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Original Contribution

Cathepsins D and L reduce the toxicity of advanced glycation end products

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ABSTRACT

Advanced glycation end product-modified proteins are known for accumulating during aging and in several pathological conditions such as diabetes, renal failure, and neurodegenerative disorders. There is little information about the intracellular fate of endocytosed advanced glycation end products (AGEs) and their influence on proteolytic systems. However, it is known that the lysosomal system is impaired during aging. Therefore, undegraded material may accumulate and play a considerable role in the development of diverse diseases. To investigate if AGEs can be degraded and to test whether they accumulate because of impaired lysosomal proteases we studied the effects of advanced glycation end products on the endosomal–lysosomal system. Five different types of AGEs were generated by bovine serum albumin incubation with glyoxal, methylglyoxal, glucose, fructose, and ribose. The first experiments revealed the uptake of AGEs by the macrophage cell line RAW 264.7. Further investigations demonstrated an increase in cathepsin D and L activity and an increase in mature cathepsins D and L. Increased activities were accompanied by the presence of more lysosomes, measured by staining with LysoTracker blue. To specify the roles of cathepsins D and L we used knockout cells to test the roles of both cathepsins on the toxicity of advanced glycation end products. In summary we conclude that both cathepsins are required for a reduction in advanced glycation end product-induced cytotoxicity.

Advanced glycation end products (AGEs) are the final products of the nonenzymatic reaction of reducing sugars and reactive aldehydes with proteins, lipids, and nucleic acids. More precisely, AGEs are formed in the so-called "Amadori reaction" by irreversible conjugation of reducing sugars and other reactive carbonyl compounds with amino groups or side chains of proteins [1] or adduct formation between carbonyl group-containing molecules and lipids and nucleic acids [2], leading to a diverse range of stable, heterogeneous structures. To date more than a dozen AGEs have been determined in various tissues in humans [3]. In addition, AGEs have been widely detected in various foods, including bakery products and full-fat milk, formed by heating [4]. Recently, it has been shown that the accumulation of AGEs is a major pathogenic process in diabetes in which blood sugar is increased and therefore the glycation reaction is accelerated [5,6]. However, AGEs are not only involved in diabetes, they are also formed in physiological aging, in senile cataract, arteriosclerosis, and neurodegenerative disorders [7-9] such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis [10]. Neurodegenerative diseases are characterized by continuous dysfunction of neuronal cells resulting in their own cell death. One of the major dysfunctions is the reduced ability to degrade undesirable and pathological toxic proteins during aging. Cells begin to lose the capacity to get rid of these molecules and finally they form higher molecular aggregates [11]. As AGEs are found in diverse diseases and because some of the effects of AGEs have been shown to be lethal [12] it is of great interest to reduce the amount of AGEs in vivo. Hence, it is essential to investigate possible AGE-degradation pathways.

The degradation of a diversity of macromolecules, including proteins, lipids, nucleic acids, and carbohydrates, which are internalized from the extracellular space by endocytosis and transferred by fusion with phagosomes, takes place in the endosomal–lysosomal system. Additionally, lysosomes are responsible for the degradation of cytoplasmic molecules through autophagy [13]. Another important intracellular protease is in the proteasome, which degrades moderately modified proteins. Heavily modified proteins are likely to be processed by the lysosomal proteases [14]. Experiments confirmed that AGEs are able to undergo receptor-mediated endocytosis by scavenger receptors [15]. Receptor-mediated endocytosis is initiated by binding of a soluble

Abbreviations: AGE, advanced glycation end product; AMC, 7-amino-4-methylcoumarin; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAMP1, lysosomal-associated membrane protein 1; MOCA, (7-methoxycoumarin-4-yl) acetyl; MOCA-GKPILFFRLK (Dnp)-RNH_2, MOCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (dinitrophenyl)-Arg-NH_2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; z-FR-AMC, z-Phe-Arg-AMC.

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ligand to a receptor, followed by the internalization of the receptor–ligand complex. The road map for the passage of endocytosed material is well established [16]. The vesicles move within the cell through a series of fusion and budding events of other vesicles, which are known as early and late endosomes and lysosomes, accompanied by a progressive acidification of the compartments [17]. During the fusion and acidification steps dissociation of the ligand from the receptor occurs and the receptor is recycled to the membrane, whereas the ligand is transported for degradation or modification within endosomes and subsequently lysosomes [18].

Lysosomes were discovered by Christian de Duve in 1955 and are known for containing more than 50 different acid hydrolases—proteases and peptidases, glycosidases, nucleases, sulfatases, lipases, and phosphatases—which degrade most types of biological macromolecules [19,20]. Functional deficiencies of lysosomal hydrolases can result in lysosomal storage diseases, which are characterized by intralysosomal deposition of macromolecules and disturbance of lysosomal function [20]. Lysosomes are involved in various cellular processes such as endocytosis and phagocytosis, as mentioned before. Other processes are autophagy, membrane repair, cell signaling and cell death, pathogen defense and antigen presentation, and bone and tissue remodeling [21]. All these complex functions make it obvious that lysosomes are more than just the wastebaskets of the cell. They are rather a central compartment in cell metabolism.

