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Cathepsin D is one of the major enzymes involved in intracellular degradation of AGE-modified proteins

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Abstract
Oxidized and cross-linked modified proteins are known to accumulate in ageing. Little is known about whether the accumulation of proteins modified by advanced glycation end products (AGEs) is due to an affected intracellular degradation. Therefore, this study was designed to determine whether the intracellular enzymes cathepsin B, cathepsin D and the 20S proteasome are able to degrade AGE-modified proteins *in vitro*. It shows that AGE-modified albumin is degraded by cathepsin D, while cathepsin B was less effective in the degradation of aldehyde-modified albumin and the 20S proteasome was completely unable to degrade them. Mouse primary embryonic fibroblasts isolated from a cathepsin D knockout animals were found to have an extensive intracellular AGE-accumulation, mainly in lysosomes, and a reduction of AGE-modified protein degradation compared to cells isolated from wild type animals. In summary, it can be assumed that cathepsin D plays a significant role in the removal of AGE-modified proteins.

Keywords: *Advanced glycation end products, protein degradation, lysosome, proteasome, proteases, cathepsins*

Abbreviations: *BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MCA, 7-amino-4-methylcoumarin; 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-2H-tetrazolium bromide]-reduction; SUC-LLVY-MCA, succinyl-leu-leu-val-tyr-7-amino-4-methylcoumarin; z-FR-MCA, z-phe-arg-MCA.*

Introduction

In the Maillard reaction, accessible free amino groups of proteins are sensitive to react with reducing sugars or aldehyde/ketone adducts to form Schiff base and Amadori products [1]. This non-enzymatic reaction includes several glycation and oxidation processes. After some time, Amadori products undergo further modifications and irreversible so-called advanced glycation end products (AGEs) are produced [1].

High protein turnover and a short half-life of many intracellular proteins can make them less prone to accumulation of AGE-modified proteins and therefore these molecules might escape from the accumulation of glycated adducts. However, under certain conditions like ageing and some pathological conditions such as hyperglycaemia, especially long lived extracellular proteins lead towards enhanced glycation reactions. However, not only the half-life of proteins is important for the modification with AGEs, but also structural pre-requisites, as recently shown [2].

In the case of modification, AGEs alter the structure and function of proteins and may therefore

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contribute to the pathogenesis and diverse complications observed in diseases. In fact, several pathologies are known for their accumulation of modified proteins in the extracellular space, like arteriosclerosis, cataract formation and additionally in ageing [3].

Cellular interactions with AGE-modified proteins are known to provoke a number of biological responses, not only endocytosis, but also induction of cytokines and growth factors, which are linked to the development of the pathologies mentioned above [1]. Endocytosis itself is well characterized and mediated by several receptors. However, the exact receptor system, which is involved in the specific intracellular uptake of AGEs, depends on the cell type. Several AGE-binding proteins have been identified, like the RAGE (*r*eceptor for *a*dvanced *g*lycation *e*nd products) receptor [4,5], CD36 (a scavenger receptor which belongs to scavenger receptor family type B) [6], macrophage scavenger receptor A I and II (belonging to the scavenger receptor family type A) [7,8] and the receptor complex p60, p90 and galectin-3 [7,9]. Some of them are responsible for endocytosis and/or for the specific cell signalling triggered by binding to AGEs. For instance, the scavenger receptor CD36 has been identified as playing an important role in the uptake of oxidized protein substrates and AGE-modified albumin [10]. There are some further investigations which describe an observed uptake via receptors like CD36 and macrophages scavenger receptor A and subsequent degradation of ^{125}I-AGE-BSA [11,12]. For the RAGE receptor it still remains to be determined if it is involved in endocytosis or if its role is limited to signal transduction.

Even though there are specific receptors which may fulfil the endocytosis of AGEs, it is obscure if AGE-modified proteins can be degraded and to what extent. Only a few data exist about a possible proteolytic process of AGEs [11,12], but the exact mechanisms are inexplicit. Based on these observations, it can be assumed that, after the specific recognition by receptors and internalization, extracellular glycated proteins can be removed. However, it is not known which proteases are involved in this possible process.

Therefore, we designed this study to investigate the role of several proteases in the degradation of AGE-modified albumin. Two proteolytic systems can be taken into considerations, the lysosomes and the proteasome. Once compounds from the extracellular space are internalized, they are located in early and late endosomes, followed by the formation of terminal lysosomes which are specific for their decline in the internal pH and the liberation of active proteases [13]. Lysosomes are responsible for the degradation of diverse materials from extracellular space through endocytosis or phagocytosis, as well as from the cytoplasm through autophagy [14]. The main proteases in lysosomes are the cathepsins, which can be sub-divided into three groups according to the amino acid that confers the catalytic activity in the active site. The 11 human cysteine cathepsins are cathepsins B, C, F, H, K, L, O, S, V/L2, W and X/Z/P. The amino acid aspartic acid characterizes cathepsin D and serine cathepsins are cathepsin A and G [15].

The cathepsins participate in numerous physiological processes such as protein degradation, regulation of signal transduction and hormone processing, antigen presentation and storage of cellular waste products and therefore play a key role in normal cell functions [16–18]. Numerous studies indicate that disturbed protein degradation leads to lysosomal aggregate–accumulation during ageing and these structures are a common feature in neurodegenerative diseases [19–21].

Another proteolytic complex, located in the cytosol and nucleus, is the proteasome [22]. The proteasome is responsible for the degradation of intracellular damaged proteins and general protein turnover [23]. Two forms of the proteasome exist. The 20S proteasome is ubiquitin- and ATP-independent and involved in 70–80% of the degradation of mildly oxidized proteins, whereas the 26S proteasome, formed upon the association of the 19S regulatory complex with the 20S catalytic core, is in general associated with ubiquitin- and ATP-dependent proteolytic pathways [23,24]. An age-related decline in the protease activities of the 20S and 26S proteasome has been reported to contribute to the accumulation of highly oxidized and aggregated proteins in the cytosol [25].

To explain the role of several proteolytic systems, we decided to use the proteasome, as well as some cathepsins (cathepsin D and cathepsin B), as their role in the degradation of age-related plaques, especially amyloid-beta protein, is well described [26–28]. We compared the obtained results with some proteases from the gastro-intestinal system, as it is known that AGEs can be taken up via nutrition [29]. Based on the first results of this study, we used mouse embryonic fibroblasts isolated from lysosomal enzyme cathepsin D knockout animals to further clarify the role of this enzyme in the degradation of AGE-modified proteins. In addition to that we tested whether the uptaken AGE-modified proteins are mainly located in the endosomal-lysosomal compartment.

Materials and methods

Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, foetal calf serum, penicillin (10 000 E) and streptomycin (10 000 µg/ml) (P/S) were purchased from Biochrom (Berlin, Germany). LysoTracker Blue DND-22 was from Molecular Probes, Invitrogen (Karlsruhe, Germany). Other chemicals were of the best grade available from Sigma-Aldrich (St. Louis, MO, USA) or Roth (Karlsruhe, Germany).

Preparation of AGE-modified bovine serum albumin

Sugar-modified bovine serum albumin (BSA) was prepared as previously described by Stolzing at al. [30] and aldehyde-modified BSA was produced according to Nagai et al. [31] and Mikulikova et al. [32]. Briefly, 1 mM of fatty acid-free bovine serum albumin (BSA) was dissolved in 0.5 M sodium phosphate buffer (PBS, pH 7.4) with various concentrations of D-glucose, D-fructose, D-ribose, glyoxal and methylglyoxal. These preparations were sterilized by ultra filtration and incubated at 37°C for 6 weeks (sugars) or 1 week (aldehydes), followed by dialysis against PBS over 24 h. During this incubation time, oxygen was present as it may also play a role in the formation of AGEs *in vivo*. Metal ions were present in the substrates in just a very small amount (under 10 ppm) according to the certifications of analyses. AGE-modification grade of BSA was determined by optical density, fluorescence and cross-linking according to Stolzing et al. [30] and protein carbonyl formation was measured as described by Buss et al. [33] with modifications by Sitte et al. [34]. Additionally, protein content was measured by the Bradford assay, using BSA as standards.

