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Research Paper

High membrane protein oxidation in the human cerebral cortex

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ABSTRACT

Oxidative stress is thought to be one of the main mediators of neuronal damage in human neurodegenerative disease. Still, the dissection of causal relationships has turned out to be remarkably difficult. Here, we have analyzed global protein oxidation in terms of carbonylation of membrane proteins and cytoplasmic proteins in three different mammalian species: aged human cortex and cerebellum from patients with or without Alzheimer's disease, mouse cortex and cerebellum from young and old animals, and adult rat hippocampus and cortex subjected or not subjected to cerebral ischemia. Most tissues showed relatively similar levels of protein oxidation. However, human cortex was affected by severe membrane protein oxidation, while exhibiting lower than average cytoplasmic protein oxidation. In contrast, ex vivo autooxidation of murine cortical tissue primarily induced aqueous protein oxidation, while in vivo biological aging or cerebral ischemia had no major effect on brain protein oxidation. The unusually high levels of membrane protein oxidation in the human cortex were also not predicted by lipid peroxidation, as the levels of isoprostane immunoreactivity in human samples were considerably lower than in rodent tissues. Our results indicate that the aged human cortex is under steady pressure from specific and potentially detrimental membrane protein oxidation. The pronounced difference between humans, mice and rats regarding the primary site of cortical oxidation might have contributed to the unresolved difficulties in translating into therapies the wealth of data describing successful antioxidant neuroprotection in rodents.

Introduction

Oxidative stress and redox dysregulation are biochemical characteristics of aging and many neurodegenerative disorders [1–4]. For instance, increased steady-state levels of oxidative end products have been reported for the majority of neurodegenerative diseases. However, only few of the clinical studies pursuing antioxidant regimens have ended with a positive outcome, and the benefit was usually modest [2,5]. Thus, it is possible that some of the functionally relevant targets of oxidation in these diseases are still to be discovered.

The potential biochemical victims of redox failure in the aged or injured human brain are diverse. Results from post-mortem studies have indicated that in Alzheimer's disease, above-normal levels of oxidation in proteins can occur on soluble proteins as well as protein filaments and aggregates [6]. Various side chains as well as the protein backbone may be affected. Specifically, protein tyrosine nitration [7], dityrosine formation [7], glycoxidation [8] and protein carbonyl accumulation [9,10] have been described. Moreover, significant damage to nucleic acids has been demonstrated [10–12]. Notably though, effect sizes in many of these investigations have been relatively small [9,10].

Integral membrane proteins have rarely been studied as separate entity in brain protein oxidation studies, despite the fact that various observations indicate a special role for these proteins as targets of oxidation. For instance, membrane proteins of the inner mitochondrial membrane massively accumulate the antioxidant amino acid methionine as a means of autoprotection [13], whereas other redox-active amino acids are avoided in an oxygen usage-dependent manner [14,15]. In senescence accelerated-prone mice, spin labeling studies have indicated a selective, oxidative stress-induced alteration of synaptosomal membrane protein structure [16]. Moreover, model studies in vitro [17] and in cultivated cells [18,19] have suggested the occurrence and relevance of one-electron transfer reactions from within the lipid bilayer onto the

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lateral surfaces of the embedded membrane proteins. After all, the fact that only lipophilic antioxidants show relevant neuroprotective capacity in cell culture [19–21] might not only be due to their selective prevention of lipid peroxidation, but also due to their selective prevention of integral membrane protein oxidation. Hence, we have investigated the extent of membrane protein oxidation in neural tissues from mice, rats, and humans, in order to achieve a basic characterization of its occurrence in the central nervous system.

Materials and methods

Human Alzheimer's disease tissue

Post-mortem tissue samples from neocortex and cerebellum of three non-demented individuals (Hu1–Hu3) and three Alzheimer's disease patients (Hu4–Hu6) were obtained from the Brain Bank of the Paul Flechsig Institute for Brain Research at the University of Leipzig. The diagnosis of definite Alzheimer's disease was based on the presence of neurofibrillary tangles and neuritic plaques in hippocampus and neocortex and met the criteria of the National Institute on Aging (NIA) and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [22]. All cortical tissues were from Brodman area 22 of the temporal lobe. Alzheimer's disease cases were Braak stage V/VI, controls were either free of discernible neuropathological changes or possessed only a few isolated neurofibrillary tangles [23]. Terminal cause of death was bronchopneumonia in all cases. Further diagnoses and post mortem intervals (PMI) were: Hu1: age 78 y, male, PMI 24 h; nicotine abuse, arteriosclerosis. Hu2: age 84 y, female, PMI 36 h; chronic alcoholism. Hu3: age 82 y, male, PMI 46 h; arteriosclerosis. Hu4: age 69 y, male, PMI 40 h. Hu5: age 64 y, female, PMI 32 h. Hu6: age 88 y, female, PMI 48 h. Regarding the potential influence of the additional diagnoses in the non-demented individuals on brain protein oxidation, no data from human samples seem to exist. Consideration of plasma markers indicates that chronic alcohol abuse might be associated with elevated protein oxidation, whereas smoking appears to have a minor effect at most [24]. In rodent models, all conditions have been linked with variable manifestations of oxidative stress in the periphery. However, major effects on the brain have not been reported.

