Identification of AGE-modified Proteins in SH-SY5Y and OLN-93 Cells

ANDRÉ K. LANGER, H. FAI POON, GERALD MÜNCH, BERT C. LYNN, THOMAS ARENDT and D. ALLAN BUTTERFIELD

*Nachwuchsgruppe 1, Interdisciplinary Centre of Clinical Research (IZKF), University of Leipzig, 04103 Leipzig, Germany; †Department of Neuroanatomy, Paul-Flechsig-Institut, University of Leipzig, Germany; 4Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506, USA; Mass Spectrometry Facility, University of Kentucky, Lexington, KY, USA; 3Comparative Genomics Center, James Cook University, Townsville, Australia. Andre.Langer@web.de; dabcnas@uky.edu

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The formation of "Advanced Glycation End products" (AGEs) is an inevitable consequence of mammalian glucose metabolism. AGE-mediated protein-protein crosslinks lead to detergent-insoluble and protease-resistant protein aggregates, and in Alzheimer's disease (AD) extra cellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) have been shown to contain AGEs. However, to date little is known concerning the most prevalent protein-targets of AGE modification under normal, non-pathological conditions. Here, a combination of 2D-electrophoresis, Western blotting and mass spectrometry has been used to identify preferentially AGE-modified proteins in oligodendrocyte (OLN-93) and neuroblastoma cell lines (SH-SY5Y) in standard culture. Proteomics analysis identified a total of eight targets with structural, metabolic and regulatory function, three of which (β-actin, β-tubulin and eukaryotic Elongation Factor 1-α) were common to both cell lines. Based on results from prior studies, modification of these proteins may lead to a loss of function. Consequently, the identification of targets for these proteins is of particular interest for a better understanding of the consequences of AGE-modification in aging, neurodegenerative diseases and diabetes.

INTRODUCTION

Glycation is a common, non-enzymatic, post-translational modification of proteins and is an unavoidable feature of physiological metabolism because the disintegration of sugars leads to reactive decomposition products that can attach to proteins. This modification occurs through a non-enzymatic reaction initiated by the primary addition of a reductive sugar to the amino groups of proteins (Monnier and Cerami, 1981). This first reaction step is reversible. Thereafter, rearrangements yield intermediates called "Amadori products".

Further oxidation, dehydration, condensation, fragmentation and cyclization reactions lead to the formation of Advanced Glycation End products (AGEs). There are numerous pathways suggested for these reaction steps which, although not yet fully defined, are known to lead to the irreversible formation of AGEs. A great variety and an increasing number of defined but heterogeneous AGE structures have been demonstrated (Thornalley, 1998; Ahmed and Thornalley, 2002; Hasenkopf et al., 2002; Nagai et al., 2002). The amino acid side-chains of lysine and arginine are thought to be most susceptible to AGE-modification (Münch et al., 2003). Some of the resultant adducts are protein-protein-crosslinks including pentosidine, methylglyoxal lysine dimer (MOLD), glyoxal lysine dimer (GOLD) or vespertlysin (Horie et al., 1997; Nakamura et al., 1997; Miyata et al., 1998; Münch et al., 1999). Some AGE crosslinked protein aggregates are detergent-insoluble and protease-resistant (Kikuchi et al., 2003).
AGE-modified, but not crosslinked, proteins must be repaired, replaced and/or degraded (Thoralley, 1998); however, glycation also alters the biological activity (Giardino et al., 1994; Kil et al., 2004; Lee et al., 2005; Yan and Harding, 2005) of proteins and their degradation processes (Kikuchi et al., 2003). The alteration of AGE-modified proteins' degradation process can even lead to accumulation of those modified proteins that are not crosslinked. Therefore, protein glycation may affect any fundamental process of cellular metabolism over time (Kikuchi et al., 2003). Since a degree of AGE modification is a consequence of metabolism, AGES tend to accumulate on long-lived proteins.

In humans, AGE-modified proteins are observed in proteins (such as collagen-fibres) (Verzar, 1964), beta-amyloid plaques and in the vicinity of microglia and astrocytes in Alzheimer's patients (Sasaki et al., 1998; de Arriba et al., 2003). In this context, AGES appear to be responsible for induction of pro-inflammatory responses leading to the up-regulation of cytokines, free radical production and impairment of glucose utilization (Loske et al., 1998; Münch et al., 1998; Lue et al., 2001; Dukic-Stefanovic et al., 2003).

It was proposed that these chemical modifications of proteins are a fundamental mechanism of aging (Maillard Hypothesis of Aging), even though they may not determine life span (Baynes, 2002). Therefore, the AGE-modification of proteins may play an important role in basic aging mechanisms, while also underlying age-associated diseases such as Alzheimer's disease (AD). Increased glyoxidative damage has recently been reported in AD-brain samples (Pamplona et al., 2005).

AGE research in the last few years has identified: many novel AGE-structures (Thomalley et al., 2003), on which amino acid side chains they appear (Dukic-Stefanovic et al., 2002; Brock, et al., 2003), how cells react to direct stimulation by AGES (Dukic-Stefanovic et al., 2003; Gasic-Milencovic et al., 2003) and what percentage of the proteome will be glycated in an age-related manner (Poggioli et al., 2002).

