Advanced Glycation End Products (AGEs) in Säugerzellen: Abbau, Akkumulation und Zelluläre Reaktionen

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MEINEN ELTERN

INHALTSVERZEICHNIS

ABBILDUNGSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

AGEs	Advanced Glycation End Products
ATP	Adenosin-5´-Triphosphat
BSA	Bovines Serumalbumin
CD36	Cluster of Differentiation 36
CEC	S-Carboxyethyl-Cystein
CEL	N_{ε}-Carboxyethyl-Lysin
CMC	S-Carboxymethyl-Cystein
CML	N_{ε}-Carboxymethyl-Lysin
DAPI	4´,6´-Diamidin-2-Phenylindol
3-DG-H	3-Desoxyglucoson-abgeleitetes Hydroimidazolon
DOLD	3-Desoxyglucoson-Lysin-Dimer
GAPDH	Glycerinaldehyd-3´-Phosphat-Dehydrogenase
GDP/GTP	Guanosin-5´-Diphosphat/Guanosin-5´-Triphosphat
G-H	Glyoxal-abgeleitetes Hydroimidazolon
GOLD	Glyoxal-Lysin-Dimer
IFN-γ	Interferon-γ
LMP2	Low Molecular Weight Protein 2
LMP7	Low Molecular Weight Protein 7
LPS	Lipopolysaccharide
MECL1	Multicatalytic Endopeptidase Complex-Like 1
MG-H	Methylglyoxal-abgeleitetes Hydroimidazolon
MHC	Haupthistokompatibilitätskomplex (*Major Histocompatibility Complex*)
MOLD	Methylglyoxal-Lysin-Dimer
mRNA	Messenger RNA
MSR-A	Macrophage Scavenger Receptor-A
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazoliumbromid
NADPH	Nicotinamidadenindinukleotidphosphat
NF-kB	Nuclear Factor 'Kappa-Light-Chain-Enhancer' of Activated B-Cells
PBS	Phosphatgepufferte Salzlösung
PCR	Polymerase-Kettenreaktion (*Polymerase Chain Reaction*)
PKC	Proteinkinase C
qPCR	Quantitative PCR
RAGE	Receptor for Advanced Glycation End Products
RNA	Ribonukleinsäure
ROS	Reaktive Sauerstoffspezies (*Reactive Oxygen Species*)
SDS-PAGE	Natriumdodecylsulfat (*Sodium Dodecyl Sulfate*)-Polyacrylamidgelelektrophorese
STAT1	Signal Transducer and Activator of Transcription 1
THP	Tetrahydropyrimidin
TNF-α	Tumornekrosefaktor-α

VORBEMERKUNG

Die hier vorgelegte Arbeit wurde unter Betreuung von Prof. Dr. Tilman Grune am Lehrstuhl für Biofunktionalität und Sicherheit der Lebensmittel am Institut für Biologische Chemie und Ernährungswissenschaft der Universität Hohenheim begonnen. Aufgrund der Neuberufung von Prof. Dr. Tilman Grune wurde die Arbeit am Lehrstuhl für Ernährungstoxikologie des Instituts für Ernährungswissenschaften an der Friedrich-Schiller-Universität Jena fertig gestellt.

Teile der vorgestellten Arbeit wurden auf mehreren Tagungen und Kongressen präsentiert. Zudem wurden bestimmte Abschnitte der Arbeit in verschiedenen wissenschaftlichen Fachzeitschriften veröffentlicht.

1 EINLEITUNG

1.1 Advanced Glycation End Products (AGEs)

1.1.1 Die Maillard-Reaktion

Louis Camille Maillard machte 1912 eine bedeutungsvolle Entdeckung. Beim Erhitzen von Aminosäuren und Zucker auf über 100°C bildeten sich nach einigen Stunden braungefärbte, aromatisch riechende Produkte [1]. Diese nach L.C. Maillard bezeichnete „Maillard-Reaktion“ findet ohne jegliche Einwirkung von Enzymen statt. Da es sich hierbei um Reaktionen von Aminen mit reduzierenden Zucker oder anderen Carbonylverbindungen handelt, hat sich im Laufe der Zeit der Begriff „Glykierung“ oder „nicht-enzymatische Glykosylierung“ durchgesetzt.
Maillard-Produkte sind heterogene stabile Verbindungen, die über mehrere Phasen gebildet werden und bis dato nur teilweise charakterisiert sind [2]. Da jedoch die mehrstufigen Reaktionen sehr komplex sind, ist die Einteilung nicht als Dogma anzusehen. Vielmehr sind die Übergänge zwischen den Phasen fließend.

1.1.1.1 Die frühe Phase

In der frühen Phase kondensiert die Aminogruppe mit der Carbonylkomponente zu einem Glykosylamin, das auch als N-Glykosid bezeichnet wird. Besonders häufig treten diese reversiblen Reaktionen an Lysin- und Arginin-Seitenketten und an N-terminalen Aminogruppen in Erscheinung [3]. Der Stickstoff der Aminogruppe reagiert hierbei unter einem nukleophilen Angriff mit dem Kohlenstoff der Carbonylgruppe. Als Produkt entsteht ein instabiles Imin, das auch als Schiffsche Base bezeichnet wird (Abb. 1) [4]. Anschließend wird der Stickstoff protoniert und es entstehen folgende Mesomere: ein Immonium- und ein Aminocarbenium-Ion [4]. Diese Mesomere sind elektrophil. Somit wird die Hydroxylgruppe an dem benachbarten Kohlenstoff azidifiziert und ein Proton abgespalten. Das daraus gebildete Enaminol tautomerisiert zu einer 1-Amino-1-Desoxyketose, dem Amadori-Produkt [4]. Die Heyns-Umlagerung erfolgt aus einer Ketose-Reaktion analog der Amadori-Umlagerung. Als Produkt entsteht hierbei eine 2-Amino-2-Desoxyaldose [5]. Diese Amadori- oder Heyns-Produkte werden als „frühe Glykierungsprodukte“ bezeichnet [6]. Im Unterschied zu den Produkten der weiterführenden und finalen Phase sind diese Verbindungen noch farblos [6].

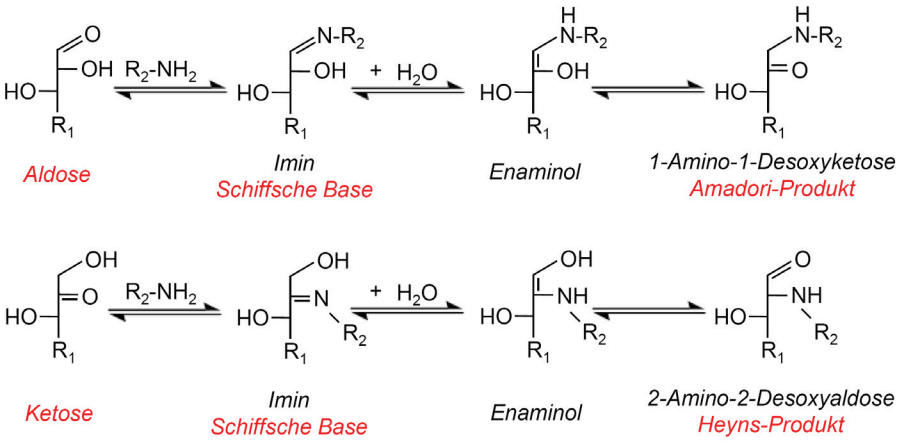

Abbildung 1: Die frühe Phase der Maillard-Reaktion

Bildung von Amadori- und Heyns-Produkten aus Aldosen und Ketosen. Modifiziert nach [4,6].

1.1.1.2 Die weiterführende Phase

In der weiterführenden Phase werden die Produkte aus der frühen Phase teilweise abgebaut und modifiziert. Es entstehen hierbei reaktive Intermediate, die sogenannten Desoxyosonen (Abb. 2) [7].

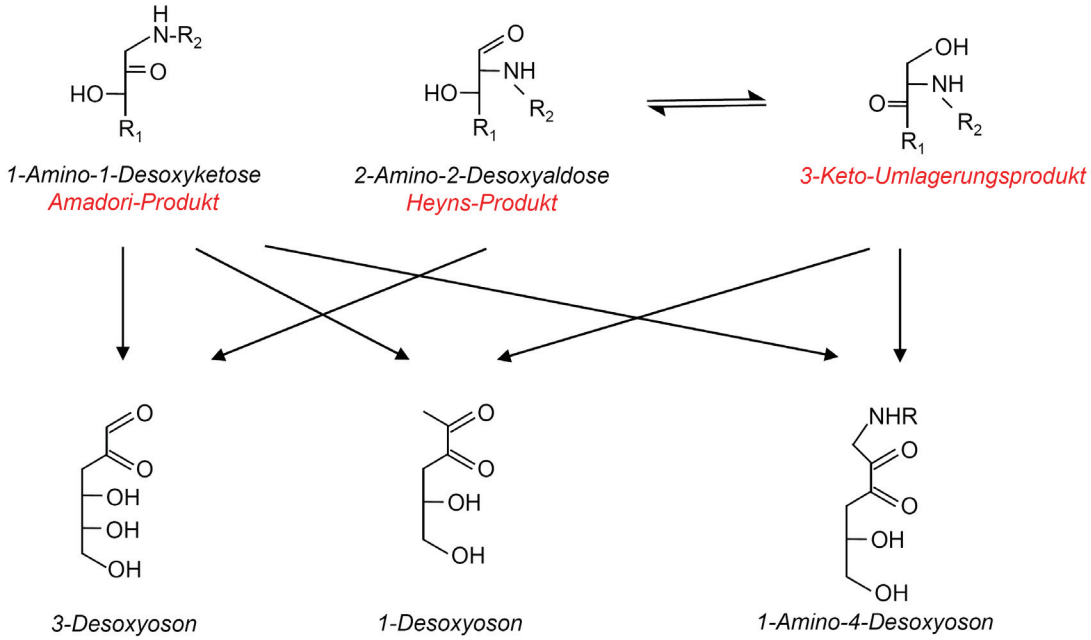

Abbildung 2: Die weiterführende Phase der Maillard-Reaktion

Bildung von Desoxyosonen aus Amadori- und Heyns-Produkten. Modifiziert nach [7].

Die Desoxyosonen können unter einer Retroaldolspaltung oder oxidativ induzierter Kettenbrüche kürzerkettige α-Dicarbonylverbindungen bilden [7]. Wichtige Vertreter dieser α-Dicarbonylverbindungen sind die Aldehyde Glyoxal und Methylglyoxal [8].

Zudem unterliegen sie weiteren Oxidations-, Isomerisations-, Zyklisierungs- und Dehydrierungsreaktionen. Die daraus resultierenden Zwischenprodukte sind ebenfalls sehr reaktiv und können weitere der oben genannten Reaktionen eingehen [8]. Zusätzliche Bindungen und Zyklisierungsreaktionen führen letztendlich zu einer Vielzahl von heterozyklischen Verbindungen, die als Vorstufen der Melanoidinen bekannt sind [7].

1.1.1.3 Die finale Phase

In der finalen Phase werden aus den Produkten der weiterführenden Phase durch Polymerisationen und Vernetzungen die höhermolekularen Melanoidine gebildet [9]. Diese Melanoidine werden als „fortgeschrittene Glykierungsprodukte“ bezeichnet [10]. Der international verwendete Begriff dieser Glykierungsprodukte “*advanced glycation end products* (AGEs)“ hat sich auch im deutschen Sprachraum etabliert. Die Reaktionen, die zur Entstehung der Melanoidinvorstufen führen, sind schon hinreichend charakterisiert [8]. Die späteren Stadien der Maillard-Reaktion sind jedoch bis heute nur sehr lückenhaft dokumentiert. Einige bereits identifizierte AGEs sind in Abbildung 3 gezeigt.

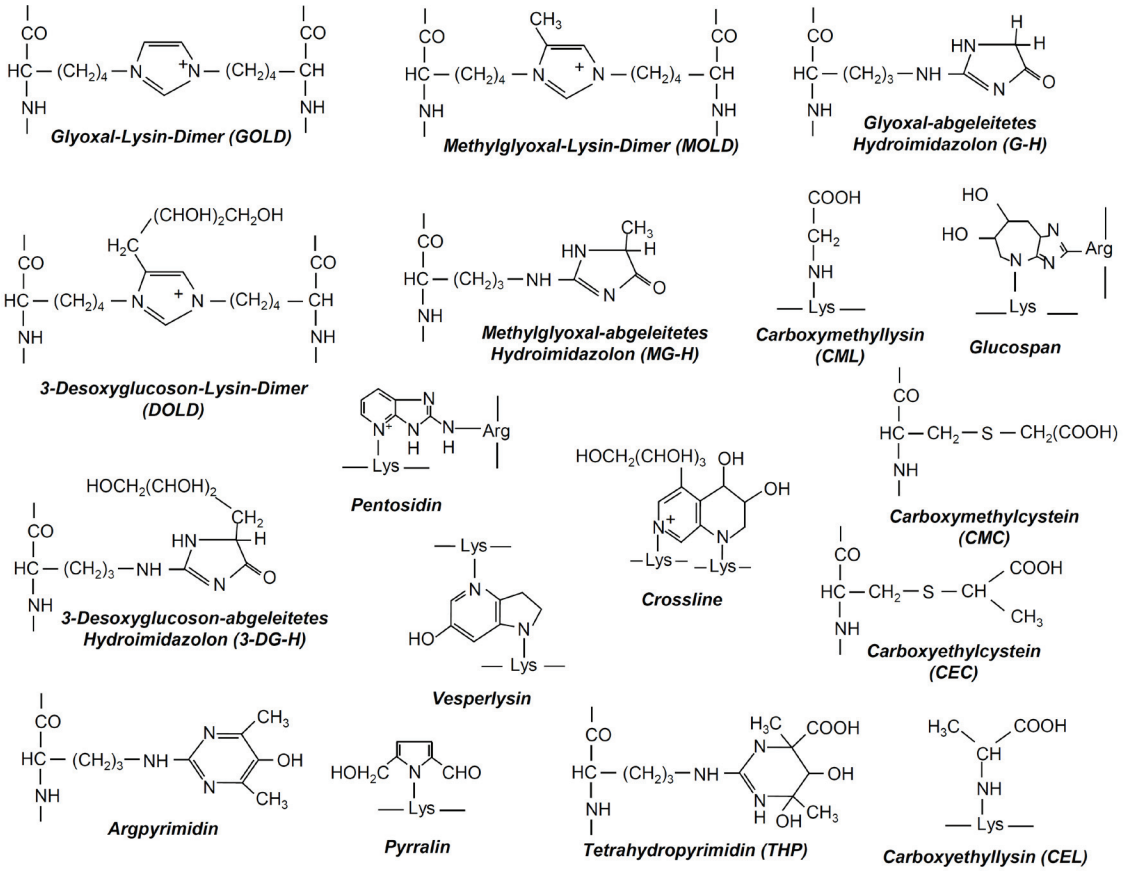

Abbildung 3: Strukturen einiger identifizierter AGEs

Modifiziert nach [11,12].

Neben der Maillard-Reaktion können andere Reaktionswege zur Bildung der AGEs beitragen. So führen beispielsweise die Autooxidation von Glucose und die Peroxidation von Lipiden zu reaktiven Dicarbonylverbindungen, die unter oxidativen Stress für die Bildung von AGEs beschrieben wurden [13]. Ein weiterer gut untersuchter Mechanismus zur Bildung der AGEs ist der Polyol-Stoffwechselweg. Hierbei wird Glucose in Sorbitol und anschließend in Fructose enzymatisch konvertiert. Die Fructose-Metaboliten bilden α-Oxoaldehyde, die mit Aminosäuren-Seitenketten zu AGEs reagieren [14]. Zusammenfassend existieren mindestens vier verschiedene Reaktionswege, auf denen AGEs entstehen können: die Maillard-Reaktion, die Oxidation von Glucose, die Peroxidation von Lipiden und der Polyol-Stoffwechselweg. In Anbetracht dieser unterschiedlichen komplexen Reaktionswege ist es nicht verwunderlich, dass die AGEs enorme strukturelle Unterschiede aufweisen. Unterteilt werden kann die AGE-Entstehung zusätzlich in nicht-oxidative und oxidative Reaktionswege. In letzterem Fall verwendet man die Bezeichnung „Glykoxidation“. Ein glykoxidativer Reaktionsweg kann durch Anwesenheit von Übergangsmetallen beschleunigt werden. Hierbei reagiert das instabile Imin unter Anwesenheit von Sauerstoff zu reaktiven Oxoaldehyden [15].

AGEs können Querverbindungen zwischen Proteinen ausbilden und aufgrund ihrer Struktur autofluoreszieren. Beispiel hierfür ist Pentosidin [16]. CML und Pyrralin sind hingegen Beispiele für nicht-fluoreszierende AGEs, die keine Querverbindungen ausbilden [17].

1.1.2 AGEs in Lebensmitteln

Maillard-Produkte nehmen in der Lebensmitteltechnologie einen bedeutenden Stellenwert ein. Als Aromastoffe bestimmen sie den Genusswert zahlreicher Lebensmittel. Hierfür sind diverse heterozyklische und nicht-zyklische Verbindungen verantwortlich, die ab der weiterführenden Phase der Maillard-Reaktion gebildet werden [18]. Auch Bräunungsreaktionen beim Backen und Rösten sind auf Maillard-Reaktionen zurückzuführen [18]. Das Ausmaß und der Gehalt dieser aromatischen und geschmackvollen Produkte sind für das jeweilige Lebensmittel spezifisch und von der Lebensmittelindustrie erwünscht.

Die Maillard-Reaktion kann direkt die nutritiven Eigenschaften eines Lebensmittels beeinflussen. Dabei führen chemische Modifikationen dazu, dass essentielle Aminosäuren schlechter verfügbar werden [18]. Dadurch wird der Nährwert eines Lebensmittels gemindert. Für gesunde Erwachsene mit einer gemischten Kost stellt dies keine Probleme

dar. Steht jedoch nur eine begrenzte Anzahl von Lebensmitteln zur Verfügung, z.B. in der Säuglingsnahrung, sollte bei der Verwendung von Technologien auf schonende Verfahren geachtet werden [19].

Bisher wurden nur einige wenige AGE-Strukturen in Lebensmitteln identifiziert und quantifiziert. Dazu zählt das Amadori-Produkt Fructoselysin und die AGEs GOLD, MOLD, DOLD, G-H, MG-H, 3-DG-H, CML, CEL, Pentosidin, Argpyrimidin, THP und Pyrralin (Abb. 3) sowie das niedermolekulare Melanoidin Pronyllysin [11,12,20]. Bei einer gewöhnlichen westlichen Ernährung wird eine tägliche Zufuhr von ca. 1,2 g Amadori-Produkte (am Beispiel von Fructoselysin) und 25-75 mg AGEs (hauptsächlich CML und Pyrralin) vermutet [20]. Allgemein lassen sich hohe Konzentrationen an AGEs in Lebensmitteln erwarten, die hoch erhitzt werden und reich an Proteinen, freien Aminosäuren und Monosacchariden sind. Zu diesen Lebensmitteln zählen Milch und Milchprodukte, Brot und Backwaren, Kaffee und Bier [9]. Eine Studie aus dem Jahre 2004 zeigte überraschenderweise, dass Lebensmittel mit hohem Protein- und gleichzeitig hohem Fettgehalt die höchsten AGE-Gehalte aufweisen, während Lebensmittel mit hohem Kohlenhydrat- und niedrigem Fettanteil nur geringe AGE-Konzentrationen beinhalten [21]. Vermutlich führt die Anwesenheit von Fett während der Temperatureinwirkung zur Lipidperoxidation und folglich zur Quelle von freien Radikalen und reaktiven Dicarbonylverbindungen, die wiederum die Glykierung von Aminogruppen begünstigen [21]. Allgemein ist der AGE-Gehalt abhängig von der Temperaturhöhe, der Dauer der Temperatureinwirkung, dem Feuchtigkeitsgrad und der Zusammensetzung der Lebensmittel [21].

Studien zu AGEs in Lebensmitteln und deren Auswirkungen auf den Organismus berichten von positiven als auch negativen Effekten. Ames *et al.* konnten zeigen, dass Melanoidine die Anzahl der gesundheitsfördernden Darmbakterien, wie beispielsweise Anaeroben und Bifidobakterien, erhöhen können, indem sie deren Wachstum stimulieren [22]. Übereinstimmende Resultate erzielte die Studie von Borrelli *et al*. Hierbei unterstützten Melanoidine aus der Brotkruste das Wachstum einiger Bifidostämme [23]. Ausgehend von diesen Studien ist eine präbiotische Wirkung der Melanoidinen vorstellbar. Negative Effekte der Melanoidinen und der AGEs in Lebensmitteln werden mit den gesundheitsschädlichen Auswirkungen der im Organismus gebildeten AGEs in Verbindung gebracht.

1.1.3 Endogene AGE-Bildung

Innerhalb der letzten 20 Jahre rückte die AGE-Bildung *in vivo* zunehmend in den Fokus zahlreicher Studien. Ausschlaggebend hierfür war die Entdeckung, dass Hämoglobin durch nicht-enzymatische Glykosylierungen, vorwiegend in Diabetes Mellitus erkrankten Personen, modifiziert werden kann [24]. Die N-terminale Aminosäure Valin in der β-Kette des Hämoglobins (Hb) dient hierbei als Angriffspunkt für Glucose und die modifizierte Form des Hämoglobins nach Glykierungsreaktionen wird HbA_{1C} bezeichnet [24]. Bei gesunden Personen liegen etwa 5 % des Hämoglobins glykiert vor, wohingegen bei Diabetikern je nach Glucosespiegel 12 % oder mehr des gesamten Hämoglobins modifiziert sind [25]. Entsprechend der Halbwertszeit des Hämoglobins von 100-120 Tagen kann das Ausmaß des glykierten Hämoglobins als Marker für die Blutzuckereinstellung bei Diabetikern genutzt werden [25]. Zahlreiche Studien stellten fest, dass AGEs auch während des physiologischen Alterungsprozesses akkumulieren [26,27]. Zusätzlich werden AGEs in höheren Konzentrationen mit zahlreichen Erkrankungen, beispielsweise Katarakt, Arteriosklerose, nephrologische Störungen, sowie mit neurodegenerativen Erkrankungen in Verbindung gebracht [3,28,29]. Immunohistochemische Analysen humaner arteriosklerotischer Läsionen konnten extrazelluläre AGE-Ablagerungen sowie massive intrazelluläre AGE-Vorkommen in Schaumzellen, die aus fettbeladenen Makrophagen und glatten Muskelzellen entstehen, nachweisen [30].

Eine nicht-enzymatische Glykosylierung von Proteinen führt *in vivo* oft zu deren Funktionsbeeinträchtigung oder Funktionsverlust. Da die weiterführende und finale Phase der Maillard-Reaktion unter physiologischen Bedingungen relativ langsam ablaufen, sind besonders Proteine mit einer längeren Lebensdauer von einer Glykierungsreaktion betroffen. Als Beispiel lassen sich hier Proteine der extrazellulären Matrix und Hämoglobin, Albumin, Kristallin, sowie die Apoproteine der cholesterinhaltigen Lipoproteine nennen [31-33].

Die Untersuchung von AGE-modifizierten Proteinen im alternden Gehirn ist von besonderer Bedeutung. Untersuchungen zu modifizierten Proteinen in Gehirnschnitten von Alzheimer Patienten konnten zeigen, dass es sich bei den Aggregaten um glykoxidierte Proteine handelt [34]. AGEs unterstützen die extrazellulären Ablagerungen von β-Amyloidproteinen, den sogenannten senilen Plaques, die vor allem bei der Alzheimerischen Erkrankung zu finden sind [35]. Zudem verstärken AGEs die Bildung neurologischer Fibrillen aus Tau-Proteinen, die intrazellulär als histologische Merkmale

dieser Erkrankung auftreten [36]. Sowohl senile Plaques als auch die fibrillären Bündel aus Tau-Proteinen führen zu synaptischen Störungen, indem sie die Signalweiterleitung zwischen den Neuronen unterbinden und den neuronalen Zelltod induzieren [37]. AGEs können zudem Mikroglia, die inflammatorischen Zellen im Gehirn, aktivieren und sowohl oxidativen Stress als auch die für neurodegenerativen Erkrankungen typischen Entzündungsreaktionen auslösen [38].

1.1.4 Beitrag der exogenen AGEs zum endogenen AGE-Spiegel

Kontrovers diskutiert wird bis heute, inwiefern exogene AGEs den endogenen AGE-Spiegel beeinflussen können. In einer Studie mit 90 gesunden Probanden korrelierte nach dreitägiger AGE-Aufnahme der AGE-Plasmaspiegel mit der Menge an aufgenommenen AGEs. Einer Gruppe wurden Lebensmittel mit geringem AGE-Gehalt zugeteilt und wie vermutet verringerte sich der AGE-Plasmaspiegel [39]. Diese Ergebnisse können durch weitere Studien bestätigt werden [40,41]. Über welche Mechanismen die Absorption der AGEs im Intestinaltrakt verläuft, ist noch nicht exakt erforscht. Studien berichten über einen Peptidtransporter, den PEPT1, der Pyrallin absorbieren könnte [42]. Hierzu fehlen jedoch noch aussagekräftige Untersuchungen.

Nicht nur Studien zur Bioverfügbarkeit sondern auch zur Eliminationskinetik wurden in den letzten Jahren durchgeführt. In der Untersuchung von Forster *et al.* wurde nach Einnahme von Salzstangen, die reich an Pentosidin und Pyrralin sind, 50 % der gesamten aufgenommenen Menge an Pyrralin und 60 % an Pentosidin über eine Zeitdauer von drei Tagen im Urin gemessen [43]. Eine weitere Studie untersuchte die Wiederfindung der AGEs am Beispiel von Pyrallin bei normaler westlicher Ernährung im Vergleich zur AGE-reduzierter Kost. Hierbei wurde eine Reduktion der renalen Ausscheidung an Pyrallin von durchschnittlich 4,8 mg/Tag bei normaler Kost auf ungefähr 0,3 mg/Tag bei AGE-reduzierter Kost beobachtet [44]. Hieraus lässt sich schließen, dass eine gesunde Nierenfunktion für die Ausscheidung der AGEs essentiell ist. Da AGEs als heterogene Substanzklasse zahlreichen Modifikationen unterliegen können, die zu stark vernetzten Produkten führen, ist es weiterhin unklar, inwiefern AGEs zuerst prozessiert werden müssen um renal ausgeschieden werden zu können.

1.2 Zelluläre Reaktionen ausgelöst durch AGEs

1.2.1 AGE-Rezeptoren

Extrazellulär gebildete AGEs können mit einer Vielzahl von Rezeptoren auf Zelloberflächen interagieren, darunter der Rezeptorkomplex Oligosaccharyl-Transferase 48 (OST-48, auch AGE-R1 oder p60 bezeichnet), 80K-H Phosphoprotein (auch AGE-R2 oder p90 genannt) und Galectin-3 (AGE-R3) [45-47]. Weitere Rezeptoren sind der Makrophagen-Scavenger-Rezeptor Typ A (MSR-A) und andere Scavenger-Rezeptoren wie beispielsweise CD36 (*cluster of differentiation 36*), der Scavenger-Rezeptor Typ BI [48], LOX-1 (*Lectin-like oxidized low-density lipoprotein receptor-1*) [49] und FEEL-1 und -2 (*fasciclin EGF-like, laminin-type EGF-like, link domain containing scavenger receptors 1 and 2*) [50], sowie der RAGE (*receptor for advanced glycation end products*) [51]. Welches Rezeptorsystem für die bestimmten zellulären Reaktionen verantwortlich ist, wird zurzeit von verschiedenen Instituten erforscht. Einige dieser Systeme sind hauptsächlich an der Endozytose von AGE-modifizierten Proteinen involviert, während andere Systeme vielmehr in der Aktivierung von Signalwegen beteiligt sind [52]. Der Scavenger-Rezeptor CD36 sowie der MSR-A sind beispielsweise für die endozytotische Aufnahme von oxidierten Proteinen und AGE-modifiziertem Albumin verantwortlich [53,54].