Lysosomal hydrolases are expressed both ubiquitously and in a tissue- and cell-type-specific manner [21]. Additionally, some lysosomal hydrolases (e.g., cathepsin D) are expressed constitutively or in an inducible manner [22]. The cathepsin D gene promoter has a mixed structure showing multiple GC boxes that characterize the expression of housekeeping genes and TATA sequences for the expression of regulated genes [22]. Cathepsins are subdivided into three groups depending on the amino acids located in their active sites that perform catalytic activity: aspartyl cathepsins (cathepsins D and E), cysteine cathepsins (cathepsins B, C, F, H, K, L, N, O, S, T, U, W, and X), and serine cathepsins (cathepsins A and G). Cathepsins differ in their tissue distribution, substrate specificity, and function [23]. The major lysosomal aspartic endoproteinase cathepsin D (EC 3.4.23.5) is a member of the pepsin family of proteinases [24]. It degrades peptide bonds flanked by hydrophobic amino acids in a pH optimum between 3.5 and 4.5 [25]. The cysteine cathepsins, e.g., cathepsin B (EC 3.4.22.1) and the endopeptidase cathepsin L (EC 3.4.22.15), are papain-like enzymes. This enzyme class is responsible for normal cellular functions such as continuous protein turnover, antigen processing [26], and bone remodeling [27]. Additionally they are involved in pathological processes such as tumor invasion and metastasis [28] and play an important role concerning joint and bone diseases to which osteoporosis, rheumatoid arthritis, and osteoarthritis belong [29]. Cathepsin L is the most powerful lysosomal cysteine proteinase against protein substrates and it is specific for hydrophobic amino acids. Both cathepsin L and cathepsin D are involved in the initiation of lysosomal proteolysis [30].

Cathepsins are synthesized as preprocathepsins and transferred cotranslationally into the rough endoplasmic reticulum. The pre-signal peptide is cleaved off and sugars are attached at the specific glycosylation sites on the resulting procathepsins leading to the formation of mannose 6-phosphate (M6P) residues. The M6P-containing procathepsins are passed into the Golgi complex and bind to M6P-specific receptors. The receptor–procathepsin complex is finally passed in clathrin-coated vesicles to the endosomes. After the fusion with late endosomes the receptor–procathepsin complex dissociates and the receptor is recycled to the Golgi apparatus. Finally, procathepsins are converted into active cathepsins through proteolytic removal of the propeptide in the acidic environment of late endosomes and lysosomes [31,32].

As phagocytosis and degradation of abnormal proteins are two of the main mechanisms of limiting pathologies associated with the accumulation of damaged proteins it is important to study the effects of AGEs on proteolytic systems. We previously reported that the proteasomal system is not involved in the degradation or processing of AGEs [33]. Furthermore we discovered a functional role of cathepsin D that is implicated in attenuating AGE accumulation [33]. The aim of this study was to find out whether there is any resemblance between the roles of cathepsins L and B and that of cathepsin D.

Additionally, we expected to discover some more functional characterizations of lysosomal proteins that will help to deepen generally the understanding of lysosomes in cellular metabolism.

Materials and methods

Materials

RPMI medium 1640, Dulbecco's modified Eagle's medium (DMEM), penicillin (10,000 E)/streptomycin (10,000μg/ml), and fetal bovine serum were purchased from Biochrom (Berlin, Germany). LysoTracker Blue DND-22 was purchased from Molecular Probes Invitrogen (Karlsruhe, Germany). Pepstatin A was bought from Enzo Life Sciences (Loerrach, Germany). D-[1-^{14}C]Glucose (250μCi) was purchased from PerkinElmer (Rodgau, Germany). Other chemicals were of the best grade available from Sigma–Aldrich (Taufkirchen, Germany) or from Carl Roth (Karlsruhe, Germany). RAW 264.7, a murine macrophage cell line, and fibroblasts were used for cell culture experiments. The mouse embryonic cathepsin D-deficient and cathepsin L-deficient fibroblasts were prepared as described previously [33,34].

Preparation of AGE-modified bovine serum albumin

D-Glucose-, D-fructose-, and D-ribose-modified bovine serum albumin (BSA) was produced as described by Stolzing et al. [35], and glyoxal- as well as methylglyoxal-modified BSA was prepared according to Nagai et al. [36] and Mikulikova et al. [37]. Briefly, 1mM fatty acid-free, endotoxin-free BSA was dissolved in 0.5M sodium phosphate buffer (PBS; pH 7.4) with 250mM sugars or 20mM aldehydes. These preparations were sterilized by ultrafiltration and incubated at 37°C for six weeks (sugars) or one week (aldehydes). To remove unbound sugars or aldehydes the preparations were dialyzed against PBS for 24h followed by the measurement of protein content with the Bradford assay. To verify AGE formation the preparations were assayed for their optical density and fluorescence according to Stolzing et al. [35]. In this work we used the same advanced glycation end products that were characterized for their optical density, fluorescence, protein cross-linking, and protein oxidation in one of our publications [33]. [^{14}C]Glucose-modified BSA was prepared as described above, and 50μCi (1.85 mBq) was added to the preparation. After six weeks of incubation the solution was dialyzed against PBS to remove unbound radioactive and nonradioactive glucose.

The final concentrations of AGEs used in these studies were 25mM sugar-modified albumin and 2mM aldehyde-modified albumin. These AGE concentrations contained 100μM proteins. To apply the same protein concentration to each sample, we used fresh BSA for the control samples and BSA that was incubated under the same conditions as AGE-modified albumin for the BSA samples. We characterized fresh and incubated BSA according to the method described by Stolzing et al. [35]. As the incubated BSA showed some characteristics of aggregates in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autofluorescence we calculated our results in comparison to fresh BSA. We included the BSA sample (incubated BSA) in our experiments to see if the observed effects were due to aggregated BSA alone or due to advanced glycation end products.

Cell culture

The RAW 264.7 cell line was grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 1% penicillin/