However, in the majority of cases it remains unclear what the direct consequences of AG modification on any particular protein are, and more importantly whether distinct proteins may be particularly susceptible to AGE modifications in non- or pre-pathological situations. Since this information is essential for our understanding of the relevance of AGE modification in the process of aging and disease, we have undertaken the proteomics identification of the most prevalent targets of AGE modification during routine culture of oligodendroglia and neuroblastoma cell lines. Our results reveal target proteins with structural, metabolic and regulatory function, and a high degree of consistency between the cell lines studied.

**MATERIALS AND METHODS**

**Production of Advanced Glycation End Products and Antiserum**

The AGES for raising the antisera AL2003-F and AL2003-G were produced by incubation of 0.89 µM Keyhole limpet hemocyanin (KLH) either with 1 mM glucose or 1 mM fructose at 50°C in 100 mM phosphate buffered saline (PBS) (pH 7.4) for 12 weeks. Unbound sugars were removed by extensive dialysis with distilled water. The concentration of protein AGES were measured by a BCA-test and adjusted to 1 mg/ml. AGE-solutions (500 µl) were mixed with Adjuvanz (Imject® Alum, Pierce, Bonn, Germany) (500 µl) and injected into Chinchilla bastard rabbits. After four weeks the animals were reboosted with another injection of AGE/Adjuvanz Solution.

One week later, the first serum was taken. The animals were then reboosted every two weeks and each time the following day serum was taken. After the 5th rebooster ing, the animals were sacrificed to obtain the final serum which was exclusively used in this investigation.

The polyclonal anti-KLH-AGE antibody (pk 2188-90) was a kind gift of Roche Diagnostics, Penzberg, Germany (Stein, Kientsch-Engel), and it has been used in previous studies (Richter et al., 2005).

**Cell Culture**

Human neuroblastoma cells (SH-SY5Y) and rat oligodendroglia cells (OLN-93, an appreciated gift from C. Richter-Landsberg, University of Oldenburg, Germany) were grown in cell culture plates (Greiner, Frickenhausen, Germany) with DMEM supplemented with 10% fetal calf serum including 20 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂/95% oxygen. Afterwards, the cells were centrifuged and stored as cell pellets at -20°C.

**Sample Preparation**

The samples used for 2D gel-electrophoresis were prepared as previously described (Poon et al., 2004). Briefly, cells were homogenized in a lysis buffer (pH 7.4, 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH₂PO₄, 0.6 mM MgSO₄ and 0.5 mg/ml leupeptin, 0.7 µg/ml pepstatin, 0.5 µg/ml trypsin inhibitor, and 40 µg/ml PMSF) by sonication on ice. Homogenates were centrifuged at 15,800g for 5 min to remove membrane lipids and debris. The supernatant was isolated and protein concentration determined by the BCA method.
(Pierce, Rockford, IL, USA). Protein (200 μg) was precipitated by addition of ice-cold 100% trichloroacetic acid (final concentration: 15%). The pellets were washed with 1:1 (v/v) ethanol/ethy alcohol solution. Afterwards, the samples were dissolved in 25 μl of 8 M urea (Bio-Rad). The samples were then mixed with 185 μl of rehydration buffer (8 M urea, 20 mM dithiothreitol, 2.0% (w/v) CHAPS, 0.2% Biolytes, 2 M thiourea and bromophenol blue).

Two-dimensional Gel Electrophoresis
Electrophoresis in the first-dimension was performed as previously described (Poon et al., 2004). A sample solution (200 μl) was applied to a ReadyStrip™ IPG® strip (Bio-Rad) and the strips were soaked in the sample solution for 1 h (passive loading). The strip was then actively rehydrated in the protein IEF cell (Bio-Rad) for 16 h at 50V (active loading). Finally, isoelectric focusing was performed and the strip was stored at -80°C until the second dimension electrophoresis.

For the second dimension, IPG® Strips, pH 3-10, were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. Linear gradient precast criterion Tris-HCl gels (8-16%) (Bio-Rad) was used to perform second dimension electrophoresis. SDS-Page Standards for SYPRO™ Orange Stain Broad Range Standard (Bio-Rad) were run along with the sample at 200V for 65 min.

Preparation for Spot Picking
The gel was incubated in fixing solution (7% acetic acid, 10% methanol) for 30 min after the second dimension electrophoresis. Approximately 80 ml of SYPRO-Rubystain™ (BioRad) was used to stain the gel at room temperature for 18 hours. Each gel was destained in deionized water overnight and afterwards stored at 4°C until spot picking. Gels were scanned for analysis on a UV-Scanner (Ex. 470 nm, Em. 618nm, Molecular Dynamics, Sunnyvale, CA, USA) and stored at 4°C until spot picking.