Der RAGE ist ein Mitglied der Immunglobulin-Superfamilie und ein Multi-ligandenrezeptor, an den sich neben AGEs auch β-Amyloid, Amyloid-Fibrillen, Amphoterin und die proinflammatorischen S100/Calgranuline binden können [55]. Vermutlich ist er primär an der Signaltransduktion beteiligt. Als membranständiger Rezeptor besteht er aus einer einfachen Transmembran-Helix, die die extrazelluläre Domäne mit dem kurzen intrazellulären Bereich verbindet. Die extrazelluläre Domäne ist aus drei immunglobulinähnlichen Einheiten aufgebaut. Von dem N-terminalen Ende ausgehend besteht der extrazelluläre Bereich aus einer V(*variable*)-Domäne, gefolgt von den C1(*constant 1*)- und C2(*constant 2*)-Abschnitten. Die N-terminale V-Domäne ist distal von der Plasmamembran gelegen [56]. Studien mit fluoreszenzmarkierten RAGE konnten zeigen, dass dieser nicht als Monomer in der Plasmamembran vorhanden ist. Er agglomeriert vielmehr mit weiteren RAGE-Molekülen und bildet größtenteils Dimere aus [57].

Als Multiligandenrezeptor erkennt er gemeinsame Merkmale bzw. Strukturen der unterschiedlichen Liganden. Diese gemeinsamen Merkmale konnten mittels Röntgen-kristallographie, Kernspinresonanzspektroskopie und biochemischen Daten ausfindig

gemacht werden. Somit ist bekannt, dass die Ligandenbindung bei neutralen pH-Wert durch elektrostatische Interaktionen initiiert wird [58]. Im Zuge der AGE-Entstehung wird die negative Gesamtladung durch chemische Modifikationen (z.B. Carboxymethylierungen) erhöht [58]. Der RAGE beinhaltet in seiner V-Domäne eine hohe Anzahl an Arginin- und Lysin-Seitenketten, die bei neutralem pH-Wert eine positive Ladung tragen [58]. Die positiv-geladenen Aminosäuren-Seitenketten der V-Domäne und die negativ-geladenen Aminosäuren-Seitenketten an der Oberfläche der AGEs und weiteren Liganden bieten somit eine optimale Voraussetzung für die Rezeptor-Liganden-Interaktion.

Der RAGE ist ein zentrales Signalmolekül in der angeborenen Immunantwort und an der Entstehung und der Aufrechterhaltung von Entzündungsreaktionen beteiligt. Diese Entzündungsreaktionen beinhalten diabetische Komplikationen [59], diverse chronische Entzündungen [60], Arteriosklerose [61], Neurodegenerationen und onkologische Erkrankungen [62]. RAGE wird überwiegend in Monozyten/Makrophagen, mesangialen Zellen, vaskulären glatten Muskelzellen, endothelialen Zellen und neuronalen Zellen exprimiert [63,64]. Der RAGE-Beitrag zur endozytotischen AGE-Aufnahme ist bis heute umstritten.

Der MSR-A-Rezeptor wurde ursprünglich als spezifischer Rezeptor für acetyliertes LDL (*low density lipoprotein*) beschrieben [65]. MSR-A ermöglicht die endozytotische Aufnahme von acetyliertem und oxidiertem LDL. Diese Aufnahme führt zur Akkumulation von Cholesterinestern und letztendlich zu einem Prozess der in der Arteriosklerose als Schaumzellbildung bekannt ist [65]. Einen Zusammenhang zwischen AGEs und dem MSR-A Rezeptor konnte von Sano *et al.* gezeigt werden, indem in MSR-A knock-out Makrophagen eine Reduktion der AGE-Aufnahme um bis zu 65 % im Vergleich zu Wildtyp-Makrophagen detektiert werden konnte [66]. Die übrige endozytotische AGE-Aufnahme lässt vermutlich auf weitere Rezeptoren schließen. Nichtsdestotrotz kann der MSR-A Rezeptor als einer der wichtigsten endozytotischen AGE-Rezeptoren in Makrophagen bezeichnet werden. MSR-A ist außer in Makrophagen und Makrophagen-abstammenden Zellen auch in intimalen vaskulären glatten Muskelzellen, nicht aber auf medialen glatten Muskelzellen, und in sinusoidalen endothelialen Leberzellen und Kupfferzellen zu finden [67,68]. Die Rezeptoren OST-48 und 80K-H scheinen als AGE-Rezeptoren für eine Aktivierung von Signalwegen eine untergeordnete Rolle zu spielen. Eine Bindung von Antiseren gegen OST-48 und 80K-H konnte AGE-induzierte zelluläre Reaktionen, wie beispielsweise eine Zytokinfreisetzung, eine verstärkte Ausbildung der

extrazellulären Matrix und Chemotaxis, nicht unterbinden [69,70]. Die Rolle von Galectin-3 ist noch wenig charakterisiert. Es gibt jedoch Hinweise, dass auch dieser Rezeptor an der Endozytose von AGE-modifizierten Substraten involviert ist [71]. Ebenso gering ist die Datenlage zu dem LOX-1 Rezeptor. Dieser endotheliale Rezeptor bindet primär oxidiertes LDL. Zudem wurde auch eine Bindung an AGEs beschrieben [49].

1.2.2 AGE-induzierte Signalwege

AGE-induzierte Signalwege sind häufig auf die Interaktion mit dem RAGE zurückzuführen. Die Aktivierung von RAGE führt zu diversen Signalkaskaden, abhängig von den interagierenden Liganden, dem Zelltyp und seiner Umgebung [72]. Die unterschiedlichen Signalkaskaden umfassen die Aktivierung von Ras-extrazellulärer signalregulierender Kinase 1/2 (ERK 1/2) [73], CDC42/Rac [74], Stress-aktivierender Protein Kinase/c-Jun-NH2-terminaler Kinase (SAPK/JNK), p38 Mitogen-aktivierender Protein (MAP) Kinase [62] und die Phosphatidylinositol-3 Kinase [75] (Abb. 4). Diese Signaltransduktionswege aktivieren Transkriptionsfaktoren wie NF-kB (*nuclear factor 'kappa-light-chain-enhancer' of activated B-cells*) [76], CREB (*cAMP response element-binding*) Protein [73] oder Mitglieder der STAT (*signal transducers and activators of transcription*) Familie [77]. Während einer Inflammation induzieren RAGE-Liganden eine gesteigerte Expression des RAGE-Rezeptors. Diese positive Rückkopplung wird über die Aktivierung des NF-kB gesteuert und konvertiert eine vorübergehende pro-inflammatorische Reaktion in einen chronischen pathophysiologischen Zustand [78].

Die Aktivierung des NF-κB induziert die Expression von Adhäsionsmolekülen, Wachstumsfaktoren (z.B. TGF-β (*transforming growth factor-β)*) und pro-inflammatorischen Zytokinen (z.B. Interleukin-6 und Tumornekrosefaktor-α) [79,80]. Überdies scheint RAGE an zahlreichen zellulären Effekten beteiligt zu sein, oft auch in gegensätzlicher Weise. Als Beispiel hierfür sind Amphoterin, ein RAGE-Ligand der für das Neuritenwachstum verantwortlich ist, und β-Amyloid, ein RAGE-Ligand der für die Pathogenese der Alzheimerischen Erkrankung zuständig ist, zu nennen. Amphoterin bindet an RAGE und induziert ein Neuritenwachstum, das die Differenzierung von Neuronen unterstützt, während die Bindung von β-Amyloid eine zelluläre Produktion von reaktiven Sauerstoffspezies (*reactive oxygen species*, ROS) induziert, die oxidativen Stress und Zytotoxizität bewirkt [63,81]. Diese Gegenüberstellung zeigt deutlich, dass die RAGE-vermittelte Signaltransduktion ligandenabhängig ist.

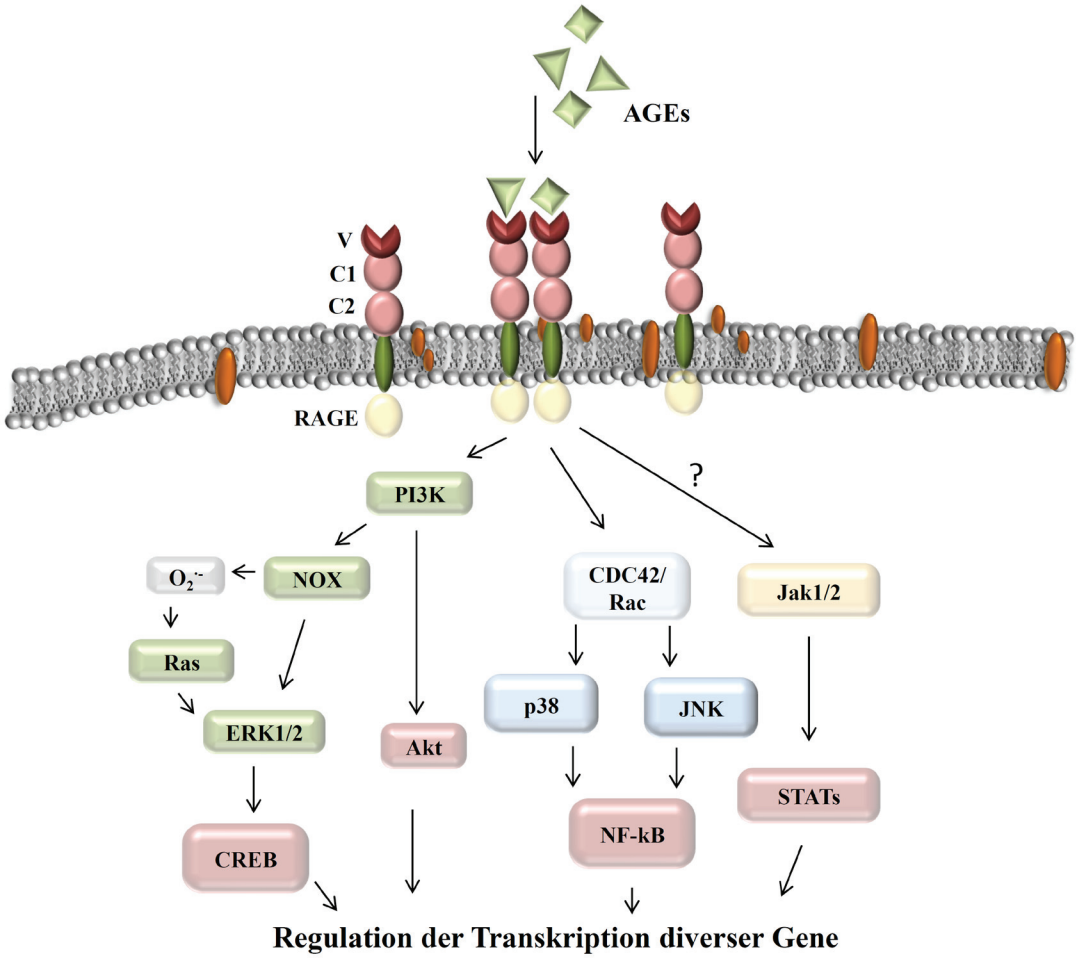

Abbildung 4: AGE-RAGE Interaktion und die Aktivierung von Signalwegen
Modifiziert nach [55,74,75,77].

1.2.3 AGE-induzierte ROS-Produktion

Eine natürliche Folge des aeroben Lebens ist die Entstehung freier Sauerstoffradikale und anderer Oxidantien aus dem für die Respiration benötigten Sauerstoff. Diese Verbindungen sind meist hoch reaktiv und induzieren zelluläre Schäden, indem sie mit Proteinen, Lipiden und Nukleinsäuren reagieren [82]. Zur Abwehr dieser Verbindungen entstanden im Laufe der Evolution zahlreiche endogene und exogene Schutzmechanismen. Die endogenen Schutzmechanismen sind antioxidativ wirksame Enzymsysteme, wie die Superoxiddismutase, die Katalase und die Glutathionperoxidase. Zu den exogenen Schutzmechanismen zählen die Vitamine C und E, sowie Carotinoide und zahlreiche sekundäre Pflanzenstoffe [83].

Der Begriff „oxidativer Stress“ wird definiert als ein Verschieben in der Balance zwischen Oxidantien und Antioxidantien zugunsten der Oxidantien [84]. Altersassoziierte verminderte Aktivität antioxidativer Enzyme wird mit erhöhtem oxidativen Stress in Zusammenhang gebracht [85]. Durch eine verringerte antioxidative Aktivität, und

infolgedessen erhöhtem oxidativen Stress, werden verstärkt reaktive Sauerstoffspezies gebildet, die unter anderem Carbonylgruppen induzieren können. Diese Carbonylgruppen sind bekanntlich sehr reaktiv und können mit den Aminosäuren-Seitengruppen der Proteine unter glykoxidativen Stress reagieren. AGEs können somit unter oxidativem Stress verstärkt entstehen, zudem können sie eine ROS-Produktion induzieren [86].

Die ROS-Produktion kann intrazellulär auf vielen verschiedenen Ebenen stattfinden. Beispielsweise reagiert die NADPH-Oxidase (NOX) der Phagozyten (z.B. Neutrophile und Makrophagen), ein enzymatischer Multiproteinkomplex, auf Stimulanzien mit einer ROS-Antwort. Zu den zahlreichen Untereinheiten der NOX zählen die membranständigen Komponenten gp91^{phox} und p22^{phox}, die zusammen das Flavocytochrom b_{558} bilden (phox kennzeichnet *NOX of phagocytes*). Die gp91^{phox} Einheit beinhaltet das katalytische Zentrum und wird als NOX2 bezeichnet [87]. Zu dieser NOX-Untereinheit existieren vier weitere Homologen. NOX 1 ist in Fibroblasten und epithelialen Zellen zu finden. NOX 2 ist überwiegend in Phagozyten vertreten, während NOX 3 in der fetalen Niere, NOX 4 weitverbreitet beispielsweise in Osteoklasten, Niere und Augen, sowie NOX 5 in Milz und Großhirn detektiert wurden [88-90]. In nicht-aktivierten Phagozyten befinden sich die vier weiteren Komponenten der NADPH-Oxidase, p47^{phox}, p67^{phox}, p40^{phox} und Rac, im Zytosol [87], während das Flavocytochrom b_{558} in der Membran von intrazellulären Granula gespeichert ist (Abb. 5) [91]. Nach Aktivierung der Phagozyten fusionieren die intrazellulären Granula, die das Flavocytochrom b_{558} enthalten, mit den neu gebildeten Phagosomen. Anschließend assoziiert der zytoplasmatische Proteinkomplex mit dem Flavocytochrom b_{558} [87]. Voraussetzung für die Bindung des zytoplamatischen Proteinkomplexes an die membranständigen Komponenten der NAPDH-Oxidase ist eine Phosphorylierung von p47^{phox}. Die Phosphorylierung führt zu einer Konformationsänderung des p47^{phox} und ermöglicht dadurch eine Interaktion mit dem p22^{phox} [92]. Es ist denkbar, dass weitere zytosolische NOX-Komponenten aktiviert werden und die Interaktion mit dem membranständigen Komplex dadurch initiiert wird. Ein genauer Mechanismus hierzu ist jedoch noch nicht ausreichend erforscht. Der aktivierte NADPH-Oxidase-Komplex überführt Elektronen von NAPDH auf Sauerstoff unter Bildung von Superoxidanionen ($O_2^{\cdot-}$) (Abb. 5). Diese Superoxidanionen werden entweder spontan oder enzymatisch durch die Superoxiddismutase zu Wasserstoffperoxid (H_2O_2) reduziert. H_2O_2 kann in Anwesenheit von Eisen- oder Kupferionen in der Haber-Weiss-Reaktion zu Hydroxylradikalen reagieren [93]. Phagozyten exprimieren zudem eine

Myeloperoxidase (MPO), die eine Bildung des hochreaktiven Hypochlorids (HOCl) aus H_2O_2 katalysiert [93].

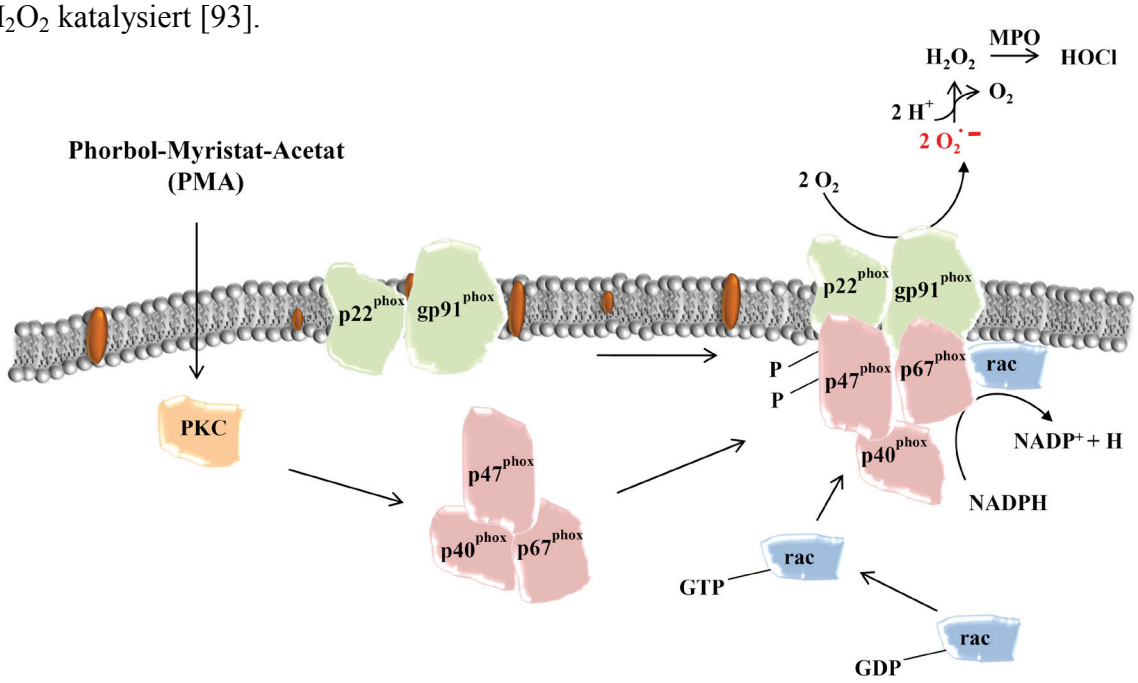

Abbildung 5: Die Aktivierung der NADPH-Oxidase

Die Aktivierung der NADPH-Oxidase erfolgt am Beispiel von PMA, einem Phorbol-Ester, der in der vorliegenden Arbeit verwendet wurde. PMA aktiviert direkt die Proteinkinase C (PKC), die anschließend beispielsweise $p47^{phox}$ phosphoryliert und die Translozierung der zytosolischen Komponenten zu den membranständigen Einheiten induziert. Alternativ kann die PKC durch Diacylglycerin aktiviert werden. PMA aktiviert vorwiegend die PKC-ζ, wobei auch die PKC-Isoformen α, β und δ, je nach Zelltyp und Stimulierung, die NADPH Oxidase aktivieren können. Modifiziert nach [87,94,95].

Diese ROS-Produktion dient während der Phagozytose zur Abtötung von Pathogenen. Überwiegt die Produktion der reaktiven Substanzen den zytoprotektiven Systemen, so entstehen oxidative Schäden an Makromolekülen. Als Konsequenz müssen diese geschädigten Produkte beseitigt werden. Mäßig oxidierte Proteine können teilweise wieder enzymatisch in ihre Ausgangsstruktur überführt werden [96]. Ist eine Reparatur nicht möglich, müssen die Proteine zum Erhalt der zellulären Funktionen abgebaut werden. Hierfür existieren verschiedene Proteasen: die lysosomalen Proteasen und das proteasomale System. Zusätzlich befinden sich im Zytosol noch calciumabhängige Proteasen (Calpaine) und in den Mitochondrien die Lon-Protease, die einen Proteinabbau bewerkstelligen können [97]. Ein proteolytischer Abbau ist bei schwerwiegendem oxidativem Stress oft nur noch eingeschränkt möglich [98]. Eine höhermolekulare Aggregatbildung aufgrund hydrophober und elektrostatischer Interaktionen und Quervernetzungen durch Reaktionen mit weiteren Proteinen und nicht-proteinartigen Komponenten behindern aufgrund ihrer Struktur den Zugang zum aktiven Zentrum der

Enzyme [99]. Als Folge können diese nicht-abbaubaren Proteinaggregate die Funktionen der Zellen und des jeweiligen Gewebes stark beeinträchtigen.

1.3 Das proteasomale System

Das proteasomale System ist für die normale Zellfunktion unerlässlich. Die Hauptaufgabe des Proteasoms besteht darin, zytoplasmatische oder nukleär lokalisierte, geschädigte oder ungeschädigte Proteine abzubauen [100]. Zudem sind Substrate für das proteasomale System kurzlebige regulatorische Proteine, die in der Zelldifferenzierung, in dem Zellzyklus, in der Transkription und in der Apoptose involviert sind [101,102].

Das Proteasom besteht aus einer zentralen Kerneinheit, dem 20S Proteasom, und zahlreichen regulatorischen Komponenten, durch deren Anlagerung an das 20S die Spezifität und Aktivität des Proteasoms beeinflusst wird [100]. Die 20S Kerneinheit bildet die strukturelle Basis aller Proteasomen-Formen. Ihre zylinderähnliche Struktur setzt sich aus zwei α- und zwei β-Ringen zusammen, wobei die β-Ringe zentral gelegen sind und von den α-Ringen nach außen hin abgegrenzt werden (Abb. 6). Jeder Ring besteht aus sieben Untereinheiten (α1-α7 und β1-β7). Die α-Ringe binden die regulatorischen Komponenten und unterstützen die Aufnahme der Proteine ins Innere des Proteasoms. Zusätzlich verschließen die α-Ringe im inaktiven Zustand den Zugang zum katalytischen Zentrum. Über den N-Terminus der α3-Untereinheit, der mit den weiteren α-Untereinheiten interagiert, wird die Öffnung zum aktiven Zentrum reguliert. Eine Deletion des N-Terminus der α3-Untereinheit führt demnach zur dauerhaften Öffnung des Zugangs zum katalytischen Zentrum [103]. Im Innern des Proteasoms findet die Hydrolyse der Proteine durch die β-Untereinheiten statt. Dabei unterscheiden sich die Aktivitäten der konstitutiven Form und der durch spezifische Substanzen induzierbaren Form des Proteasoms.

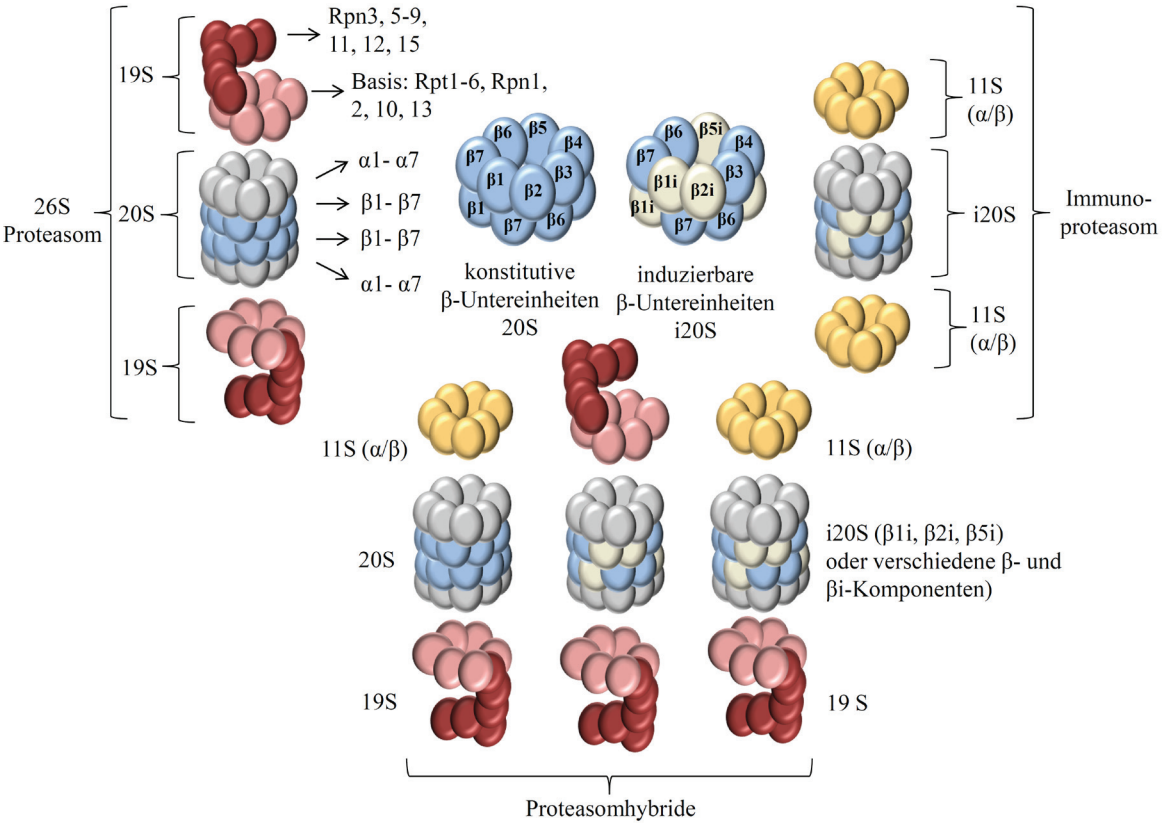

Abbildung 6: Das proteasomale System

Das 26S Proteasom setzt sich aus der 20S Kerneinheit und zwei 19S Regulatoren zusammen, die mit den α-Untereinheiten des 20S interagieren. Das 20S Proteasom bildet eine zylinderähnliche Struktur aus 4 Ringen mit jeweils 7 Untereinheiten. Der 19S Regulator besteht aus einer Basis und einem oberen, äußeren Abschnitt. Die konstitutiven β-Untereinheiten der Kerneinheit zählen zu dem 20S Proteasom. Die induzierbaren β-Untereinheiten in der Kerneinheit (i20S) bilden zusammen mit den 11S Regulatoren das Immunoproteasom. Der zytoplasmatische 11S Regulator setzt sich aus α- und β-Einheiten zusammen, während der im Nukleus lokalisierte 11S Regulator aus γ-Untereinheiten gebildet wird (nicht gezeigt). Modifiziert nach [100,104-106].

