (Fig. 2A, B, C) from the tissue array samples. Significant labeling in the white matter and often extending well into the grey matter of axons was found in every case of autism examined. Often, cell bodies were also labeled (Fig. 2D). This pattern of staining was not seen in any control case, age-matched or even older cases. Thus, this pattern of staining is apparently a hallmark of autistic brain.

Strong staining in the white matter and grey matter in a representative autism case is readily apparent in the larger tissue specimens viewed at lower magnification (Fig. 3B) in contrast with a comparable section of control brain (Fig. 3A). Adjacent serial sections of another case of autism show that the cellular localization of CEP (Fig. 3C) is greatly reduced following adsorption with its antigen (Fig. 3D), confirming the specificity of the antibody. Other well characterized markers of oxidative damage are also localized to similar structures in many cases of autism. In autism brain, heme oxygenase-1 (Fig. 4E) and iso[4]LGE₂ (Fig. 4F) accumulates within the same processes as CEP- modified proteins (Fig. 4D), compared to control cases (Fig. 4A, B, C) stained for the same markers.

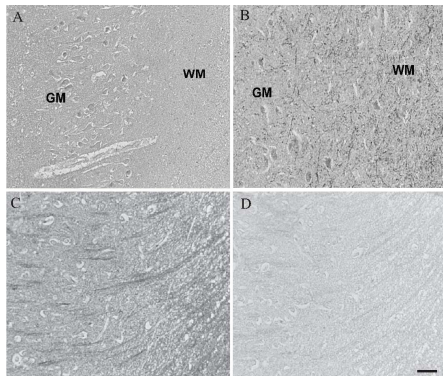


Fig. 3: At lower magnification, the accumulation of CEP-modified protein is readily apparent in the white matter and extends into the grey matter throughout the cortex in cases of autism (C). A similar area in an age-matched control case shows no CEP (A). Following adsorption with antigen, the staining is greatly reduced (D) when compared to the positive staining on an adjacent serial section (C). Scale bars= 50 μ m.

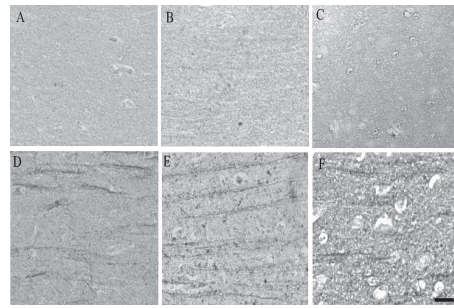


Fig. 4: The cellular processes in autism brain labeled with CEP-modified protein (D) also accumulate heme oxygenase-1 (E) and iso[4]LGE₂-protein adduct (F). These markers are not present in specific structures in any corresponding controls (CEP-A, heme oxygenase-1-B, iso[4]LGE₂-protein adduct-C). Scale bar = 50 μ m.

Using Western blot analysis, a band around 200 kDa is seen to contain CEP-modified protein (Fig. 5). In striking contrast, a 17 year control shows no CEP immunoreactivity. Given that anti-CEP demonstrated extensive staining of neuronal processes, we suspect that this 200 kDa band represented neurofilament heavy subunit (NFH). To further confirm the association of CEP and neurofilaments, an immunoprecipitation experiment was performed. The CEP antibody was used to immunoprecipitate CEP-modified proteins from autism brain homogenates. Probing the resultant blot with SMI32, an antibody against NFH, indeed shows an immunoreactive band at approximately 200 kDa, confirming that NFH is indeed a major target of CEP modification in autism brain (Fig. 5).

Levels of CEP and iso[4]LGE₂-protein adducts in human blood were measured by competitive ELISA using polyclonal anti-CEP and anti-iso[4]LGE₂-KLH antibodies respectively. We examined plasma from 27 patients with diagnosed ASD and 11 healthy controls with matched ages. There were no significant differences in the mean levels of CEP adduct between the plasma of ASD, 124.5 ± 57.9 pmol/mL, and the plasma of age-matched healthy controls, 110.4 ± 30.3 pmol/mL, $p = 0.45$. Nor were there statistically significant differences in the mean levels of iso[4]LGE₂ protein adduct between ASD, 16.7 ± 5.8 nmol/mL, and controls, 13.4 ± 3.4 nmol/mL, $p = 0.088$.