Degradation of AGE-modified BSA by proteases

Measures of 250 mM of glucose-, fructose- or ribose-modified albumin as well as 20 mM of glyoxal- or methylglyoxal-modified albumin were incubated with the pure proteasome (20S), cathepsin D, cathepsin B, pepsin, trypsin, chymotrypsin and proteinase K. This method was modified according to Reinheckel et al. [35]. The final concentration of the enzymes and substrates was previously tested out in order to determine the optimal degradation rate. This optimal degradation rate was calculated as 30% from the maximum degradation rate of unmodified albumin, where further increased enzyme concentrations did not show any influence. Based on this 30% degradation rate of unmodified albumin, we could easily see any possible difference in the degradation (increased or decreased) of AGE-modified albumin. The ratio of enzyme:substrate for 30% degradation was determined for each enzyme.

The samples were incubated for 2 h at 37°C, followed by the measurement of free NH_2, detected via fluorescamine [35]. The fluorescence intensity was measured at 360 nm excitation and 460 nm emission in a fluorescence reader and the NH_2-concentration was calculated using glycine as standards.

Cell culture

Mouse embryonic fibroblasts (MEF) were obtained from cathepsin D deficient mice and wild-type littermates backcrossed to the C57BL/6 genetic background [36,37]. Briefly, the MEF were prepared from individual embryos at embryonic day 13.5. The head and internal organs were removed and the torso was minced and dispersed in 0.25% trypsin (Gibco/Invitrogen, Karlsruhe, Germany) for 15 min at 37°C. Cells were collected by centrifugation, plated on plastic cell culture dishes and grown to confluence in DMEM containing 10% inactivated foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. At this stage, cell numbers were determined by counting trypan blue excluding viable cells and this number was set as reference for determination of population doublings. They were passaged every 2 weeks and medium was changed twice weekly. Cells of a population doubling level 5–18 were used for the experiments.

Proteasomal and lysosomal activity

Activity of the 20S proteasome was assayed as described previously by Stolzing et al. [30] with minor modifications. In brief, cells were washed twice with phosphate buffered saline (PBS) and detached using trypsin/EDTA. The cell suspension was centrifuged for 5 min at 900 × g and lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM magnesium chloride, 1 mM EDTA and 1.7 mM DTT) was added to the pellet. Cells were lysed using a syringe, followed by repeated freeze–thaw cycles. Afterwards, cell lysates were centrifuged at 14 000 × g for 30 min and the supernatant was used for the determination of protein content with Bradford assay and proteasomal activity. For proteasomal activity, the supernatant was incubated in 225 mM Tris buffer (pH 7.8), 45 mM potassium chloride, 7.5 mM magnesium acetate, 7.5 mM magnesium chloride and 1 mM DTT. As a substrate for the chymotrypsin-like proteasomal activity the fluorogenic peptide suc-LLVY-MCA was used. Samples were incubated for 30 min at 37°C, followed by MCA liberation measurement with a fluorescence reader at 360 nm excitation and 460 nm emission. Free MCA was used as standards for quantification. The 20S proteasome was isolated according to Hough et al. [38].

Measurement of the lysosomal activity was previously described by Sitte et al. [39]. This method is similar to the above one for proteasomal activity except for the preparation of the cell lysates. Cells were harvested and incubated for 1 h in 1 mM DTT at 4°C under vigorous shaking. Lysates were sonicated for 2 min using SONOPLUS GM70 on ice. The cell lysates were incubated in 50 mM sodium acetate, 8 mM cysteine-hydrochloride and 1 mM EDTA pH 4.0. For lysosomal cysteine cathepsins (mainly cathepsin B and L) the substrate z-FR-MCA (Bachem, Germany) was used and the MCA liberation was measured with a fluorescence reader at 360 nm excitation and 460 nm emission.

For cathepsin D and E activity, the substrate MOCA-GKPILFFRLK(Dnp)-RNH_2 (Biomol, Germany) was used and MOCA liberation was measured at 360 nm excitation and 460 nm emission. The liberation of MCA and MOCA was calculated by a calibration curve with MCA or MOCA (Biomol, Germany) standards.

Cell viability

The assessment of viability, based on the mitochondrial function, was performed by the reduction of soluble MTT (3-[4,5-dimethylthiazol-2yl]-2-5-diphenyltetrazolium bromide) into an insoluble formazan reaction product [40]. For this assay, cells were incubated for 24 h with different concentrations of AGE-modified albumin, followed by replacement of medium with MTT (0.17 mg/ml in medium). After 2 h incubation, cells were solubilized in dimethylsulphoxide (DMSO), 10% sodium dodecyl sulphate (SDS) and 0.6% acetic acid. The optical density of the formazan product was measured at 590 nm with a microplate reader. Viability is expressed in percentage to wild type control (100%) with the same amount of pure BSA.

Flow cytometry

For the detection of autofluorescence in AGE-treated wild type and knockout fibroblasts, cells were incubated for 24 h with glyoxal- or methylglyoxal- (2 mM aldehyde concentration), as well as glucose-, fructose- or ribose-modified (each sugar in a 25 mM concentration) albumin. Cells (1×10^6) were washed three times with ice-cold PBS, scraped and suspended in PBS. The cells were analysed by flow cytometry using a FACScan cytometer (Epics XL, Beckman Coulter, CA, USA). A total of 20 000 cells were counted for each FACS analysis. As a control, cells without addition of AGE-modified albumin were measured, in order to determine the autofluorescence of untreated cells.

Immunofluorescence microscopy

Cells were incubated with glyoxal-modified BSA (glyoxal concentration: 2 mM) for 7 h, followed by an addition of 75 nM LysoTracker Blue DND-22 (Molecular Probes, Invitrogen) according to the manufacturer's instruction. After 1 h incubation, cells were washed twice with ice-cold PBS and examined on a fluorescence microscope (Zeiss 'Axioplan' fluorescence microscopy). Due to the fact that LysoTracker Blue and the autofluorescence of AGEs had different excitation/emission filter sets, it was possible to analyse both channels. An overlay of the different records was done with the software program Adobe Photoshop CS3.

Statistical analysis

Data are presented as mean ± SD. Significance of differences was tested using ANOVA, Bonferroni's multiple comparison test, considering $p < 0.05$ as significantly different.