Western Blot and Immunoreactivity
After 2D-electrophoresis, gels, filter tissues and the nitrocellulose membrane were equilibrated in transfer buffer (80 ml 10x Trisglycin, 200 ml methanol, 720 ml distilled water) for 15 min. The proteins were then transferred to nitrocellulose papers (Bio-Rad) using the Transblot-Blot® SD semi-Dry Transfer Cell (Bio-Rad) at 15V for 2-3 h. The membranes were blocked either with 3% BSA (antisera F&G) or with 10% Rotiblock (Carl Roth, Karlsruhe, Germany) for the KHL-AGE 2188-90-antibody. The membranes were briefly washed with PBST (F&G) or 100 mM TBS (KHL-AGE 2188-90) for 2 x 10 min.

The primary antisera F and G were diluted 1:1000 in 50 mM PBS and incubated with the nitrocellulose membranes overnight at 4°C. The KHL-AGE 2188-90 (Roche) was diluted 1:1250 and required 3 days incubation time at 4°C with gentle shaking on a rocker. Afterwards the membranes were washed 3 x 15 min in PBST (F&G) or TBS (KLH). As a secondary antibody, a goat anti-rabbit alkaline phosphatase-conjugated IgG antibody (Sigma, St Louis, MO, USA), diluted 1:30,000 was applied and incubated for 2 h. Membranes were washed three times with PBST. The resultant stain was developed by the addition of SigmaFast (BCIP/NBT) tablets.

Data Analysis
Blots were scanned with Microtek Scanmaker 4900 (Carson, CA, USA), and PD-Quest software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyse the scanned blots and gels to find co-localised spots. Only spots that were positive for all three antibodies (KHL 2188-90, AL2003-F, AL2003-G) were relocated on the gel, and marked for spot picking. Also, we use PD-Quest to perform the optical measurement of the spot-density of gels and blots to generate the relative AGE-modified protein levels.

Spot Picking and In-Gel-Digestion
All working steps were performed under a hood (Forma Scientific Hood, Marietta, OH, USA). Samples were prepared using techniques described by Jensen et al. (1999), later modified by Thongboonkerd et al. (2002). Briefly, the protein spots were excised with a clean blade and transferred into clean micro-centrifuge tubes. They were then washed with 0.1M ammonium bicarbonate (NH₄HCO₃) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in a flow hood. The protein spots were then incubated with 20 μl of 20 mM dithiothreitol (DTT) in 0.1M NH₄HCO₃ at 56°C for 45 min. Afterwards, the DTT solution was removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH₄HCO₃. This solution was incubated at room temperature in the dark for 30 min. The iodoacetamide was then removed and replaced with 0.2 ml of 50 mM NH₄HCO₃ and incubated at room temperature for 15 min, after which 200 μl of acetonitrile was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel
pieces were rehydrated with 20 ng/μl modified trypsin (Promega, Madison, WI) in 50 mM NH₄HCO₃ with the minimal volume to cover the gel pieces. The gel pieces were minced into smaller pieces and incubated at 37°C overnight in a shaking incubator.

**Mass-Spectrometry**

All mass spectra reported in this study were acquired from the University of Kentucky Mass Spectrometry Facility (UKMSF) as previously described (Poon et al., 2004). A Bruker AutoFlex MALDI TOF (matrix assisted laser desorption and ionization with time-of-flight) mass spectrometer (Bruker Daltonic, Billerica, MA, USA) operated in the reflectron mode was used to generate peptide mass fingerprints. The MALDI and tandem spectra used for protein identification from tryptic fragments were searched within the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com).

**RESULTS**

The aim of this study was to determine which proteins are most sensitive to AGE-modifications. Here we report the identification of 8 AGE modified proteins based on Western blot analysis and subsequent immunohistochemical detection of AGE-modifications in two different cell lines.

**Choice of Cells**

The OLN-93 rat oligodendroglia were used as a model system for glia cells, because it was shown that untreated astroglia-rich cell primary cultures contain small amounts of Carboxymethyl lysine (CML)-positive cells, which were shown to be oligodendroglia cells (positive for oligodendroglia-marker Gal-C). These cells were strongly marked by CML-antibodies, suggesting a high level of CML-modified proteins in these cells (Pawlowski and Dringen, 2003).

The human SH-SY5Y neuroblastoma cell line was chosen here because when undifferentiated, this line very probably contains a proteome representative of many types of neurons. Additionally it is a widely used neuronal cell line in neurobiology research.

**Identification of AGE-modified Proteins**

The cells were grown, harvested and processed under conditions optimized to provide the maximum protein yield from the cell. With this sample preparation method, both membrane and cytosolic proteins are recovered. However, most of the membrane proteins are lost when samples are applied to the first dimension of the 2D-gel-electrophoreses because they do not enter the IPG-strip as well as hydrophilic ones (Görg et al., 2000).

For each cell line, five independent 2D gels were run, and Western blotting was performed on three of them using the three different anti-AGE antibodies. Gels and blots were scanned and compared. Only spots which were recognized by all three AGE-antibodies (KLH-2188-90, AL2003-F, and AL2003-G) were localized in the gel and selected for MS-analysis to minimize cross-reactivity effects.