1.3.1 Die konstitutive Form des Proteasoms

Die konstitutive Form des Proteasoms beinhaltet die enyzmatisch aktiven β1-Y, β2-Z und β5-X Untereinheiten und den 19S Regulator (Abb. 6). Zwei 19S Regulatoren bilden zusammen mit der 20S Kerneinheit das 26S Proteasom.

Die β1-Untereinheit verfügt über eine peptidyl-glutamyl-peptid-hydrolase (PGPH) oder caspaseähnliche Aktivität, die für die Spaltung von sauren Aminosäuren verantwortlich ist. Die β2-Untereinheit weist eine trypsinähnliche Aktivität auf, die die Spaltung basischer Aminosäuren katalysiert, während die β5-Untereinheit eine chymotrypsinähnliche

Aktivität beinhaltet, die wiederum Peptidbindungen an neutralen und hydrophoben Aminosäuren proteolytisch spaltet [100].

Bereits entfaltete Proteine können ohne Regulatoreinheit in das Innere des Proteasoms gelangen. So wurde beschrieben, dass eine Oxidation von Proteinen eine Konformationsänderung der Proteinstruktur zufolge hat. Durch die Oxidation gelangen hydrophobe Aminosäuren, die sich unter normalen Bedingungen im Proteininneren befinden, an die Oberfläche der Proteine. Infolgedessen wird eine Erkennung durch die α-Untereinheiten des 20S Proteasoms ermöglicht [107]. Diese Eigenschaft hängt jedoch von dem Modifikationsgrad der Oxidation ab, da stärker oxidierte Proteine vermutlich durch ihre hydrophoben Oberflächen mit anderen Proteinen interagieren und Aggregate bilden, die vom 20S Proteasom aufgrund ihrer Struktur und Größe nicht mehr in das Innere des Proteasoms und somit ins aktive Zentrum aufgenommen werden können [107].

Durch Anlagerung des 19S Regulators wird das Spektrum der abzubauenden Proteine erweitert. Der Regulator erkennt Proteine, die im Vorfeld für den Abbau markiert wurden. Diese Markierung erfolgt durch eine ATP-abhängige Übertragung von Ubiquitin, einem 8,5 kDa kleinen Signalmolekül, auf die für den Abbau vorgesehenen Proteine [100]. Katalysiert wird diese Markierung durch ein komplexes Enzymsystem [100]. Im ersten Schritt wird das Ubiquitin durch E1-Enzyme unter Verwendung von ATP aktiviert und anschließend zu den E2-Enzymen transportiert. Der E2-Ubiquitin-Komplex bindet an E3-Enzyme, die mit ihrer Ubiquitin-Ligase-Aktivität das Ubiquitin aus dem E2-Ubiquitin-Komplex auf Lysin-Seitenketten von Proteinen übertragen. An das proteingebundene Ubiquitin können weitere Ubiquitin-Monomere hinzugefügt werden. Der 19S Regulator erkennt die Polyubiquitinkette und leitet die Substrate in entfalteter Form dem Proteasom zu [100]. Dieser Regulator ist in mindestens 18 Untereinheiten unterteilt, die wiederum in zwei Subtypen eingeteilt werden können. Die Basis besteht aus sechs unterschiedlichen ATPasen der AAA-Familie und weiteren nicht-ATPase Einheiten [108]. Diese Basis bindet an die außen gelegenen α-Untereinheiten der 20S Kerneinheit und veranlasst die ATP-abhängige Öffnung des katalytischen Zentrums [108]. Der obere Abschnitt des 19S Regulators besteht aus den nicht-ATPase Einheiten, die für die Bindung der ubiquitinierten Substrate und der Enzyme für den Abbau, sowie für das Recycling von Ubiquitin, verantwortlich sind [103].

1.3.2 Die induzierbare Form des Proteasoms

Die induzierbare Form des Proteasoms, auch Immunoproteasom oder Immunoform des Proteasoms genannt, entsteht durch einen Austausch der konstitutiv exprimierten katalytischen Untereinheiten aufgrund unterschiedlicher Stimulanzien, wie beispielsweise den Interferonen (IFN), Tumornekrosefaktor-α (TNF-α) und Lipopolysacchariden (LPS) [109]. Darüber hinaus kann eine Induktion der immunoproteasomalen Subtypen aus Hitzeschock-Reaktionen [110], durch Arsentrioxid [111,112] und Stickstoffmonoxid [113], erfolgen. Dabei werden die β1-, β2- und β5-Untereinheiten in der 20S Kerneinheit durch die jeweiligen immunoproteasomalen Untereinheiten *low molecular weight protein 2* (LMP2 oder β1i), *multicatalytic endopeptidase complex-like 1* (MECL1 oder β2i) sowie der *low molecular weight protein 7* (LMP7 oder β5i)-Untereinheit ersetzt (Abb. 6). Zusätzlich binden 11S Regulatoren (PA28) an die 20S Kerneinheit. Die 11S Regulatoren bilden zusammen mit den induzierbaren β-Untereinheiten das aktive Immunoproteasom (Abb. 6). Der 11S Regulator besteht aus mehreren Untereinheiten, die als α-, β- oder γ-Untereinheit charakterisiert sind und die sich in ihrer zellulären Lokalisation unterscheiden. Im Zytoplasma vorkommend ist der heterodimere 11Sα/β-Komplex und überwiegend im Nukleus befindet sich der 11Sγ-Komplex [100]. Der heterodimere 11Sα/β besteht aus drei α- und vier β-Untereinheiten, die sich zu einer ringförmigen Struktur zusammenfinden. Durch die Anlagerung des 11S an die 20S Kerneinheit wurde eine verstärkte Hydrolyse von Peptiden beobachtet [114]. Auch hier ist jedoch eine Entfaltung von Proteine erforderlich, um diese in das Innere des Immunoproteasom-Komplexes zu translozieren [115].

Am besten charakterisiert ist die Induktion der immunoproteasomalen Untereinheiten durch Interferon-γ (IFN-γ). Eingeleitet wird diese Induktion durch die Bindung an seinen spezifischen Rezeptor, dem IFN-γ-Rezeptor. Diese Bindung induziert die Phosphorylierung der Rezeptoruntereinheiten durch die Januskinasen (Jak). Bei dieser Aktivierung entsteht an der Rezeptoruntereinheit 1 (*interferon-gamma receptor 1,* IFNGR1) die Bindungsstelle für den Transkriptionsfaktor *signal transducer and activator of transcription 1* (STAT1) [116]. STAT1 bindet über seine *src homology 2* (SH2)-Domäne an IFNGR1 und die Tyrosin-Aminosäure an der Position 701 wird phosphoryliert. STAT1 wird dadurch aktiviert und schließt sich als Homodimer mit einem weiteren phosphorylierten STAT1 zusammen. Für die Dimerisierung von STAT1 ist die Phosphorylierung an Tyrosin 701 von Bedeutung. STAT1 kann zudem an Serin 727 phosphoryliert werden, hierbei wird jedoch vermutet, dass diese Aktivierung über einen

anderen Signalweg als Jak1/2 erfolgt [117]. Das Homodimer aus aktiviertem STAT1 interagiert mit dem Transkriptionsfaktor *interferon regulatory factor-1* (IRF-1), die beide anschließend in den Nukleus translozieren. Aktiviertes STAT1 bindet im Nukleus an die *gamma-activated sequence* (GAS)-Elemente, die sich in der Promotorregion des zu exprimierenden Gens befinden [118]. IFR-1 bindet hingegen an das *interferon-stimulated response element* (ISRE), welches in unmittelbarer Nachbarschaft zur GAS-Sequenz lokalisiert ist [118]. Die Transaktivierung von IRF-1 durch aktiviertes STAT1 führt hierbei zu einer verstärkten Synthese der immunoproteasomalen Untereinheiten (β1i, β2i, β5i, 11Sα und 11Sβ) [116,119].

Das Immunoproteasom ist verstärkt an der Prozessierung von Peptidstücken beteiligt, die für die Antigenpräsentation bei der adaptiven Immunantwort vorgesehen sind. Die Peptidstücke mit 8-10 Aminosäuren werden von dem Immunoproteasom ins Zytosol entlassen, wo sie an die *transporter associated with antigen processing* (TAP1/TAP2) Moleküle binden [119]. Die Peptidfragmente gelangen dann über die TAP1/TAP2 Moleküle in das Endoplasmatische Retikulum. Dort werden die MHC (*major histocompatibility complex*)-I-Moleküle mit den Peptidfragmenten beladen und über den Golgi-Apparat in sekretorischen Vesikeln an die Zelloberfläche zur Antigenpräsentation transportiert [119].

Insbesondere die Aktivität des Immunoproteasoms ermöglicht eine effizientere Freisetzung von MHC-I-präsentierenden Antigenen aus Pathogenen und intrazellulären Proteinen, die an der Zelloberfläche von zytotoxischen T-Zellen erkannt werden [120]. Nicht-infizierte Zellen induzieren jedoch die Bildung des Immunoproteasoms ohne dass eine erhöhte Antigen-Prozessierung notwendig ist [120]. Zudem ist bekannt, dass die konstitutiven Untereinheiten des proteasomalen Systems in nahezu allen Zellen des Organismus exprimiert werden, wobei die immunoproteasomalen Untereinheiten in geringen Mengen konstitutiv in immunrelevanten Zellen oder Geweben und aufgrund von Stimulanzien wie IFN-γ oder TNF-α in größeren Mengen und in den meisten anderen Zellen zu finden sind [121]. Die induzierbare β5i/LMP7-Untereinheit wurde außerdem in Abwesenheit von Zytokinen in vielen Zellen und Geweben in geringen Mengen detektiert [121]. Zusätzlich zeigt eine aktuelle Studie, dass das Immunoproteasom für den Erhalt der Protein-Homöostase und der Zellvitalität unter Zytokin-induziertem oxidativem Stress verantwortlich ist [121]. Aufgrund dieser Beobachtungen kann auf weitere Funktionen, die nicht mit einer Antigen-Prozessierung assoziiert sind, geschlossen werden. Allerdings ist die Datenlage hinsichtlich dieses Zusammenhangs noch nicht ausreichend gedeckt.

1.4 Das lysosomale System

Lysosomen sind intrazelluläre membranumschlossene Organellen, die mit einer Vielzahl von Hydrolasen ausgestattet sind. Diese Hydrolasen umfassen Proteasen, Peptidasen, Lipasen, Glykosidasen, Nukleasen, Sulfatasen und Phosphatasen [122]. Ein Großteil dieser Enzyme besitzt ein pH-Optimum zwischen 4,5-5,0. Dieser intralysosomale pH-Bereich wird durch Protonen aufrechterhalten, die über membranständige V-ATPasen ins Lumen transportiert werden [122]. Lysosomen nehmen eine bedeutende Rolle im Abbau von extrazellulären aber auch intrazellulären Makromolekülen ein. Extrazelluläres Material kann über die Pino-, Phago- oder rezeptorvermittelte Endozytose aufgenommen werden. Als Autophagie wird die Aufnahme von Zytosol bzw. zellulären Organellen bezeichnet [123]. Internalisiertes extrazelluläres Material ist zunächst in den frühen Endosomen lokalisiert, die im nachfolgenden Verlauf durch Fusionen mit weiteren Vesikeln die späteren Endosomen bilden. Im Laufe der Endosomenreifung sinkt der pH-Wert und die lysosomalen Proteasen werden aktiviert [124]. Die wichtigsten lysosomalen Proteasen sind die Cathepsine (griechisch: „kathepsein“ verdauen). Je nach Aminosäure, die im aktiven Zentrum lokalisiert ist, werden die Cathepsine in Cystein-Cathepsine (Cathepsin B, C, F, H, K, L, N, O, S, T, U, W und X), in Aspartat-Cathepsine (Cathepsin D und E) und in Serin-Cathepsine (Cathepsin A und G) unterteilt [124]. Zunächst werden die Cathepsine als inaktive Proenzyme in den Lysosomen gespeichert und bei Bedarf durch ein Absinken des pH-Wertes und der Aktivierung weiterer Enzyme aktiviert. Die drei Cathepsine B, D und L werden als die Hauptproteasen in humanen Lysosomen angesehen [125].

Cathepsin B (EC 3.4.22.1) gehört zu den Cysteinproteasen und besitzt neben seiner Funktion als Endopeptidase auch eine Aktivität als Carboxypeptidase [126]. Angesichts seiner strukturellen Homologie zu der in Pflanzen vorkommenden Protease Papain wird Cathepsin B, als auch Cathepsin L, zur Familie der papainähnlichen Proteasen gezählt [127]. Dem Cathepsin L (EC 3.4.22.15) wird innerhalb der Cysteinproteasen die bedeutendste Rolle in der lysosomalen Proteolyse von endozytierten Proteinen zugesprochen [128]. Cathepsin D (EC 3.4.23.5) gehört zur Pepsin-Familie und wird in die Aspartatproteasen eingestuft. Es besitzt eine starke Aktivität als Endopeptidase, die bevorzugt Peptidbindungen zwischen hydrophoben aromatischen Aminosäuren spaltet [129]. Cathepsin D wird ubiquitär exprimiert und ist primär am lysosomalen Abbau von Proteinen beteiligt. Zudem wirkt es proteolytisch an der Aktivierung weiterer Proenzymen mit [129].

2 ZIELSETZUNG DER ARBEIT

Innerhalb der letzten 100 Jahre konnte die mittlere Lebenserwartung der Menschen in den westlichen Ländern enorm gesteigert werden. Allerdings korreliert das zunehmende Lebensalter mit der Entstehung altersbedingter Erkrankungen. Aus diesem Grunde ist die Erforschung neurodegenerativer Erkrankungen, ihre Pathogenese, sowie die Möglichkeiten zur Beeinflussung unerlässlich [130]. Eine Vielzahl von neurodegenerativen Erkrankungen basieren auf Funktionsstörungen im Nervensystem, die größtenteils durch Ablagerungen von Stoffwechselprodukten in Form von Plaques entstehen können [131]. Dabei handelt es sich um Akkumulationen dysfunktioneller Proteine, die AGE-Modifikationen aufweisen können. Es ist bis heute nicht ausreichend bekannt, ob das Auftreten AGE-modifizierter Proteine durch eine unzureichende proteolytische Aktivität während der Alterung ausgelöst wird. Immer mehr Befunde belegen, dass AGEs im alternden Gehirn an oxidativem Stress und chronischen Entzündungen beteiligt sind. Zudem konnten immunohistochemische Untersuchungen an Gehirnschnitten von Alzheimer Patienten eine Kolokalisation von reaktiven Mikroglia und AGE-modifizierten Proteinen nachweisen [132]. In Zellkulturmodellen sezernieren aktivierte Mikroglia nach AGE-Stimulierung verschiedene Zytokine und reaktive Sauerstoff- und Stickstoffspezies. Vermutlich führt eine dauerhafte Aktivierung mikroglialer Zellen durch inflammatorische Stimulanzien (z.B. AGEs) zur zellulären Seneszenz und somit zum Funktionsverlust. Folglich können veränderte Mikrogliaaktivitäten zur Entstehung von neurodegenerativen Erkrankungen beitragen. Andererseits kann eine nicht-angemessene Aktivität dieser Zellen langfristig zur Akkumulation dysfunktioneller Proteine beitragen. Im Rahmen der eigenen Untersuchungen galt es Mechanismen zu identifizieren, wie die Akkumulation dieser AGE-modifizierten Proteine verhindert werden kann. Ein weiterer Schwerpunkt bildete die Charakterisierung zellulärer Reaktionen, die durch akkumulierte AGEs ausgelöst werden können.

Im Rahmen dieser Arbeiten wurden folgende Aufgabenstellungen bearbeitet:

- Zu Beginn wurde anhand der aktuellen wissenschaftlichen Datenlage der Zusammenhang zwischen oxidativen Proteinmodifikationen und deren Abbau durch das proteasomale System in **Publikation I** erläutert. Einen Schwerpunkt bildeten hierbei die Regulation des proteasomalen Systems und dessen Zusammenhang mit neurodegenerativen Erkrankungen.

- In ersten experimentellen Arbeiten wurden verschiedene Proteasen (Proteasen aus dem Gastrointestinaltrakt, lysosomale Proteasen und das proteasomale System) auf ihre proteolytischen Eigenschaften im AGE-Abbau analysiert (**Publikation II**).

- Desweiteren wurde die Eigenschaft des Cathepsin D mittels eines knock-out Zellmodells charakterisiert (**Publikation II**). Zusätzliche Untersuchungen anhand dieses Zellmodells sollten die Rolle des Cathepsin D in der Reduktion der AGE-Akkumulation bestätigen (**Publikation II**).

- Im weiteren Verlauf wurden die beiden wichtigsten lysosomalen Enzyme, das Cathepsin D und das Cathepsin L, in ihrer Expression und Aktivität nach AGE-Stimulation untersucht (**Publikation III**).

- Bedingt durch den Einfluss der AGE-modifizierten Proteine auf das lysosomale System wurden Untersuchungen in Cathepsin D und Cathepsin L knock-out Zellen durchgeführt, um deren Notwendigkeit verifizieren zu können (**Publikation III**).

- Darüber hinaus wurde der Einfluss der AGEs auf das proteasomale System untersucht. Die Beurteilung erfolgt hierbei ebenfalls über Aktivitäts- und Proteinexpressions-untersuchungen (**Publikation IV**).

- Einen weiteren Schwerpunkt bildeten die Untersuchungen zur Induktion des Immunoproteasoms nach AGE-Inkubation. Hierbei wurde die Aktivierung des Signalweges ausgehend von dem in der Signaltransduktion involvierten Rezeptor bestimmt (**Publikation IV**).

- Bedingt durch die Beeinflussung der Cathepsine und des proteasomalen Systems sollten des Weiteren anhand primärer mikroglialer Zellen, isoliert aus jungen, adulten und alten Mäusen, die Eigenschaften der AGEs bezüglich zellulärer Seneszenz und Veränderungen der zellulären Funktion während der Alterung ermittelt werden (**zusätzliche Daten**).

3 PUBLIKATIONEN

3.1 Publikation I: Oxidative protein damage and the proteasome.

Stefanie Grimm, Annika Höhn, Tilman Grune. *Amino Acids,* 2012, 42: 23-38

Oxidative Proteinmodifikationen, induziert durch freie Radikale, werden mit dem Auftreten zahlreicher Erkrankungen assoziiert. Zudem spielen oxidierte Proteine während des Alterungsprozesses eine bedeutende Rolle. Für den Abbau dieser geschädigten Proteine stehen verschiedene intrazelluläre proteolytische Systeme zur Verfügung. Es ist bekannt, dass oxidierte Proteine bevorzugt durch das proteasomale System hydrolysiert werden können. Dieser Abbau ist jedoch nicht uneingeschränkt möglich. Studien der letzten Jahre zeigen, dass die proteasomale Aktivität verschiedenen Regulationen unterliegt und zudem durch das Ausmaß der Proteinoxidation beeinflusst werden kann. So führt eine anhaltende Belastung mit Oxidatien zur verstärkten Proteinoxidation und gleichzeitig zu einer Verringerung der proteolytischen Empfindlichkeit. Aktuelle Studien deuten darauf hin, dass neben dem proteasomalen System auch die Autophagie, nicht nur in der Beseitigung geschädigter Organellen, sondern zusätzlich in der Eliminierung von Proteinen, eine Rolle spielt. In dem vorliegenden Übersichtsartikel wurde unter Verwendung aktueller Studien die Rolle des Proteasoms in der Proteolyse von oxidativ geschädigten Proteinen erläutert. Besonders hervorgehoben wurde der Einfluss des Proteasoms auf die Pathogenese neurodegenerativer Erkrankungen.

Eigenanteil:

- Literaturrecherche
- Erstellung des Übersichtsartikels
- Anfertigung der Abbildungen und der Tabelle

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REVIEW ARTICLE

Oxidative protein damage and the proteasome

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Abstract Protein damage, caused by radicals, is involved in many diseases and in the aging process. Therefore, it is crucial to understand how protein damage can be limited, repaired or removed. To degrade damaged proteins, several intracellular proteolytic systems exist. One of the most important contributors in intracellular protein degradation of oxidized, aggregated and misfolded proteins is the proteasomal system. The proteasome is not a simple, unregulated structure. It is a more complex proteolytic composition that undergoes diverse regulation in situations of oxidative stress, aging and pathology. In addition to that, numerous studies revealed that the proteasome activity is altered during life time, contributing to the aging process. In addition, in the nervous system, the proteasome plays an important role in maintaining neuronal protein homeostasis. However, alterations in the activity may have an impact on the onset of neurodegenerative diseases. In this review, we discuss what is presently known about protein damage, the role of the proteasome in the degradation of damaged proteins and how the proteasome is regulated. Special emphasis was laid on the role of the proteasome in neurodegenerative diseases.

Keywords Protein oxidation · Protein degradation · Proteasome · Neurodegeneration

Abbreviations

AD	Alzheimer's disease
ATP	Adenosine triphosphate
γ-IFN	Interferon-gamma
HD	Huntington's disease
HSP	Heat shock protein
MHC-I	Major histocompatibility complex class I
PARP	Poly-(ADP-ribose) polymerase
PHF	Tau-based paired helical filaments
PD	Parkinson's disease
ROS	Reactive oxygen species
UCH-L1	Ubiquitin carboxyl terminal hydrolase L1

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Introduction: protein oxidation

Free radicals and other oxidants are produced as metabolic by-products in an oxygen-containing environment by a large number of physiological and pathophysiological processes. Sources of radicals include electron leakage from the mitochondrial electron transport chain, the generation of hydroxyl radicals by Fenton-type reactions and the production of superoxide, hydrogen peroxide and hypochlorite as a consequence of several enzymatic reactions. An imbalance between the formation and detoxification of reactive oxygen species (ROS) can lead to oxidative stress and ROS react with all sorts of biological molecules with the consequence of a loss of function or induction of undesirable effects (Davies 2005).

As the process of protein oxidation is very complex, there is no satisfactory scheme for classification of oxidative modifications. However, it can be helpful in separating the reactions into two categories, those that modify protein backbones (polypeptide chain) and those that alter side chains of amino acids (Stadtman and Levine 2000). Modifications of protein backbones are characterized by

fragmentation of polypeptide chains (Davies 1987) and alterations in the side chains of amino acids result in a large variety of different products (Stadtman and Levine 2003). Among them, the most frequently determined products are protein carbonyls (Shringarpure and Davies 2002).

The oxidation process can also be distinguished in direct or indirect reactions. The indirect reaction is often called a secondary mechanism in which reactive species react primarily with non-protein components, such as lipids, carbohydrates and nucleic acids (Davies 2003) and such reactive products are able to react in turn with proteins and form numerous adducts via covalent cross-linking of amino acid side chains (Grune et al. 2001; Levine 2002). Owing to extensive modification and adduct formation of proteins, highly polymerized protein aggregates are formed under certain conditions and often described as lipofuscin, ceroid and advanced glycation endproducts—pigment-like fluorophores (Yin 1992, 1996).

To maintain normal cellular homeostasis and to avoid excessive accumulation of impaired and unprofitable proteins, it is necessary to subject such proteins to a degradation process. In a cell, several proteolytic systems are available including the autophagy–lysosome system, mitochondrial proteases, calcium-dependent proteases and the proteasomal system (Knecht et al. 2009).

In this review, we summarize reports published about the proteasomal function and its role in protein degradation.

The two major proteolytic systems: proteasome and autophagy

The major proteases responsible for protein degradation in mammalian cells are the cytosolic calpains, the lysosomal cathepsins and the proteasomes. The calpains are calcium-regulated papain-like and thiol-dependent proteases responsible for the degradation of substrates involved in signal transduction, cell cycle progression and cell mobility, for review see, Croall and Ersfeld (2007). A specific role for calpains in the degradation of oxidized proteins has not been demonstrated, thus they will not be discussed in this review.

The proteasomal system is located in the cytosol and the nucleus. Interestingly, this system is responsible for the degradation of more than 70–80% of intracellular proteins, mainly short-half life proteins such as newly synthesized, misfolded and regulatory proteins (Rock et al. 1994). In addition, damaged proteins and proteins that are out of function are substrates for the proteasomal system (Coux et al. 1996; Davies 2001; Jung et al. 2009).

The lysosome-linked autophagy system is considered to be also a degradation system of cytoplasmic constituents, including entire organelles and proteins, by their engulfment (Fig. 1). This type of autophagy is called macroautophagy. Other types of autophagy are microautophagy, which involves a nonspecific engulfment of cytoplasm and chaperon-mediated autophagy (Orenstein and Cuervo 2010). It was believed some years ago that autophagy is an unspecific removal of material. However, some recent studies have revealed that autophagy can function as a selective degradation process. These studies detect the involvement of some ubiquitin-binding receptors (e.g. p62 and NBR1) in the autophagic clearance of protein aggregates (Kirkin et al. 2009; Pankiv et al. 2007). Unlike the other forms of autophagy (macro- and microautophagy) in which parts of cytoplasm are engulfed, chaperon-mediated autophagy is selective for cytosolic proteins. Substrate proteins bear a targeting motif that is recognized by cytosolic chaperone complexes that deliver substrates to lysosomes. Furthermore, chaperones facilitate substrate unfolding, modulation of substrate interaction with the lysosomal membrane and, therefore, chaperones support the translocation of the substrate across the lysosomal membrane. However, the contribution of various autophagy pathways in the degradation of oxidized proteins remains to be investigated. Some recent papers indicate that there is an interaction of lysosomal–autophagy and the proteasome by the deacetylase HDAC6 (histone deacetylase 6, a microtubule-associated deacetylase); however, this requires also further investigations (Lamark and Johansen 2009; Pandey et al. 2007). HDAC6 is localized in the cytosol and recent studies found out that HDAC6 controls the fusion of autophagosomes with lysosomes (Lee et al. 2010). The formation of autophagosomes is the first step in autophagy and sequesters the substrates for degradation. In the next step, autophagosomes need to fuse with lysosomes. Interestingly, HDAC6 has an intrinsic ubiquitin-binding activity and associates with the cytoskeleton to facilitate the fusion of autophagosomes including ubiquitin-containing proteins with lysosomes (Kwon et al. 2007; Zhang et al. 2007). It is currently speculated that an impaired proteasomal function leads to a compensatory induction of autophagy (Iwata et al. 2005), but this interesting hypothesis has not been proofed yet. Further studies are necessary to investigate the new aspects in oxidized protein and protein aggregate clearance by autophagy, as well as the regulation/interaction or compensation of the proteasomal system combined with autophagy.