Results

Characterization of advanced glycation end products

In the Maillard reaction—also called browning process—mainly proteins react with sugars or aldehydes to form Schiff bases, followed by Amadori products. These early products are converted into advanced glycation end products after some incubation, which can be characterized physico-chemically using absorbance, fluorescence, intra- and intermolecular cross-linking and finally by the formation of the oxidation product protein carbonyls (Figure 1). Figure 1A shows the optical density of AGE-modified albumin, depending on the browning reaction, after 6 weeks incubation of sugars and 1 week of aldehydes with BSA. Traces of oxygen and metal ions were not removed, but during the incubation no additional oxygen was allowed. The light grey column represents BSA protein incubated without sugars and was taken as a control. All the other modifications were calculated according to the BSA control. We detected an increase in the optical density, on average more than 2-fold higher than in the BSA control solution based on the absorbance properties of the AGE-adducts. In Figure 1B, the fluorescence of different modified BSA at 460 excitation and 530 nm emission is demonstrated. This fluorescence qualification can be attributed to the formation of ring structures during the browning reaction [34,41]. In further analysis we tested whether the incubation of sugars or aldehydes with BSA resulted in an increase in protein cross-linking (Figure 1C). For this, we performed an electrophoresis and detected that ribose-, fructose- and methylglyoxal-modified albumin formed high-molecular-weight protein aggregates. In contrast to them, glucose- and glyoxal-modified albumin showed less formation of aggregated proteins. As it is known that the AGE formation might be accompanied by oxidation reactions, we additionally investigated the formation of a protein oxidation product, the protein carbonyl groups (Figure 1D). As demonstrated, there is an increase in protein carbonyl groups after modification of BSA with sugar or aldehydes as compared with pure albumin.

Degradation of AGE-modified proteins by intracellular and gastro-intestinal proteases

In the following we tested whether these formed albumin-AGEs can be degraded by major intracellular proteases. In Figure 2 the results of degradation

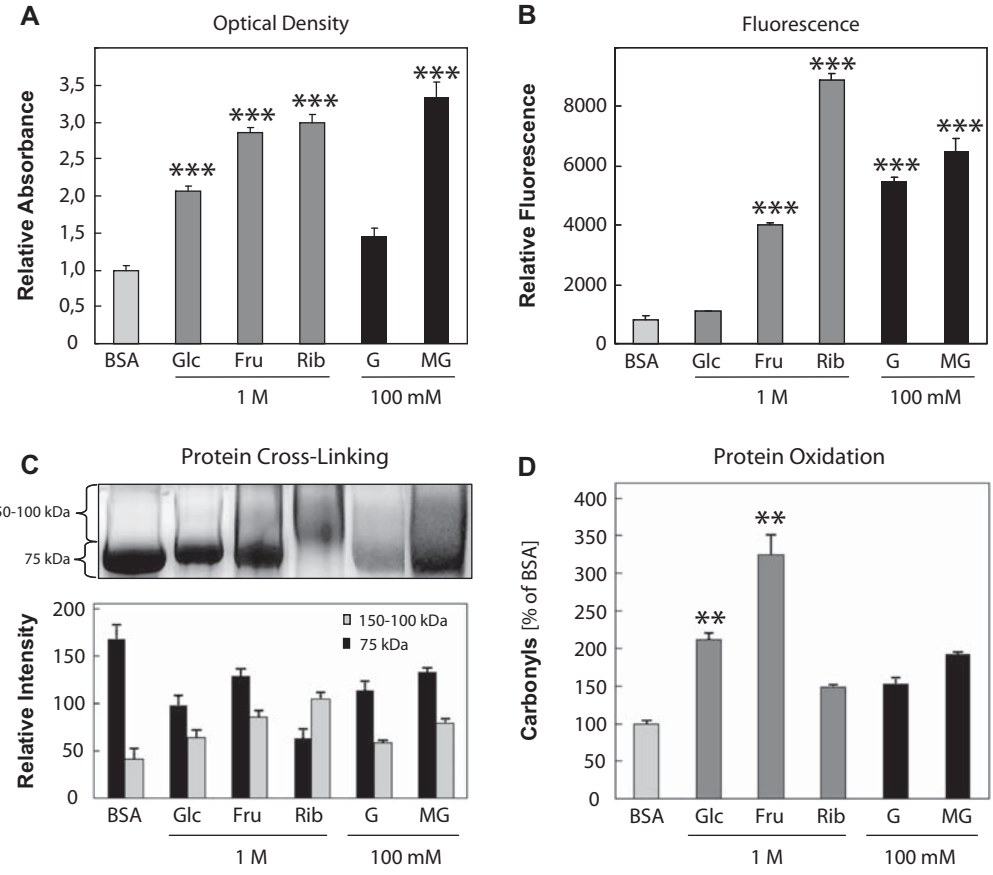

Figure 1. Physico-chemical characterization of advanced glycation end products (AGE). AGEs are formed by incubating bovine serum albumin (BSA) with the indicated concentrations of glucose (Glc), fructose (Fru), ribose (Rib), glyoxal (G) and methylglyoxal (MG) as described in the Methods section. The results of the browning reaction were determined by measuring the absorbance at 300 nm (A). Further the AGE-modified albumin were characterized by fluorescence at 460 excitation and 530 nm emission (B), the formation of intermolecular cross-links by electrophoresis (C) and the formation of protein-bound carbonyl groups (D). For the calculation in (C), we determined the pixel intensities for two areas of each lane on the gel. The first area included the field of 100–150 kDa and the second region was set at ~ 75 kDa. All data reflect the mean ± SD of the independent experiments. Statistical significance between BSA and BSA-modified AGEs is indicated as $^{***}(n=3,\ p < 0.001$, ANOVA, Bonferroni's multiple comparison test) and $^{**}(n=3,\ p < 0.01$, ANOVA, Bonferroni's multiple comparison test).

attempts of three different intracellular proteases, including 20S proteasome (Figure 2A) and the lysosomal aspartic pepsin like-protease cathepsin D (Figure 2B) and the cysteine protease cathepsin B (Figure 2C) are shown. As shown in Figure 2A, native bovine serum albumin (BSA) represents a very poor substrate for the 20S proteasome. Interestingly, also the modified BSA could not be degraded by this proteolytic system. For positive control of the activity of the 20S proteasome, we compared the degradation of oxidized BSA to non-oxidized BSA, and we could observe a significant 5-fold higher degradation rate of oxidized BSA compared to non-oxidized BSA. Next we determined the degradation of AGE-modified albumin by cathepsin D after determining the optimal degradation rate (see Methods) (Figure 2B) and cathepsin B (Figure 2C). Both enzymes are able to degrade sugar- and aldehyde-modified BSA to some extent. Although these proteases did not show an enhanced degradation rate, was demeonstrated for the 20S proteasome in the case of oxidized proteins, there is some remaining degradation for the sugar-modified BSA and in the case of cathepsin D also for the aldehyde-modified BSA. In any case this degradation is much more effective than that of the 20S proteasome. Since cathepsin D is more effective than cathepsin B in the case of aldehyde-modified BSA, we concluded that cathepsin D is one of the major enzymes involved in the degradation of AGE-modified proteins.

Since we were successful in determining cathepsin D as a potential enzyme degrading intracellular AGE-BSA we decided to test whether other proteases are also able to do so. So we used some gastro-intestinal proteases, as it is known that AGEs can also be taken up via nutrition [29] and proteinase K as a widely experimentally used serine protease with a broad substrate specificity.

The results of the degradation ability of pepsin, trypsin, chymotrypsin and proteinase K are shown in Figure 3. Both pepsin and proteinase K are able to degrade AGE-modified albumin (Figures 3A and D, respectively), however, trypsin and chymotrypsin exert a lower degradation-capability (Figures 3B and C, correspondingly).