Anti-CEP autoantibody titer in plasma from 26 ASD and 5 healthy controls was measured by ELISA. There were no significant differences in the mean levels of anti-CEP autoantibody titer between the plasma of ASD, 1.2 ± 0.7 , and the plasma of age-matched healthy controls, 1.2 ± 0.3 , $p = 0.9$. Nor were there statistically

significant differences in the mean levels of anti-iso[4]LGE₂ autoantibody titer between ASD, 1.3 ± 1.6 , and controls, 1.0 ± 0.9 , $p = 0.6$.

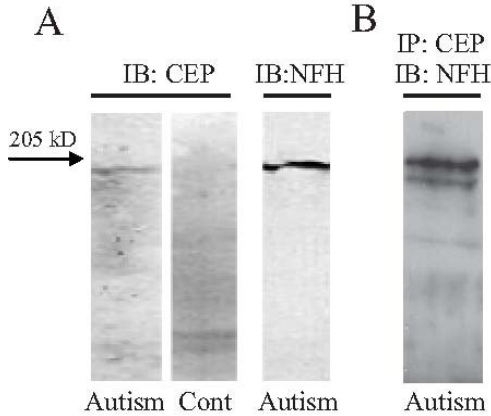


Fig. 5: Western blot analysis shows CEP-modified protein in autism and not in control brain, corresponding to the band recognized by a monoclonal antibody to NFH. Further confirming the association with NFH, following immunoprecipitation with anti-CEP antibody, the blot was probed with SMI32 antibody specific for NFH.

A strong correlation exists in CEP and iso[4]LGE₂-protein adduct autoantibody titer (Fig. 6), $R = 0.76$, $p < 0.001$. Fig. 7 shows differences in levels of lipid oxidation protein adducts, grouped according to birth events of ASD patients with no events, born premature or other events such as C-section, cord wrapped and low birth weight.

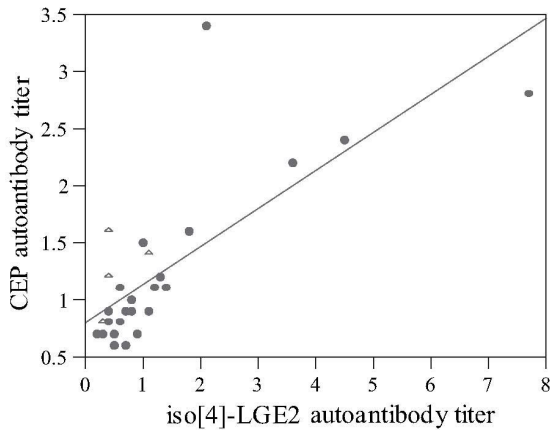


Fig. 6: Correlation between CEP and iso[4]LGE₂-protein adduct autoantibody titer in human plasma from ASD (λ , $N = 26$) and normal controls (Δ , $n = 5$), $R = 0.76$, $p < 0.001$.

A number of inflammatory and neurodegenerative disorders have been linked with protein modification by reactive nitrogen species including ocular inflammation, atherosclerosis, retinal ischemia, lung infection, cancer, Parkinson's disease, and Alzheimer's disease^[20]. Measurement of NO₂Tyr, a posttranslational modification of proteins generated by reactive nitrogen species, serves as a quantitative index of nitrative stress in vivo. In this study, there were no differences between the ratio of NO₂Tyr/Tyr in plasma from 27 ASD patients ($7.81 \text{ E}^{-06} \pm 3.29 \text{ E}^{-06}$) and that from 11 healthy controls ($7.87 \text{ E}^{-06} \pm 1.62 \text{ E}^{-06}$) (data not shown).

