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Oxidative modification of proteins in the frontal cortex of Alzheimer's disease brain

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Abstract

There is a large body of evidence highlighting the importance of oxidative stress in the pathogenesis of Alzheimer's disease (AD). We have previously standardised a method that can be applied to study oxidative changes in individual brain proteins by using two-dimensional oxyblots (Korolainen MA, Goldsteins G, Alafuzoff I, Koistinaho J, Pirttilä T. Proteomic analysis of protein oxidation in Alzheimer's disease brain. Electrophoresis 2002;23(19):3428–33). Here we have identified proteins that exhibited oxidative changes in AD when compared to age-matched controls and these protein changes have been further examined in relation to the neuropathological data. Indeed, several Tris–HCl soluble proteins tended to be less oxidised in AD when compared to controls. Two enzymes, mitochondrial glutamate dehydrogenase and cytosolic malate dehydrogenase, were increased in amount but showed significantly decreased degree of oxidation in AD brains when compared to controls. Furthermore, some changes related to the amounts or oxidation statuses of proteins were associated with the duration of the clinical impairment and also with the neuropathology. These results do not contradict the hypothesis of increased oxidative stress in AD but may represent co-existing compensatory changes in response to oxidative stress.
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1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly [22]. The classical neuropathological hallmarks of AD brains are extracellular deposits of beta-amyloid (βA) containing plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. However, these changes are not specific for AD and have also been reported in brains from cognitively unimpaired individuals [9,19]. Moreover, the relationship between amyloid plaques, NFTs and neuronal cell loss has remained unclear. Many recent studies have indicated that the pathogenesis of AD is a complex and heterogeneous process. In addition to the formation of amyloid plaques and NFTs; gliosis, chronic inflammatory reactions, excitotoxic damage and oxidative stress all appear to contribute to the progression

Abbreviations: AD, Alzheimer's disease; AAT, aspartate aminotransferase; βA, beta-amyloid; CA, carbonic anhydrase; 2D, two-dimensional; 2-DE, two-dimensional gel electrophoresis; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenyl hydrazine; NFT, neurofibrillary tangle; GDH, glutamate dehydrogenase; GFAP, glial fibrillary acidic protein; MDH, malate dehydrogenase; ROS, reactive oxygen species

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of AD. Thus, the cause(s) of the progressive cell death and loss of memory and cognitive functions resulting in profound dementia are still poorly understood.

Recent studies suggest that abnormal protein oxidation may play an important role in AD. Exposure to reactive oxygen species (ROS) is unavoidable when an organism exists in an aerobic environment. Therefore, ROS pose as a constant threat to proteins, RNA, DNA and lipids. Oxidative stress becomes harmful when the production of ROS exceeds the cell's antioxidant capacity. Increased oxidative stress during aging may lead to accumulation of oxidised proteins [6]. As a consequence, proteins become resistant to proteosomal degradation. On the other hand, oxidative stress is also known to enhance protein turnover accompanied by a specific removal of oxidised proteins [59]. The removal of oxidised proteins has been considered to be impaired during aging due to decreased proteosomal activity [58]. Therefore, increased oxidative stress may lead to alterations in both oxidative modification and turnover of proteins. There is a growing body of evidence indicating that oxidative damage contributes to the early pathogenesis of AD [29,47,53]. Oxidative damage has been associated with aggregation of proteins, calcium dysregulation, mitochondrial malfunction, chronic inflammation, altered antioxidant function and accumulation of redox-active metals [5,10,25,61]. Furthermore, it has been claimed that there is a link between oxidative stress, the levels of cholesterol, and the formation of βA plaques [51].

ROS causes an oxidation of amino acid residue side-chain hydroxyls, converting them into ketone or aldehyde derivatives [6]. Thus, measurement of these carbonyl groups is one way to assess the extent of oxidation of proteins. In AD, several proteins have been identified as targets of oxidative stress. Immunohistochemical studies have described an increase in carbonyl formation in AD brain [60]. However, in that study, no oxidatively modified proteins were actually identified. Other workers have detected a band of oxidised protein at 78 kDa on 1D oxyblots in AD plasma [18]. A recent study revealed that several isoforms of fibrinogen α-chain precursor protein and of α-1-antitrypsin exhibited a greater specific oxidation in AD plasma [17]. Furthermore, cytosolic creatine kinase BB isoform, β-actin, glutamine synthase, ubiquitin carboxy-terminal hydrolase L-1, dihydropyrimidinase-related protein 2 and alpha-enolase are also known to be targets of protein oxidation in AD brain [1,2,12,13].

Two-dimensional gel electrophoresis (2-DE) offers an efficient tool to simultaneously separate and quantify pathological changes in proteins in complex biological samples. Posttranslational modifications such as phosphorylation and oxidation of proteins can be identified by 2D immunoblotting. We have previously standardised a 2D oxyblot method and used it to reveal changes in the oxidation status of soluble proteins in the frontal cortex of AD patients and age-matched controls [31]. In this study, we have further identified the proteins exhibiting significant changes in their carbonyl content and analysed their correlation with the neuropathological data.

2. Materials and methods

2.1. Human brain samples and neuropathology

The post-mortem tissue was obtained from the Kuopio Brain Bank, Kuopio University Hospital. All patients were hospitalised prior to death and evaluated clinically. Frontal cortices of AD patients were selected based on the severity of dementia and the absence of histological changes other than those related to AD. Prior to death, a neurologist had investigated the patients, and the clinical diagnosis of AD was based on the NINCDS-ARDRA [42] and the DSM-III-R criteria (American Psychiatric Association 1987). Frontal cortices of control cases were selected based on age-matching. Further, the retrospective evaluation of medical records did not reveal any signs of cognitive impairment or other neurological deficits. A total of 19 patients were available for the analysis, these being 10 AD patients (age = 83.3 ± 2.4) and nine age-matched controls (age = 82.0 ± 2.4). Seven of the AD patients and one of the controls carried one or two copies of the apolipoprotein E ε4 allele [26,64]. Patient demographics and clinical information are given in Table 1.