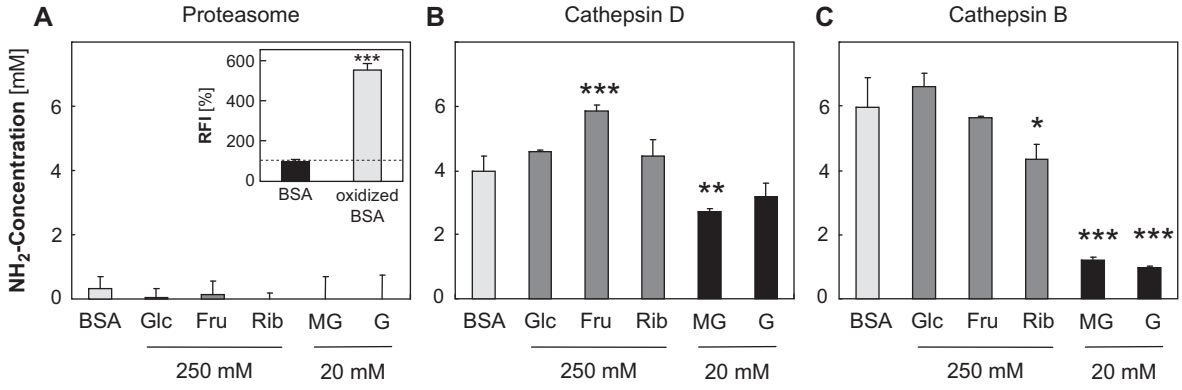

Figure 2. Degradation of AGE-modified albumin by intracellular proteases. Glucose (Glc), fructose (Fru) and ribose (Rib)-modified albumin (grey columns), methylglyoxal (MG) and glyoxal (G)-modified albumin (black columns) as well as unmodified albumin (BSA, 1 mg/ml, light grey columns) were incubated with isolated 20S proteasome (A), cathepsin D (B) and cathepsin B (C). The proportion of enzyme and substrate was established in additional experiments, taking a maximal degradation as readout (data not shown), described in Methods section. The enzyme-to-substrate ratio depicted here was in (A) 1:15 (w/w), in (B) 1:25 (w/w) and (C) 1:5 (w/w). The samples were incubated for 2 h at 37°C, followed by the measurement of free NH_2 as an indicator of protein degradation, as described in Methods. The inserted diagram in (A) shows the proteasomal degradation of oxidized and non-oxidized BSA, whereas oxidized protein was calculated according to the 100% control (light grey column). All data reflect the mean ± SD of four independent experiments. Statistical significance between BSA and BSA-modified AGEs is indicated as $^{***}(n=3,\ p < 0.001$, ANOVA, Bonferroni's multiple comparison test), $^{**}(n=3,\ p < 0.01$, ANOVA, Bonferroni's multiple comparison test) and $^{*}(n=3,\ p < 0.05$, ANOVA, Bonferroni's multiple comparison test).

Degradation of glyoxal-modified albumin by cathepsin D

In our *in vitro* assays we were able to show that several proteases are able to degrade AGE-modified BSA. Most importantly, we could identify cathepsin D as a potential intracellular protease responsible for the AGE-protein degradation. Therefore, we were wondering if the observed degradation of AGE-BSA is dependent on the degree of modification and of the time of the enzyme action. As demonstrated in Figure 4A, we could find that high amounts of glyoxal-modification to albumin (to 100 mM) can also be degraded by cathepsin D. It seems that there is no strong modification-dependent decrease in the degradation capability. Furthermore, we incubated glyoxal (20 mM)-modified albumin for 2, 8 and 24 h with cathepsin D and determined the degradation rate and the remaining protein cross-links (Figures 4B and C). The major degradation takes place within the first 2 h, as only some small amounts of protein remained. Further incubation (8 or 24 h) did not influence the breakdown of this modified albumin dramatically.

Characteristics of wild type and cathepsin D knockout fibroblasts

After showing that cathepsin D is able to degrade AGE-BSA almost independently from the degree of modification in the time scale of several hours, we raised the question of whether this is also true in living cells. Our working hypothesis was that cathepsin D is playing a major role in the degradation of AGE-proteins. Consequently, a lack of cathepsin D should lead to a dramatic impairment of AGE-protein breakdown and perhaps to an accumulation of such modified proteins, most likely in the endosomal–lysosomal compartment.

Therefore, we used isolated murine embryonic fibroblasts from wild type and cathepsin D knockout animals to test in these cells the fate of AGE-BSA. In both cell clones we tested first the cell proliferation, detecting that that cathepsin D knockout cells exhibit a lower population-doubling rate (PD) compared to wild type cells (Figure 5A). After ~ 90 days wild type cells passed into a stationary phase. As the population curve was nearly linear between PD 5 until PD 18 (equivalent to day 20 until day 60), only cells in this time period were used for the experiments.

Furthermore, we tested the activity of the major proteolytic systems in both cell types. As expected, the lysosomal activity, measured by a substrate for cathepsin D, was significantly reduced in cathepsin D knockout cells (Figure 5C), indicating that malfunction of this enzyme has an influence on the activity of lysosomes. Interestingly, we also found that the lysosomal activity for cathepsin B and L as well as the 20S proteasomal activity was somewhat decreased in these cells (Figures 5B and D).

Cell viability after incubation with different concentrations of modified albumin

Before starting to investigate the accumulation of AGE-BSA in the used cell clones, we studied the influence of the different modified BSA in several concentrations on cell viability (Table I). Wild type cells, incubated with unmodified BSA specified at a concentration of 0 mM treatment as it was not