Structure and complexity of the proteasome

Proteasomes are multi-catalytic protein complexes that are present in the cytosol, associated with centrosomes and cytoskeleton, whereas some are associated with the

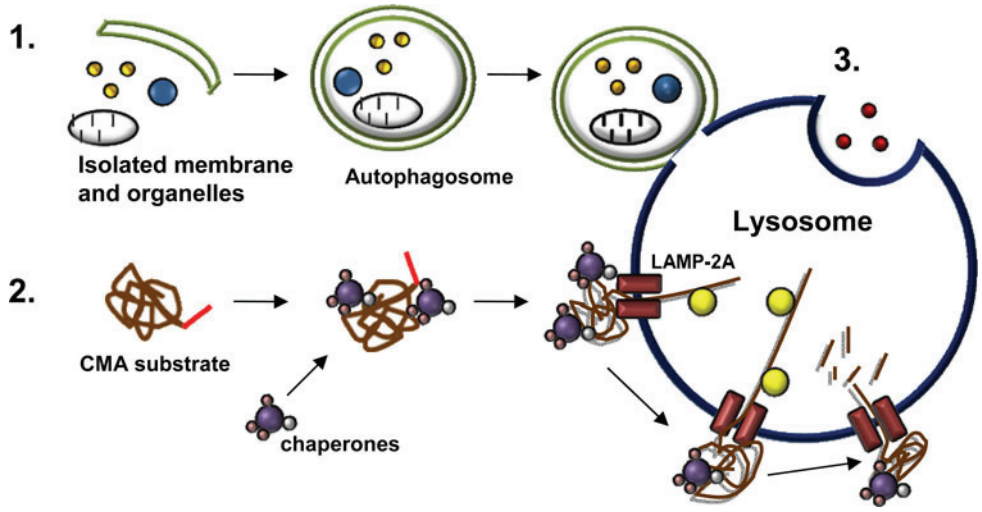

Fig. 1 Lysosome-linked autophagy system. The lysosome-linked autophagy system consists of three different types: (1) macroautophagy, (2) microautophagy and (3) chaperon-mediated autophagy (CMA). Substrates bearing a targeting motif that is recognized by a chaperone complex delivers them to the lysosomes. At the membrane substrate binds to monomeric forms of lysosomal-associated membrane protein-2A (LAMP-2A). This binding induces the multimerisation of membrane-associated proteins into a higher ordered complex that facilitates the translocation of the substrate. In lysosomes, several proteolytic enzymes exist that are involved in the degradation of the substrate

endoplasmic reticulum. Proteasomes are also present in the nucleus, localized in euchromatin regions and on the periphery of the heterochromatin (Jung et al. 2009).

The proteasomal system consists of the so-called 20S catalytic 'core' proteasome and several regulatory proteins (Keller et al. 2002; Rubinsztein 2006).

The 20S 'core' proteasome has a barrel shape and consists of two outer alpha rings and two inner beta rings with seven different subunits in each ring. Each subunit is transcribed from a distinct gene that is independent from the other subunit genes and all subunits form a complex with a total mass of 670–700 kDa (Jung et al. 2009). The alpha subunits are responsible for the recognition of substrates or attachment of regulatory subunits (Coux et al. 1996; Keller et al. 2000a). The main task of the beta-subunits is the proteolytic degradation of proteins and polypeptides. In each beta ring, the three subunits β1, β2, and β5 are responsible for the catalytic activity. These subunits exhibit the peptidyl-glutamyl-peptide-hydrolyzing (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities (Coux et al. 1996; de Vrij et al. 2004). The peptidyl-glutamyl-peptide-hydrolyzing activity is also known as caspase-like activity (de Vrij et al. 2004). The mentioned subunits are the constitutive ones, but there are also inducible ones with different activities and kinetics (Ding and Keller 2001b). It is important to point out that there might be both, constitutively expressed proteasome subunits as well as inducible subunits at the same time, and therefore, alterations in the proteasomal activities are possible (Ding and Keller 2001b). The subunits β1i, β2i and β5i ("i" for inducible) are expressed under cytokine induction and built into proteasomes instead of their constitutive counterparts. The 20S proteasome, formed under the influence of cytokines, is then often called 'immunoproteasome' as it is part of the immune defense (Goldberg et al. 2002). The replacement of the catalytic active subunits is suited for producing peptides able to bind MHC class I.

The two major forms of proteasome complexes are 20S and 26S, which co-exist in eukaryotic cells. The 'core' 20S proteasome is ATP independent, whereas the 26S form is ATP dependent (approximately 2,100 kDa) (Jung et al. 2009) (Fig. 2).

The 26S proteasome consists of the 20S core proteasome with the 19S regulatory subunit at each end of the hollow barrel (Ciechanover 1994; Jung et al. 2009). The central element for recognition of target proteins by the 26S proteasome is the covalent linkage of polyubiquitin to the substrates. The polyubiquitin chain serves as a binding moiety for the 26S proteasomal degradation (see below).

The 19S regulatory complex, also called PA700, is composed of approximately 19 subunits of varying molecular masses (25–110 kDa). The subunits belong to one of the two groups, ATPases or non-ATPases. Rpt/Rpn nomenclature distinguish between the AAA ATPase subunits (Rpt = *R*egulatory *p*article *t*riple-A protein) and non-AAA ATPase subunits (Rpn = *R*egulatory *p*article *n*on-ATPase). Six subunits belong to the AAA ATPase family (Rpt1, Rpt2, Rpt3, Rpt4, Rpt5 and Rpt6), whereas the non-ATPase subunits are Rpn1, Rpn2,…until Rpn13 (Li and DeMartino 2009; Nickell et al. 2009). Rpn10 is described for its binding capacity of polyubiquitin and

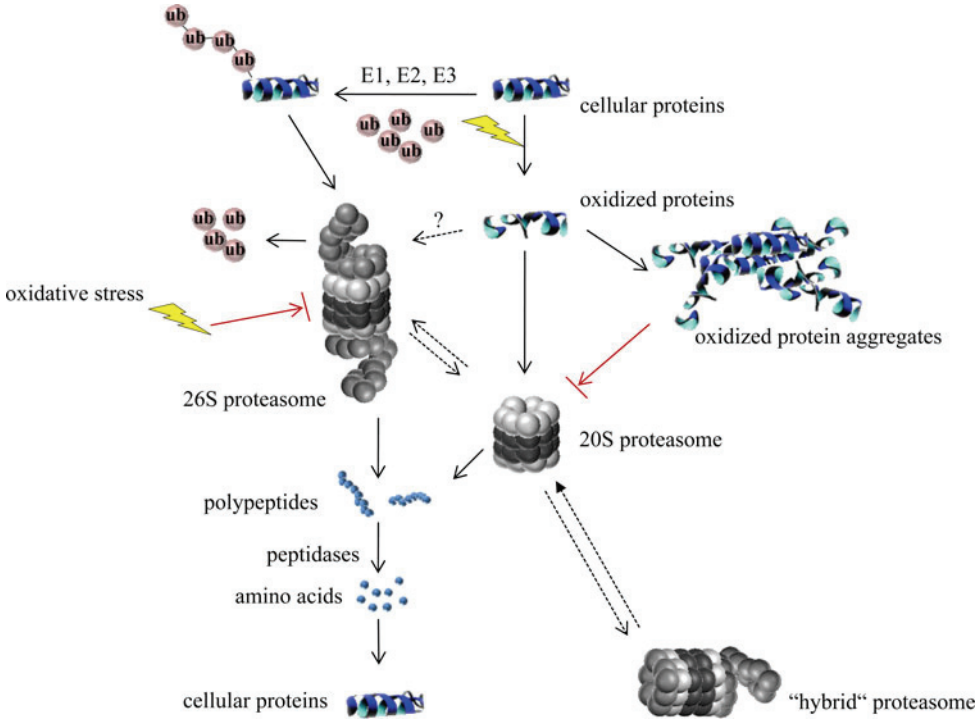

Fig. 2 Degradation by the proteasome. Many substrates of the 26S proteasomes are short-lived, undamaged proteins. They need an external signal in form of a polyubiquitin chain to ensure their recognition by the 19S regulators of the 26S proteasomes. The ubiquitin pathway includes ubiquitin activation (E1) and ubiquitin transfer (E2 + E3). Following an oxidant attack, cellular proteins are oxidized and partially unfolded exposing their "inner" hydrophobic residues to the outer side. The 20S proteasome can recognize such modified proteins and degrade them into peptides. Released peptides can further be degraded by peptidases in the cytosol to amino acids and those are used for protein synthesis again

Rpn11 displays deubiquitinating activity (Deveraux et al. 1994; Verma et al. 2002). Rpn11 cleaves polyubiquitin chains from the proteins and the subunit Uch37 (Rpn13) cleaves ubiquitin monomers from the polyubiquitin chains (Lam et al. 1997). The role of the remaining Rpn-subunits is to date not exactly clear.

The general structure of the 19S is termed "base" and "lid". The "base" region contains eight subunits, including six AAA ATPases (Rpt1–Rpt6) and two non-ATPases (Rpn1 and Rpn2). The six ATPases form a ring that binds to the heptameric α-ring of the 20S proteasome. The lid complex contains the remaining subunits and it is linked to the base via Rpn10. The 19S regulator uses ATPases to utilize energy from ATP hydrolysis for its activity, like removal of the polyubiquitin chain and unfolding the substrate (Gorbea et al. 1999). The binding of the 19S component leads to a configuration change of the N-terminal ends of α-subunits (described more in detail in the chapter regulation of proteasomal activity). This configuration change results in an open access pore (Powell et al. 2007) and the unfolded protein is then inserted into the catalytic core of the 20S proteasome.

PA28 (also called 11S, REG or its analog in *Trypanosoma brucei*: PA26) is another regulator and a heteroheptameric ring-shaped complex with approximately 200 kDa. The PA28 regulator does not recognize ubiquitin or utilize ATP and it is associated with the α-subunit-ring of the 20S core proteasome (Ma et al. 1992). Despite there are three different subunits that are described for PA28 regulator (PA28α, PA28β and PA28γ), only two varieties exist. Interferon-γ (γ-IFN) induces the synthesis of the PA28α and PA28β and these subunits are found principally in the cytoplasm (Rechsteiner et al. 2000). When compared with the so far known two γ-IFN-induced subunits of the PA28 regulator, the subunit PA28γ is largely confined to the nucleus of mammalian cells and is not regulated by γ-IFN (Goldberg et al. 2002). Importantly, due to γ-IFN, the three inducible β-subunits are also up-regulated (see above). The function of the PA28 regulator is still somewhat obscure. Presumably, the 20S proteasome gets activated through an allosteric effect of the PA28 binding.

As mentioned above, the proteasome is located in the cytosol and nucleus. Another regulator, called PA200 is found in the nucleus in three different forms (PA200i, PA200ii and PA200iii). Only PA200i seems to bind to the 20S proteasome and is involved in DNA repair, presumably by recruiting proteasomes to DNA double-strand breaks (Blickwedehl et al. 2007; Ustrell et al. 2002).

Therefore, the 20S proteasome is just a core particle of a whole system of regulatory factors also interacting with other pathways, including the ubiquitin pathway, chaperones and heat-shock proteins (Ciechanover 1994; Coux et al. 1996; Peters 1994).

Regulation of proteasomal activity

The entrance to the active center of the proteasome located in the inner cavity is thought to be tightly controlled by a gating mechanism. This mechanism protects proteins from

spontaneous degradation and defines the rate at which substrates enter the proteasomal cylinder. The N-termini of the alpha subunits hide the access to the channel of the free 20S proteasome; therefore, entrance to the barrel can only be achieved by structural rearrangement of alpha subunits. This conformational change (initiated by activators or regulatory particles) causes a maximal opening of the cylinder form and substrates are able to enter the catalytic sites (Groll et al. 2000), but only in an unfolded state as the diameter of the opened hollow cylinder is approximately 13 Å (Groll et al. 2005; Groll and Huber 2003; Smith et al. 2007).

The 19S regulatory particle is required for the degradation of ubiquitinated substrates. The attachment of the ATPase-containing 19S particle to the surface of the 20S α-ring regulates the gated channel, activates proteolysis by unfolding ubiquitinated substrates, removes ubiquitin and facilitates the translocation of substrates into the proteolytic chamber (Smith et al. 2007). Other regulatory particles can also influence the proteolytic activity in an ATP-independent manner, for example, the non-ATPase activators PA28 and PA26 complexes (Rechsteiner et al. 2000).

Proteasome in Archaea are somewhat different from that in eukaryotes. Crystal structures of the 20S proteasomes in archaeal organisms found the proteasome in an open state (Lowe et al. 1995). Later studies reported a closed gate (Rabl et al. 2008). Despite the appearance of an open gate in some studies, also regulatory complexes were found in these organisms (such as the proteasome-activating nucleotidase, PAN) (Smith et al. 2005). One would think that dynamic conformation of the α-subunit tails may partially restrict the entrance of the pore. However, it is not entirely clear why eukaryotic 20S require an ordered, closed-gate conformation, albeit in archaeal 20S, the flexible N-terminal residues are sufficient to restrict passage of most proteins. One possibility is that eukaryotes contain in several cells native unfolded proteins or functionally important peptides that are able to pass a flexible gate as compared to archaea. For the latter one, the flexible gate is sufficient to form a barrier for protein substrates. Therefore, PAN may act as a regulator due to anchoring these residues in a stable, open conformation.

This may also be valid for bacteria, as their first eight amino acids in the α-subunit ring are disordered, showing an open pore (Kwon et al. 2004). The corresponding regulatory particle is the AAA ATPase ring complex and probably this stimulates proteolytic activity by attaching firmly α-subunit tails to generate a static, open pore (Zwickl et al. 1999).

In eukaryotes, the N-termini of the seven α-subunits point towards the center of the ring, sealing the entrance of the 20S core proteolytic channel. The tail of α3 points directly across the surface of the α-ring and maintains close contact to the other α-subunits. Importantly, truncation of the tail region in α3-subunit in yeast (the α3ΔN mutant) results in an open 20S core proteasome, indicating the pivotal role of α3 (Groll et al. 2000). The importance of these N-termini regions can be assumed as they are highly conserved across all eukaryotes (Bajorek and Glickman 2004). The conserved region in the N-terminal segments of most α-subunits is the short sequence: Tyr8-Asp9-Arg10 or "the YDR motif" which is thought to be responsible for the interaction between the α-subunits in forming the gate. In order for substrates to enter the proteolytic chamber and for products to exit the chamber as well, the gate of the 20S must be opened. Therefore, the interaction of the anchored tails in the closed formation has to be rearranged and a stable open conformation should be formed.

The interaction of the α-subunits is modified by the regulatory particles. The 19S regulatory particle in eukaryotes and the homologous PAN ATPase complex in archaea contain a conserved C-terminal region, the hydrophobic-tyrosine-X motif that presumably triggers gate opening. It could be demonstrated in studies with the archaeal PAN's that this regulatory particle enables gate opening by binding to the 20S in pockets between α-subunits. There, the peptides with HbYX interact with conserved regions in the α-subunits, the YDR motif, following by a rotation in the α-subunits and a movement of a reverse-turn loop that stabilizes the open gate (Rabl et al. 2008). In eukaryotic 19S, three of the six ATPases contain this conserved HbYX motif (Smith et al. 2007). Especially C-terminal peptides from the 19S ATPases subunits Rpt2 and Rpt5 which contain the HbYX motif, induce gate opening (Rabl et al. 2008) and a limited subset of Rpt subunits has been found to come in contact with a α-subunit, for example Rpt2-α4, Rpt4-α2, Rpt4-α4, Rpt4-α6, Rpt4-α7, Rpt5-α2, Rpt6-α1 and Rpt6-α2 (Rabl et al. 2008).

So far, it is unclear whether activators, such as PA26/28 open the channel in a similar way to PAN or 19S as they do not possess the HbYX motif that causes α-subunit rotation. The group of Hill demonstrated that an activation loop in PA26 stabilizes the open conformation by a displacement of a critical reverse-turn loop in the α-subunit which involves Pro17 (Forster et al. 2005).

As mentioned before, binding of the regulatory proteins at the end of the 20S core proteasome induces not only gate opening, but also affect proteolytic activities as these proteins are known to be involved in the recognition and unfolding of protein substrates. Owing to this they make protein degradation more efficient but it is important to point out that 11S and 19S cap structures do not possess any proteolytic activity towards the substrate (Harris et al. 2001).

Notably, some substrates, especially hydrophobic ones can activate gate opening and facilitate, therefore, their

own degradation (Kisselev et al. 2002). Presumably, sequence motifs in the substrate may also interact with residues in α-subunits as described above and promote gate opening. Interestingly, the pore region of the 20S exposes only hydrophobic or negatively charged side chains of amino acids in the closed state and no positively charged groups are located on the surface of the α-ring (Unno et al. 2002). Thus, this may explain the hypothesis why unstructured substrates with hydrophobic or positively charged amino acid side chains interact with α-subunits in the gating region and facilitate their own translocation in the inner catalytic chamber of the 20S possibly without any regulators. However, this may also depend on the structure of the substrates as they should be partially unfolded to enter the 20S catalytic chamber and further investigations should to done to verify this hypothesis.

An additional reason for gating, not mentioned yet, is the regulation of the products exit the proteasome which is thought to be slow. It is possible that under normal conditions, product release is slowly to increase the proteolytic digestion and decrease the average peptide length. Under certain conditions (e.g. during inflammation), it might be beneficial to produce peptides with other length. A difference in the open state increases hereby the exit rate of peptides with an appropriate length for antigen-presentation. This gating mechanism is maybe further influenced by posttranslational modifications of subunits.

Many studies found potential sites that can be modified posttranslationally. For instance, in a number of core and regulatory subunits are potential phosphorylation sites—tyrosine and serine/threonine—located (Fujiwara et al. 1989; Haass et al. 1989; Heinemeyer et al. 1994; Tanaka et al. 1990). Several subunits in the 19S particle including Rpt2, Rpt3, Rpt4, Rpt6 and Rpn8 have been shown to be phosphorylated (Mason et al. 1998). In mammalian cells, it could be confirmed that the two α-subunits C8 and C9 are phosphorylated (Castano et al. 1996; Mason et al. 1996). The exact function of this modification is still not clear, however, it can be suggested that the activity of these subunits might be influenced by phosphorylation (Bose et al. 1999). Bose et al. assume that due to phosphorylation, the conformation of diverse core and regulatory subunits might influence the activities of the whole proteasomal complex. To determine if phosphorylation of proteasome subunits regulates enzymatic activity, a study from Mason et al. (1996) applied immunoprecipitation of proteasomes from human embryonic lung cells (L132) in the absence of protease inhibitors. Phosphorylation sites of C8 and C9 lie at the ends of the cylindrical structure (Mason et al. 1996), therefore, it can be suggested that phosphorylation of these subunits may regulate the association of regulatory proteins to the core proteasome.

Beside this, *N*-acetylation (Claverol et al. 2002), glutathionylation (Demasi et al. 2003) and glycosylation (Zhang et al. 2003a) of subunits are reported to influence their activities. Despite these facts, less is known about the posttranslational subunit modifications and the influence on proteasomal function. Further investigations should be performed before making any conclusions of the physiological significance of these observations.

In summary, there are several factors that influence proteasomal activity. Subunits are influenced by phosphorylation and/or acetylation (Bose et al. 1999; Claverol et al. 2002). In addition, some environmental factors are able to enhance the 20S proteasomal activity, like mild heat shock (Beedholm et al. 2004) and repeated freeze–thaw cycles (Bajorek and Glickman 2004). These are able to induce structural changes that open the α-ring of the 20S proteasome. Other studies have demonstrated that many subunits of the 11S and 19S caps contain binding sequences for heat shock proteins (HSP) implicating a possible role for HSPs in proteasome function (Luders et al. 2000). It can be assumed that HSPs may play a role in the upregulation of proteasome activity observed following mild oxidative stress. Ding et al. could demonstrate that HSPs are necessary for maintaining proteasome activity during oxidative stress (Ding and Keller 2001a). Neuronal SH-SY5Y cells were used stably transfected with HDJ-1, a member of the human heat shock family, and exposed to paraquat, H_2O_2 and $FeSO_4$ to induce oxidative stress. The degree of ROS formation in the transfected cells was the same as compared to untransfected cells; however, cells with higher HDJ-1 expression demonstrated a higher resistance towards oxidative stress. A lower impact on mitochondrial function and proteasomal activity in the transfected cells was detected (Ding and Keller 2001a). Although these data indicate a possible role of HSPs in maintaining proteasomal activity during oxidative stress, the exact mechanism has not been determined. The authors suggest that increased HSP levels delay the deleterious effects of oxidative stress on proteins (e.g. formation of aggregates) as it is proposed that HSPs play an important role in protein folding and recruitment of substrates to the proteasomes. However, it should be critical noted that these findings are not sufficient for evidence and more studies should be done to support this suggestion.

Beside the above-mentioned mechanisms by which the proteasome activity is increased, there are also some specific inhibitors of activation which can be used to study the function of proteasome. Proteasomal inhibitors are natural and synthetic ones, like lactacystin (from fungi), TMC-95 A-D and epoxymicin (from bacteria) (Fenteany et al. 1995; Kohno et al. 2000; Loidl et al. 2000) derivates of peptide-aldehydes, like leupeptin, MG-132 and MG-115 and calpain inhibitor I and II (Harding et al. 1995; Lee and

Goldberg 1996; Rock et al. 1994). Additional substances are mentioned that form covalent adducts and inhibit proteasomal activity, like peptide vinyl sulfones, chlormethyl ketones, diazomethyl ketones and α',β'-epoxyketones (Groll and Huber 2004; Rydzewski et al. 2006; Savory et al. 1993; Verdoes et al. 2007). An antibacterial peptide, PR39, induces an allosteric change of the mammalian proteasome. This causes a reduced binding of the 19S regulator protein and reduced proteolytic activity (Gaczynska et al. 2003). The heat shock protein 90 (HSP 90) is although known to be a negative regulator of the proteasome (Conconi and Friguet 1997). The overall consequence of proteasome inhibition is a decrease in protein breakdown leading to a rapid accumulation of proteins. Furthermore, the activity of the proteasome is also decreased in pathological conditions (Keck et al. 2003; Keller et al. 2000a) like neurodegenerative diseases (see last chapter of the review).

Proteolytic degradation by the proteasome

The degradation of many intracellular proteins, such as cyclins, transcription factors, other short-lived regulatory proteins and damaged proteins (Bose et al. 1999) require an initial ubiquitination, followed by the recognition of the 26S proteasome (Ciechanover 1994; Jung et al. 2009).

Ubiquitin, a 76-amino acid protein, found in the cytosol and nucleus and is expressed in all eukaryotic cells (de Vrij et al. 2004). It is generally attached to substrate proteins through the formation of a covalent peptide bond involving ε-amino groups of lysine residues within the substrate and the carboxyl-terminal glycine residue of ubiquitin (Pickart and Fushman 2004). The initial ubiquitin molecule can serve as the target of a second one, whose carboxyl-terminal glycine is attached to a lysine within the first ubiquitin molecule. This reaction continues until the ubiquitin chain, anchored to the substrate, is formed (Glickman and Ciechanover 2002).

The polyubiquitin chain formation is accomplished by several enzymes (E1, E2 and E3). The ubiquitin-activating enzyme (E1) activates in the first step ubiquitin by an ATP-dependent formation of a thiolester with the cysteine of E1, therefore, allows a covalent bond formation. The activated ubiquitin is transferred to one of the ubiquitin-conjugating enzymes (E2) and afterwards this E2 is involved in the assembling of the ubiquitin chain with the help of an additional ubiquitin ligase (E3) (Glickman and Ciechanover 2002). Chains of four or more ubiquitin-residues appear to form a recognition signal that enable substrates to be shuttled to the proteasome by some proteins (e.g. chaperones) (Richly et al. 2005) and recognized by the 19S regulators of the 26S proteasome. An additional factor E4 was described to play a role in the formation of high-molecular mass ubiquitin conjugates (Koegl et al. 1999).

Proteolysis of proteins by the ubiquitin-26S proteasomal system can be thought to consist of the following steps (Richly et al. 2005 and Bercovich et al. 1997): (1) chaperone-mediated substrate presentation (this is optional, depending on the substrate), (2) polyubiquitin-substrate recognition by the 19S subunits, (3) cleavage of polyubiquitin chains to separate the substrate, (4) substrate unfolding, (5) translocation of the substrate into the 20S core proteasome, (6) cleavage of the substrate and (7) release of peptides. Step (3) and (4) can occur in either order as it is not clearly determined yet, which step is first.

However, the proteasomal system is also able to cleave not-ubiquitinated proteins. This is catalyzed by the 20S core proteasome, although this pathway is believed to be largely underestimated in the past (Asher et al. 2005; Baugh and Pilipenko 2004; Jariel-Encontre et al. 2008; Moorthy et al. 2006; Sdek et al. 2005). It remains still unclear whether the ubiquitin-independent protesomal degradation is catalyzed by the free 20S proteasome or whether some regulators are involved. Some catalytic problems in the ubiquitin-independent proteasomal degradation remain unresolved, as the gating mechanism, the substrate unfolding and translocating to the catalytic center. Because only unfolded proteins can enter the catalytic core, it seems that unfolded, non-ubiquitinated proteins can be degraded by the free 20S core proteasome under stress conditions, when the regulatory degradation process is abated. This can reduce the amount of energy used (less ATP is consumed) under certain stress conditions, for instance, prolonged starvation, oxidative stress and heat-induced damage (Bajorek et al. 2003).

However, certain non-ubiquitinated proteins are also substrates for the 26S proteasomes, the best studied example is the ornithine decarboxylase (ODC) (Murakami et al. 2000). ODC binds to antizyme (an endogenous protein inhibitor), which is thought to be a mediator of ubiquitin-independent proteasomal degradation (Mangold et al. 2008). The antizyme probably induces a conformational change of ODC and permits interaction of ODC-C-terminus with the 19S regulator (Murakami et al. 2000; Zhang et al. 2004). In addition, this antizyme-induced ODC degradation can be competitively inhibited by polyubiquitin chains, indicating that the same subunit recognizes both (Zhang et al. 2003b).

It was mentioned in the former section that cytokines, especially IFN-γ, can induce special forms of the proteasome. To recapitulate, IFN-γ induces the expression of different catalytic subunits (LMP2, LMP7 and MECL1) and these subunits replace the constitutively expressed β1, β2 and β5 subunits in the 20S particle. The incorporation of the inducible subunits LMP2 and LMP7, as well as

MECL1, enhances the peptidase activity of the proteasome and increases the release of small peptide products which can be represented by the MHC 1 complex. Peptides with an average length of approximately 2–24 amino acids residues leave the proteasome and are further hydrolyzed in the cytosol by several intracellular peptidases (cytosolic endo- and exopeptidases) to single amino acids which are used for protein synthesis again or to peptides which are presented as antigens by the MHC-I complex (Goldberg et al. 2002; Rock and Goldberg 1999; Tanaka and Kasahara 1998). Because of its role in antigen presentation, proteasomes containing the subunits LMP2, LMP7 and MECL1, instead of β1, β5 and β2, respectively, are called "immunoproteasomes" (Goldberg et al. 2002).

Recognition of oxidized proteins by the proteasome

Despite the fact that regulatory proteins influence proteasomal activity, approximately 30% of the 20S core proteasomes are not bound to any regulatory particle (Tanahashi et al. 2000). There is evidence that proteins can be degraded by the 20S core proteasome without the requirement of ubiquitin conjugation, reviewed in Jariel-Encontre et al. (2008).

It is known that the proteasomal system is the major proteolytic pathway responsible for the removal of oxidized proteins (Breusing and Grune 2008).