Mouse aging cohort

A cohort of C57BL/6J mice was established and aged under standard animal care conditions. After weaning, male and female animals were caged separately in groups of maximum 5 littermates per cage, with free access to food and water, residing in temperature-, humidity-, and light-controlled rooms (12 h light/dark cycle). Young (Ms1–Ms3) and old (Ms4–Ms9) mice were caged individually 1 week before sacrifice by cervical dislocation. The removed brains were flash-frozen in isopentane/dry ice, wrapped in parafilm, individually placed in small plastic boxes and thereupon stored at −80 °C. To match the human tissues analyzed, cortex and cerebellum were chosen for the determination of membrane protein oxidation. Animal parameters were: Ms1: age 152 d, male, weight 27 g. Ms2: age 150 d, male, weight 30 g. Ms3: age 150 d, female, weight 27 g. Ms4: age 651 d, male, weight 34 g. Ms5: age 642 d, female, weight 26 g. Ms6: age 808 d, female, weight 28 g. Ms7: age 794 d, male, weight 34 g. Ms8: age 651 d, female, weight 27 g. Ms9: age 651 d, male, weight 34 g.

Bilateral carotid artery occlusion (BCAO) in rats

BCAO with hemorrhagic hypotension was applied to male, 10–12 week old Sprague–Dawley rats as described [25]. After 2 h, the animals were sacrificed, and the anatomical regions of interest were dissected and frozen at −80 °C. As only the hippocampus is selectively affected by cell loss in this model of cerebral ischemia, cortex and hippocampus (in lieu of cerebellum) were further processed for biochemical analysis. Non-ischemic, naive rats (R1–R3) of the same cohort were used as controls for the cerebral ischemia rats (R4–R7).

Membrane protein preparation

Human, mouse and rat brain samples were fractionated by differential centrifugation. Samples of ~100–200 mg were homogenized on ice with a Potter-Elvehjem apparatus in 6 volumes of buffer A, containing 5 mM TRIS pH 7.4, 1 mM EDTA, 1 mM DTT, 10 μM phenothiazine, and 1 × protease inhibitor cocktail (Sigma-Aldrich). Following 60 passages through a 25-gauge needle and brief sonication (3 × 5 s), the homogenate was mildly centrifuged (800*g* for 10 min at 4 °C) to remove any protein aggregates and insoluble material. The supernatant was then ultracentrifuged (100,000*g* for 90 min at 4 °C) to generate membrane (pellet) and cytoplasmic (supernatant) fractions, which were quality controlled by Western blotting against the marker proteins Na^+/K^+-ATPase (ATP1A1) and superoxide dismutase (SOD1) using monoclonal antibodies (anti-Na^+/K^+-ATPase α1 subunit from Novus Biologicals, 1:1000; anti-SOD1 from Epitomics, 1:1000). A selection of human and rat brain samples were also fractionated by phase separation with Triton X-114 [26], yielding similar results. Protein contents of all obtained fractions were determined using the bicinchoninic acid method (Pierce), which was compatible with the employed buffer and reagent concentrations.

Protein carbonyl immunoblotting

Protein carbonyls were quantified by derivatization with 2,4-dinitrophenyl hydrazine (DNPH). Subcellular fractions containing 8 μg protein were adjusted to identical volume with buffer A, supplemented with 0.5 volumes of buffer B (180 mM TRIS pH 7.4, 30% sucrose, 6% SDS), mixed, and incubated with 0.5 volumes of freshly prepared DNPH derivatization solution (10 mM DNPH in 2 M HCl) for 20 min at room temperature. After neutralization with 0.33 volumes of 3 M TRIS base, the samples were supplemented with 0.33 volumes of loading buffer (200 mM TRIS pH 6.8, 40% glycerol, 20% β-mercaptoethanol, 8% SDS, 0.04% bromophenol blue), separated by SDS-PAGE in a 10% gel (acrylamide:bis-acrylamide 29:1) and transferred onto nitrocellulose membranes following standard protocols. After 1 h incubation with 5% fat-free dry milk in TBST, the membranes were probed with anti-2,4-dinitrophenyl (anti-DNP) antibody (1:1000; from Invitrogen) in TBST at 4 °C overnight. The membranes were washed three times with TBST, incubated for 1.5 h with peroxidase-conjugated donkey anti-rabbit antibody (1:10,000; from Jackson Immunoresearch) at room temperature, washed three times with TBST again, and visualized by enhanced chemiluminescence. Ponceau staining (0.1% Ponceau S in 5% acetic acid) of the blots was performed as loading control.

Anti-isoprostane enzyme immunoassay

The levels of anti-isoprostane immunoreactivity were determined by competitive enzyme immunoassay (Cayman Chemical), employing an antiserum that has been shown to be specific for 8-iso prostaglandin $F_{2\alpha}$ against a considerable number of likely competitors (http://www.caymanchem.com/catalog/516351). Still, it cannot be excluded that it may have cross-reacted with oxidized, isoprostane-like derivatives of fatty acids other than arachidonic acid, particularly docosahexaenoic acid, which is an abundant, but