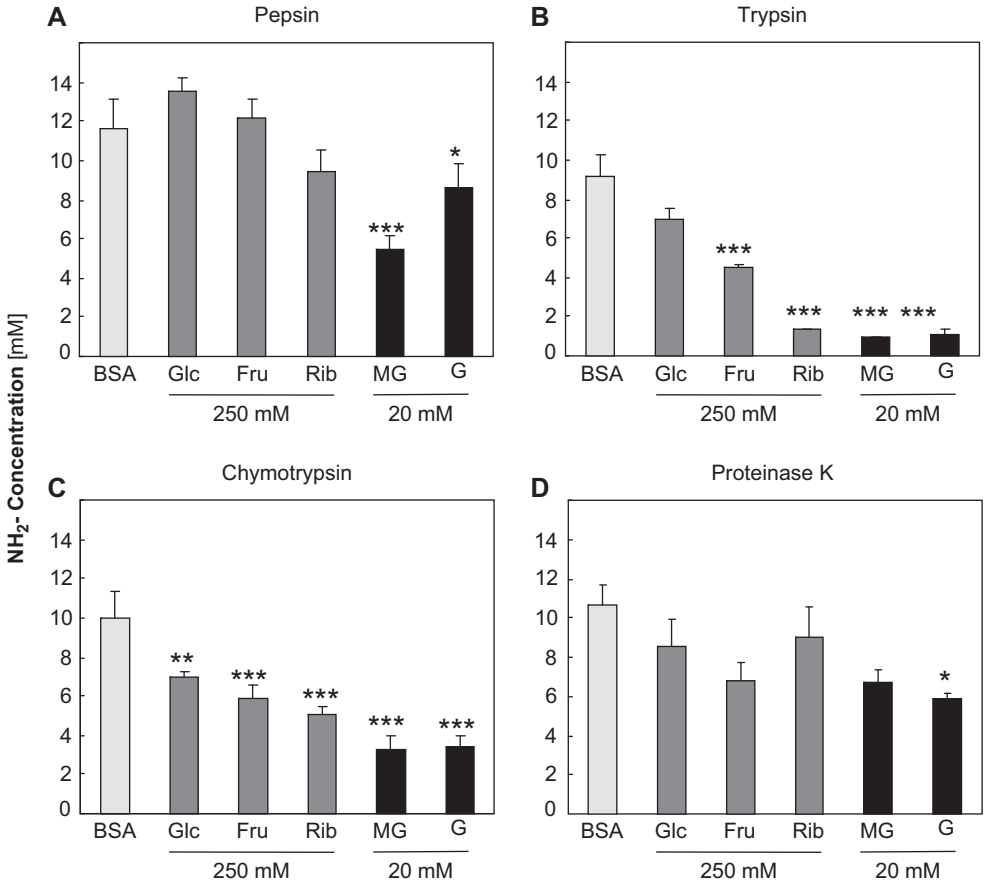

Figure 3. Degradation of AGE-modified albumin by extracellular proteases. Glucose (Glc), fructose (Fru) and ribose (Rib)-modified albumin (250 mM), methylglyoxal (MG) and glyoxal (G)-modified albumin (20 mM) as well as unmodified albumin were incubated with pepsin (A), trypsin (B), chymotrypsin (C) and proteinase K (D). The proportion of enzyme and substrate was in (A) 1:20 (w/w), (B) 1:25 (w/w), (C) 1:25 (w/w) and (D) 1:100 (w/w). These mixtures were incubated for 2 h at 37°C, followed by the measurement of free NH_2 as an indicator of protein degradation. All data reflect the mean ± SD of four independent experiments. Statistical significance between BSA and BSA-modified AGEs is indicated as $^{***}(n=3, p < 0.001$, ANOVA, Bonferroni's multiple comparison test), $^{**}(n=3, p < 0.01$, ANOVA, Bonferroni's multiple comparison test) and $^{*}(n=3, p < 0.05$, ANOVA, Bonferroni's multiple comparison test).

incubated with sugars or aldehydes, were defined to 100% viability. The cells were incubated with albumin modified by AGE to various degrees for 24 h followed by the MTT assay. We could demonstrate that none of the used AGE-modified albumin suppressed cell viability, neither in wild type nor in knockout fibroblasts to a great extent. None of the modified aldehydes were able to decrease the viability by more than 30%. Such moderately influenced cell viability was observed after treatment with ribose- and glyoxal-modified albumin. Additionally, we could not see any difference between the two cell clones in cell viability. Thus, these different modified BSA could be used in further cell-culture studies.

Accumulation of modified albumin in wild type and cathepsin D knockout fibroblasts

An accumulation of AGEs was determined in wild type and cathepsin D knockout cells, using the autofluorescence of AGEs. For each sample, 20 000 cells were detected by flow cytometry and measured for their specific AGE-emission capability. Notably, cathepsin D knockout cells exerted an almost 1.5-fold higher autofluorescence than the wild type cells from the same population doubling rate without incubation of AGE-modified albumin (indicated as '0 h' sample). This increased autofluorescence indicates perhaps an accumulation of oxidized cross-linked material resulting from normal metabolism, which is not degraded due to the lack of cathepsin D activity.

Interestingly, if cathepsin D knockout fibroblasts were incubated with 2 mM glyoxal- or methylgloxal-modifed albumin, a significant increase in the accumulation of autofluorescent AGEs was observed (Figures 6A and B). This time-dependent accumulation was more pronounced in the knockout cells compared with the wild type fibroblasts, whereas this difference was not seen in glucose- and fructose-modified albumin, whereas ribose-modified BSA showed the same accumulation as the aldehyde-modified albumin. This is in accordance with the differences in fluorescence intensity of the modified BSA (see Figure 1).

Table I. Cell viability after incubation with different concentrations of modified albumin.

Concentration (mM)	Ribose-AGE		Fructose-AGE		Glucose-AGE	
	Wt	D –/–	Wt	D –/–	Wt	D –/–
0	100.0	98.5	100.0	99.6	100.0	99.4
5	77.4	76.0	84.9	84.6	92.7	95.3
25	71.4	71.1	87.8	97.1	93.3	87.3
100	83.7	73.9	92.7	103.5	86.4	92.1

	Glyoxal-AGE		Methylglyoxal-AGE	
	Wt	D –/–	Wt	D –/–
0	100.0	98.5	100.0	99.6
2	77.4	76.0	84.9	84.6
10	71.4	71.1	87.8	97.1

Viability of wild type (Wt) and cathepsin D knockout cells (D –/–) after 24 h incubation with modified albumin was measured by the MTT assay. As a control, wild type cells incubated with non-modified albumin (0 mM) were defined as 100% alive, whereas the other samples were calculated according to the control.

Localization of glyoxal modified albumin in fibroblasts

Since we found in untreated cathepsin D knockout fibroblasts and in the fluorescent AGE-loaded cells a higher autofluorescence than in the corresponding wild type cells, it seemed important to find out whether this increased autofluorescence is due to accumulating cross-linked material. Since it is known that such material is accumulating in the lysosomes [42], we performed a fluorescence microscopic investigation of the cells, studying the localization of glyoxal-AGEs in cells. For this, we incubated wild type and cathepsin D knockout cells for 7 h with glyoxal-BSA modified with 2 mM of glyoxal. The results obtained from fluorescence microscopy confirmed that cathepsin D knockout cells treated with AGE-modified albumin exert a higher autofluorescence, as measured by FACS analysis (Figure 7). Therefore, the amount of autofluorescent AGEs in these impaired cells revealed that this phenomenon was due to an enhanced AGE-accumulation in the cytosol or lysosomes (Figure 7B). In order to elucidate the localization of AGE-accumulation, we used the lysosomal dye LysoTracker Blue and stained lysosomes in wild type and cathepsin D knockout cells (Figure 7C). An overlay of Figures 7B and C, resulting in colour addition, indicated the co-localization of lysosomes and AGEs (shown as white regions) (Figure 7D). This co-localization is more obvious in knockout cells, as the amount of AGE-accumulation is significantly higher. Despite the fact that AGEs are localized in lysosomes, not every lysosome in cells was loaded.