Irrespectively of whether the 20S, 26S, the immunoproteasome or other hybrid proteasomes are involved in proteolysis, substrates must be able to enter the catalytic core in an almost unfolded state, as folded aggregates cannot entry the narrow barrel structure of the 20S. It is reasonable to assume that mildly oxidized proteins are suitable substrates whereas heavily oxidized proteins are not suitable for proteasomal degradation as they form stable aggregates as a result of covalent crosslinks, hydrophobic interactions and disulfide bounds (Davies 2001). Partially unfolded, mildly oxidized proteins expose hydrophobic amino acid residues from the interior of the protein to the outside (Lasch et al. 2001). This is important for binding of the substrate and gating of the proteasome and for degradation itself, as two of the six catalytic active sites from the β-subunit prefer hydrophobic amino acids. This "chymotrypsin-like" (β5) subunit cleaves after hydrophobic amino acids. Two of the β-subunits ("trypsin-like" (β2)) cleave after basic residues and the last two catalytic subunits ("peptidyl glutamyl peptide hydrolyzing activity" or "caspase activity" (β1) cleave after acidic amino acid (Goldberg et al. 2002). Oxidized proteins undergo structural arrangement and are usually less folded as compared to their non-oxidized structures (Lasch et al. 2001; Guedes et al. 2009). Therefore, such modifications allow better substrate recognition by the proteasome and their further translocation into the catalytic core.

It was postulated a long time ago that ATP and ubiquitin are not required for the degradation of oxidatively modified proteins, and the 26S proteasomal activity is decreased during oxidative stress, whereas the 20S proteasome is unaffected (Reinheckel et al. 1998, 2000). Therefore, it was assumed (and later shown) that the 20S proteasome is sufficient to degrade oxidatively damaged and misfolded proteins. According to Pacifici et al. (1993), there are at least three advantages of this specific degradation of oxidatively damaged proteins by an ATP-independent way: (1) the relatively fast removing of oxidized proteins, (2) the prevention of accumulation of oxidized proteins and further cross-linking to other proteins and (3) the release of undamaged amino acids for translation of new proteins. However, recent studies revealed a possible involvement of ubiquitin and 26S proteasomal system in the stress response to oxidation (Medicherla and Goldberg 2008), although, a clear ubiquitination of oxidized proteins was not shown. However, whether the 26S-ubiquitin pathway plays some role in the removal of oxidized proteins, remains to be clarified, since Shringarpure et al. (2003) clearly excluded a role of ubiquitin. One possible explanstion would be differences in the regulation of protesomal pathways in yeast and mammalian cells, as described for the inducible proteasomal subunits and the PARP-mediated proteasome activation. It remains possible that both 20S and 26S-ubiquitin degradation pathways are involved in the degradation of oxidized proteins.

The relationship between protein oxidation and the proteolysis of mildly oxidized proteins is extensively investigated and discussed (Grune et al. 2003; Jung et al. 2007). It could be demonstrated in red blood cells (Davies 1993) and rat liver cells (Grune et al. 1995) that damaged proteins (e.g. intracellular proteins that are metabolically radiolabeled, hemoglobin and superoxide dismutase) due to mild oxidative stress, are selectively recognized by the 20S proteasome and can, therefore, be degraded in an ATP-independent way.

It is well known that mildly oxidized proteins can be degraded well, whereas extensively oxidized proteins are resistant against the proteasomal system due to a higher tendency of the formation of protein aggregates (Grune et al. 2004). Therefore, strong oxidative stress or strong denaturizing conditions lead to low protein degradation by the proteasome. It is known that aggregates are poor substrates for proteasomal degradation (Friguet and Szweda 1997). The mechanism remains speculative, but it was proposed as aggregates cannot penetrate into the narrow cylinder of the 20S proteasome because of their extensive cross-linking. However, earlier steps in the UPS pathway may also be influenced, presumably the binding of E3 and

processing of the aggregate by the 19S regulator. As lysine side chains are often susceptible for oxidative modifications during oxidative stress (Guedes et al. 2009) and lysine residues are known to be the binding site for ubiquitin (Pickart and Eddins 2004), it can be assumed that modified lysine side chains cannot be linked with ubiquitin.

Recent studies report that the removal of highly oxidized protein aggregates from the cytosol is carried out by autophagy. Rodgers et al. demonstrated that the degradation of the more highly modified aggregate dopa-containing proteins began to switch from proteasomal to lysosomal pathways. This may reflect the point at which some of the modified proteins are no longer substrates for the proteasomes. Indeed, there is still a gap of knowledge about the interaction of proteolytic pathways in the degradation of oxidized proteins. It is likely that the removal of oxidized proteins involves a combination of pathways and that the relative contribution of the proteasomes depends on the type and extent of that modification (Jariel-Encontre et al. 2008).

When the aggregates cannot be degraded, neither by the proteasome nor by lysosomes, the material is stored intracellular, accumulate during aging and moreover inhibits the proteasome (Keck et al. 2003; Sitte et al. 2000; Stadtman 1992). Initial aggregates can be further oxidatively modified and it is proposed that they grow towards higher molecular weight and insoluble aggregates (Grune et al. 2004) in a complex stochastic progress during cellular life.

As the proteasomal system is responsible for the degradation of more than 70–80% of normal and abnormal intracellular proteins, aberration of this system may lead to the dysfunction in cellular homeostasis and the development of multiple diseases (e.g. neurodegenerative diseases) (Grune et al. 1996).

Protein damage and the proteasome in aging and neurodegenerative diseases

Mammalian brain shows high oxygen consumption and low activities of antioxidative enzymes like glutathione peroxidase and reductase, catalase and superoxide dismutase (Dringen et al. 2000; Kish et al. 1992). Owing to this, the brain is susceptible for oxidative stress and damage of molecules, including proteins. As mentioned above, oxidatively damaged proteins tend to form aggregates.

Another reason for accumulation of proteins, especially during aging, is an ineffective proteasome–ubiquitin and autophagy pathway. It is also well investigated that ROS inhibit the proteasome (Friguet and Szweda 1997; Grune 2000; Reinheckel et al. 1998) and as the levels of ROS have been suggested to increase during aging (Beckman and Ames 1998), they can contribute to the malfunction of the proteasome via direct and indirect effects. The indirect modification of protein and lipids are known to generate undegradable substances, which accumulate in cells (Grune et al. 1997). These non-degraded substances are mainly found in cells which cannot divide the accumulated and stored materials during cell division and pass half of the material to each daughter cell. Therefore, postmitotic cells like neurons might be especially vulnerable to the accumulation of protein aggregates. It has to be pointed out that protein aggregates influence numerous cellular signaling pathways not only by influencing the proteolytic system. It is also described that these accumulated materials exert toxic activities against neurons and, therefore, contributes to age-associated alterations and perhaps to the onset of neurodegenerative diseases (Barnham et al. 2004).

The involvement of accumulated and aggregated oxidatively modified proteins in the aging process is well investigated (Sitte et al. 2000; Viteri et al. 2004). Moreover, the ubiquitin–proteasome activity declines with aging and is also diminished in various areas that are affected by neurodegenerative diseases (Keck et al. 2003; Keller et al. 2000a, b, 2002; Lopez et al. 2000). The consequence of this decline in ubiquitin–proteasome activity is likely to be a less efficient clearance of proteins. Presence of mutant and/or aggregated proteins can deteriorate the impaired proteasome efficiency by blocking entrance to the proteasome and consequently further reducing proteasomal activity (Ciechanover and Brundin 2003). This hypothesis of blocking or binding of non-degradable protein aggregates and cross-linked proteins to the proteasome perhaps makes the degradation of other misfolded or damaged proteins less effective, and might result in an additional accumulation of undegraded materials in the cell and a vicious circle occurs. This condition might have dramatic effects on cellular aging and cell viability. However, recent studies have shown that some accumulated proteins can also be degraded via autophagy (Williams et al. 2006), therefore, reducing the level of aggregated proteins and attenuating their toxicity. Despite this probably compensatory pathway aggregated proteins are often found in neurodegenerative diseases.

An exact investigation of these aggregated proteins revealed that they include proteasomal components like ubiquitin conjugates and/or inclusion bodies associated with ubiquitin. Some of these deposits are hallmarks of neurodegenerative diseases, therefore, it is plausible that the ubiquitin–proteasome system is involved in the disease onset (de Vrij et al. 2004) as it is described that in almost all neurodegenerative diseases higher amounts of proteins are in a polyubiquitinated state. However, this should be regarded critically, as there is increasing evidence that autophagy can compensate an impaired proteasomal

system; reviewed in Nedelsky et al. (2008); Pandey et al. (2007). An essential link between these systems is described before (chapter: oxidative protein modifications in cells) and thought to be regulated via HDAC (Pandey et al. 2007).

Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis show intracellular deposits of aggregated and misprocessed proteins in specific brain regions (Barnham et al. 2004; Bossy-Wetzel et al. 2004; Ciechanover and Brundin 2003; Emerit et al. 2004), perhaps leading to a loss of neurons in particular areas (Table 1). In some of the several neurodegenerative diseases, a single gene mutation leads to an abnormal protein structure that cannot be degraded by the proteasome. In AD, a significant decrease in proteasomal activity was observed in different regions of the brain and this impairment could not be explained by a decreased proteasome expression (Keller et al. 2000a). Typical physiologic hallmarks in AD are two kinds of aggregates, the extracellular accumulation of β-amyloid plaques and the intracellular formation of paired helical filaments (PHFs) and neurofibrillary tangles consisting of hyperphosphorylated tau proteins (Lee et al. 2001). The reason for this hyperphosphorylation is, however, still unknown. It is also described that these deposits consists of ubiquitinated molecules and are poor substrates for the proteasome (de Vrij et al. 2004). The amount of tau-based PHFs and neurofibrillary tangles in neurons and the extracellular β-amyloid plaques correlate with the degree of Alzheimer dementia (Fein et al. 2008; Naslund et al. 2000). The activity of the proteasome was inhibited in the presence of β-amyloid peptide (Cecarini et al. 2008). Owing to this inhibiting effect on the proteasome activity, an impact on neurons can be assumed. Also a pharmacological inhibition of the proteasome is sufficient to induce neuronal degeneration and neuronal death (McNaught et al. 2010; Sun et al. 2006). Therefore, a dysfunction of the proteasomal system leading to protein aggregation or caused by protein aggregates has detrimental effects on neurons resulting in neurodegenerative diseases like AD. Keck et al. (2003) found out that this proteasome dysfunction is due to an inhibitory binding of tau-based PHFs to proteasomes. This study could confirm other authors reported a decreased proteasome activity in AD brain (Keller et al. 2000a; Oh et al. 2005). In addition to the impaired proteasomal activity, no impaired proteasomal expression was detected by Keck et al., therefore, it can be assumed that the impaired proteasomal function in AD is the consequence of PHF formation (Keck et al. 2003).

Insoluble protein aggregates presumably result from structural changes in the molecule that prevent their recognition or degradation and from malfunction or overload of the proteasomal system.

Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) is an enzyme that hydrolyses polyubiquitin to generate monoubiquitin. Under oxidative stress, UCH-L1 is oxidatively modified and the hydrolase activity is decreased (Choi et al. 2004). Therefore, oxidative modification of UCH-L1 depletes the availability of free ubiquitin and impairs protein degradation in cells. Consequently, protein aggregates might be formed. Indeed, loss of activity of UCH-L1 in AD brain is consistent with the observed increased protein ubiquitinylation, accumulation of proteins and decreased proteasome activity (Butterfield and Boyd-Kimball 2004) and increased levels of oxidized UCH-L1 are also found in AD (Castegna et al. 2002).

In addition to UCHL1, the enzymatic activity of the proteasome machinery can directly be affected in aging. Investigations with rat liver, isolated from old animals, could show that the enzymatic activity of peptidyl-glutamyl peptide hydrolase was 50% lower in comparison to the liver isolated from young rats (Conconi et al. 1996). Furthermore, an age-related decrease in proteasome expression can be postulated. Indeed, the expression of proteasomes in keratinocytes and epidermis culture is down-regulated with age (Petropoulos et al. 2000). Anselmi et al. 1998 and Bulteau et al. 2000 reported a modification of at least two proteasomal subunits during the aging process (Anselmi et al. 1998; Bulteau et al.

Table 1 Protein aggregates found in neurodegenerative diseases

Disease	Protein aggregates	Symptoms	References
Alzheimer's disease	β-Amyloid plaques, neurofibrillary tangles, paired helical filaments	Neuronal loss	(Avila 2006; Hernandez et al. 2005)
Parkinson's disease	Lewy bodies (α-synuclein, parkin)	Neuronal loss	(Chau et al. 2009; Moore 2006)
Huntington's disease	Polyglutamine inclusions (Huntingtin)	Neuronal dysfunction/loss	(Bennett et al. 2007)
Amyotrophic lateral sclerosis	Cu, Zn superoxide dismutase 1 (SOD1) aggregates	Motor neuron sclerosis	(Cookson et al. 2002)
Prion disease	Mutant prion protein	Neuronal dysfunction/loss	(Kristiansen et al. 2007)

2000). Therefore, structural changes in the subunits and a decreased or modified expression can contribute to age-related decrease in proteasomal activity as well.

Several mutations in the ubiquitin–proteasomal system are also involved in the pathology of the wide range of neurodegenerative diseases. An autosomal recessive loss-of-function in parkin, an E3 ligase, is found to cause PD (Gasser 2009). In addition, mutations in the gene encoding UCH-L1 have been implicated in the families with PD (Kabuta and Wada 2008). This UCH-L1 might not only function as an ubiquitin hydrolase, which cleaves ubiquitin chains to free ubiquitin monomers, but also as an ubiquitin ligase (Liu et al. 2002). The recessive deletion of a part of the UCH-L1 gene causes deleterious ubiquitinated intraneuronal aggregates (Walters et al. 2008). The main histological markers in PD are intracellular formed Lewy bodies (LB). These LB are mainly formed by α-synuclein that is as in most of all neurodegenerative diseases hyperphosphorylated (Chau et al. 2009). Another protein, that forms aggregates in PD, is parkin (Moore 2006).

These examples for primary genetic deficiencies involved in the pathogens of neurodegenerative diseases are however not explicit enough to make a conclusion whether proteasomal dysfunction is an early causal factor in proteinopathies in vivo in which there are no primary defects in the ubiquitin–proteasome pathway.

Although the perturbations in the proteasome pathway lead to pleiotropic effects on neurons including cell death, one of the early effects is believed to be synaptic malfunction (Selkoe 2002) as it is known that the proteasome regulates presynaptic protein turnover and synaptic efficacy (Speese et al. 2003). However, it is not clear whether aberration in proteolysis plays a causative role or only a secondary role in the pathogenesis. Taken together, these data reflect that inhibition of the proteasome system occurs during aging and may be a source of neuronal morbidity during the aging process and age-related diseases, such as neurodegenerative diseases.

It should be mentioned here that the second proteolytic machinery in cells, the autophagy system, may also play an important role in the onset of neurodegenerative diseases. Unfortunately, we are just in the beginning to understand autophagy and its interaction with the proteasomal system. Many studies are necessary to understand this complex co-operation.

Further aims are probably therapeutic modulation of proteolytic systems, thus enhancing the degradation of misfolded proteins and preventing diseases related to protein accumulation. Although many inhibitors of the proteasome are available, no effective drug exist that can enhance the function of the proteasome. Several steps in the ubiquitin proteasome pathway could be therapeutically influenced: (1) up-regulation of proteasomes to compensate the proteasome inhibition, (2) stimulation of substrate recognition by proteasomes, (3) stimulation of the 20S activity and (4) increase in the chaperone activity (Upadhya and Hegde 2005). The role of the proteasome in neurodegenerative diseases is an intensive area of research worldwide, not only for therapeutic reasons but although for understanding its complex tasks. Despite the fact that aggregated proteins exert negative effects, there is increasing evidence that they may activate autophagy to remove the affected protein (Keller et al. 2004; Ravikumar et al. 2004), but this role needs further investigations.

In summary, it appears likely that an impaired proteasome system and aggregated proteins contribute or perhaps trigger the aging and neurodegeneration process. It is assumed that in the longer term, aggregation may exceed clearance of proteins by proteasome or autophagy and cause cell death. Furthermore, similar to the proteasome, the chaperone system is also thought to deteriorate with age (Soti and Csermely 2003). When this system is affected, an increase in aggregates can be supposed. The cumulative effect of inefficient chaperone and proteasomal activity, and a malfunction of mitochondria (leading to lower ATP levels and increased ROS concentrations resulting in oxidative damage of proteins, lipids and nucleic acids) produce a vicious cycle in the dysfunction of cellular metabolism. The interplay between these systems and their overlapping is a complex cellular function. However, understanding this interplay is perhaps the only way to find efficient and curative medicines for the different neurodegenerative diseases.

Summary

The proteasome system is a complex system interacting with many other cellular systems to fulfill all the different tasks in diverse cells. It is abundantly clear that the ubiquitin–proteasome system is of central importance in eukaryotic cell physiology. Recently, many studies have been conducted to reveal new insights into this complex system. For instance, advantages are made in the discovering of proteasome-activator functions. However, this is still a research field to be investigated, especially in combination with the function of the chaperone and autophagy system. It is well established that mildly oxidized proteins capable of being unfolded are degraded by the proteasome. More and more evidence exist that extensively modified proteins aggregate and are directed towards the lysosome for degradation. However, the fate of oxidized proteins in lysosomes should be further investigated. It is assumed that oxidized proteins in lysosomes can probably induce a chain of events, especially in the presence of transition metals

(Höhn et al. 2010). Further studies may prove insight into this complex pathway.

Owing to the complex tasks of the proteasome system, it is obvious that its malfunction may result in numerous pathologic processes, among them neurodegenerative diseases. Moreover, proteasomal activity becomes altered in the "healthy" aged CNS. In young brain, the proteasome is able to degrade a vast contingent of proteins, including mildly oxidized proteins. However, in the aged brain, the activity of the proteasome may become inefficient. It can be assumed that impaired proteasome function contributes to aging through deleterious intracellular protein accumulation. The proteasome however seems to be a potential therapeutic target in the modulation of disease activity in neurodegeneration. An activation of this proteolytic system seems to be a promising way against age-related diseases, however, much more knowledge about the regulation of the proteasome and autophagy are necessary.

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3.2 Publikation II: Cathepsin D is one of the major enzymes involved in intracellular degradation of AGE-modified proteins.

Stefanie Grimm, Lisa Ernst, Nicole Grötzinger, Annika Höhn, Nicolle Breusing, Thomas Reinheckel, Tilman Grune. *Free Radical Research*, 2010, 44(9): 1013-1026

AGE-modifizierte Proteine treten vorwiegend während der Alterung und in zahlreichen pathophysiologischen Zuständen, wie beispielsweise der Arteriosklerose, Diabetes Mellitus, renaler Dysfunktionen und neurodegenerativen Erkrankungen in Erscheinung. Es ist bisher nicht bekannt, inwiefern dieses Auftreten durch eine beeinträchtigte Proteasefunktion begründet ist. In der vorliegenden Arbeit wurden Untersuchungen zum enzymalen Abbau von AGE-modifizierten Proteinen durchgeführt. Dabei wurden intrazelluläre Proteasen (lysosomale Cathepsine und das Proteasom) und Proteasen aus dem Gastrointestinaltrakt verwendet. Einen Abbau AGE-modifizierter Proteine konnte durch die Proteasen Pepsin und Cathepsin D festgestellt werden. Die intrazelluläre Bedeutung des Cathepsin D wurde anschließend in einem knock-out Modell bestätigt. In Cathepsin D knock-out Fibroblasten wurde eine signifikant verstärkte Akkumulation AGE-modifizierter Proteine, insbesondere in den Lysosomen, detektiert. Zusammenfassend zeigt eine intakte Cathepsin D Funktion eine verringerte AGE-Akkumulation.

Eigenanteil:

- Herstellung von AGE-modifiziertem BSA
- Anteilige Durchführung der proteolytischen Untersuchungen mittels eines Fluorescamin-Assays
- Planung und Durchführung aller weiteren Versuche:
  Zellkultur, Bestimmung der lysosomalen und proteasomalen Aktivität, Zellvitalitätsassay, Bestimmung der Aufnahme AGE-modifizierter Proteine
- Statistische Auswertung, Interpretation und Darstellung aller Ergebnisse, Verfassung des Manuskriptes

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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 $\pm$ 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

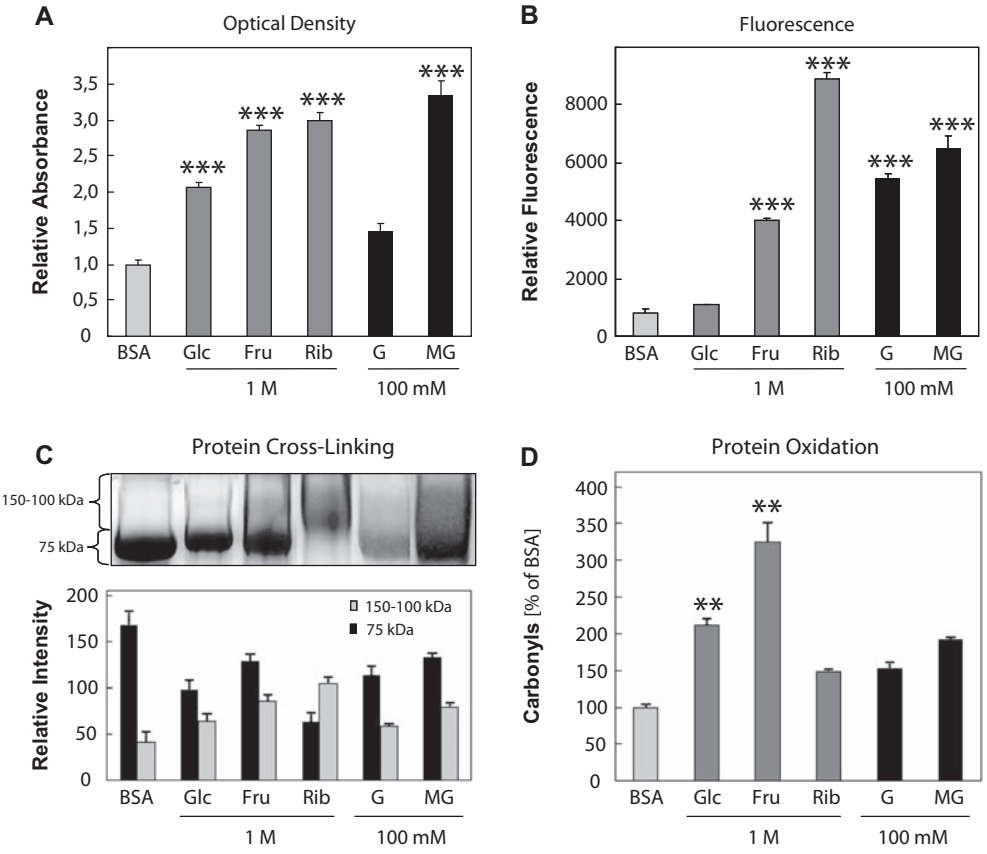

Figure 1. Physico-chemical characterization of advanced glycation end products (AGE). AGEs are formed by incubating bovine serum albumin (BSA) with the indicated concentrations of glucose (Glc), fructose (Fru), ribose (Rib), glyoxal (G) and methylglyoxal (MG) as described in the Methods section. The 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).

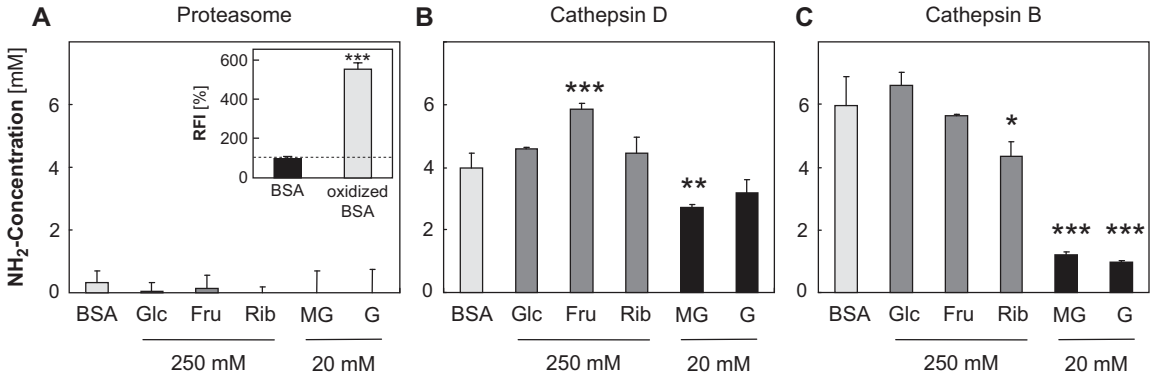

Figure 2. Degradation of AGE-modified albumin by intracellular proteases. Glucose (Glc), fructose (Fru) and ribose (Rib)-modified albumin (grey columns), methylglyoxal (MG) and glyoxal (G)-modified albumin (black columns) as well as unmodified albumin (BSA, 1 mg/ml, light grey columns) were incubated 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

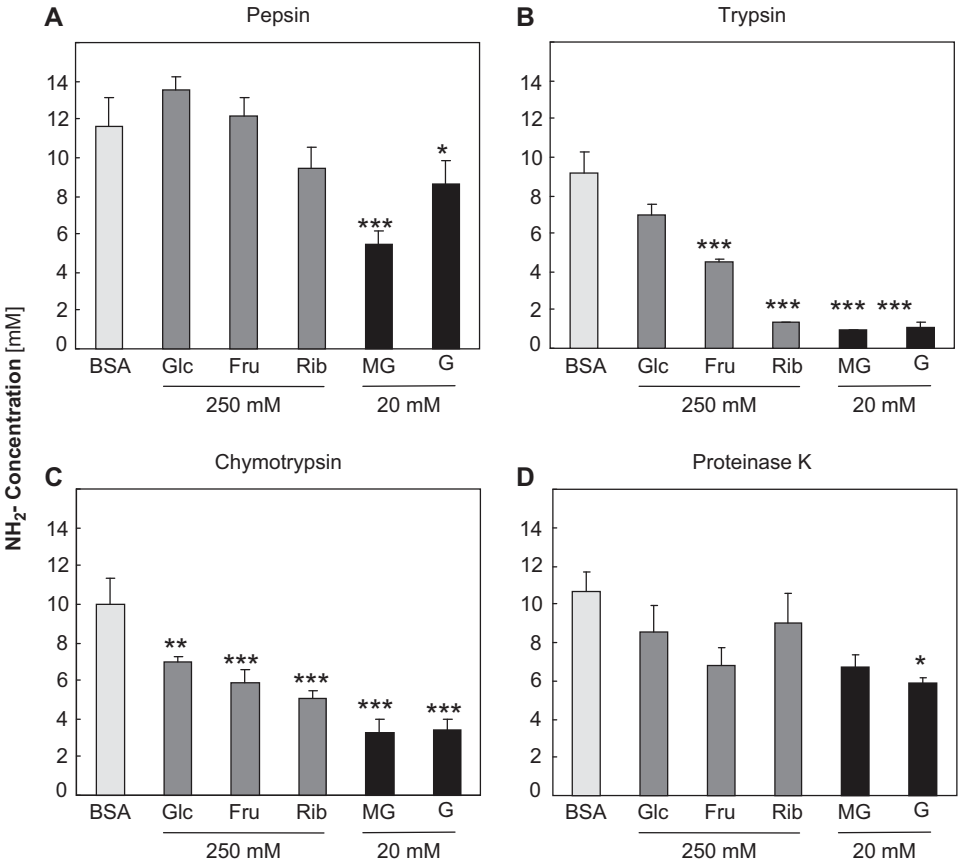

Figure 3. Degradation of AGE-modified albumin by extracellular proteases. Glucose (Glc), fructose (Fru) and ribose (Rib)-modified albumin (250 mM), methylglyoxal (MG) and glyoxal (G)-modified albumin (20 mM) as well as unmodified albumin were incubated with pepsin (A), trypsin (B), chymotrypsin (C) and 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.