**AGE-modified Proteins in OLN-93 Rat Oligodendroglia**

Figure 1A shows a 2-D Gel of the proteome from OLN-93 oligodendroglia. Proteins were stained by the fluorescent stain Sypro Ruby, which exhibits very high sensitivity for proteins in gels without interfering with the Mass-Spec procedure (Steinberg et al., 1996; Talent et al., 1998; Butterfield et al., 2003). As many as two thousand spots were detectable on some gels, representing a great variety of proteins in the cell lysate of OLN-93 cells. The spot pattern is spread over the whole blot, indicating that proteins with diverse masses (10-200 kDa) and with a range of pH optimum (3-10) were realized.

Figures 1 B-D show the Western-blot results. Only spots that were marked by all three antibodies as AGE-positive were taken into account and picked out of the gel for mass-spectrometry. The chosen targets were numbered from 1-10.

Table I summarizes the mass-fingerprint results for the OLN-93 cell line. For each protein the spot-number, the resulting Mowse (from the Mascot search engine that uses mass spectrometry data to identify proteins from primary sequence databases) scores and the known cellular function are described in the table. All proteins identified in this way were unequivocally aligned to proteins in the database.

**AGE-modified Proteins in SH-SY5Y Neuroblastoma**

In SH-SY5Y cells, 9 protein targets (of which 5 could be identified) were consistently immunostained by all three AGE antibodies. Three out of these 5 proteins (beta tubulin, beta-actin and εEF-1α) were also AGE-positive in OLN-93 cells. Additionally we found α-enolase and the zeta polypeptide. Figure 2A shows the 2D gel and images B-D show the immunostained Western blots. Again, only spots marked by all three antibodies were taken into account, leading to 9 targets for mass-spectrometry.

In table II, the Mascot search results for the nine investigated SH-SY5Y spots and their cellular function are listed. Again the search results were highly significant, leading to an unequivocal identification.

Table I Mass-fingerprint results from OLN-93. The identified proteins and their cellular function are listed. Score for Mass-Spectrometry based on significance ($p < 0.05$). Mouse scores greater than 58 are significant.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>identified protein</th>
<th>cellular function</th>
<th>Mouse score significance level &gt; 58</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>beta tubulin</td>
<td>cytoskeleton</td>
<td>104</td>
</tr>
<tr>
<td>2</td>
<td>beta actin</td>
<td>cytoskeleton</td>
<td>116</td>
</tr>
<tr>
<td>3</td>
<td>not identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>alpha enolase</td>
<td>glycolysis enzyme</td>
<td>153</td>
</tr>
<tr>
<td>5-8</td>
<td>not identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>zeta polypeptide</td>
<td>activates tyrosinkinase</td>
<td>144</td>
</tr>
<tr>
<td>10</td>
<td>eEF 1-alpha-1</td>
<td>protein translation</td>
<td>90</td>
</tr>
</tbody>
</table>

Table II Mass-fingerprint results from SH-SY5Y. The identified proteins and their cellular function are listed. Score for Mass-Spectrometry based on significance ($p < 0.05$). Mouse scores greater than 63 are significant.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>identified protein</th>
<th>cellular function</th>
<th>Mouse score significance level &gt; 63</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + 2</td>
<td>eEF 1-alpha-1</td>
<td>protein translation</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>beta actin</td>
<td>cytoskeleton</td>
<td>145</td>
</tr>
<tr>
<td>4</td>
<td>beta tubulin</td>
<td>cytoskeleton</td>
<td>167</td>
</tr>
<tr>
<td>5</td>
<td>TCTP</td>
<td>cell cycle</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>eIF-5A</td>
<td>initiation of translation</td>
<td>86</td>
</tr>
<tr>
<td>7</td>
<td>not identified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 + 9</td>
<td>cyclophilin A</td>
<td>immuno-suppressant</td>
<td>124</td>
</tr>
</tbody>
</table>
Table III  Percentage of lysine, arginine, β-sheets and α-helix in identified proteins of OLN-93 cells. The total amount of amino acids and the % content of lysine and arginine were determined by the mascot-sequence. The % content of β-sheets and α-helix were determined by the predicted secondary structure (JPRED).

<table>
<thead>
<tr>
<th>protein</th>
<th>total A.A</th>
<th>% of Lys</th>
<th>% of Arg</th>
<th>β-sheet</th>
<th>α-helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>beta tubulin</td>
<td>444</td>
<td>3.4</td>
<td>5.0</td>
<td>16.2</td>
<td>35.6</td>
</tr>
<tr>
<td>beta actin</td>
<td>375</td>
<td>5.1</td>
<td>4.8</td>
<td>23.2</td>
<td>27.2</td>
</tr>
<tr>
<td>alpha enolase</td>
<td>434</td>
<td>10.2</td>
<td>3.7</td>
<td>17.7</td>
<td>40.6</td>
</tr>
<tr>
<td>14-3-3 zeta</td>
<td>245</td>
<td>8.2</td>
<td>4.5</td>
<td>11.0</td>
<td>58.4</td>
</tr>
<tr>
<td>eEF-1</td>
<td>462</td>
<td>8.3</td>
<td>3.9</td>
<td>30.7</td>
<td>19.0</td>
</tr>
</tbody>
</table>