According to the dissection protocol used in the Kuopio University Hospital, the brains were weighed, evaluated for grossly detectable lesions and vessel abnormalities. The right hemisphere was fixed in 10% buffered formalin for at least 1 week and cut in coronal slices of 1 cm thickness. Brain specimens were taken from 15 standard cortical and subcortical regions, embedded in paraffin and cut into 7 μm-thick sections that were stained routinely applying haematoxylin and eosin and modified Bielschowsky silver impregnation. Further, additional immunohistochemical stainings were applied [33,35,49,50,52]. For electrophoreses, tissue samples were obtained from the left hemisphere, frontal cortices (Broadman area 9). The fresh brain specimens were stored immediately after dissection at −70 °C until use.

For the assessment of AD pathology, subjects were classified into neuropathological groups as recommended by the guidelines established by Braak and Braak [8] and by Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [43]. All AD patients fulfilled the histopathological criteria of AD proposed by CERAD and they all had numerous NFTs in the frontal cortex, i.e. the isocortical stage of the disease according to Braak and Braak stages [8]. The age-matched controls did not show any NFTs in frontal cortex, i.e. stages I–IV [8,9] according to Braak and Braak and they were all classified as normals (a or b) according to CERAD criteria.

2.2. Quantitative pathology and immunohistochemistry

The βA aggregates, glial fibrillary acidic protein (GFAP) and HLA DR expression in the grey matter were visualized using immunohistochemical methodology on sections from frontal cortices [33,52]. In our laboratory, immunohistochemical methods for the detection of βA aggregates,

Table 1
Patient demographics

	Clinical diagnosis	ApoE ε4 allele	Age at onset (years)	Age at death (years)	Duration of CI (years)	MMS	Cause of death	Brain weight (g)	PMD (h)	Detected pathologies			
										Tau (Braak)	βA (fraction)	α-synuclein	Vascular
1	Control	–		83		24	Cor insufficiency[a]	1245	31	1	1.3	–	+
2	Control	–		75		28	Cor infarct	1500	10	1	0.4	+	–
3	Control	–		79		26	Cor infarct	1660	24	1	0.0	–	–
4	Control	–		76		29	Cor insufficiency	1295	3	2	0.0	–	–
5	Control	–		84		NA	Cor infarct	1100	5	4	1.6	–	–
6	Control	+		76		NA	Cor infarct	1300	22	1	3.5	–	+
7	Control	–		84		NA	Cor infarct[b]	1130	7	1	0.0	–	–
8	Control	–		83		26	Cor infarct	1550	8	1	4.3	–	–
9	Control	–		98		NA	Cor insufficiency[c]	1115	9	2	0.0	–	+
10	AD	–	68	76	8	0	Pneumonia	1055	6	6	1.0	++	–
11	AD	+	66	75	9	0	Pneumonia	1130	7	6	2.6	++	–
12	AD	+	62	80	18	0	Pneumonia	1245	4	5	3.6	–	–
13	AD	+	76	86	10	10	Pneumonia	1310	3	5	0.2	–	–
14	AD	–	90	102	12	0	Pneumonia	1110	2	5	3.3	–	–
15	AD	–	73	82	9	0	Cor infarct	980	3	5	1.8	–	–
16	AD	+	67	81	14	0	Pneumonia	1175	3	5	3.9	–	+
17	AD	+	77	86	9	0	Pneumonia	990	4	5	4.0	+	–
18	AD	+	67	83	16	0	Pneumonia	1045	9	5	13.1	+	–
19	AD	+	69	82	13	0	Pneumonia	1070	12	5	6.9	–	–

βA (fraction): beta-amyloid load given as percentage of beta-amyloid staining in a microscopic field; duration of CI: duration of clinical impairment; MMS: mini mental state; NA: not available; PMD: post-mortem delay.

[a] Non-fatal additional microscopic infarct not related to the cause of death.

[b] Non-fatal additional small ocult meningiothelic tumor not related to the cause of death.

[c] Non-fatal limited acute vascular infarct in the watershead region in the right hemisphere not related to the cause of death.

GFAP as a marker of reactive astrocytes and HLA DR as a marker of reactivated microglia have been repeatedly used [33,49,50]. For immunohistochemical stainings, we used monoclonal antibody to human βA, at a dilution of 1:100 (DAKO A/S, Glostrup, Denmark, M872), monoclonal antibody to human HLA DR, clone CR3/43 at a dilution of 1:100 (DAKO, M775), polyclonal antibody to cow GFAP (DAKO, Z0334) at a dilution of 1:1000 and a strepavidin-alkaline phosphatase system (Histomark Kit, 71-00-39). The reaction product, streptavidin–biotin complex, was visualized by using Vector-Red (Vector Labs, SK-5100). The quantification of GFAP and HLA DR expression was performed by using Quantimet 570 Image Analysis system (Leica Ltd., Cambridge, England). The expression was estimated on five randomly selected fields in frontal cortices. The subpial region as well as the border zone between white and grey matter were excluded. The staining was evaluated per standard unit field (0.5 mm^2) and evaluated as stained area fraction [49]. The quantification of βA expression was performed under light microscopy at 40× magnification by using an NIH Image system for PC [3]. The βA expression (Table 1) was estimated in frontal cortices within the total thickness of grey matter on three by chance selected fields with the βA load being reported as stained area fraction.