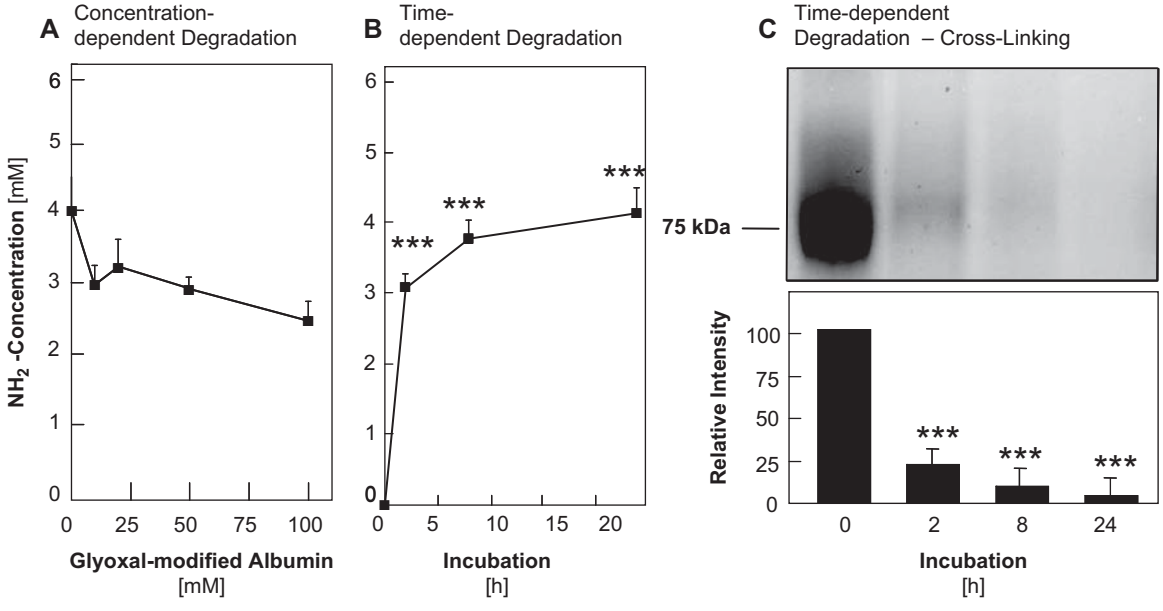

Figure 4. Degradation of glyoxal-modified albumin by cathepsin D. (A) The modification-dependent glyoxal-modified albumin degradation after 2 h incubation with cathepsin D at 37°C. BSA was modified by the indicated concentrations of glyoxal as described in the Methods section. The ratio of enzyme-to-substrate was 1:25 (w/w). The content of free NH_2 was determined as a marker of protein degradation described in Methods. (B) The time-dependent degradation of 20 mM glyoxal-modified albumin by cathepsin D over a time period of 24 h. The content of free NH_2 was determined as a marker of protein degradation. '0 h' shows the sample without any degradation by cathepsin D. (C) The time-dependent degradation of the same conditions described in (B). Electrophoresis was used to determine the extent of the remaining protein. A SDS-PAGE system using a 10% gel was performed and detected by Coomassie staining. The intensities of the lanes around 75 kDa were determined by the software program Corel Photopaint 11. The '0 h' sample was set as 100% intensity, whereas the other probes were calculated in relation to the control. Statistical significance between undegraded glyoxal-modified albumin ('0') and glyoxal-modified albumin incubated with cathepsin D is indicated as $^{***}(n=3, p < 0.001$, ANOVA, Bonferroni's multiple comparison test).

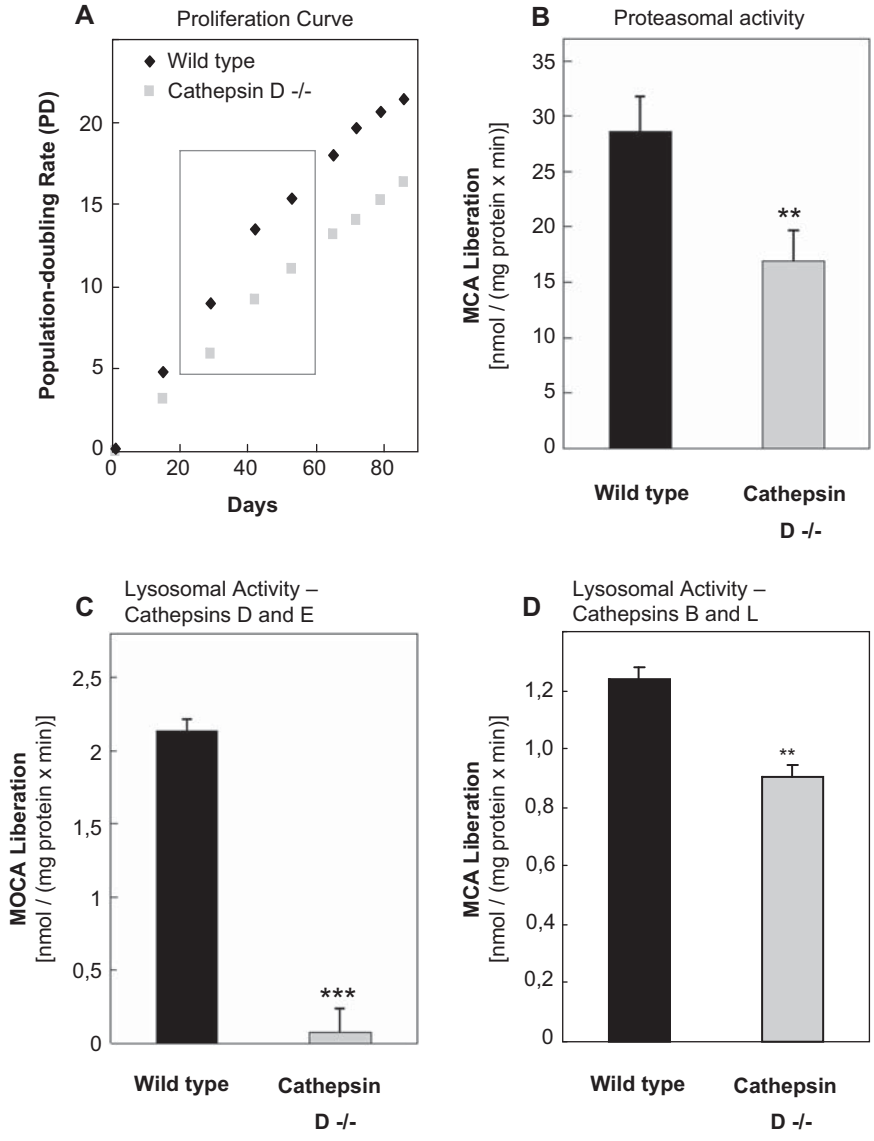

Figure 5. Characteristics of wild type and cathepsin D knockout fibroblasts. (A) The proliferation curve of mouse embryonic fibroblasts isolated from wild type and cathepsin D knockout animals, measured as population doublings (PD) over 90 days. For the experiments, cells between PD 5 and 18 were used (indicated by the rectangle). (B) The MCA liberation from the fluorogenic substrate suc-LLVY-MCA, which characterizes the chymotrypsin-like activity of the β5-proteasomal sub-unit, is shown. (C) The lysosomal activity of cathepsin D and E was measured with the substrate MOCA-GKPILFFRLK(Dnp)-RNH_2 by the MOCA liberation and (D) the fluorogenic peptide z-FR-MCA, which is cleaved mainly by cathepsin B and L was used to characterize the lysosomal activity of wild-type and cathepsin D knockout cells. Statistical significance between the lysosomal and proteasomal activity of wild type and knockout cells is indicated as $^{**}(n=3, p < 0.01$, ANOVA, Bonferroni's multiple comparison test) and $^{***}(n=3, p < 0.001$, ANOVA, Bonferroni's multiple comparison test).

Discussion

Cells which are very rarely (or never) replaced during the lifetime of an organism are sensitive for accumulation of biological waste materials like lipofuscin, irreversible damaged mitochondria and aberrant proteins such as AGEs and other indigestible protein aggregates [43,44]. This leads to a functional impairment and at worst to cell death [44]. This progressive decline is known as one of the major factors in ageing [43–45].

Additionally to ageing, the formation of indigestible protein aggregates is involved in a number of age-related diseases [43,45]. For instance, Alzheimer's disease is characterized by the aggregation of the tau-protein in neurofibrillary tangles and the formation of extra-neuronal β-amyloid plaques [46]. AGEs are found on many age-related protein aggregates, such as amyloid plaques and neurofibrillary tangles, and it could be shown by others that plaque formation is significantly accelerated by cross-linking through AGEs [47]. The consequence of AGE-mediated protein cross-linking is the contribution to age-related malfunction of cells and tissues [43].