The Role of Protein Abundance

Of all Western blots shown, that seen in figure 1C shows the highest level of immunoreactivity which is caused by many different AGE modified proteins. It is important to note that there were many strong AGE-positive protein-spots in the area of pl 7-8 and molecular weight 25-50 kDa, whereas only a few, small protein spots were detected in the gel (1A). This observation illustrates that some low abundance proteins were strongly AGE-positive, even when they were unaccounted for in this investigation, because they were not recognized by all three antibodies. On the other hand, not every highly abundant protein is modified by AGEs. Note for example three strong spots at 80-110 kDa and 6-5.5 pH in the gel (FIG. 1A) which cannot be seen in any blot.

In order to address this point in more detail, we performed an optical-density measurement with PDQuest on the identified spots. Figures 3 and 4 show the staining-density of the different antibodies divided by the protein level of the gel-spot. These values indicate the unit of AGE-modification per unit protein. Additionally, they show data is normalized on the actin value to allow comparison of the different antibodies. The eEF-1, beta-tubulin, TCTP and Cyclophilin A are therefore realized as highly AGE-modified by all antibodies with minor exceptions. Comparing these data with the percentile content of Arg and Lys in the protein sequence (Tables III and IV), eEF-1 yielded the highest level of Arg and Lys and AGE-modification.
Relative AGE-modified protein levels of the OLN-93 proteome

![Bar chart showing relative AGE-modified protein levels for OLN-93 proteome](image)

FIGURE 3  Relative AGE-modified protein levels of the OLN-93 proteome. Optical density measurement was performed on gel and blots with PD-Quest. Shown here is the staining-density of the different antibodies, divided by protein level of the gel-spot (measured values are normalized to the value of actin).

Relative AGE-modified protein level of the SH-SY5Y proteome

![Bar chart showing relative AGE-modified protein levels for SH-SY5Y proteome](image)

FIGURE 4  Relative AGE-modified protein levels of the SH-SY5Y proteome. Optical density measurement was performed on gel and blots with PD-Quest. Shown here is the staining-density of the different antibodies, divided by protein level of the gel-spot (measured values are normalized to the value of actin). The eEF-1 (spots 1+2) and cyclophilin A (spots 8+9) bars where calculated as means of the two spots.
TCTP and Cyclophilin A have a higher Arg and Lys content and a higher level of modification than actin, but beta-tubulin is mostly shown to be more highly modified than actin, but having a smaller content of the relevant amino acids.

The data shown indicate that various proteins are affected by AGE modification in the tested cell lines. Most identified proteins were ubiquitous, and three of them were found to be AGE-positive for both cell lines. The AGE-modifications mostly take place in proteins connected with cell growth, cell metabolism, cytoskeleton and cell organization.

DISCUSSION

AGE-modification Targets Only a Subset of Proteins
In this report, we have identified 8 targets of protein modification by AGEs. For SH-SY5Y-cells, we found eukaryotic elongation factor 1 alpha 1 (eEF-1α), beta-actin, beta-tubulin, translationally-controlled tumor protein (TCTP), eukaryotic initiation factor 5A (eIF-5A) and cyclophilin A to be AGE-modified. In OLN-93 cells, again beta-tubulin, beta-actin and eEF-1α were modified by AGEs and furthermore, it could be shown that α-enolase and the zeta-polypeptide pore AGE-modifications. Most of these proteins are abundantly expressed.

We showed here that the pattern of the cell lysates (gel) and the pattern of AGE-modifications detected by the three antibodies on Western blots could be aligned to locate spots shown to be AGE-positive in the gel. But gel and blots were not super-imposable, pointing out that glycol-oxidative modification targets only a restricted set of proteins. Other authors have shown similar results (only a sub-set of proteins were modified) on AGE-modified proteins in peripheral blood lymphocytes (Poggioli et al., 2002).

Different AGE-Structures are Detected.
For our immunoassay, we use three different antibodies and picked only protein-spots that were marked to be AGE-positive by all of them. Since AGE-adducts are a wild-spread group of various structures, we will get at least some of the most common structures, because presumably all antibodies will recognize them. Additionally, some non-specificity can be eliminated by having three differently raised antibodies against three different epitopes. The fact that each antibody gives us a different protein-pattern supports this point of view. Even though the specific AGE-structure cannot be identified, using three antibodies can ensure the protein is AGE-modified. This approach gives us a good working base to find the main targets for AGE modifications.

Implications on the used Model System
In this work, cell-lines where used as a model system for the brain to address the question of which proteins are most sensitive for AGE-modifications, and the results of current study will be form a foundation for future studies that compare stressed vs non-stressed situations on the same and additional cell-lines. These studies are currently are in progress in our laboratory.