2.3. Sample preparation for electrophoresis

Brain samples were homogenised in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA and protease inhibitor cocktail Complete (Roche Molecular Systems, Almeda, CA, USA). Soluble fractions containing all Tris–HCl soluble proteins including cytosolic proteins were separated by centrifugation at $13800 \times g$ at 4 °C for 15 min prior to precipitation of proteins as described earlier [31].

2.4. 1D immunoblotting

In order to further estimate the activation of astrocytes in the tissue samples used for oxyblots, we quantified soluble GFAP. Additionally, in order to further examine some of the protein changes obtained by oxyblotting, we also quantified the total levels of soluble cytosolic malate dehydrogenase (MDH1) and glutamate dehydrogenase (GDH). Briefly, proteins were separated on a one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylinidene difluoride (PVDF) membranes as previously described [31] with certain modifications. Samples were homogenised and precipitated in the same way as for 2-DE in order to separate the soluble proteins, which were then dissolved in the SDS-containing Laemmli sample buffer prior to electrophoresis [36]. Proteins were separated on a 10% SDS-PAGE and electrotransferred to PVDF membranes. For the detection of GFAP, a dilution of 1:3000 of rabbit anti-cow GFAP (DAKO, Z0334) and a dilution of 1:500 of fluorescent Cy5-labelled secondary anti-rabbit antibody made in goat (Jackson Immunoresearch Laboratories, West Grove, PA, USA, 011-170-003) were used. For the detection of MDH1, a dilution of 1:1500

of sheep anti-pig heart MDH (Rockland, Gilpertsville, PA, USA, 100-601-145) and a dilution of 1:500 of fluorescent Cy5-labelled secondary anti-sheep antibody made in rabbit (Jackson Immunoresearch Laboratories, 313-175-003) were used. Finally, for the detection of GDH, a dilution of 1:1500 rabbit anti-bovine liver GDH (Rockland, 100-4158) and a dilution of 1:500 anti-rabbit antibody made in goat (Jackson Immunoresearch Laboratories, 011-170-003) were used. Fluorescence signals were detected by fluoroimager Storm 860 (APBiotech, Uppsala, Sweden) at 635 nm. The light intensities of proteins were measured by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The relationship between the levels of soluble GFAP and protein changes was calculated by Pearson's correlation test (SPSS Inc., Chicago, IL, USA). Mann-Whitney's *U* test was used to compare the total levels of soluble MDH1 and GDH in AD when compared to controls (SPSS Inc.).

2.5. 2D oxyblots and protein identification

2D oxyblots were performed as previously described [31]. Shortly, equal amounts of soluble proteins were derivatised with 2,4-dinitrophenyl hydrazine (DNPH) (Sigma, St. Louis, MO, USA) and separated by 2-DE. Proteins were then electrotransferred to PVDF membranes and stained first with Sypro Ruby fluorescent stain followed by the detection of hydrazone groups with an anti-dinitrophenyl (DNP) antibody (DAKO, V0401). Fluorescence intensities of detected proteins on membranes were analysed by using ImageMaster 2D Elite, Version 3.1. software (APBiotech). Mann-Whitney's *U* test was used to compare normalised volumes of light intensities of the amount of protein-bound carbonyls and the amount of each protein. The degree of oxidation was calculated as the ratio of protein-bound carbonyls to the amount of protein for AD and control brains. Furthermore, the relationship between post-mortem delay, duration of the disease, brain pathology and protein changes was calculated by Pearson's correlation test (SPSS Inc.).

For protein identification 2-DE, was performed as previously described [31] with certain modifications. Protein extracts were not treated with DNPH. Further, 2D gels were stained with Sypro Ruby gel stain after electrophoresis according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Protein spots were cut out from the gels on a UV-table, and in-gel digested [46,55,57]. Proteins were reduced and alkylated before digestion with trypsin (Sequencing Grade Modified trypsin: Promega, Madison, WI, USA) overnight at 37 °C. The peptides were extracted twice with 150 μl of 5% formic acid in 50% acetonitrile, the extracts were pooled and dried in a vacuum centrifuge. Alternatively, the peptides were extracted by adding 20–50 μl of 5% formic acid to the digestion mixture and incubated for 15 min at 37 °C, this being followed by direct desalting. Desalting was performed with μ-tips [34] containing Oligo R3-material (PerSeptive Biosystems, Framingham, MA, USA). The detailed protocols for in-gel digestion and desalting can be found at webpage, http://www3.btk.utu.fi:8080/Genomics/Proteomics/Protocols.

The resulting peptides were analysed with a Voyager DE PRO MALDI-TOF mass spectrometer (ABI/Perseptive Biosystems) in the positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix. The mass spectra were internally calibrated with autoproteolytic trypsin fragments, 842.50 and 2211.10 Da. Database searches were performed by using the Mascot Search engine (http://www.matrixscience.com). Alternatively, the peptide mixture was analysed by automated nanoscale capillary LC–MS/MS using an Ultimate^{TM} capillary LC system, and Famos autosampler (LC Packings, The Netherlands) coupled to a quadrupole time-of-flight mass spectrometer (Q-Star Pulsar, ABI/MDS-SCIEX, Toronto, Canada). Reversed phase separations were carried out using a 75 μm × 15 cm Pepmap^{TM}-column (LC Packings, The Netherlands) at a flow rate of 200 nl/min. Solvent A was 0.1% formic acid/5% ACN and solvent B was 0.1% formic acid/95% ACN. Peptide separation was carried out with a linear gradient of 5–60% solvent B in 80 min. LC–MS/MS data analysis was performed with software BioAnalyst 1.0 and database searches with PepSea Server Version 2.2.1.7.

3. Results

3.1. 2D oxyblotting and identification of proteins

As previously demonstrated, about 150 proteins and more than 100 oxidised proteins were visualised and analysed on double stained PVDF membranes in both AD and control cases by 2D image analysis (Figs. 1 and 2) [31]. A total of nine proteins that exhibited statistically significant changes in AD brains were excised from the 2D gels and identified by mass spectrometry and database searches (Table 2).

