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

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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.

Keywords: Advanced glycation end products; AGEs; Alzheimer disease; SH-SY5Y cells; OLN-93 cells; β-actin; β-tubulin; Eukaryotic elongation factor 1-α; Aging; Neurodegenerative disease; 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 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 vesperlysine (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).

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AGE-modified, but not crosslinked, proteins must be repaired, replaced and/or degraded (Thornalley, 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 glycoxidative 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 (Thornalley *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 AGE 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 Antisera

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 reboostering, 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% CO_2/95% 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 KH_2PO_4, 0.6 mM $MgSO_4$ 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 15,800*g* for 5 min to remove membrane lipids and debris. The supernatant was isolated and protein concentration determined by the BCA method