However, it has to be pointed out, that this used model system has a couple of implications. First, in order to keep a cell-line in a preferable state, one has to stay with higher concentrations of glucose than physiological for brain cells, because cell-lines have a much higher energy demand than native brain cells. Therefore, the glucose concentration used here is perhaps more comparable to a diabetes situation than to that of a healthy or AD-brain. A higher concentration of glucose presumably leads to a higher level of AGE-modifications compared to an in vivo situation.

Second, cells in culture are exposed to higher oxygen-concentrations than in vivo, because they have a higher demand of oxygen generated by a higher energy-metabolism and both presumably support oxidative modifications on proteins as well as oxidative stress.

On the other hand, cell-culture cells have a short life before they divided, compared to brain cells which live a long life in stable conditions, but being slowly modified by AGEs over the years. Therefore, investigating the cell-culture system here may provide information of accelerated aging in a non-stressed condition.

Evidence of Relative Modification-levels and Percentage Amount of Relevant Amino Acids
The calculation of the optical-density measurements of western blot and gel lead to an approximate quantification of the degree of AGE-modification relative to protein levels. The eEF-1, β-tubulin, TCTP and cyclophilin A were realized to be highly AGE-modified. To discover if this result may be based on common structural characteristics of this proteins, we did a bioinformatics-based search [JPRED: http://www.compbio.dundee.ac.uk/~www-jpred/] on the identified proteins, to get information about the secondary structure content and their amount of Lys- and Arg-residues. The majority of proteins that were observed to be glycated contain an N-terminal methionine capping, leading to a relatively long half-life (n-end rule; Bachmair et al., 1986). Additionally, the identified proteins have a comparably higher lysine content (natural frequency of lysine is 5.9%; Fasman, 1989) and were predicted to have β-sheets. Predominantly β-sheet proteins are found to be protease-resistant (e.g., prion protein; Selvaggi et al., 1993) and hence stable. Since the
identified proteins are mainly stable, they are exposed to potential glycation for a longer period of time, get presumably more glycated, and therefore become recognized by all antibodies.

A possible exception to this is the 14-3-3 zeta-polypeptide which has predominantly helical content and therefore might be less stable, but contains relatively high lysine. Beta-actin and tubulin have a standard amount of lysine. These proteins are part of cytoskeletal elements which are relatively stable and therefore may elongate the half-life of these proteins, leading to a longer exposition to glycation processes.

Possible Consequences of AGE-modification on Protein Structure and Function
Since enzyme function depends on protein structure, the loss of structure presumably caused by some AGE-modification would be critical for a particular enzyme, even in cells under "normal conditions", as investigated here. On the other hand, many AGE-modifications may not become functionally relevant. They occur on residues and/or to parts of the protein with no critical relevance to function. Also it should be mentioned that protein modifications by AGE refer to a variety of AGE-adducts linked to different amino acid residues, and therefore may produce different functional effects. Also the possibility of constitutive function of AGE-modified protein in cellular metabolism cannot be ignored. Nevertheless, clear indications of altered enzyme-activity caused by AGE-modifications have recently been shown (Bousova et al., 2005). Therefore, it is important to know the main protein targets for AGE modification in cells cultured in normal conditions.

AGE-modified Proteins Involved in Translation
The initiation phase of translation in eukaryotes is complex, and involves numerous factors. In addition, it is the main point of regulation of mRNA transcripts (Hershey, 1991; Kozak, 1999; Caraglia et al., 2001).

We show that the eukaryotic translation initiation factor 5A (eIF-5A) is modified in SH-SY5Y cells (FIG. 2, spot 6). The eIF-5A is an 18 kDa (Caraglia et al., 2001) protein which promotes the formation of the first peptide bond between methionine (amino acid of the start codon) and the first amino acid of the coding sequence (Hershey, 1991), and is therefore very important for initiating translation.

The protein eIF-5A is additionally required for cell proliferation (Bevec et al., 1994; Ganoza et al., 2002). Diminution of eIF-5A activity leads to a reversible arrest of the cells in the G1-phase of the cell cycle and inhibits the growth of mammalian cells (Park et al., 1984; Park, 1987; Caraglia et al., 2001; Clement et al., 2003). This might be connected to the postulated re-entry of AD-neurons into the cell cycle, stopping at the G1-phase (Arendt, 2002; Ueberham et al., 2003).

The eukaryotic elongation factor 1 (eEF1, FIG. 2: spots 1+2; FIG. 1: spot 10) catalyzes the binding of aminoacyl-tRNA to the ribosome. It has a very strong promoter which assures high translation levels (Ejiri, 2002). Elongation factor 1a is ubiquitous and highly abundant (1-2% of total cell protein).

Alterations or dysfunction of elongation factors were associated with several diseases such as cancer, AD and diabetes. In the case of AD, no correlation with EF-1α has been reported so far, but the translocation activity of EF-2 has been shown to be disabled (Johnson et al., 1992). An unbalanced expression of EF-1α compared to the other EF-1 subunits is reported in diabetes, which is supposed to compensate for the reduced protein synthesis associated with this disease. It remains unclear if these changes are causes or consequences of these diseases (Reynet and Kahn, 2001).