3.2. Neuropathology

We also examined the relationship in the AD samples between post-mortem delay, brain pathology, soluble GFAP and protein changes. Braak and Braak staging and βA load were used to estimate tau and amyloid pathology, respectively. Inflammatory events were characterised in AD patients immunohistochemically by labelling reactive astrocytes (mean stained area fraction ± S.E.M. = 0.6 ± 0.2) and activated microglia (mean stained area fraction ± S.E.M. = 0.5 ± 0.2). Since the GFAP staining was not performed for control brains, we estimated the astroglial activation by measuring the levels of soluble GFAP in tissue homogenates by 1D immunoblotting (Fig. 3). Several different isoforms and degradation products of GFAP were detected between 40 and 50 kDa in both AD and control subjects. Fluorescence inten-

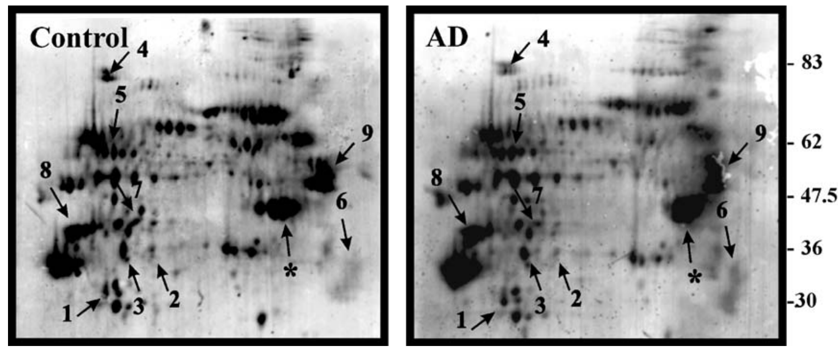

Fig. 1. About 150 soluble frontal cortex brain proteins were visualized on PVDF membranes with Sypro Ruby protein staining. The numbers indicate the proteins selected for identification and the asterisk an example of proteins that did not reach the sufficient resolution to be oxidatively characterised.

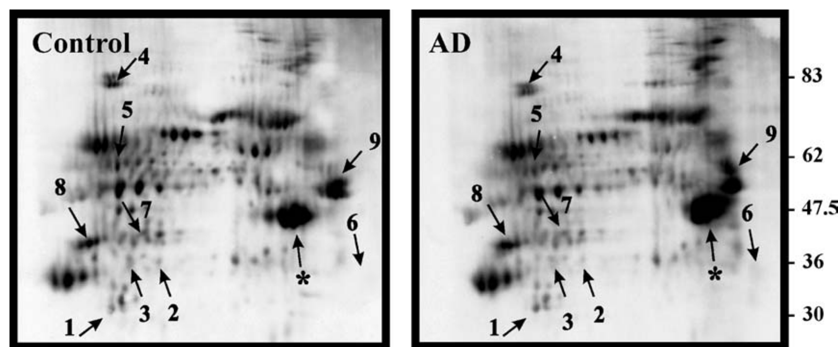

Fig. 2. About a 100 soluble oxidised proteins can be detected on anti-DNP oxyblots. The numbers indicate the proteins selected for identification and the asterisk an example of proteins that did not reach the sufficient resolution to be oxidatively characterised.

Table 2
Summary of the protein identification data

No.	Protein	SWISS-PROT Acc#	Theor. M_r	Theor. pI	No. of peptides	Seq. cov. (%)	Identification method	Functional role
1	Carbonic anhydrase Ii,	P00915	29153	7.20	5	25	MALDI	Acid-base transporter
2	Malate dehydrogenase 1 (MDH1a)	P40925	36523	5.92	5	19	MALDI	Metabolism
3	Malate dehydrogenase 1 (MDH1b)	P40925	36620	7.37	20	49	LC-MS/MS	Metabolism
4	Aconitase, mitochondrial	Q99798	82405	6.61	9	14	MALDI	Metabolism
5	Glutamate dehydrogenase	P00367	56875	6.72	10	22	MALDI	Metabolism
6	14-3-3 Protein zeta/delta	P29312	27916	4.54	14	55	LC-MS/MS	Cell signaling
7	Aldolase C	P09972	39830	6.41	10	35	MALDI	Glycolysis
8	Aldolase A	P04075	39720	8.39	7	17	MALDI	Glycolysis
9	ATP synthase beta chain, mitochondrial	P06576	56525	5.26	10	24	MALDI	ATP synthesis

All the proteins that were identified by MALDI and by Mascot Search engine fulfilled the criteria of being significant ($p < 0.05$) according to the probability based mowse score.

Identification of proteins is indicated by SWISS-PROT accession numbers; M_r: theoretical molecular weight; pI: theoretical isoelectric point; No. of peptides: number of peptides matched; Seq. cov.: sequence coverage of matched peptides.

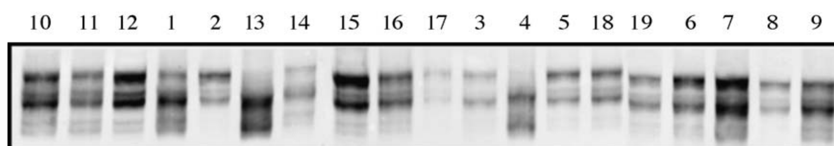

Fig. 3. Several different isoforms and degradation products of soluble GFAP were detected between 40 and 50 kDa in both AD and control subjects by 1D immunoblotting. Numbering indicates the patients presented in Table 1.