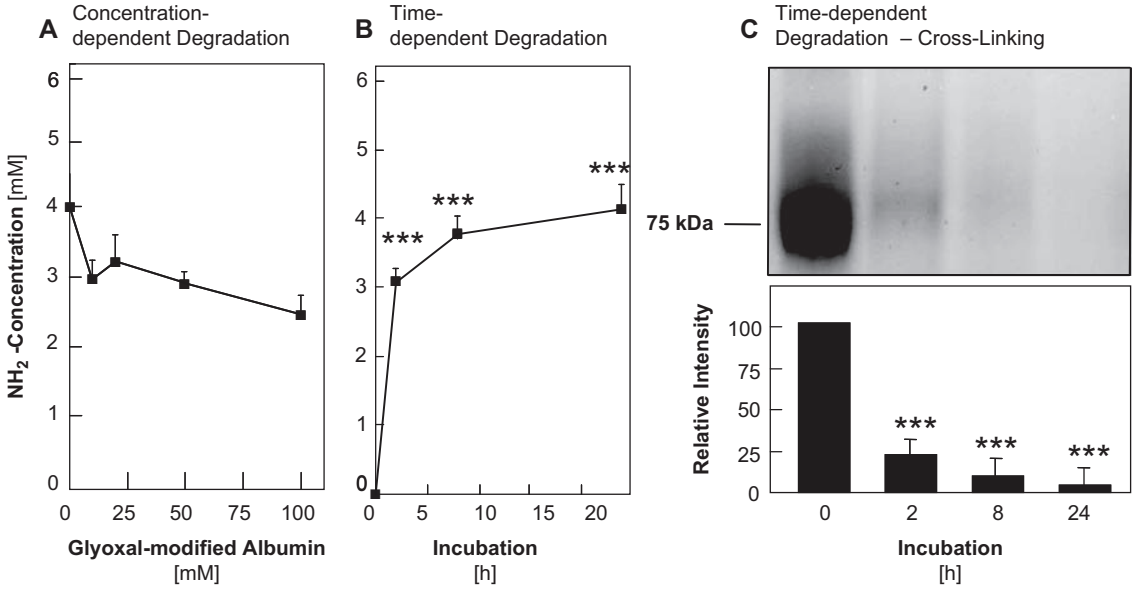

Figure 4. Degradation of glyoxal-modified albumin by cathepsin D. (A) The modification-dependent glyoxal-modified albumin degradation after 2 h incubation with cathepsin D at 37°C. BSA was modified by the indicated concentrations of glyoxal as described in the Methods section. The ratio of enzyme-to-substrate 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).

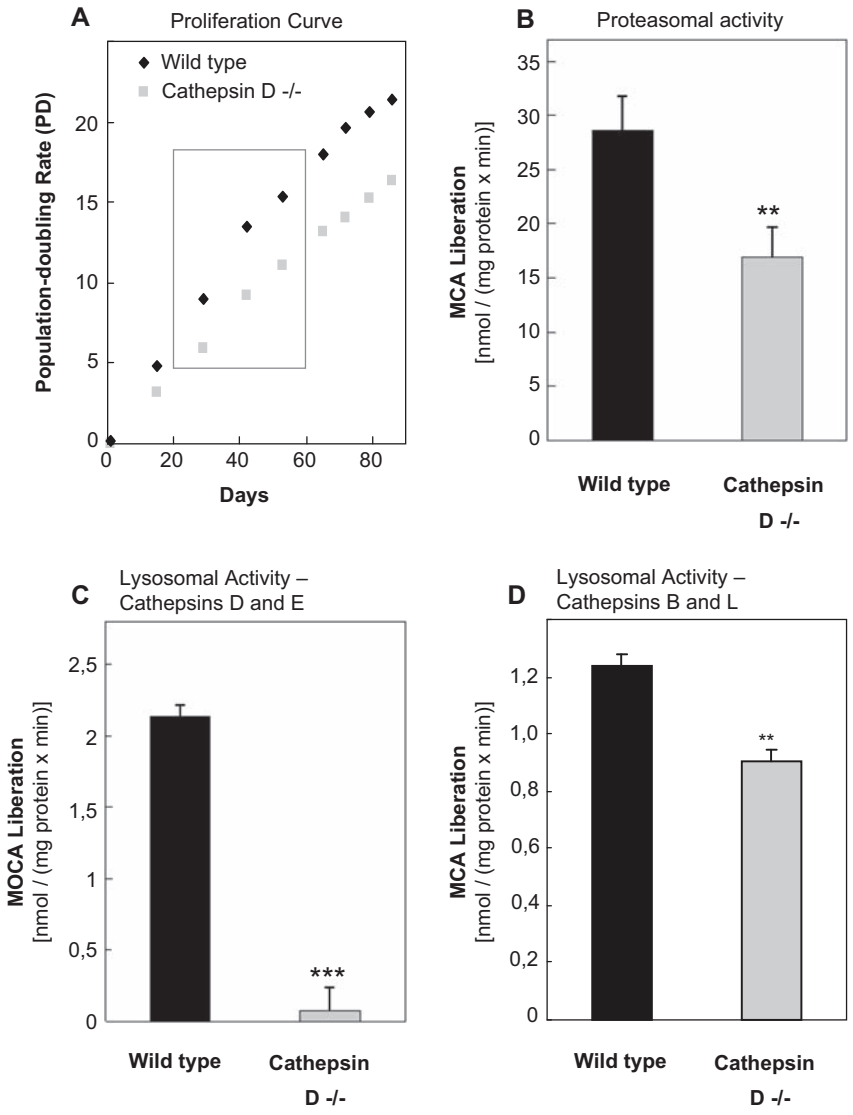

Figure 5. Characteristics of wild type and cathepsin D knockout fibroblasts. (A) The proliferation curve of mouse embryonic fibroblasts isolated from wild type and cathepsin D knockout animals, measured as population doublings (PD) over 90 days. For the experiments, cells between PD 5 and 18 were used (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].

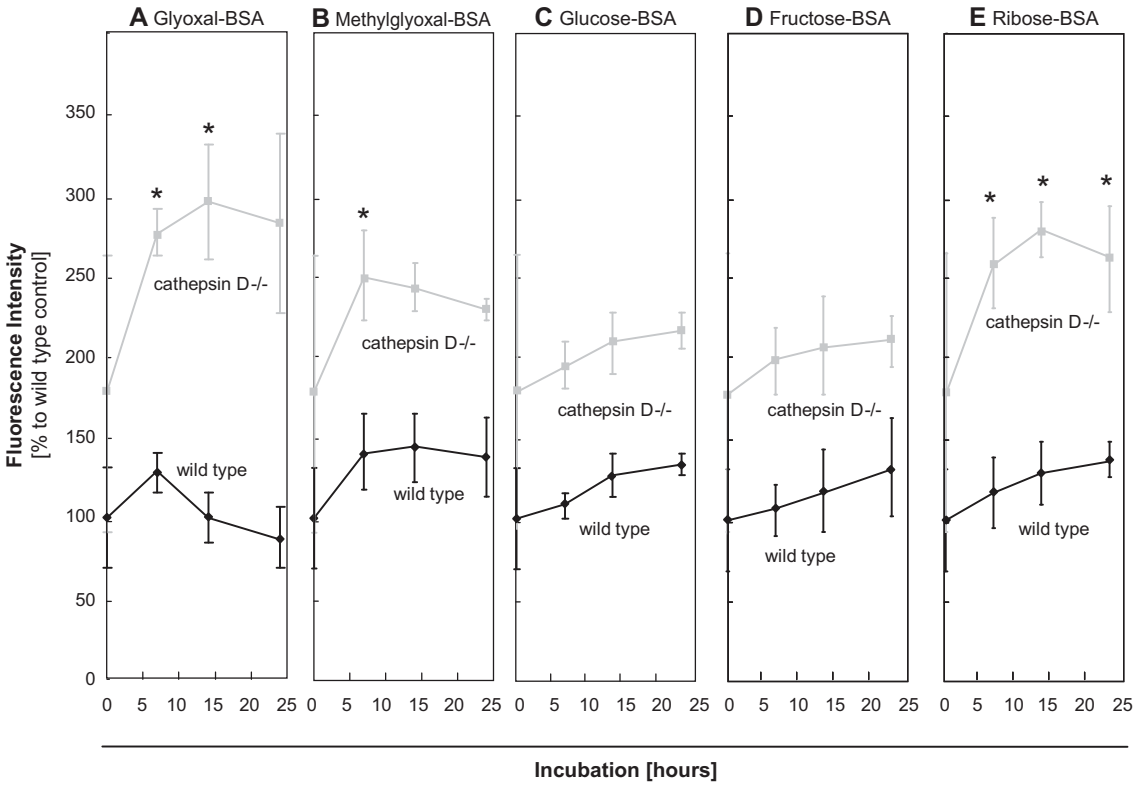

Figure 6. Accumulation of modified albumin in wild type and cathepsin D knockout fibroblasts. The fluorescence of fibroblasts was measured with flow cytometry (488 nm excitation/620 nm emission). The autofluorescence of wild type fibroblasts and without addition of AGE-modified albumin was set as 100% 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

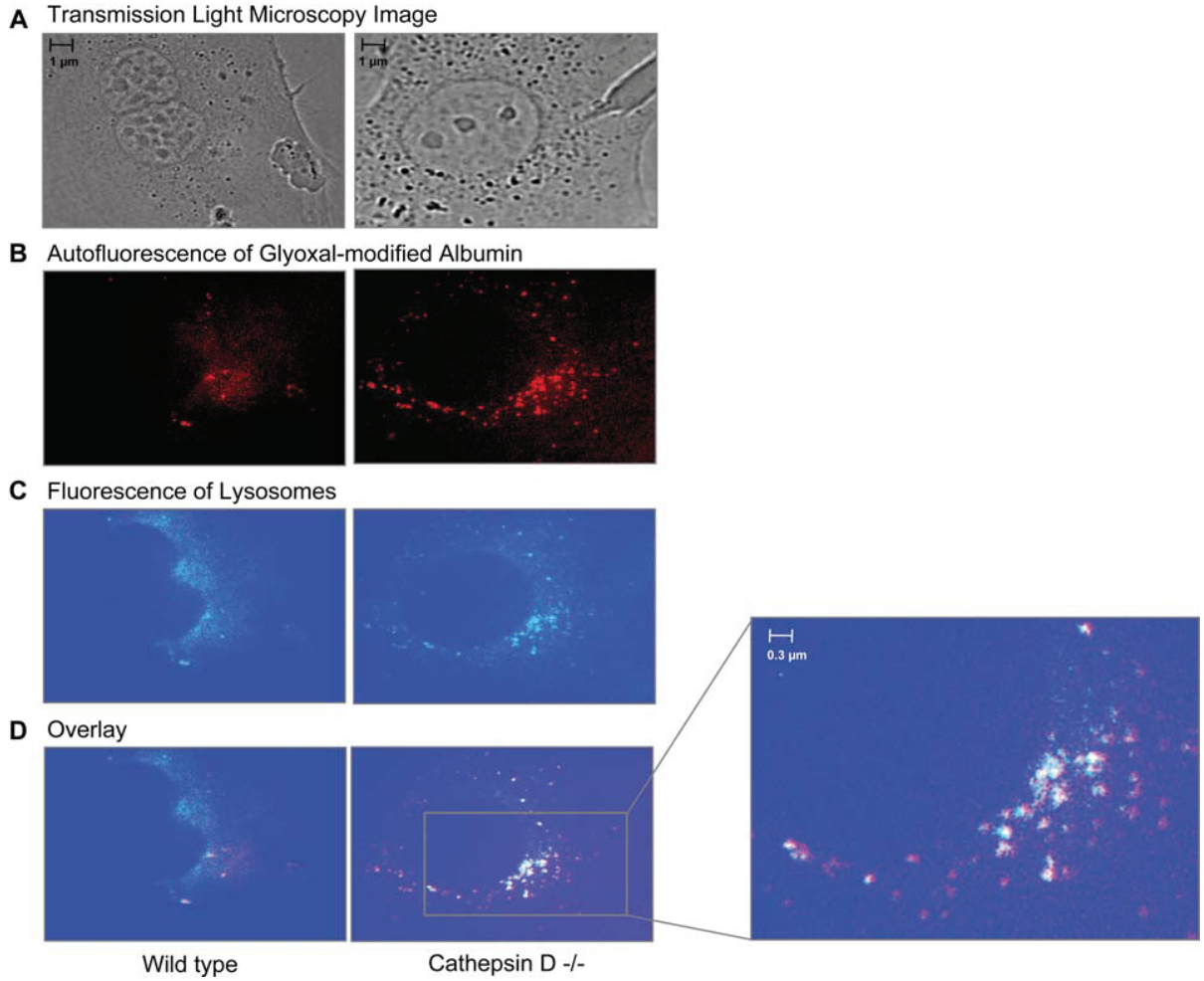

Figure 7. Localization of glyoxal-modified albumin in fibroblasts. Wild-type and cathepsin D knockout cells were incubated with 20 mM glyoxal-modified BSA for 7 h. (A) The transmission light microscopy image. The autofluorescence of glyoxal-AGEs in cells was investigated by fluorescence microscopy (B). 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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3.3 Publikation III: Cathepsin D and L reduce the toxicity of advanced glycation end products.

Stefanie Grimm, Melanie Horlacher, Betül Catalgol, Annika Hoehn, Thomas Reinheckel, Tilman Grune. *Free Radical Biology & Medicine*, 2012, 52(6): 1011-23

Vorangegangene Untersuchungen zeigten, dass Cathepsin D in dem Abbau und somit in der Reduktion akkumulierter AGE-modifizierter Proteine involviert ist. Da neben Cathepsin D auch das Cathepsin L als bedeutende Protease im Abbau endozytierter Proteine charakterisiert ist, ergab sich daraus die Hypothese, dass Cathepsin L möglicherweise einen Einfluss auf den AGE-Stoffwechsel ausübt.
Aktivitätsassays zeigten eine signifikant erhöhte Cathepsin D- sowie Cathepsin L-Aktivität während einer Inkubation mit AGE-modifiziertem Material. Diese Steigerung beruht zum einen auf eine erhöhte Lysosomenanzahl, zum anderen auf eine verstärkte Prozessierung der Procathepsine in reife Cathepsine. Zur Beurteilung dieser Beobachtungen wurden Cathepsin D und Cathepsin L knock-out Zellen verwendet. Einen synergistischen Effekt beider Enzyme wurde durch die Inhibierung der Cathepsin D Aktivität in Cathepsin L knock-out Zellen erzielt. Hierbei konnte eine signifikant gesteigerte Akkumulation der AGE-modifizierten Proteine festgestellt werden. Interessanterweise wurden in Zellen mit Cathepsin D- und L-Defizienz eine verringerte Zellvitalität nach AGE-Behandlung mit gleichzeitiger erhöhter ROS-Produktion detektiert. Aus diesen Ergebnissen lässt sich ableiten, dass Cathepsin D und Cathepsin L für das Überleben der AGE-exponierten Zellen von Bedeutung sind, indem sie die AGE-Toxizität und die AGE-Akkumulation reduzieren.

Eigenanteil:

- Planung aller Versuche, Zellkultur
- Quantifizierung der AGE-Aufnahme, der lysosomalen Aktivität, der Vitalität und der ROS-Produktion
- Anteilige Durchführung der Gen- und Proteinexpressionsuntersuchungen
- Statistische Auswertung, Interpretation und Darstellung aller Ergebnisse, Verfassung des Manuskriptes

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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/

streptomycin. Fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were kept at 37°C in 5% CO_2 and 95% humidity. RAW cells were passed twice a week when they reached confluence at 80%, whereas fibroblasts were passed every two weeks and medium was changed three times a week. Cells were plated at a density of 2×10^5 in RPMI culture medium (RAW cells) or DMEM (fibroblasts) with reduced fetal bovine serum (FBS) (2%) for three days. Afterward freshly dissolved BSA, incubated BSA, and AGE-modified albumin were delivered to the cells in reduced-FBS medium for up to 72h.

Cell viability and proliferation

RAW cells were treated with the indicated concentrations of AGEs as described under Preparation of AGE-modified bovine serum albumin and Cell culture. The assessment of the reduction of soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) by mitochondrial succinate dehydrogenase into an insoluble formazan reaction product is an indicator of mitochondrial function and therefore of cell viability [38]. For this assay cells were treated with AGEs as described above followed by replacement of medium with 0.17mg MTT per milliliter of new medium. After 2h of incubation the cells were solubilized in dimethyl sulfoxide, 10% SDS, and 0.6% acetic acid. The optical density of the formazan product was measured with a microplate reader at 590nm. Viability of untreated cells was expressed as 100%.

Preparation of cell lysates and measurement of lysosomal activity

For the determination of lysosomal activity and the preparation of cell lysates for immunoblot 2×10^6 cells were harvested and incubated for 1h in 1mM dithiothreitol at 4°C under vigorous shaking. Lysates were sonicated for 2min using Sonoplus GM70 (Bandelin, Berlin, Germany) on ice. Afterward the cell lysates were centrifuged at 14,000*g* for 20min and the supernatant was used for the determination of protein content by Lowry DC assay (Bio-Rad, Munich, Germany), the measurement of lysosomal activity, and the expression of lysosomal proteins.

To measure lysosomal activity cell lysates were incubated in 50mM sodium acetate, 8mM cysteine–hydrochloride, and 1mM EDTA at pH 4.0. The substrate MOCA-GKPILFFRLK (Dnp)-RNH_2 (Biomol, Hamburg, Germany) was used for lysosomal aspartate cathepsins (mainly cathepsins D and E), and the substrate z-FR-AMC (Enzo Life Sciences) was used to measure cysteine cathepsins (mainly cathepsins B and L).

The liberation of the fluorophores AMC and MOCA was measured by a fluorescence reader at 360nm excitation and 460nm emission. The liberation of AMC and MOCA was calculated by a calibration curve made using AMC or MOCA (Biomol) standards.

Quantification of lysosomes by flow cytometry

Assessment of lysosomal concentration was performed in RAW 264.7 cells by a single staining method using LysoTracker blue and flow cytometry analysis. Cells were plated and treated with AGEs as described previously. After 72h of incubation the medium was replaced by fresh medium containing 100 nM LysoTracker blue. The cell suspension was harvested after 30min, washed two times with ice-cold PBS, and resuspended in PBS for analysis. LysoTracker emission was detected by a Beckman Coulter (Krefeld, Germany) Cell Lab Quanta SC-MPL flow cytometer with a mercury arc lamp (365nm) for excitation. Emission was measured using a 450/55nm band pass filter.

Immunoblot analysis

To detect protein expression of lysosomal-associated membrane protein 1 (LAMP1), cathepsin D, cathepsin L, and cathepsin B, control or treated cells were harvested and lysed and protein concentration was measured as described above. Fifty micrograms of protein extract was subjected in Laemmli buffer to SDS–PAGE. After electrophoresis the separated proteins were transferred to polyvinylidene difluoride membranes. Blots were blocked in 5% nonfat milk/TBS buffer (0.02M Tris base, pH 7.6, 0.14M sodium chloride, 0.1% Tween 20) at room temperature for 1h followed by incubation with primary antibodies that were diluted according to the recommendations of the manufacturer under constant agitation for another hour. The following primary antibodies were used: anti-LAMP1 (Abcam, Cambridge, UK), anti-cathepsin D (Epitomics, Burlingame, CA, USA), anti-cathepsin L (Acris Antibodies, Hiddenhausen, Germany), anti-cathepsin B (BioVision, Mountain View, CA, USA), and anti-glyceraldehyd-3-phosphate dehydrogenase (GAPDH) (Novus Biologicals, Cambridge, UK). After being washed three times, the blots were incubated with the appropriate secondary antibody, goat anti-rabbit or rabbit anti-mouse (Calbiochem Merck Chemicals, Darmstadt, Germany), conjugated with peroxidase for 1h. Blots were washed and exposed to the substrate LumiGlo Reagent (Cell Signaling Technology, Danvers, MA, USA). Kodak Biomax XAR films were developed and analyzed by the determination of the pixel intensities.

Quantitative PCR

Total RNAs were extracted from cells using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Hilden, Germany). Total RNA (1μg) was transcribed into cDNA using the cDNA Synthese Kit (Bio-Rad). A standard real-time PCR with a total volume of 25μl was performed with 1μl of the reverse transcriptase reaction mixture in 35 cycles, each at 94°C for 30s, followed by 60°C for 30s, continuing at 72°C for 45s, in IQ SYBR Green Supermix (Qiagen) and 100 nM specific primer. The thermal cycler for quantitative real-time PCR was the Rotor Gene-6000 Corbett Research cycler (Qiagen). The primers were synthesized by biomers.net (Ulm, Germany) according to the following sequences: LAMP1, 5′-GTCCTCATTGCCTACCTCATT-3′ and 5′-CCTCCCTTCCACACCTATCTA-3′; cathepsin D, 5′-GCAGTGTTTCA-CAGTCGTCTT-3′ and 5′-CATAGGTGCTGGACTTGTCAC-3′; cathepsin L, 5′-CGAAGGACGGATCTTGTAAAT-3′ and 5′-GCGTCCATAGCAACA-GAAATA-3′; cathepsin B, 5′-AAGAGAAGAGGACCCTGAACA-3′ and 5′-TTCATGTACCCAAGGAAGACA-3′; GAPDH, 5′-AACCTGCCAAGTAT-GATGACA-3′ and 5′-CATACCAGGAAATGAGCTTGA-3′; 18s rRNA, 5′-ATTAAGTCCCTGCCCTTTGAT-3′ and 5′-AGTCAAGTTCGACCGTCTTCT-3′; and actin-β, 5′-TTCCTTCTTGGGTATGGAATC-3′ and 5′-GCTAGGAGCCA-GAGCAGTAAT-3′. The LAMP1 PCR product was 137bp, the cathepsin D 128bp, the cathepsin L 136bp, and the cathepsin B 156bp long. The GAPDH PCR product was 192bp, 18s rRNA was 134bp, and actin-β was 178bp long. Quantification of gene expression was based on the cycle threshold (C_t) value for each sample considering PCR efficiency based on the relative expression software tool REST 2008 (Corbett Life Science, Qiagen). Three different housekeeping genes were used and RNA integrity was checked with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Boeblingen, Germany). All experiments included negative controls consisting of no cDNA for each primer pair.

Immunofluorescence microscopy

Cells were incubated for 2h with 150μM pepstatin A, and glyoxal–AGE was added to the culture medium for 24h followed by the addition of 75 nM LysoTracker Blue DND-22 according to the manufacturer's instructions. After 1h of incubation the cells were washed twice with ice-cold PBS and examined on a fluorescence microscope (Carl Zeiss Axiovert 100M, Jena, Germany).

Determination of reactive oxygen species with 2′,7′-dichlorofluorescein diacetate

Intracellular reactive oxygen species (ROS) production was measured using the sensitive fluorescent probe 2′,7′-dichlorofluorescein

diacetate (DCF). Fibroblasts were seeded at a density of 2×10^5 per well in a 24-well plate. After 48h, the cells were incubated with 100 μM *N-tert*-butyl-α-phenylnitrone (PBN) or medium control for 2h, followed by incubation with 150μM pepstatin A for another 2h before glyoxal–AGE was added to the samples for 24h. DCF was added to the culture medium at a concentration of 5μM for 45min. Cells were washed two times with PBS and the detection of ROS was carried out in PBS with a fluorescence reader (excitation 485nm, emission 528nm).

Statistical analysis

All experiments were performed at least in triplicate. The statistical analysis of the data was performed using one-way ANOVA followed by the post hoc Tukey significant difference test. Data are presented as means ± SD and statistical analysis was performed using GraphPad Prism version 5.

Results

Uptake of advanced glycation end products in RAW 264.7 macrophages

As described above, results from in vivo and in vitro studies demonstrate a role for AGEs in modulation of immune response, especially during aging. To further explore the effects of AGEs on the proteolytic mechanisms, we first investigated the uptake of these aggregates into the macrophage cell line RAW 264.7 with different methods. We prepared radioactively labeled glucose–AGEs as described under Materials and methods and incubated RAW cells with 25mM [^{14}C]glucose-modified albumin for 1, 24, 48, and 72h, followed by intensive washing steps in PBS. Cells were lysed in 1mM dithiothreitol as previously described and radioactivity was measured in a scintillator (Beckman Coulter LS6500). Although within this setup it is not possible to have an appropriate control (no glucose), one is able to follow the uptake of [^{14}C]glucose-modified albumin for 72h. During the first 24h, there is a significant increase in intracellular accumulation of glucose-modified albumin and this continues throughout the following days (Fig. 1A). To further support the intracellular accumulation of various AGEs the uptake was measured with a fluorescence reader and microscope, as some AGE structures have the ability to emit light. Figs. 1B and C illustrate the uptake of AGEs after chronic (72h) incubation. A significant increase in the autofluorescence of AGE-treated cells was measured using a fluorescence reader (Fig. 1B) and fluorescence microscopy (Fig. 1C) compared to untreated cells. Statistical significance between incubated BSA and AGE-treated cells was observed for glyoxal-, methylglyoxal-, and ribose-modified albumin.

Activity of lysosomal proteases and the amount of lysosomes after AGE incubation

Because our original goal was to measure AGE influence on lysosomal protein degradation we measured cathepsin L and B activity after AGE incubation with the specific substrate z-FR-AMC. A trypan blue exclusion assay showed no influence on viability and proliferation of cells after AGE incubation for 72h (data not shown). An increase in the activity in glyoxal- and glucose-modified albumin-treated samples is demonstrated in Fig. 2A. After 72h we could measure a significant increase in both AGE-treated samples. Additionally, we measured cathepsin L and B activity after 72h in BSA- and AGE-treated samples. As demonstrated in Fig. 2B there was a significant increase in cathepsin L and B activity in AGE-treated samples compared to control (fresh protein) or incubated BSA treatment.

Additionally, we measured cathepsin D and E activity, and for those cathepsins also we could determine a significant increase in the activity after 72h of glyoxal- and glucose-modified albumin incubation (Fig. 2C). To compare these observed effects with different AGEs we incubated the macrophages with sugar- and aldehyde-modified albumin and incubated BSA for 72h (Fig. 2D). A significant increase in cathepsin D and E activity was observed compared to control and BSA-incubated cells, too. As significant effects were observed after 72h, we decided to continue our experiments with this incubation time.

To investigate if the increased activity is due to more lysosomes we quantified the expression of LAMP1 at the protein and mRNA levels (Figs. 3A and B). For both parameters we could not measure any significant increase in LAMP1. Although LAMP1 is widely used as an indicator of lysosomal content, there might be a discrepancy between LAMP1 and the lysosomal amounts, in other words, the composition of the lysosomal membrane might vary. Therefore, we visualized late endosomes and lysosomes by LysoTracker dye (Figs. 3C–I). The signal from LysoTracker blue was measured at 370nm absorption and 420nm emission and therefore did not interfere with the autofluorescence of AGEs (460nm absorption/530nm emission). The gray curves in Figs. 3C to H represent cells incubated with fresh protein and stained with LysoTracker blue. Cells treated with incubated albumin (BSA) (Fig. 3C) and modified albumin (Figs. 3D–H) are shown with dark lines. Fig. 3I presents the quantification of the fluorescence signal. It is precisely shown that the uptake of AGEs was accompanied by an increase in lysosomal content during 72h of exposure compared to control and BSA-incubated cells. Therefore, it can be assumed that the increasing lysosomal activity is due to enhanced processing of preenzymes to active forms during endosome and lysosome maturation.