AGE-modified Cytoskeleton Proteins
All three antisera recognize actin (FIG. 1, spot 3; FIG. 2, spot 2) and tubulin (FIG. 1 spot 4; FIG. 2, spot 1) as glycated in both cell-lines. Being fundamental to the cytoskeleton, actin and tubulin make up a large part of the total cellular protein and are, therefore, highly concentrated in total lysates of living cells as used in this study.

Human actin has a molecular mass of 43 kDa (Kabsch and Holmes, 1995). Its genes encode for 6 isoforms and the β-isoform was shown to be modified in this study. It was shown that in AD-patients the mRNA concentration for β-actin is lowered in the hippocampus region, but not in the cerebellum (Vogelsberg-Ragaglia et al., 2001). Additionally, β-actin is differentially expressed in the brain specimens from control subjects compared to those of AD patients (Gutala and Reddy, 2004). β-actin is oxidatively modified in AD brain (Aksenov et al., 2001).

The actin sequence is highly conserved and many other proteins bind or interact with actin. Therefore, nearly every residue is probably crucial to some aspect of its function (Clarkson et al., 2004). As a result, an AGE-modification could easily be functionally relevant. Our investigation did not demonstrate whether modified actin is globular or bound in filaments, but even if modification does not impair the polymerization of the filaments, it could easily disturb interactions with other proteins important for stabilization of the filament, vesicle-transport, or likewise.

β-tubulin is a further cytoskeletal protein which was found to be AGE-modified in both cell lines (FIG. 1,
Tubulin has GTPase activity which impairs MT-stability and the balance of assembly/disassembly of MT is highly regulated by many factors (Downing and Nogales, 1999). Disturbance of this balance could easily lead to impairment of axonal transport as is shown to happen in AD (Kennedy and Baynes, 1984) and in diabetic neuropathy (Galbraith and Gallant, 2000).

It has been postulated that a delay of the slow axoplasmic transport is caused by non-enzymatic glycosylation/AGEs (Medori et al., 1988). For example, it has been shown that the non-enzymatic glycation of tubulin profoundly inhibited GTP-dependent tubulin polymerization, especially under the elevated glucose concentrations in diabetes mellitus (Williams et al., 1982). Moreover, it was shown in vitro by the same authors, that non-enzymatic tubulin glycosylation results in the formation of high molecular weight aggregates. The influence of non-enzymatic glycosylation on MT-assembly could be explained by the fact that some lysine residues of tubulin are critical for polymerisation (Williams et al., 1982) and that lysine residues are a specific target of AGE-modifications.

Tubulin is reported to be modified by non-enzymatic glycosylation in experimental and human diabetes (Kennedy and Baynes, 1984). It has been shown that axonal transport of tubulin (and also actin) is reduced in various diseases, including axonal degeneration (e.g., diabetic neuropathy), and with age (Galbraith and Gallant, 2000).

Hyperphosphorylation of tau is probably caused by an imbalance between phosphorylation and dephosphorylation leading to hyperphosphorylation. Hyperphosphorylated tau has been shown to be the major component of NFTs, but β-tubulin has been shown to be hyperphosphorylated in AD as well, thereby supporting the imbalance-thesis (Vijayan et al., 2001). Even when the altered phosphorylation did not seem to impair MT-assembly, it could easily constrict the interaction of MT with the associated proteins. Additionally, AGE-modified MT might therefore be unable to interact with tau, facilitating its accumulation.

Further Proteins Found to be AGE-modified
The translationally controlled tumor protein (TCTP) is AGE-modified in SH-SYSY cells (Fig. 2, spot 5). TCTP is a ubiquitously expressed protein present in diverse organisms throughout the entire animal and plant kingdom. This suggests a crucial role for this protein in cellular survival (Thiele et al., 2000; Cans et al., 2003) which isn’t recognized yet. TCTP binds to alpha and beta tubulin, as well as to microtubules (Gachet et al., 1999).

TCTP co-localizes with eEF1B-β and eEF1-A around the nucleus and near ER. This implicates a function for TCTP in translation elongation. This notion is supported by the observation that TCTP inhibits eEF1B-β-mediated GDP exchange reaction on eEF1-A (Cans et al., 2003).

TCTP binds tubulin during the cell cycle and is most active during mitosis (Gachet et al., 1999). This observation is supported by the finding that mRNA levels of TCTP are low in brain tissues, which naturally have low mitotic activity (Thiele et al., 2000; Bommer and Thiele, 2004). Here we worked with neuron and glia-derived cell lines which, of course, show high mitotic activity. The MT-binding site of TCTP contains predominantly lysine and glutamic acid residues (Gachet et al., 1999). The former could be modified by AGEs and, therefore, could alter the MT-binding capacity of TCTP.