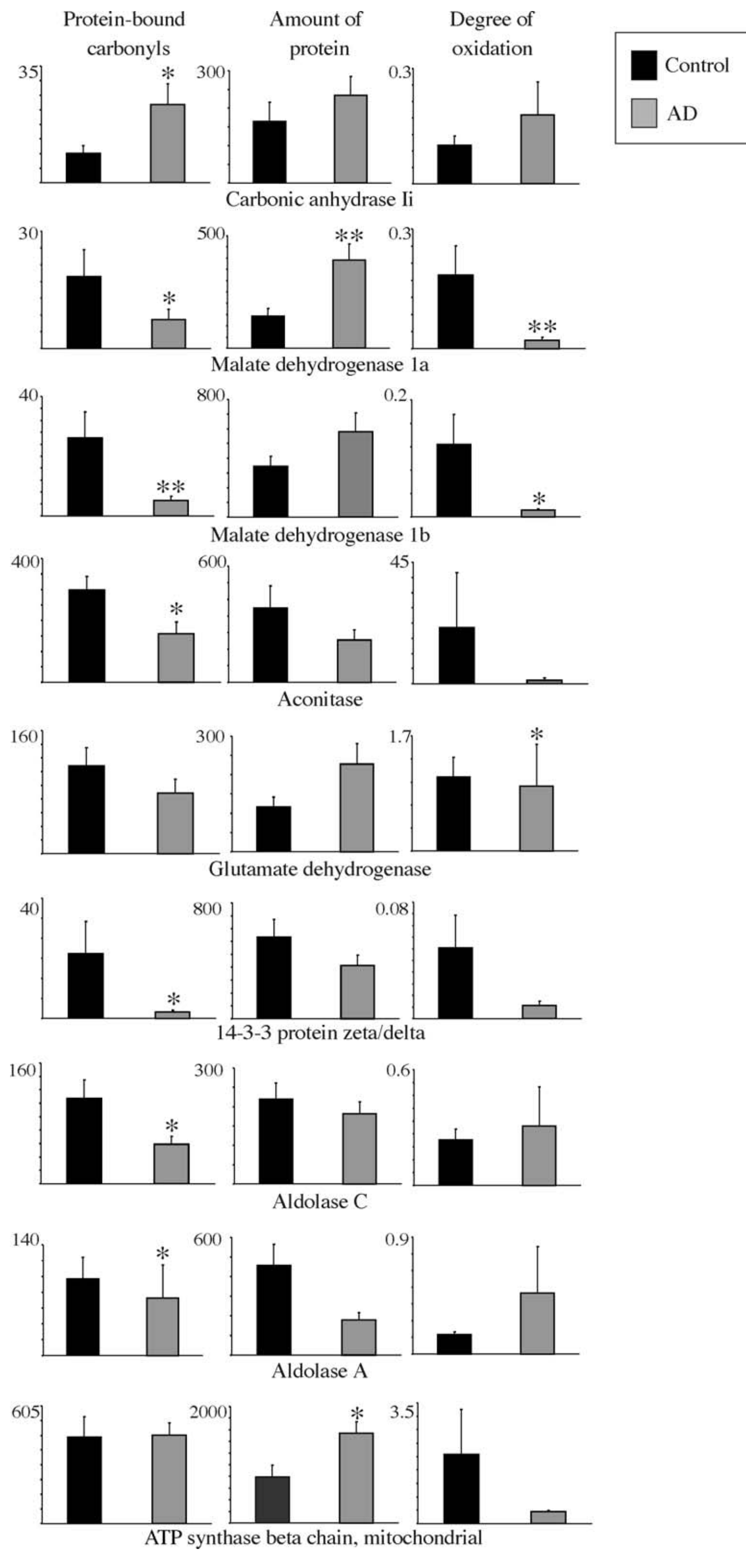

Fig. 4. Altogether nine soluble oxidatively modified protein isoforms showed quantitative differences in light intensities in AD when compared to controls. Values of light intensities are given as mean ± S.E.M. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

Table 3
Summary of the correlations of the oxidation status with the post-mortem delay and duration of clinical impairment in AD

No.	Protein	Protein-bound carbonyls (LI) PMD (h)		Amount of protein (LI) PMD (h)		Degree of oxidation (LI) PMD (h)		Protein-bound carbonyls (LI) duration of CI (years)		Amount of protein (LI) duration of CI (years)		Degree of oxidation (LI) duration of CI (years)	
		r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
1	Carbonic anhydrase Ii,	0.456	0.185	0.576	0.081	0.074	0.839	0.569	0.086	0.733*	0.016	−0.350	0.322
2	Malate dehydrogenase1 (MDH1a)	0.138	0.704	0.090	0.817	−0.022	0.956	0.144	0.692	−0.250	0.517	0.436	0.241
3	Malate dehydrogenase1 (MDH1b)	0.011	0.975	−0.168	0.643	0.0148	0.684	0.714*	0.020	−0.207	0.567	0.685*	0.029
4	Aconitase, mitochondrial	0.192	0.595	0.461	0.180	−0.258	0.473	0.184	0.610	0.025	0.945	−0.258	0.473
5	Glutamate dehydrogenase	−0.117	0.748	−0.003	0.993	0.008	0.982	0.675*	0.032	−0.627	0.052	0.682*	0.030
6	14-3-3 Protein zeta/delta	−0.131	0.718	−0.005	0.990	0.202	0.575	0.104	0.775	−0.404	0.247	0.595	0.070
7	Aldolase C	−0.076	0.834	0.646*	0.043	−0.275	0.0443	−0.371	0.291	0.036	0.922	0.171	0.637
8	Aldolase A	0.639*	0.047	−0.638*	0.047	0.734*	0.016	0.126	0.729	0.074	0.839	0.122	0.737
9	ATP synthase beta chain, mitochondrial	0.156	0.667	−0.169	0.641	0.149	0.682	−0.113	0.746	−0.067	0.853	−0.530	0.885

LI, light intensity; PMD, post-mortem delay; Duration of CI, duration of clinical impairment. * $p \leq 0.05$ and ** $p \leq 0.01$.

sities of all isoforms as well as degradation products were included in the statistical analyses performed by Pearson's correlation test.