Protein and mRNA expression of lysosomal cathepsins D, L, and B

Because we found an increase in lysosomal activity and elevated lysosomal amounts it seemed important to test whether the protein and mRNA expression of the main lysosomal proteases is similarly enhanced. In contrast to cathepsins D, L, and B, cathepsin E is not located in lysosomes [39]; therefore we decided to concentrate on cathepsins D, L, and B. The concentration of defined proteins can be enhanced in cells either as a result of increased gene expression, by the stabilization of existing proteins, or by the conversion of proforms into active forms. The processing of procathepsin D (approximately 46kDa) undergoes two steps. Procathepsin D is first converted into the single-chain form, followed by conversion into the double-chain active enzyme in the endosomal–lysosomal compartment [40]. The latter is made up of the N-terminal light chain (13kDa) and the C-terminal heavy chain (31kDa) linked by noncovalent interactions. Immunoblot analysis showed only the procathepsin form (46kDa) and the mature heavy chain (31kDa). Fig. 4A presents the protein expression of cathepsin D. The results obtained from immunoblots revealed that more mature cathepsin D is present in AGE-treated cells compared to control or BSA-treated cells. Additionally we could measure a significantly increased cathepsin D mRNA concentration in these cells (Fig. 4D). In accordance with cathepsin D, a higher amount of mature cathepsin L was observed in AGE- or BSA-exposed cells (Fig. 4B) compared to control cells. However, we were not able to measure an increased level of cathepsin L mRNA (Fig. 4E). Cathepsin B was slightly but not significantly processed into the mature form, with no effect on mRNA (Figs. 4C and F, respectively). Therefore, we concluded that the increase in the activity of lysosomal proteases is due to an increase in the amount of cathepsins D and L. However, compared to BSA-incubated cells the effect of AGEs was more obvious in the case of cathepsin D.

Effect of AGEs on cathepsin D- and cathepsin L-deficient cells

After showing that more active cathepsins D and L are present in AGE-treated RAW 264.7 cells we raised the question whether

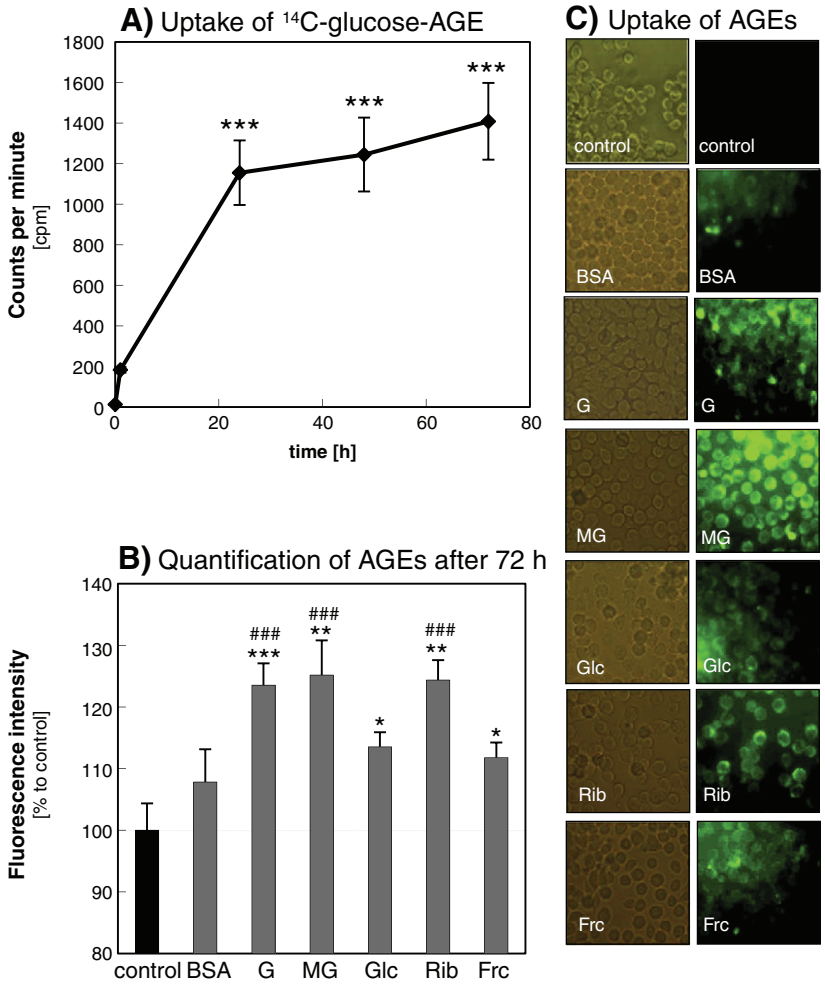

Fig. 1. Uptake of advanced glycation end products (AGEs) in RAW 264.7 cells. (A) RAW cells were incubated with [^{14}C]glucose-modified albumin over a period of 72h. After 1, 24, 48, and 72h, cells were washed and lysed and the uptake of [^{14}C]glucose-modified albumin was measured in scintillation fluid. (B) RAW cells were further incubated with fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, and glucose (Glc)-, ribose (Rib)-, or fructose (Frc)-modified albumin for 72h. After the cells were washed, the uptake of AGEs was measured by a fluorescence reader (excitation 460nm, emission 530nm). (C) Representative images made by fluorescence microscopy using the same experimental design as in (B). The left column of panel C represents cells in transmitted light, whereas the right column shows the autofluorescence of AGEs. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$; statistically significant difference compared to control. ###$p<0.001$; statistically significant difference compared to cells treated with incubated BSA.

cathepsins D and L are essential for the survival of the AGE-treated cells. We used mouse embryonic fibroblasts and verified the increase in cathepsin L (Fig. 5A) and cathepsin D (Fig. 5D) activity after AGE treatment. As we were not able to find any difference in mature cathepsin B in control versus treated cells (Fig. 4B), we further intended to exclude the influence of cathepsin B activity in AGE treatment. For this aspect we used mouse embryonic fibroblasts with cathepsin L deficiency and incubated them with AGEs as described previously and measured the cathepsin L and B activity. Fig. 5B shows no increase in cathepsin B activity. Therefore, we can support the hypothesis that the increased activity due to AGEs in the degradation of the cathepsin B and L substrates is indeed due to an increase in cathepsin L activity as shown in Fig. 5A. As the substrate for cathepsin D is additionally cleaved by cathepsin E we used cathepsin D knockout fibroblasts to exclude any cathepsin E influence in the activity. As assumed, we were not able to measure an enhanced cathepsin D and E activity in cathepsin D-deficient cells (Fig. 5F). From this aspect we can conclude that only cathepsin D activity is increased in AGE-treated cells. We further wanted to investigate if cathepsin L activity is increased in cathepsin D knockout cells with and without AGE treatment (Fig. 5C). Untreated cathepsin D knockout cells demonstrate approximately 20% less cathepsin L activity (see Fig. 5C, inset). However, in AGE-treated knockout cells the cathepsin L activity can be significantly increased compared to untreated cells. Cathepsin L seems to have an important role in the metabolism of accumulated AGEs, and the lower activity in knockout cells can be restored by AGE treatment. Conversely, we used cathepsin L knockout cells to measure cathepsin D activity in untreated versus treated cells (Fig. 5E). In untreated cells we could measure an increase in cathepsin D activity in cathepsin L knockout cells (L −/−) compared to wild type cells (inset diagram in Fig. 5E). However, cathepsin D activity was not further enhanced in cathepsin L-deficient cells after AGE treatment, perhaps because of already increased activity in untreated knockout cells compared to wild type. Despite the enhanced cathepsin L and D activity in wild type as well as the increased cathepsin L activity in cathepsin D knockout fibroblasts we could not measure a statistical significance between BSA- and AGE-treated cells.

Effect of the cathepsin D inhibitor pepstatin A on AGE-treated cells

To further explore the effects of both lysosomal enzymes on the treatment of AGEs we used pepstatin A as a cathepsin D inhibitor in combination with cathepsin L knockout cells. Cell viability assays without the inhibitor showed no effect after AGE treatment (data not shown). Pepstatin A is a tight-binding inhibitor of cathepsin D showing a low dissociation constant [41]. A double knockout of cathepsins L and

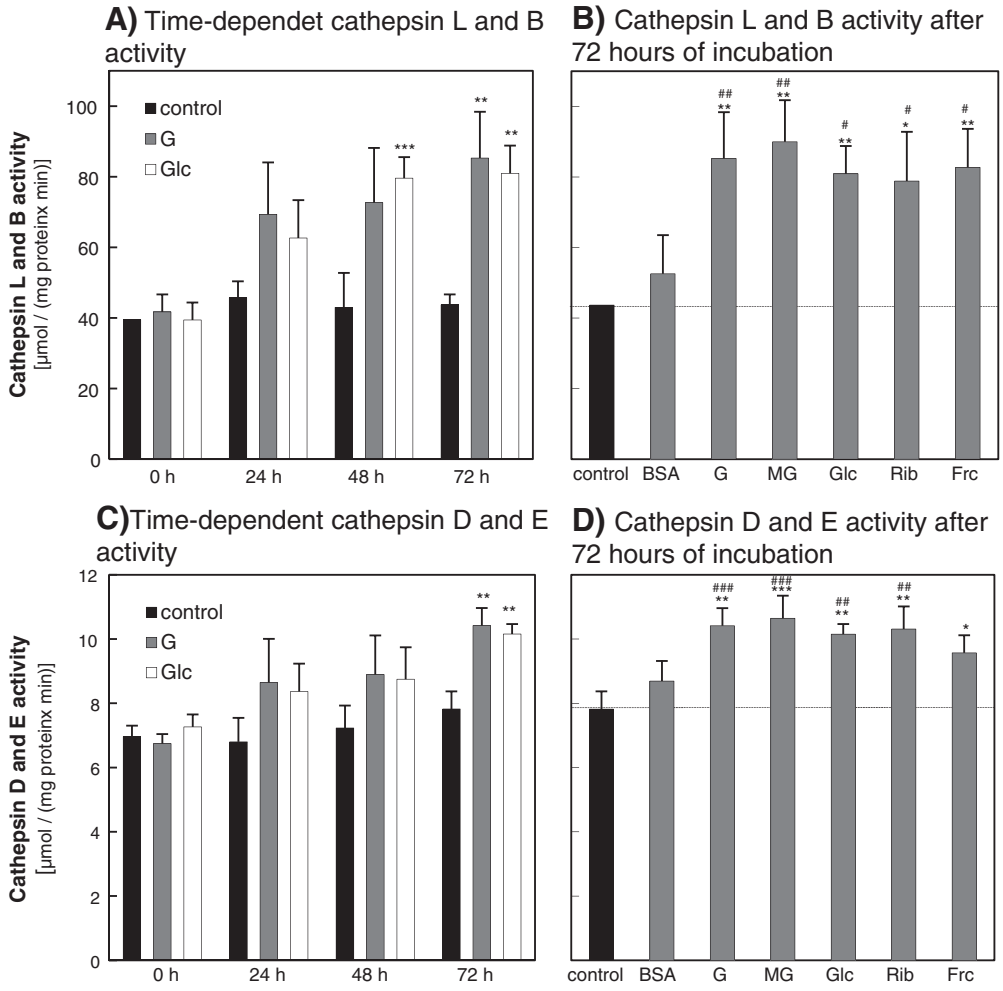

Fig. 2. Lysosomal activity after incubation with AGEs. (A) Lysosomal activity was measured for up to 72h of incubation with glyoxal (G)- and glucose (Glc)-modified albumin. (B) Cathepsin L and B activity after 72h of incubation with fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, or glucose (Glc)-, ribose (Rib)-, or fructose (Frc)-modified albumin. (C and D) Cathepsin D and E activity after time-dependent incubation of G- or Glc-modified albumin and 72h of AGE incubation in RAW 264.7 cells. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$; statistically significant difference between control (freshly dissolved BSA) and AGE-treated cells. $\#p<0.05$, $\#\#p<0.01$, and $\#\#\#p<0.001$; statistically significant difference between cells treated with incubated BSA and cells treated with AGEs.

D is not viable and therefore double-knockout mice cannot be generated. Fig. 6A presents cathepsin D and E activity in wild-type and cathepsin D knockout cells. Not more than 20% activity was seen in cathepsin D-deficient cells, presumably explicable by cathepsin E activity. Fig. 6B reveals the cathepsin D and E activity in cathepsin L-deficient cells after treatment with the cathepsin D inhibitor pepstatin A. Pepstatin A at 150µM achieved almost the same cathepsin D and E activity in cathepsin L-deficient cells compared to cathepsin D knockout fibroblasts. However, as pepstatin A also blocks cathepsin E in a concentration-dependent manner, the viability of these treated cells was reduced significantly (Fig. 7A). Cathepsin L knockout and the simultaneous inhibition of cathepsin D have a stronger influence on viability compared to cathepsin L knockout alone. In addition, concentrations beyond 150µM pepstatin A displayed no measurable cathepsin D and E activity (data not shown). The viability was significantly more reduced in the presence of AGEs compared to control or BSA-incubated cells (Fig. 7B), thus revealing the importance of cathepsins D and L for the uptake and reduced toxicity of AGEs. In further investigations we tested the uptake and accumulation of AGEs in knockout cells and in knockout cells with inhibited lysosomal proteases. We could see an increase in glyoxal-AGE autofluorescence in wild-type cells (Figs. 8A and B). In cathepsin L −/− cells the accumulation was much stronger, compared to the control (Fig. 8A). However, the accumulation of AGEs in cathepsin D-deficient (D −/−) glyoxal-treated cells was highest (Fig. 8B). All treated samples demonstrated a significant increase in glyoxal-AGE autofluorescence after 24h in the presence of pepstatin A, again revealing the important role of functional active cathepsin D in reduced accumulation of AGEs. However, as was predictable, in D −/− cells pepstatin A had no further effect. As the accumulation of glyoxal-AGE is enhanced in cathepsin L knockout cells with cathepsin D inhibition and these cells demonstrated a significantly reduced viability (Fig. 7B), we investigated whether this reduction in viability is due to a higher production of ROS in these cells. Indeed, glyoxal-AGE treatment induced a significantly increased generation of ROS in wild-type and L −/− cells (Fig. 8C). The pretreatment with pepstatin A demonstrated a significantly enhanced ROS production. Moreover, in cathepsin L knockout cells pretreatment with pepstatin A and glyoxal-AGE incubation induced a significantly enhanced generation of reactive oxygen species, compared to cathepsin L wild-type cells that were also pretreated with pepstatin A and incubated with glyoxal-AGE. To investigate if the radical scavenger PBN attenuates ROS production, we preincubated the cathepsin L knockout cells with 100µM PBN. For all samples we could observe a reduced ROS generation; however, this was significant only for glyoxal-treated cells. The PBN- and pepstatin-pretreated cells incubated with glyoxal-AGE showed an immense ROS production again (Fig. 8D).

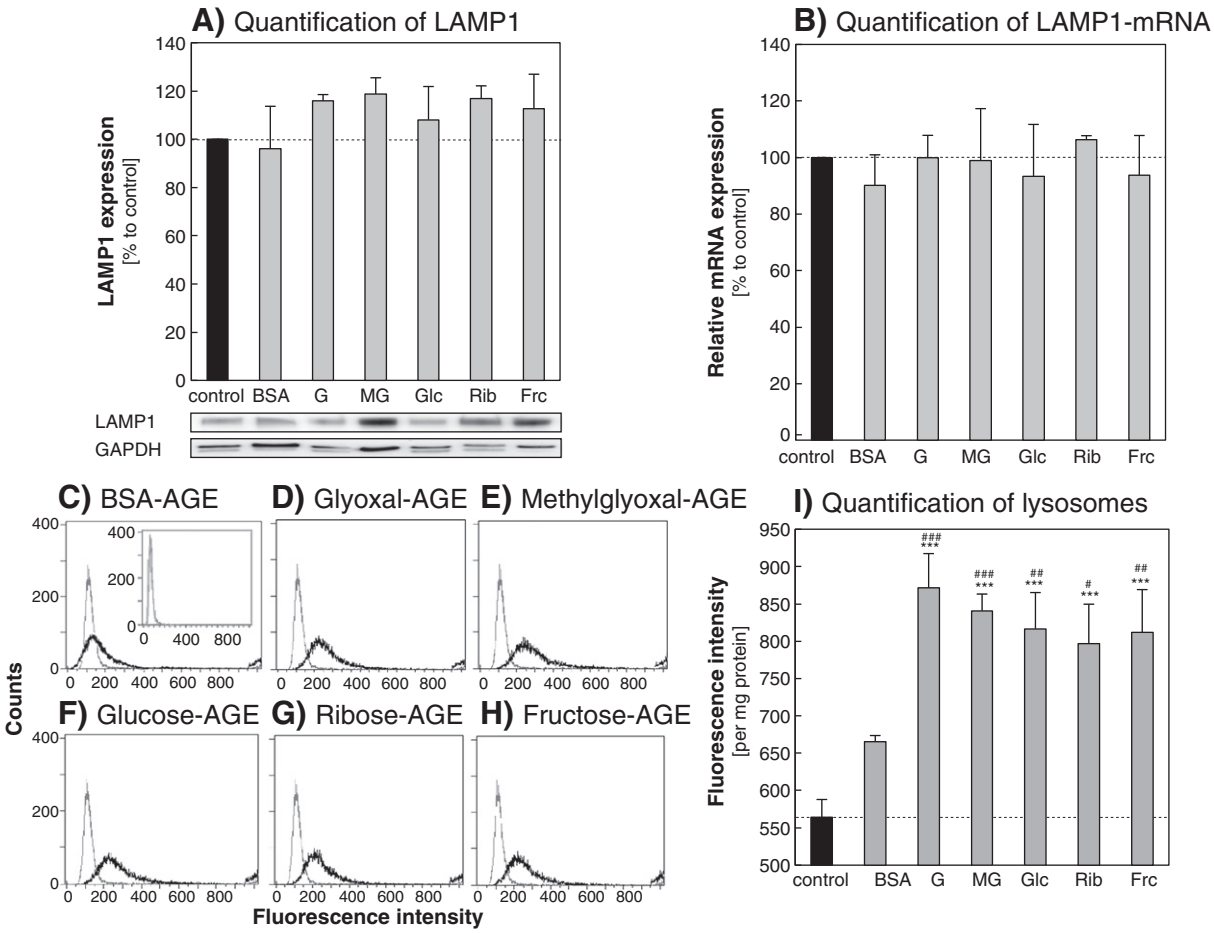

Fig. 3. Quantification of lysosomes after AGE incubation. The concentration of lysosomes was determined by the expression of (A) LAMP1 protein and (B) LAMP1 mRNA after 72h of AGE incubation. Control samples were defined as 100%, whereas the other samples were calculated according to the control. (C–I) The lysosomal amount was quantified for 72h of AGE incubation with LysoTracker blue. RAW 264.7 cells were stained with 100 nM LysoTracker and investigated with a flow cytometer. LysoTracker blue was excited (365nm) and the emission of the dye was analyzed by a 450/55 nm band pass filter. Fluorescence of control cells with fresh protein and without LysoTracker blue is indicated in the inset diagram in (C). The gray curves demonstrate control cells incubated with fresh protein and stained with LysoTracker blue, whereas the black curves display cells after AGE or aggregated BSA treatment as described above. Fluorescence intensity of the black curves was analyzed by the statistical software of the flow cytometer and is summarized in (I). ***$p<0.001$; statistically significant difference between control (freshly dissolved BSA) and AGE-treated cells. #$p<0.05$, ##$p<0.01$, and ###$p<0.001$; statistically significant difference between cells treated with incubated BSA and cells treated with AGEs.

Discussion

AGEs are formed at high rates in the metabolic syndrome and in many other diseases [5-9]. We produced five different AGE types, because these molecules constitute a broad family of glycated proteins, showing different responses [42]. AGEs produce oxygen free radicals by binding to receptors or by their internalization by inflammatory cells. So recently Guimaraes et al. showed that AGEs induce the production of ROS by the activation of NADPH oxidase [43]. In glyoxal-modified albumin-treated cathepsin L wild-type and knockout cells, we detected reactive oxygen species by the DCF assay. In cathepsin L knockout cells and in cells with inhibited cathepsin D activity we detected more ROS compared to untreated control and wild-type cells. Interestingly, cells with deficiency in cathepsin L or D that were treated with AGEs showed an enormous increase in ROS production and a massive decrease in viability. Moreover, AGEs induce the release of cytokines and contribute to the proinflammatory response [44]. Both ROS and cytokines are responsible for the cytotoxic effects toward cell death upon exposure to AGEs. To reduce these cytotoxic effects it is crucial to understand the cellular reactions triggered by AGEs. Because the production of AGEs is difficult to avoid, our aim is to investigate possible interaction pathways in their accumulation.

One mechanism to reduce the toxic effect of AGEs is their proteolytic breakdown. Recently, we found out that AGEs can be degraded by cathepsin D, whereas the proteasome was not able to degrade them [33]. Here we decided to test whether other cathepsins are also involved in AGE processing. As cathepsin L is one of the most powerful lysosomal proteases, we suggested that it also plays a role in AGE processing.

For our studies we used the macrophage cell line RAW 264.7, as these cells are immune cells that function in the clearance of infectious particles and undesired extracellular materials. An uptake of AGEs could be verified by fluorescence methods and additionally by radioactive glucose-modified albumin (Fig. 1). It is necessary to investigate the uptake of AGEs or the modification grade of AGEs not only by fluorescence methods, as only some of the various AGE structures have fluorescent properties [45]. Nonfluorescent or low-fluorescent AGEs are, for example, carboxymethyllysine, carboxyethyllysine, and pyralline [45], and it depends on the modifying agents and the terms of reaction if fluorescent or nonfluorescent products will be formed. After verifying the uptake of AGEs by the different methods we determined the influence on cell viability. We did not measure an influence on cell viability and proliferation in the presence of indicated concentrations of glyoxal- and methylglyoxal-modified albumin and glucose-, ribose-, and fructose-modified albumin for 72h. However, higher doses of modified albumin led to a significant decrease in cell viability and lower doses led to less significant results (data not shown). As significant effects were observed especially

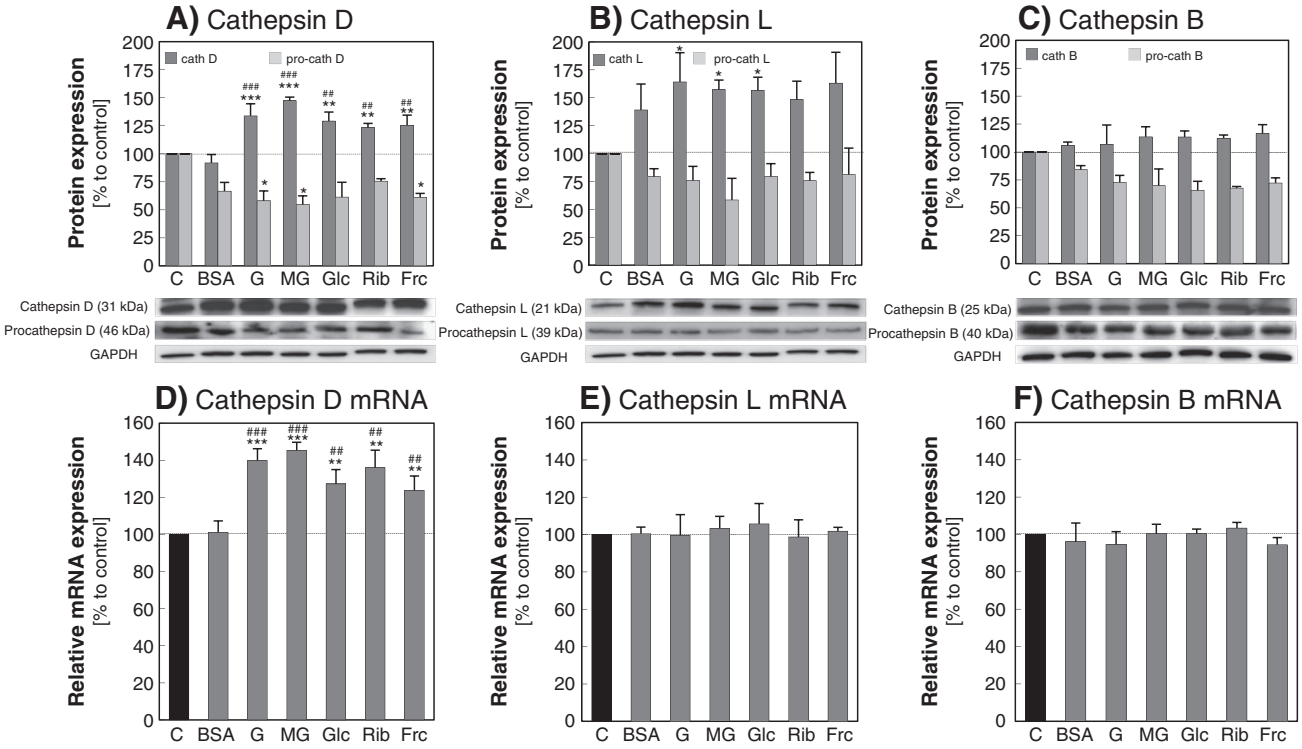

Fig. 4. Effects of AGEs on the protein and mRNA expression of cathepsins D, L, and B. RAW 264.7 cells were treated for 72h with fresh protein (C), incubated BSA (BSA), and AGEs, followed by the extraction of cell lysates and isolation of RNA. One representative blot is displayed under each respective diagram for cathepsins D, L, and B. Control cells were set at 100% and the other cells were counted in reference to the control sample. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$; statistically significant difference between control (freshly dissolved BSA) and AGE-treated cells. $\#\#p<0.01$ and $\#\#\#p<0.001$; statistically significant difference between cells treated with incubated BSA and cells treated with AGEs.

after 72h (Figs. 2A and C), we decided to continue our experiments with an incubation time interval of 72h.

The activity of cathepsins is regulated by several mechanisms, such as zymogen processing, inhibition by endogenous inhibitors, pH stability, and, to a lesser extent, regulation of synthesis [31,32]. Peptide substrates enable the distinction between various protease activities. After 72h we determined a significant increase in cathepsin L and D activity due to AGEs compared to control or BSA-incubated cells (Figs. 2B and D). However, this could not be confirmed by more lysosomes after quantification with LAMP1 (Fig. 3). LAMP1 is one of the most abundant components of the lysosomal membrane [21]. Nevertheless, the function of LAMP1 is still not known and obviously lysosomes contain various amounts of LAMP1. A knockout of the LAMP1 gene in mice causes only a mild phenotype and not an apparent lysosomal dysfunction. It can be assumed that the absence of LAMP1 may be compensated for by LAMP2. More than 50% of lysosomal membrane proteins are LAMPs [23]. However, as there are several different forms [46], the composition of LAMPs in the membrane may vary. The role of LAMP2 has been better investigated. It plays a role in chaperone-mediated autophagy, and LAMP2-deficient mice revealed a disturbance in autophagy, lysosomal enzyme targeting, and lysosomal maturation [11].