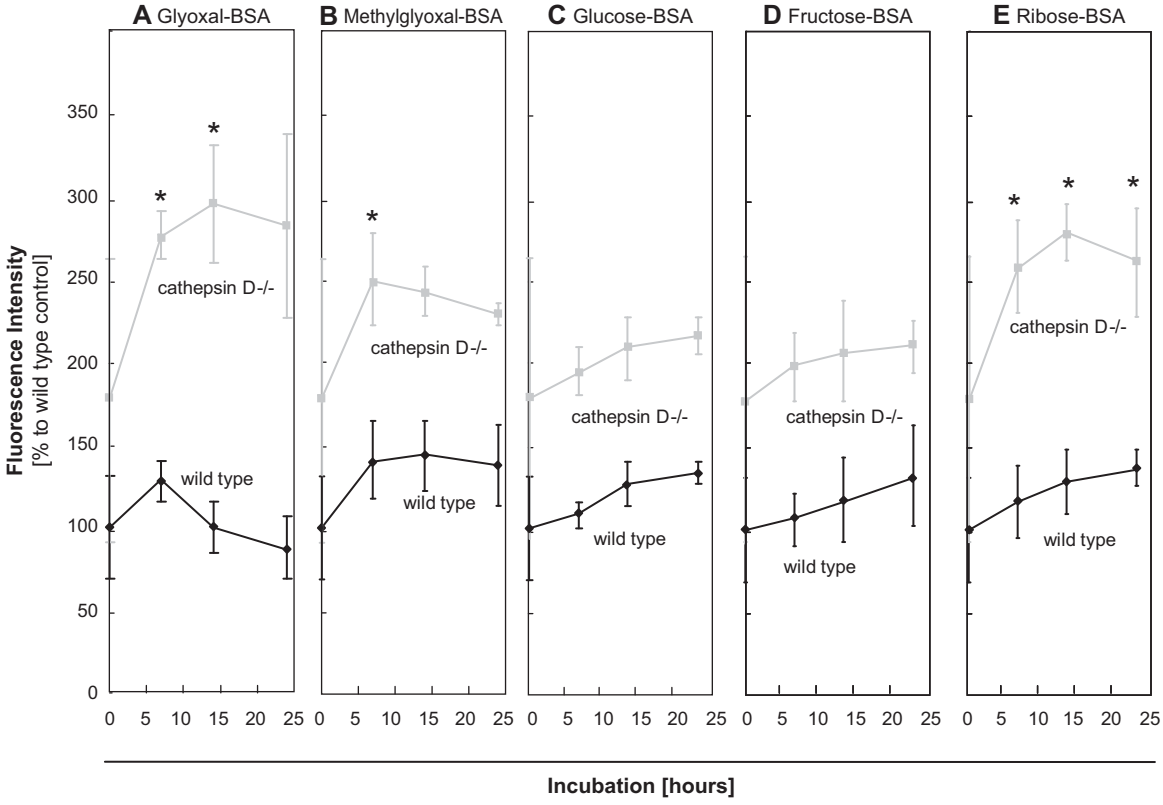

Figure 6. Accumulation of modified albumin in wild type and cathepsin D knockout fibroblasts. The fluorescence of fibroblasts was measured with flow cytometry (488 nm excitation/620 nm emission). The autofluorescence of wild type fibroblasts and without addition of AGE-modified albumin was set as 100% intensity. (A–E) The fluorescence intensity of fibroblasts after incubation with AGE-modified albumin over a time period of 24 h is demonstrated. BSA was modified with 25 mM glyoxal (A) or methylglyoxal (B), whereas 250 mM glucose (C), 250 mM fructose (D) or ribose (E) were used. Statistical significance between the fluorescence intensity of wild-type cells compared to knockout cells is indicated as $^{*}(n=3, p < 0.05$, ANOVA, Bonferroni's multiple comparison test).

It can be assumed that impaired structures, which accumulate during ageing, are due to a decreased turnover by the cellular proteolytic systems. Already 10 years ago, it was presumed by Munch et al. [47] that AGE formation causes cross-linking of proteins which are resistant to proteases and leading therefore to accumulation and protein deposition. In agreement with this hypothesis, Bulteau et al. [48] demonstrated that n(ε)-carboxymethyl-lysine (CML), a known AGE-structure, is resistant against the 20S proteasome degradation [49], a result we could confirm here. Further results were obtained from Cervantes-Laurean et al. [50] using glyoxal to modify histones, in order to investigate whether the nuclear proteasome is influenced. Glyoxal-modified histones have been found to activate nuclear proteasome activities, whereas the total cellular proteasome activity was decreased [50]. Additionally, it is reported by others that the degradation of pyralline-modified albumin, a known AGE-structure, is diminished in comparison to unmodified albumin, in a macrophage-like cell line. According to them, there was a rest activity of lysosomal enzymes of ~60% [51]. In summary, these results demonstrated a decline in proteasomal and lysosomal activity due to AGEs.

On the other hand, several reports demonstrate a potential degradation of AGE-modified proteins. Araki et al. [11] previously described an endocytotic uptake and degradation of AGEs in Chinese hamster ovary cells, over-expressing the macrophage scavenger receptor (MSR). They reported a clearly mediated endocytotic uptake and degradation of AGEs through this MSR. Saito et al. [52] used cloroquine, a membrane-diffusible reagent which raises pH in intracellular components and leupeptin, a membrane-diffusible proteinase inhibitor. They reported that both reagents significantly suppressed ^{125}I-AGE-BSA degradation and, therefore, confirmed the endocytosis and lysosomal degradation of AGE-BSA. Earlier work of our group demonstrated that internalized glycated proteins can be degraded by cells to some extent [30].

Based on these observations we characterized the degradation of various AGE-modified BSAs by several proteases. For this we chose lysosomal proteases, the 20S proteasome as well as major gastrointestinal proteases (i.e. trypsin, chymotrypsin and pepsin) and the subtilisin-like proteinase K. We could confirm the principal ability of proteases to degrade AGE-BSA. The most efficient intracellular protease was cathepsin