3.3. Oxidation status of proteins

The quantitative data of the identified proteins are presented in Fig. 4. No averaging was performed during the 2D image analyses and thus Figs. 1 and 2 are not comparable with the quantitative data presented in Fig. 4. We also analysed the correlation between protein changes and post-mortem delay and duration of the clinical impairment (Table 3).

3.3.1. Carbonic anhydrase I (CAI)

An increase in the amount of CAI, as well as in protein-bound carbonyls was found in AD when compared to controls (Fig. 4). The tendency was that CAI was more oxidised in AD. The amount of CAI was associated with the duration of the clinical impairment ($r = 0.733$, $p = 0.016$) and the amyloid load ($r = 0.806$, $p = 0.005$) (Fig. 5). A positive relationship was also found between the degree of oxidation and Braak staging ($r = 0.651$, $p = 0.042$) in AD.

3.3.2. Cytosolic malate dehydrogenase (MDH1)

Two isoforms of MDH1 (named a and b) exhibited a decrease in their oxidation status (Fig. 4). Both isoforms were less oxidised in AD when compared to controls whereas the amount was increased only for isoform a. Further, the amount of total soluble MDH1 was unchanged in AD (Fig. 6). The duration of the clinical impairment was associated with the amount of protein-bound carbonyls of MDH1b ($r = 0.714$, $p = 0.020$) and with the degree of oxidation of MDH1b ($r = 0.685$, $p = 0.029$) (Fig. 7).

3.3.3. Aconitase

There was a decrease in the total amount of aconitase and in protein-bound carbonyls in AD although the decrease in the degree of oxidation did not achieve the statistical significance (Fig. 4).

3.3.4. Glutamate dehydrogenase (GDH)

The degree of oxidation was decreased for an isoform of GDH (Fig. 4) and the amount of total soluble GDH was increased (Fig. 6) in AD patients when compared to controls. The amount of protein-bound carbonyls of GDH ($r = 0.675$, $p = 0.032$) and the degree of oxidation ($r = 0.682$, $p = 0.030$) (Fig. 7) were associated with the duration of clinical impairment. Furthermore, there was a significant correlation between the degree of oxidation and soluble GFAP levels in controls ($r = 0.699$, $p = 0.036$) whereas no relationship was found in AD patients.

3.3.5. 14-3-3 Protein zeta/delta

The amount of protein-bound carbonyls of 14-3-3 protein zeta/delta was decreased in AD although the degree of oxidation did not reach statistical significance (Fig. 4). HLA DR labelled activated microglia had an association with the amount ($r = 0.749$, $p = 0.013$) and the degree of oxidation of ($r = 0.674$, $p = 0.033$) of protein 14-3-3 zeta/delta in AD. Furthermore, an association was found between soluble GFAP levels and the amount of protein-bound carbonyls ($r = 0.686$, $p = 0.041$) in controls.

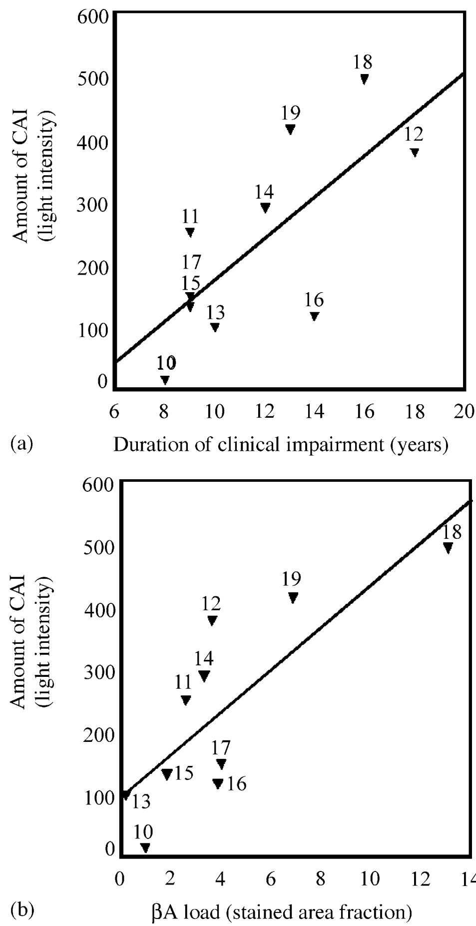

Fig. 5. The amount of CAI was associated (a) with the duration of the clinical impairment ($r = 0.733$, $p = 0.016$) and (b) with the βA load ($r = 0.806$, $p = 0.005$) in AD patients. Numbers indicate the patients presented in Table 1.

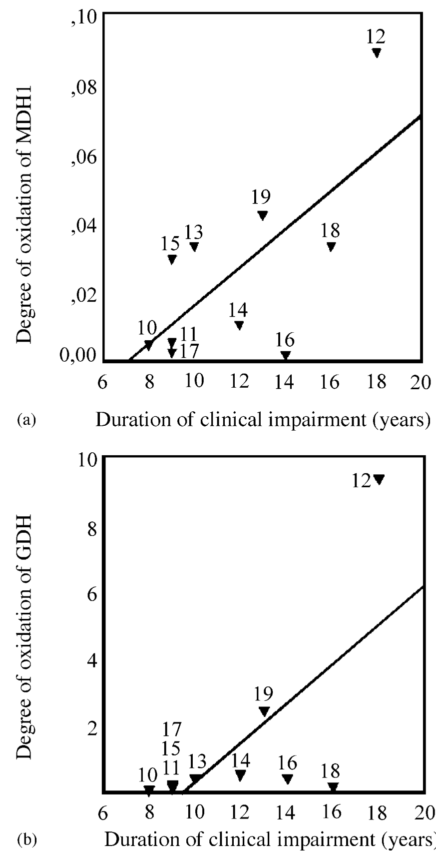

Fig. 7. The degree of oxidation of (a) MDH1b ($r = 0.685$, $p = 0.029$) and (b) GDH ($r = 0.682$, $p = 0.030$) had a significant positive correlation with the duration of clinical impairment of AD patients. Numbers indicate the patients presented in Table 1.