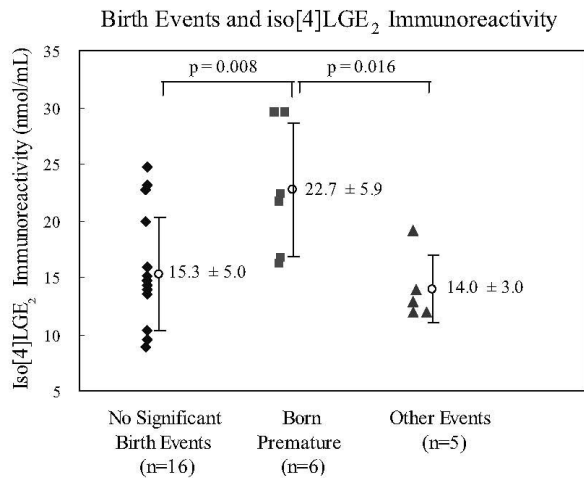


Fig. 7: Iso[4]LGE₂-protein adduct immunoreactivity grouped according to birth events of ASD patients.

DISCUSSION

There is ample evidence of oxidative injury in autistic peripheral tissues^[41, 42], however, there is little direct evidence for oxidative injury in the autistic brain. It is increasingly recognized that autism is primarily a neurodevelopmental disorder^[16, 18, 20] which may involve substantial neuronal loss in more than one brain regions^[43, 44]. Given that oxidative stress is implicated in neurodegeneration in many neurodegenerative diseases^[45] and that the developing brain appears at increased risk for oxidative damage because of an immaturity of antioxidant defenses^[46], it is of major impact to determine whether there is oxidative injury in autistic brain. In this study, we present experimental evidence of oxidative damage in the autistic brain, primarily in the white matter, in the form of increased levels of lipid derived oxidative protein modifications,

i.e., CEP and iso[4]LGE₂-protein adducts, and heme oxygenase-1.

Lipid peroxidation is a well-established mechanism of cellular injury induced by elevated oxidative stress which results in the production of lipid peroxides and their byproducts that lead to the loss of membrane functions and integrity. In view of the high omega-3 poly unsaturated fatty acid (PUFA) content of the brain, it is likely that these fats participate in brain biochemistry, physiology and functioning; and may play a role in some neuropsychiatric diseases and ageing. However, the measurement of peroxidation products of free PUFA such as MDA, HNE or lipid hydroperoxides, is limited by the relative instability of the analytes and their ready formation *ex vivo*^[47]. We recently developed and characterized a specific method to detect CEP adducts as a reliable and sensitive dosimeter for local oxidative damage which was successfully applied to the study of oxidative damage in AMD^[48, 49]. CEP adducts are uniquely generated from only the oxidation of docosahexaenoate (DHA)-containing lipids. DA is the most oxidizable fatty acid in humans. It is a minor lipid in most tissues but rich in specific regions of the brain and retina^[50, 51]. Therefore, the production and accumulation of CEP-protein adducts might be especially sensitive to oxidation and important in pathological processes in these tissues. The most noteworthy result of the present study is the discovery that significant CEP-staining is found in the white matter in every autism cases but is absent in any of the control cases (age-matched or older) examined, suggesting elevated oxidative damage in these brain regions in autism. Obviously, oxidative damage in white matter in autistic brain involves more than DHA-containing lipid since there is also elevated level of iso[4]LGE₂-protein adducts, an oxidative protein modification derived from arachidonate-containing lipids. More importantly, the notion of elevated oxidative stress in autistic brain is further supported by the increased expression of heme oxygenase-1, a widely used oxidative stress marker in various neurodegenerative diseases^[19]. The induction of heme oxygenase-1, an inducible antioxidant enzyme, is likely a response to elevated oxidative stress, which indicates that the elevated oxidative damage as evidenced by increased CEP and iso[4]LGE₂-protein adducts elicit functional consequences, e.g., by inducing an antioxidant response.