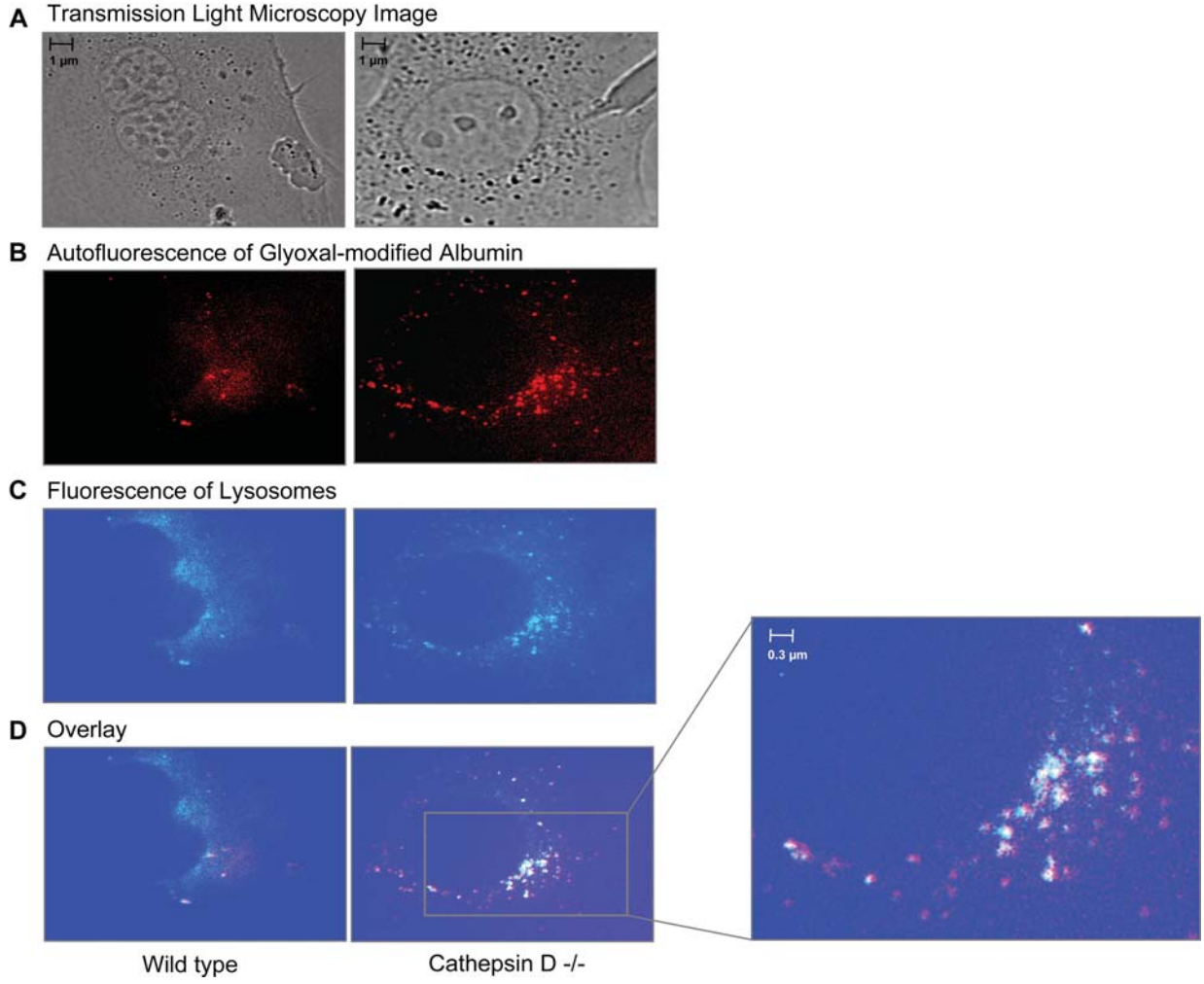

Figure 7. Localization of glyoxal-modified albumin in fibroblasts. Wild-type and cathepsin D knockout cells were incubated with 20 mM glyoxal-modified BSA for 7 h. (A) The transmission light microscopy image. The autofluorescence of glyoxal-AGEs in cells was investigated by fluorescence microscopy (B). The lysosomes in these cells were stained with 75 nM of a lysosome tracker (LysoTracker Blue DND-22) (C). (D) An overlay of lysosomal staining and autofluorescence signals. The red channel represents the autofluorescence, the light blue the lysosomes, whereas the white signal is showing the overlapping of both intensities.

D followed by pepsin and proteinase K in the degradation of AGE-modified albumin. Interestingly, pepsin and cathepsin D are structurally related and belong to the same family of aspartic proteases (Clan AA, family A1) consisting of potent endopeptidases, most of which are most active at acidic pH [15,49]. This acidic pH may not only foster the most active and stable conformation of these proteases but denaturation of AGE-modified proteins in an acidic environment is likely to increase the accessibility of peptide bonds for the cleaving proteases.

As revealed in earlier studies oxidized proteins are preferentially degraded by several proteases (i.e. trypsin, chymotrypsin, 20S proteasome) compared with their non-oxidized forms [23], we could demonstrated that the 20S proteasome degrades oxidized BSA but not AGE-modified albumin. Interestingly, oxidized proteins are often a better substrate than non-oxidized, whereas the AGE-modified proteins seem to keep almost the same proteolytic susceptibility after modification. This reflects a constant protein degradation rate and seems not to be a preferential recognition of the modified substrates. Our data of cathepsin D being one of the most suitable protease for degradation of protein aggregates are in line with previous studies showing a co-localization of cathepsin D and age-related proteins [53].

We examined further the accumulation of AGEs in fibroblasts, a well characterized cell model for the uptake of modified materials [54]. To confirm our first obtained results, we used fibroblasts with a cathepsin D knockout. As a control, we used wild type fibroblasts of the same passage. In our cells we observed a decrease in population doubling rate due to cathepsin D knockout. Furthermore, the activities of the major lysosomal proteases (cathepsin D, B and L) and the 20S proteasome were reduced. However, cathepsin D deficient fibroblasts do not show an impaired turnover of 'normal' ^{35}S-Met/Cys-labelled endogenous proteins in pulse-chase experiments [37].

The relation between malfunction of the lysosomal and the proteasomal system has not been well studied yet. Bifsha et al. [55] demonstrated, in cells exhibiting lysosomal storage disorders through deficiency of lysosomal enzymes, an accumulation of undegraded products, an increase size and number of lysosomes and a decreased proteasomal activity. Qiao et al. [56] revealed a significant down-regulation of proteasome activities in cathepsin D knockout brain tissue. In accordance to the work of Qiao et al., we also observed a proteasome activity reduction in cathepsin D knockout cells despite the fact that the amount of proteasome in wild type and cathepsin D knockout cells is unchanged (data not shown). The exact mechanism behind this phenomenon is still uncertain, but it is supposed that the accumulated storage material escapes the lysosomes and is inhibiting cytosolic processes, including the proteasome. Interestingly, cathepsin D deficiency in the knockout mice results in a severe hypotrophic and neurodegenerative phenotype that shortens the life span of these mice to 26 days [37]. In the brain a neuronal ceroid-lipofuscinosis phenotype with intracellular accumulation of autophagic and endocytic vesicles is the hallmark of this mouse strain [19,57,58]. To date several inactivating cathepsin D mutations that cause congenital neuronal ceroid-lipofuscinosis in sheep, bulldog and (most importantly) humans have been identified [20,21,59,60]. Hence, accumulation of undigested AGE-modified proteins within the cathepsin D deficient lysosomal and other cellular compartments will be part of the pathogenesis of this neurodegenerative disease.

Since AGEs are able to induce the generation of reactive oxygen species (ROS) and hence apoptosis in neuroglial cells [61], we investigated the influence of different AGE-modified albumin on the viability of wild type and cathepsin D knockout cells and could not find any harmful effect. We addressed also the question of whether AGEs are taken up and accumulate in cells. Without adding AGEs we could observe a significant higher autofluorescence in knockout cells compared to wild type cells. As numerous lysosomal storage disorders are related with lysosomal enzyme defects it can be assumed that more autofluorescent aggregates, present in cathepsin D knockout cells, are due to the impairment in the lysosomal enzymatic system [62]. Additionally, we could show that, after incubation of glyoxal- and methylglyoxal-modified albumin, there was a significant higher accumulation of these AGEs in cathepsin D knockout cells. The fate of proteins that enter cells by endocytosis has been investigated in several studies. They pass through early and late endosomes and are delivered to lysosomes [13]. We investigated, therefore, whether the autofluorescence of AGEs is changed when AGEs are removed from cell media and cells incubated over some time periods before measuring the accumulation of AGEs. No decline in accumulation was detected in this experiment, so it can be assumed that cells are unable to exocytose AGEs (data not shown). In agreement with the findings in previous studies, we found that AGEs accumulate in lysosomes. This observation was made in pyramidal neurons, which selectively accumulate AGE-containing vesicles, presumably in endosomes and lysosomes in an age-dependent manner [63,64].

In previous experiments, it was found that foetal lung cells treated with glyoxal form AGEs. It was further revealed that these cells exhibit a dramatic loss of cathepsin D activity and cathepsin D mRNA expression [65]. Cathepsin D, probably the best player in the degradation of AGE-modified proteins, may, therefore, be negatively influenced in its activity due to AGEs.

In summary, it has to be considered that glycated proteins are not always resistant to degradation. This seems to be important as glycation is an unavoidable process of post-translational protein modification. However, the efficiency of degradation depends on the degree of glycation and cross-linking of the substrate. Resistance to proteolytic removal is attributed to the result of an extensive cross-linking, as high cross-linking limits the access of proteases to their cleavage sites [22,30]. Decline of proteolytic functions due to inherited protease mutations, age-related impairment of protease biosynthesis or post-translational modification, i.e. by excessive oxidation, may further foster the accumulation of the AGE-modified proteins and eventually cause irreversible neurological and cognitive impairment.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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