3.3.6. Aldolases A and C

Total amount of aldolases and also protein-bound carbonyls were decreased in AD brains whereas there was a tendency that they would be more oxidised when compared to age-matched controls (Fig. 4). There was a significant correlation between the degree of oxidation of aldolase C and soluble GFAP levels in controls ($r = 0.646$, $p = 0.045$) whereas GFAP labelled reactive astrocytes revealed a negative correlation with the amount ($r = -0.697$, $p = 0.023$) and the degree of oxidation ($r = 0.911$, $p \leq 0.001$) in AD. Furthermore, aldolases were the only proteins that correlated with post-mortem delay (Table 3).

3.3.7. Mitochondrial ATP synthase beta chain

The ninth protein identified was ATP synthase beta chain; its amount was elevated in AD. Even though no significant changes were found in the oxidation status, the result has been included in Tables 2 and 3 and Fig. 4. This increase in the amount of ATP synthase beta chain in AD brain is convergent with a previous study performed by Tsuji et al. [63].

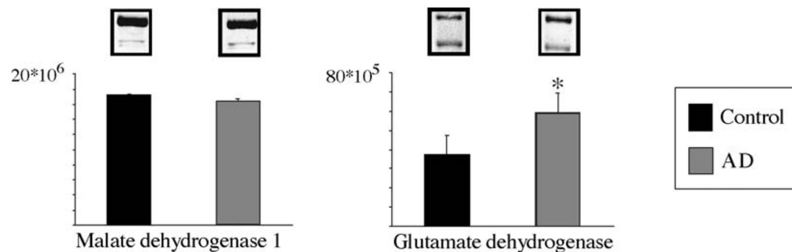

Fig. 6. Two different molecular weight groups of isoforms were detected for MDH1 at about 36 kDa and for GDH at about 60 kDa. Light intensities revealed unchanged amount of total soluble MDH1 but increased amount of total soluble GDH in AD when compared to controls. Values of light intensities are given as mean ± S.E.M. *$p \leq 0.05$.

4. Discussion

Increased oxidation evoked by ROS is associated with physiological functions during life, i.e. when considering supportive activities. However, altered oxidation status of proteins is also one of the post-translational modifications leading to a severe failure of biological functions and to cell death [7]. Measurement of protein-bound carbonyls is thought to represent a good estimate for the extent of oxidation of proteins associated with various conditions of oxidative stress, aging, physiological disorders, and disease [16,37,60]. The total amount of protein-bound carbonyls is known to be elevated in the AD brain [60]. It is important to characterise oxidation of individual proteins in order to understand the relationship between protein oxidation, protein aggregation, protein turnover and neurodegeneration. Several proteins that are targets of ROS in AD have recently been identified. We have here identified several distinctive soluble and cytosolic proteins that exhibited oxidative changes in AD brains. Interestingly, these proteins were closely involved in cell homeostatic processes and energy metabolism, and tended to be less oxidised in AD patients than in age-matched controls.

We found a total of eight proteins that exhibited changes in their oxidation status. However, the degree of oxidation was changed only in two of these proteins, both being soluble NAD+/H-dependent oxido-reductases, mitochondrial GDH and two isoforms of MDH1. These proteins are both closely linked to neurotransmitter synthesis and cellular energy metabolism via glutamate/glutamine cycling and the malate-aspartate shuttle [30,40,45]. The ninth protein identified was ATP synthase beta chain that did not exhibit oxidative changes in AD when compared to controls. The observed increase in the amount of ATP synthase beta chain in AD when compared to controls supports the finding by Tsuji et al. [63] and was included in the results.

The enzyme that functionally connects GDH and MDH1 is aspartate aminotransferase (AAT). GDH is co-localised in cells with AAT, which participates in the malate–aspartate shuttle with MDHs [30,41,65]. It is known that transaminations by AAT are involved in compensating the energy regeneration of a cell when more ATP is required [14,21,65], and in previous studies the activity of AAT has been shown to be increased in the cerebrospinal fluid of AD patients [54,62]. In addition, the GDH pathway is thought be mostly active when glucose levels are low [20], and the activity of GDH has been reported to be unchanged in brain [11,56] but elevated in plasma of AD patients [44]. We found that not only the degree of oxidation was changed for GDH but also the total amount of soluble GDH was increased in AD. These findings support the possible compensatory role of GDH in the pathogenesis of AD, potentially reflecting altered energy metabolism.

Previous studies have indicated that the activity of MDH is increased in AD brain [48] and the activity also increases with age in rat brain [38]. It must be noted that the activity of MDH is not a specific measure for the activity of MDH1. There is also a mitochondrial MDH2 that shares a similar enzymatic function but little structural homology when compared to isoform 1 [28]. We found that the degree of oxidation was decreased for two isoforms of MDH1. However, the total amount of soluble MDH1 was unchanged in AD when compared to controls indicating differences in the amounts of distinctive isoforms. One of the explanations may be aberrant post-translational modification of MDH1 in AD leading to a differential quantitative distribution of isoforms when compared to controls. In 2-DE, the isoelectric point of protein isoforms is influenced by post-translational modifications such as phosphorylation and glycosylation [39]. Further, post-translational modifications determine proteins activity state, localization, turnover and interaction with other molecules.