Our findings show Cyclophilin A as a target for AGE-modification (Fig. 2, spots 8+9). Cyclophilin A (CypA) belongs to a class of proteins called immunophilins, a group of proteins which interact with immunosuppressant drugs leading to blocking of transcriptional activation of genes responsible for the early T-cell response (IL2, IL4) (Walsh et al., 1992).

The fact that CypA is recurrent cross-species, its abundance in many different tissues and the fact that CypA has 4 different cDNAs strongly suggested an important cellular function besides that of immunosuppression (Neye, 2000). However, it is still not completely clear which function that might be. CypA has cis-trans-peptidyl-prolyl-isomerase (PPIase)-activity which supports isomerisation of cis-X-Pro bonds to trans-X-Pro bonds (Walsh et al., 1992). Arg 55 is important for substrate-stabilization during the isomerisation-process (Agarwal et al., 2004) and due to its accessible position in the molecule, it is a potential target for AGE modifications.

An influence of CypA on a signal transduction pathway activating ERK 1 and 2 has been reported, which could influence cell cycle progression (Jin et al., 2000). Also it has been demonstrated that the human homologue for paravulin (Pin 1) is essential for G2-M transition in the eukaryotic cell cycle (Göthel and Marahiel, 1999). Therefore, AGE-modifications on CypA may impair cell cycle progression.

Immunophilins are more abundant in the nervous system than in the immune system, which may explain why we found them to be AGE-positive in the neuroblastoma cell line only. Due to their participation in axonal transport and synaptic vesicle assembly, it is reasonable to postulate that immunophilins protect against abnormal protein aggregation, which has been linked to several neurodegenerative disorders. Additionally, co-localisation with NFTs has also been
We have shown that 14-3-3 proteins (zeta-isofrom) are modified by AGEs in OLN-93-cells (FIG. 1, spot 9). 14-3-3 proteins are highly concentrated in brain tissues and comprise up to 1% of its total soluble protein. They are present in the cytoplasm, plasma membrane and in intracellular organelles (Berg et al., 2003). 14-3-3 proteins normally function as dimers and have three Lys- and Arg-residues, which form a basic pocket within the acidic molecule, to facilitate dimerization (Tzivion and Avnuch, 2002). As previously mentioned, Lys- and Arg-residues are potential targets for AGE-modifications, so they might disturb dimerization of 14-3-3 proteins. This would be functionally critical because most of its more than 100 binding partners (Yaffe, 2002) need the dimer-form for interaction (Tzivion et al., 2001).

There are many reports of elevated levels of 14-3-3 proteins in the cerebrospinal fluid of patients with various neurodegenerative diseases, and 14-3-3 proteins seem to assemble in some disease-specific lesions and protein aggregates within the brain (Berg et al., 2003). The zeta isotype, found to be AGE-modified here, has been shown to be associated with tangles in AD, and it has been suggested that 14-3-3-ζ plays a causative role in the development of this neuropathy (Hashiguchi et al., 2000). Amyloid beta-peptide(1-42) addition to neuronal cultures leads to oxidative modification of 14-3-3 protein (Boyd-Kimball et al., 2005). 14-3-3-ζ causes a conformational change in the tau-molecule exposing Ser262 and Ser356 to PKA phosphorylation, both of which lie within the microtubule-binding region of tau. This finding suggests that 14-3-3-ζ regulates tau phosphorylation and microtubule dynamics in the brain. Moreover, 14-3-3-ζ induces oligomerization of its target proteins and stabilizes the bound proteins against phosphatas (Hashiguchi et al., 2000).

Alpha-enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is a 47 kDa, highly expressed cytosolic enzyme which shows age-related loss of activity which is thought to be caused by posttranslational modification coincident with aging. Its consequent monomerization (Wistow et al., 1988) results in loss of enolase activity because this enzyme is only active as a dimer (Westhead, 1966).

Enolase mRNA is nearly undetectable in cells in the stationary phase, although enolase protein levels can be high - thereby implying that the enolase protein is extremely stable. Together with its proximity to glucose and glucose-fission products, this leads to an increased possibility for enolase to be glycated. It should additionally be noted that enolase has two important lysine residues (Lys 345+396) which are critical for protein function, because they stabilize the removed proton until the reaction is complete (Pancholi, 2001). As previously discussed, lysine and arginine are the primary modification targets for AGEs and would interfere with enzyme function. In AD brain α-enolase is oxidatively modified (Butterfield et al., 2003; Butterfield, 2004).

Possible Physiological and Pathological Consequences of AGE Modifications

We have shown in the in vitro systems used here that a number of proteins are vulnerable to AGE modifications, even in an unstressed situation. Most of these proteins contain functionally relevant arginine or lysine residues at readily accessible locations and which may be potential targets for AGE modifications. Modifications at such sites will presumably have an effect on function.

We speculate that most of these modifications are consequential in the context of normal cell physiology and might reflect a neutral phenomenon related to protein ageing. However, there is increasing evidence for the involvement of AGE modifications in pathophysiological situations. Further studies are needed to characterize the influence of various factors related to patho-physiological conditions on AGE modifications of the currently identified proteins.

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