One particular interesting aspect of our finding is that these three oxidative stress markers demonstrate similar staining patterns with white matter being most intensely labeled. The striking threadlike pattern appears to be a hallmark of the autistic brain. White matter consists primarily of axons serving to connect different sections of the brain. In fact, anatomical

studies revealed abnormal alterations in white matter in autistic brain: depending on specific regions, the white matter abnormalities in autism include smaller white matter volumes in some regions such as corpus callosum^[52, 53] but also dysregulation and larger white matter volumes in other regions such as cerebellum^[54-58]. A more detailed and systematic study on CEP modification in autistic brain is thus warranted. Nevertheless, it is of interest to note that reduced functional connectivity, i.e., the degree of synchronization or correlation of the time series of the activation between the various participating cortical areas, appears to be a general characteristic of the neurobiology of neural system in autism since it occurs in executing tasks involving reasoning, language, and social judgment^[40], all the major symptom domains that define the syndrome of autism. The proximal biological causes of functional underconnectivity could be either in grey matter or white matter or both. Our finding suggests that oxidative damage to white matter may potentially contribute to the underfunctioning or disorganization of white matter tracts which could underlie the underfunctioning of the inter-regional communication process that make use of these tracts and result in functional underconnectivity.

In contrast to the brain, similar levels of CEP and iso[4]LGE₂-protein adducts and autoantibodies against them were detected in blood from both autistic individuals and normal controls. However, a subpopulation consisting of patients born prematurely showed significantly elevated iso[4]LGE₂-protein adduct immunoreactivity levels compared with patients with no birth events or other birth events. The relevance of this finding to the pathogenesis of autism is unclear. The possible generality of the phenomenon of elevated plasma iso[4]LGE₂-protein adduct levels does warrant further investigation as a heretofore unrecognized consequence of premature birth or postnatal treatment protocols. Another interesting observation, that has no evident relevance to the pathogenesis of autism, is a strong correlation between anti-CEP and anti-iso[4]LGE₂-protein adduct autoantibody titers in plasma from 26 ASD patients and 5 normal controls. This suggests that the proclivity of an individual to develop an immune response to altered self protein is similar for a diverse structural array of immunogenic modifications. A number of inflammatory and neurodegenerative disorders that have been linked with protein modification by reactive nitrogen species including ocular inflammation, atherosclerosis, retinal ischemia, lung infection, cancer, Parkinson's disease, and Alzheimer's disease^[50, 51]. We used tandem mass spectrometry to measure plasma levels of protein nitrative products (nitrotyrosine) in plasma. We found no significant differences between ASD and controls.

The exact mechanism of oxidative stress in the autism brain is not known. It may be due to increased production of pro-oxidants or neuroinflammation. Even though there is induction of heme oxygenase-1, a deficient antioxidant response could not be excluded at this point since it is possible that induction of antioxidant enzymes may not be sufficient to balance the elevated oxidative threats as is the case in AD^[59, 60]. Elevations of peripheral brain-derived neurotrophic factor BDNF^[61], which exerts potentially protective anti-oxidant influences in brain may represent another homeostatic reaction to excess oxidative stress during neurodevelopment.

Overall, it seems highly likely that oxidative injury of proteins in the brain would be associated with neurological abnormalities. Therefore, the development of an animal model of the autistic phenotype should aim to replicate the pervasive, characteristically localized, oxidative protein modifications that we have uncovered in the present investigation. Our discovery of these protein modifications in autistic brain not only support the notion that brain oxidative stress plays a role in autism, but also suggest an area of focus for future in-depth mechanistic studies on the potential involvement of NFH oxidative modification in the pathogenesis of under connectivity in autistic brain. An increased understanding of the extent and mechanisms of oxidative stress within the brain is needed, and is likely to lead to improvements in the treatment of autism.

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