There is overwhelming evidence that increased oxidative stress is a phenomenon in AD pathogenesis. Therefore, it may seem surprising that we detected a decreased, not increased, degree of oxidation of GDH and MDH1. However, these findings do not necessarily contradict the hypothesis of increased oxidative stress in AD. We did analyze the Tris–HCl soluble fraction of the brain homogenates that contains, i.e. most of the cytosolic proteins. It is well known that oxidised proteins do have a tendency to accumulate, and we did not study changes in insoluble aggregated or membrane-bound proteins since they are a challenge to the 2-DE technology. However, increased oxidative stress is not only known to lead to aggregation but also to an enhanced turnover of some proteins [59]. Moreover, it is generally assumed that decreased oxidation of proteins is associated with enhanced enzymatic functional status since oxidation most often leads to degradation or accumulation of proteins [58]. Therefore, our results that GDH and MDH1 were less extensively oxidised, with a concomitant increase in their amount, agree with some previous studies showing normal or increased activities of these enzymes in AD [11,44,48,56]. The changes of GDH may reflect decreased energy metabolism and altered glycolytic pathways that have been suggested to be involved in the pathogenesis of AD [25]. On the other hand, enhanced activity of the malate–aspartate shuttle involving both MDH1 and AAT has been associated with glutamate neurotoxicity and oxidative stress due to increased mitochondrial membrane permeability [4]. Therefore, the increased amount and decreased oxidation of these proteins may also reflect compensatory changes that are related to both increased oxidative stress and to compromised energy metabolism. Additionally, as is known in the case of AAT and the functional consequences of oxidative stress, our results most probably also reflect neurodegeneration rather than a specific event in AD.

Previous proteomic studies on human brain have characterised oxidatively damaged proteins in AD [1,2,12,13] whereas we have detected different oxidised proteins that tended to be less oxidised in AD. There are numerous factors that may explain why different targets are identified by

different proteomic studies. There is a substantial variance in the methodological procedures used in different laboratories. In example, sample preparation protocols, selected pH gradients, and gel sizes vary. Therefore, the same proteins are not always dissolved, separated well enough, or even detected in the gels of different laboratories. For example, we were not able to detect previously shown oxidative changes of creatine kinase BB and β-actin [1,2,12] because these proteins remained masked as a part of large spot on the membrane containing several different proteins (shown with an asterisk in Figs. 1 and 2). This was simply due to differences in methodological and technical procedures when carrying out 2D oxyblotting. Nevertheless, one of the advantages of proteomics is the possibility to examine different and new proteins by slightly modifying the experiment. It is evident that additional and less abundant changes in protein oxidation are to be ravelled by modifying 2-DE techniques.

Interpretation of data obtained analysing human post-mortem brain tissue must always be viewed with caution since the tissue samples are affected by different ante- and post-mortem events [27]. The comparison between different studies is also difficult because ante- and post-mortem events including the cause of death are not always reported. The post-mortem delay and the cause of death of our subjects varied and we cannot exclude the possibility that some changes may have been attributable to these factors. In fact, the post-mortem delay of three control brains was much longer when compared to other subjects. Therefore, we additionally verified the results by removing these controls (1, 3 and 6 in Table 1). The removal had no influence on the main results suggesting that the post-mortem interval has not greatly influenced the data presented. In general, most proteins are thought to be quite resistant to post-mortem delay. The amounts of some proteins are known to increase and some to decrease as a result of the post-mortem delay in rat and mouse brain [23,24]. In these studies, the changes were mostly detected after a 24 h delay or even later. Specific internal controls have been proposed for controlling the effect of post-mortem factors in mouse brain [24]. However, protein changes in human brains are undoubtedly much more difficult to predict and confirm due to the complexity of ante- and post-mortem events. Our results suggested that aldolases may be modified after death in AD but not in control brains although the delay was longer for control brains. It is possible that post-mortem changes may vary in relation to the physiological or to the pathological biochemical environment. However, post-mortem delay in our samples was reasonable when compared to other studies [15,32].

The other proteins that showed changes in carbonyl content in AD brains were carbonic anhydrase I, 14-3-3 protein zeta/delta, aconitase, aldolase A and C. However, the degree of oxidation itself did not reach statistical significance perhaps due to a concomitant change in the amount of these proteins. We also found statistically significant associations with protein changes and neuropathological data, although due to the small sample size, the significance of the relationships needs to be further evaluated. However, our results emphasize that each individual protein and even its distinct isoforms, may exhibit a distinct, characteristic response to oxidative stress. The individuality of oxidative modification may be explained by amino acid composition of proteins. On the other hand, the effect of other post-translational modifications on folding, location and function of proteins affecting the susceptibility of distinctive isoforms to oxidation are far from being understood. Thus, it is possible that changes in the protein oxidation status and the amount of protein may be strongly dependent on the stage and the duration of the clinical impairment as well as on the brain pathology of the patient. In the future it will be important to examine the less severely affected regions to determine whether the changes in the oxidation status of proteins are a generalized phenomenon or linked specifically to AD pathology. Therefore, undertaking studies with patients at an earlier stage of the disease states is of the utmost importance.

In conclusion, our results provide novel information on oxidative modification of individual proteins in AD. The most significant finding of the present study was that both GDH and MDH1 that are closely related to neurotransmitter release and energy metabolism were less oxidised in AD. These results do not oppose the hypothesis of increased oxidative stress in AD since the relation between oxidation status and function may vary in individual proteins. It is possible that the increased oxidative stress does not always lead to oxidative damage and aggregation of proteins but may also be compensatory and lead to an enhanced turnover and activity of some proteins. However, whether these types of changes in the oxidation status of proteins play a protective role or cause acceleration down the slippery slope remains unknown to AD pathogenesis.

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