It is well known that the size and quantity of lysosomes can increase dramatically in any cell type if the lysosomes accumulate non-degradable material [47]. Therefore, we decided to investigate the quantity by LysoTracker dyes, which are specific for a broad range of acidic compartments. Indeed, by adding LysoTracker blue, we observed significantly increased amounts of lysosomes after AGE treatment compared to control or BSA-incubated cells. More endosomes and lysosomes, as well as increased cathepsin D and L activity, are associated with endocytosis of AGEs and, therefore, the phagocytic function of macrophages. After the entry of substrates into the lysosomal compartment the acidic pH value induces the denaturation and therefore exposure of formerly intermolecularly located and thus buried hydrophobic areas, making these proteins much more sensitive to degradation by cathepsins D and L because of their substrate specificity against hydrophobic amino acids. The difference in the cathepsin D and L mRNA levels might be explained by the fact that the cathepsin D promoter gene has a mixed structure showing multiple GC boxes and TATA sequences. The TATA sequences are putative binding sites for the transcription factor IID [48]. Wang et al. reported that tumor necrosis factor α (TNFα) induces the expression of cathepsin D [49]. In accordance with this we could measure an increase in TNFα after AGE treatment (data not shown). An increase in protein content does not always reflect enhanced mRNA synthesis. The explanation for this is differential transcription and translation rates that account for this weak correlation. This may be especially true for zymogens, which undergo several processing steps until they reach their mature and active structure.

Owing to enhanced mature cathepsins D and L after AGE incubation we conclude that these two cathepsins play important roles in the cellular response against AGEs. Several substrates for cathepsin D have been investigated. Cathepsin D cleaves, for example, serum albumin, collagen, hemoglobin, myosin, myelin proteins, angiotensinogen, and endorphins [50]. Some substrates for cathepsin L are the same as for cathepsin D, for example, collagen, albumin, hemoglobin, and myosin. Further substrates are actin, tubulin, vimentin, elastin, and many cytosolic enzymes [30]. The activity of cathepsin B against protein substrates is low in comparison to the activity of cathepsin L (less than 10%) [51].

In general, all lysosomal enzymes are synthesized as inactive precursors, which are processed either autocatalytically or by other proteolytic active enzymes to remove the N-terminal prosequence. This prosequence is thought to block the active site, thus inhibiting proteolytic activity of the enzyme and having a function in the correct folding of the nascent protein [52]. Recently published data showed that cathepsin L is involved in cathepsin D processing to generate the light and heavy chain from the single-chain procathepsin D [53].

Because of these multiple lines of evidence that cathepsins D and L play important roles in the metabolism of AGEs we aimed to

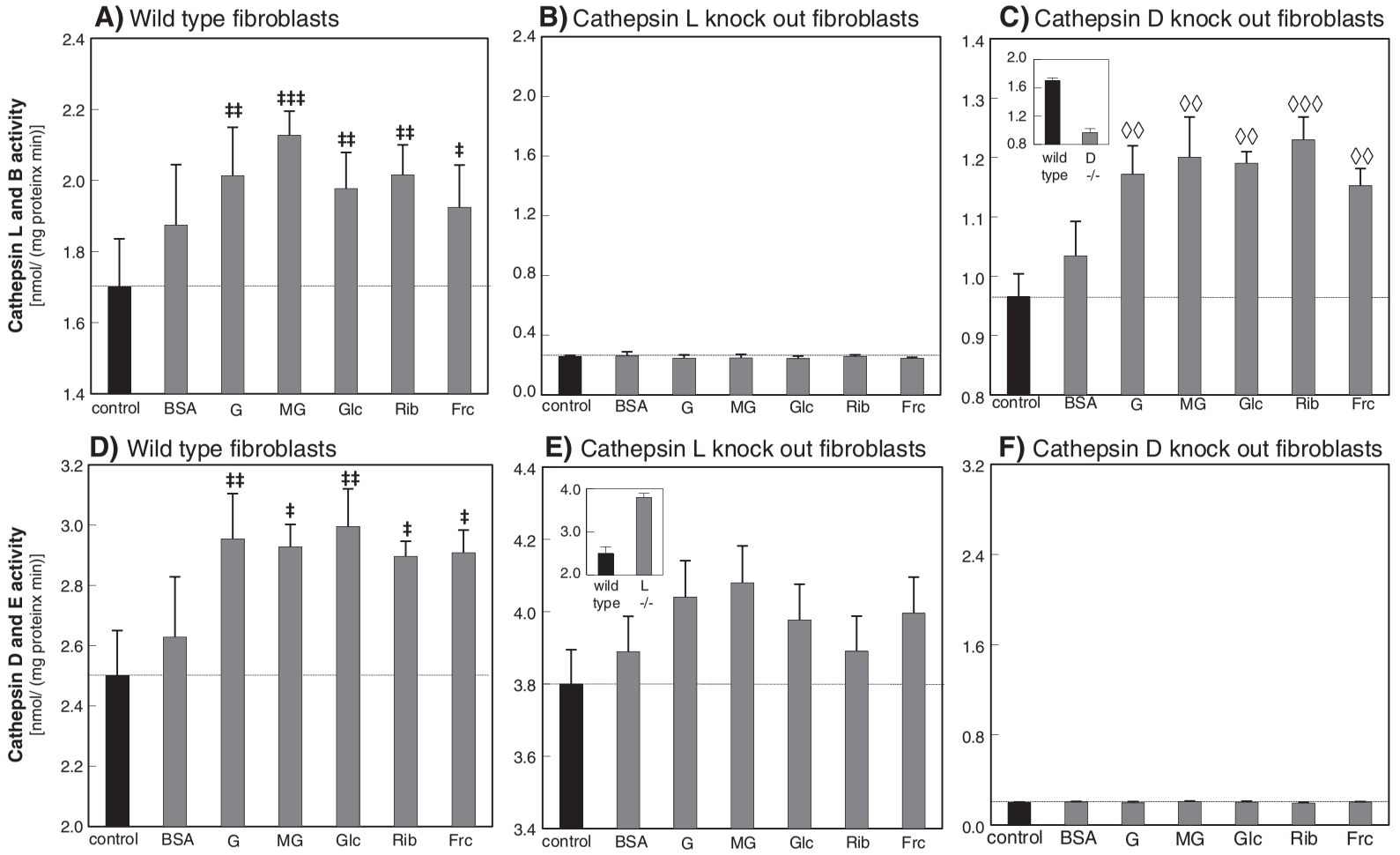

Fig. 5. Influence of AGEs on cathepsin L- and cathepsin D-deficient cells. Fresh protein (C), incubated BSA (BSA), glyoxal (G)- and methylglyoxal (MG)-modified albumin, and glucose (Glc)-, ribose (Rib)-, and fructose (Frc)-modified albumin were added to wild-type, cathepsin L-deficient, and cathepsin D-deficient cells for 72h. The activity was measured with the specific substrates for cathepsins L and B and for cathepsins D and E as described under Materials and methods in (A and D) wild-type, (B and E) cathepsin L knockout, and (C and F) cathepsin D knockout cells. The inset diagram in (C) shows cathepsin L and B activity in cathepsin D knockout (D −/−) control cells compared to wild-type control fibroblasts, whereas the inset diagram in (E) displays cathepsin D and E activity in cathepsin L knockout (L −/−) control cells versus wild-type control cells. ‡$p<0.05$, ‡‡$p<0.01$, and ‡‡‡$p<0.001$; statistically significant difference between wild-type control cells (fresh protein) and wild-type treated cells (fresh protein + AGEs). ◊◊$p<0.01$ and ◊◊◊$p<0.001$; statistically significant difference between knockout control cells (fresh protein) and knockout treated cells (fresh protein + AGEs).

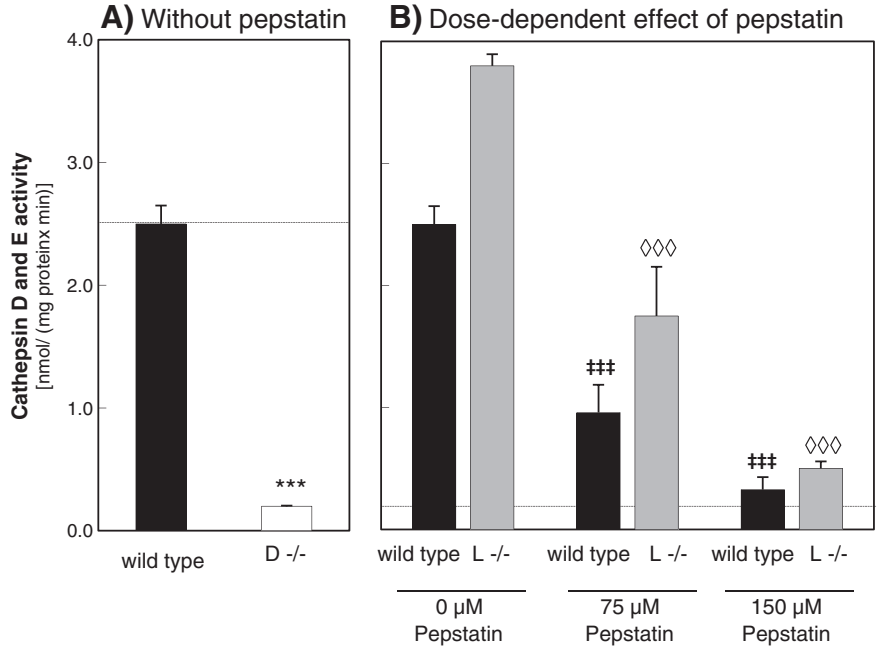

Fig. 6. Influence of various concentrations of pepstatin A on cathepsin D and E activity. Cathepsin D- and L-deficient fibroblasts as well as wild-type fibroblasts were incubated with 0, 75, and 150µM pepstatin A for 24h. All samples received 100µM fresh BSA. Cells were harvested and cell lysates were measured for their cathepsin D and E activity. (A) Cathepsin D knockout fibroblasts (D −/−) were investigated for their cathepsin D and E activity and compared to wild-type samples that were not treated with pepstatin A (black column). (B) Cathepsin D activity in cathepsin L wild-type and L knockout fibroblasts (L −/−) after incubation with 0, 75, and 150µM pepstatin A. Cathepsin D activity in D knockout cells is indicated as a solid line. ‡‡‡$p<0.001$; statistically significant difference between wild-type control cells (fresh protein) and wild-type treated cells (fresh protein + pepstatin). ◊◊◊$p<0.001$; statistically significant difference between knockout control cells (fresh protein) and knockout treated cells (fresh protein + pepstatin).

investigate their presence in cathepsin D- and L-deficient cells in more detail by using primary mouse embryonic fibroblasts from knockout and wild-type control mice as a model system. As shown before cells reacted upon AGE treatment; thus we hypothesized that it might be fatal for them if active cathepsin D or L is absent. Several laboratories have demonstrated that cathepsin D is elevated in cathepsin L-deficient systems. In mouse embryonic cathepsin L knockout fibroblasts increased amounts of cathepsin D were measured [54]. We were able to confirm this by an increase in cathepsin D activity in the same cellular model. However, as untreated cathepsin L

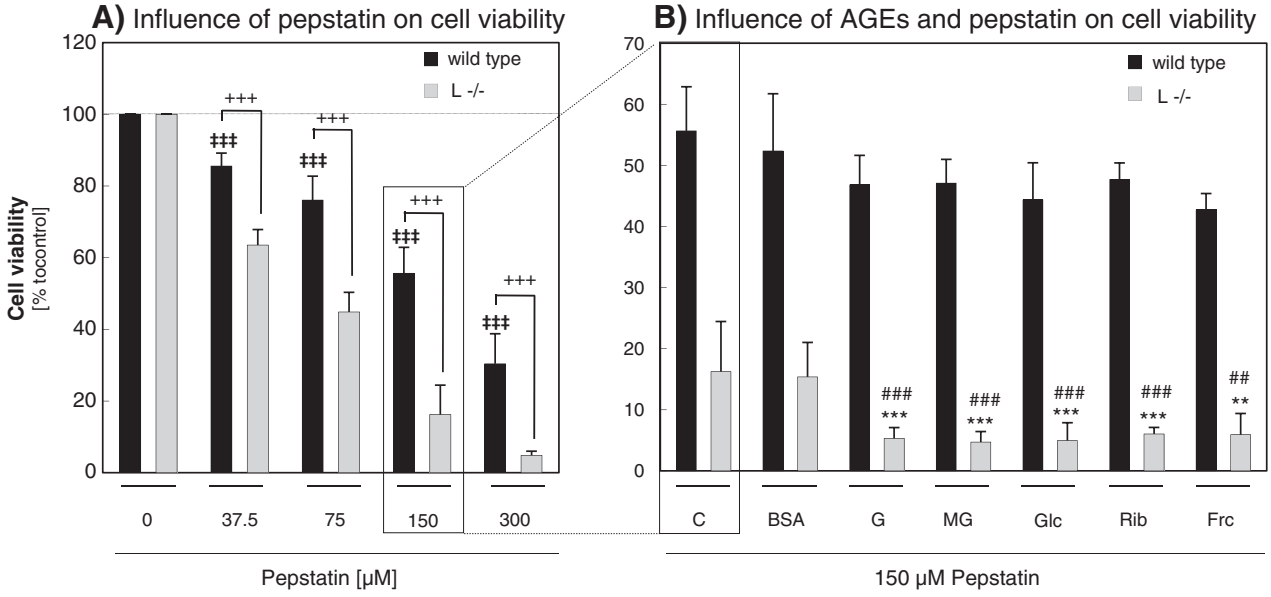

Fig. 7. Influence of various concentrations of pepstatin A on the viability of cathepsin L wild-type and knockout fibroblasts in the presence of AGEs. Cathepsin L-deficient fibroblasts as well as wild-type controls were incubated with various concentrations of pepstatin A for 24h. All samples received 100µM fresh BSA. (A) To investigate the influence on viability the cell medium was replaced with 0.17mg MTT/ml. After 2h the cells were solubilized and the optical density of the reaction product was measured at 590nm. Cells that were not treated with pepstatin A were set at 100% viable and the treated samples were calculated according to the 100% viable control cells. (B) The influence of fresh protein (C), incubated BSA (BSA), glyoxal (G)- and methylglyoxal (MG)-modified albumin, and glucose (Glc)-, ribose (Rib)-, and fructose (Frc)-modified albumin on the viability of pepstatin A-treated cathepsin L-deficient (L −/−) and wild-type fibroblasts. Cells were incubated with 150µM pepstatin A for 4h followed by addition of AGEs for 24h. Viability was measured as mentioned above. **$p<0.01$ and ***$p<0.001$; statistically significant difference between control (freshly dissolved BSA) and AGE-treated cells. ##$p<0.01$ and ###$p<0.001$; statistically significant difference between cells treated with incubated BSA and cells treated with AGEs. ‡‡‡$p<0.001$; statistically significant difference between wild-type control cells (fresh protein) and wild-type treated cells (fresh protein + pepstatin). +++$p<0.001$; statistically significant difference between wild-type and knockout cells.

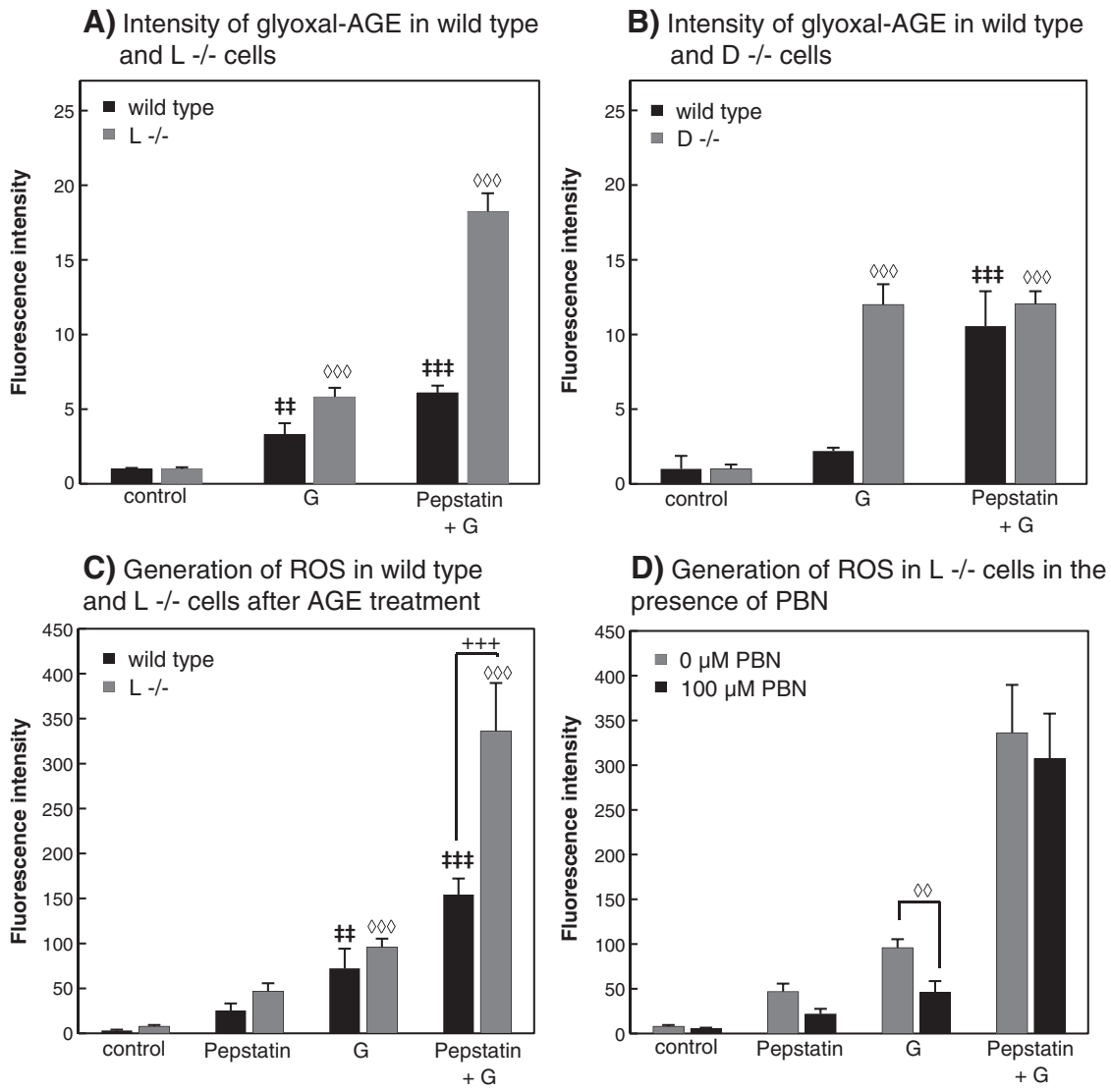

Fig. 8. Accumulation of AGEs dependent on functional active cathepsins L and D. (A and B) Wild-type and cathepsin L- and D-deficient cells were incubated with glyoxal-modified albumin with or without the presence of pepstatin A for 24h. Samples with pepstatin A were preincubated with the inhibitor for 4h before glyoxal-modified albumin (G) was added to the cell culture medium. Cells that were not treated with glyoxal-modified albumin received fresh protein. (A) shows the fluorescence intensity measured by fluorescence microscopy due to the autofluorescence of glyoxal-AGEs in wild-type and cathepsin L knockout (L −/−) fibroblasts. (B) shows the fluorescence intensity signals from wild-type and cathepsin D knockout (D −/−) fibroblasts. (C) ROS generation, measured by DCF fluorescence, after pepstatin and AGE treatment in wild-type and L −/− cells. (D) ROS generation in L −/− cells after pepstatin A and glyoxal-AGE treatment in the presence of PBN. ‡‡$p<0.01$ and ‡‡‡$p<0.001$; statistically significant difference between wild-type control cells (fresh protein) and wild-type treated cells (fresh protein + pepstatin and/or AGEs). +++$p<0.001$; statistically significant difference between wild-type and knockout cells. ◊◊$p<0.01$ and ◊◊◊$p<0.001$; statistically significant difference between knockout control cells (fresh protein) and knockout treated cells (fresh protein + pepstatin and/or AGEs or fresh protein + PBN).

knockout cells show increased cathepsin D activity, this could not be enhanced after AGE treatment. On the other hand, cathepsin L activity was decreased in cathepsin D knockout cells. Nevertheless, cathepsin L activity could be significantly increased in these knockout cells after AGE treatment. Similar to our findings, Saftig et al. have shown that in cathepsin D knockout animals bulk proteolysis was not impaired [55]. They assumed that the bulk proteolysis is maintained by the compensatory action of other lysosomal proteinases, for example, cysteine proteinases such as cathepsin B, L, S, or H [55]. We did not measure proteolysis, but we could show that the activity is increased after treatment with the potential substrate AGE for cathepsin L.

The important roles of cathepsins L and D in bulk proteolysis during aging are still under debate. It was found that cathepsin L activity is decreased in aged rat brain tissues [56]. In contrast, the cathepsin D level is significantly increased with aging. Nakanishi et al. assumed that increased cathepsin D levels are important in the degradation or modification of age-related proteins [57]. However, as it was shown that cathepsin L activity is decreased with aging, one can also hypothesize that this may contribute to the accumulation of abnormal proteins and protein deposition in age-related diseases. Indeed, alterations in protein turnover and protein degradation by lysosomes have been observed in many common diseases [58], for example, in neurodegenerative disorders such as Parkinson disease [59,60] and Alzheimer diseases [61] and additionally in many forms of cancer [62] and atherosclerosis [63]. To date it is not clear if the observed alterations (increased levels of certain cathepsins and reduced activities of other cathepsins) represent causes or secondary effects in these diseases. It is reported that α-synuclein, which accumulates in Lewy bodies in Parkinson disease, can be digested by cathepsin D [64]. It can be concluded that cathepsin D may be involved in abnormal protein processing in neurodegenerative diseases. We could also demonstrate that cathepsin D is able to degrade AGEs [33]. Indeed, increased cathepsin D activity was found in Huntington disease characterized by abnormal protein aggregation [65]. The important role of lysosomes has been reported previously. Headlam et al. have demonstrated that the activity of lysosomal cathepsins (cathepsins B and L)

is inhibited by hydroperoxide-containing oxidized proteins [66]. The group of Friguet reported a decreased proteolytic susceptibility of Nε-carboxymethyllysine-glycated glucose-6-phosphate dehydrogenase [67]. Decreased proteolytic activity due to glycated proteins is also described by Zeng et al. They demonstrated an inhibition of cathepsin B, L, and S activity in isolated form and in a macrophage cell line due to a loss of the cysteine residue that is located in the active site of the enzymes [68]. Compared to this, earlier work in our group demonstrated that AGEs exert some inhibitory effects on the proteasome and on lysosomal cathepsins [35]. Furthermore, the impairment of proteases due to advanced glycation end products also influences the turnover of other intracellular and extracellular proteins [35]. This reduction in intracellular protein turnover was also confirmed by Xiang et al. [69]. Interestingly, Sebekova et al. reported time- and dose-dependent decreased activities of cathepsins B, L, and H after incubation with AGEs. They were not able to demonstrate direct inhibitory effects of AGEs but they assumed that inhibition of cathepsin mRNA expression plays a role in decreased activities [70]. In contrast to this, we did not measure any inhibitory effects on mRNA levels. Our contradictory results may be explained by different AGE-modification grades, AGE concentrations, AGE incubation times, and cell lines. Moreover, it seems that the degradation of a complex mixture of AGE-modified proteins might be a multifactorial process involving several proteases and peptidases and probably other enzymes for non-protein moieties. Moheimani et al. demonstrated reduced proteasomal activity due to glycated proteins [71]. We were able to confirm this in our former work [33]. As both proteolytic pathways, the proteasome and the endosomal–lysosomal system, are involved in the catabolism of modified and damaged proteins, it is assumed that they act cooperatively [72,73]. Therefore the effects of glycated proteins on the lysosomal system may vary depending on the nature of the glycated proteins and conditions such as cell culture and other experimental design.

In conclusion, we demonstrate that both cathepsins D and L are necessary for the survival of cells exposed to AGE-modified proteins. In fact, this gives an insight into how the accumulation of AGEs and their toxic effects can be reduced. Further investigations should clarify the question if stimulation of cathepsins D and L, e.g., through expression, can reduce the accumulation of AGEs. This may be a basis for the development of new pharmaceutical products in the combat against neurodegenerative disorders.

Acknowledgment

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3.4 Publikation IV: Advanced glycation end products-induced formation of immunoproteasomes: involvement of RAGE and Jak/STAT

Advanced glycation end products-induced formation of immunoproteasomes: involvement of RAGE and Jak/STAT. Stefanie Grimm, Christiane Ott, Melanie Horlacher, Daniela Weber, Annika Höhn, Tilman Grune. Manuskript in Überarbeitung für *Biochemical Journal*, 2012

In dieser Studie wurde der Effekt der AGEs auf das proteasomale System untersucht. In der vorausgegangenen Publikation konnte gezeigt werden, dass die AGE-modifizierten Proteine keine Substrate für das 20S Proteasom darstellen. Mit Hilfe spezifischer Substrate für die proteasomalen katalytischen Untereinheiten konnte eine Reduktion aller Aktivitäten des konstitutiven Proteasoms durch AGE-modifiziertes Albumin nachgewiesen werden. Interessanterweise wurde während einer AGE-Exposition die Expression der konstitutiven Subtypen reduziert, während die induzierbaren Untereinheiten teilweise signifikant erhöht wurden. Die Induktion wurde über eine Phosphorylierung und somit über eine Aktivierung des STAT1-Transkriptionsfaktors eingeleitet. Im Gegensatz dazu konnte Resveratrol, das die Aktivierung des STAT1 inhibiert, die Induktion des Immunoproteasoms hemmen. Dieser Signalweg wird durch die Aktivierung des RAGE und nachfolgender Phosphorylierung von Jak2, gefolgt von STAT1, eingeleitet. Eine RAGE-siRNA und eine Inhibierung von Jak2 mittels AG-490 bestätigten diesen Signalweg. Diesbezüglich fanden nach AGE-Inkubation keine Phosphorylierung von Jak2 und keine Aktivierung von STAT1 statt und die Induktion des Immunoproteasoms wurde nicht beobachtet. Die Untersuchungen belegen erstmalig eine verstärkte Expression des Immunoproteasoms nach AGE-Inkubation unter Einbeziehung des RAGE und einer Signalkaskade über Jak/STAT.

Eigenanteil:

- Versuchsplanung, Zellkultur, Quantifizierung der proteasomalen Aktivität
- Anteilige Durchführung der Gen- und Proteinexpressionsuntersuchungen
- Durchführung aller Untersuchungen zu den Effekte der siRNA mittels Proteinexpressionsuntersuchungen
- Statistische Auswertung, Interpretation und Darstellung aller Ergebnisse, Verfassung des Manuskriptes

Advanced glycation end products-induced formation of immunoproteasomes: involvement of the receptor for AGEs and Jak/STAT

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Running head: Immunoproteasome induction via the receptor for AGEs (RAGE)

ABSTRACT

Advanced glycation end products (AGEs) accumulate in aging and several pathologies such as Alzheimer disease and diabetes. These protein products are known to inhibit proteolytic pathways. Moreover, AGEs are known to be involved in the activation of immune responses.

Here we demonstrate that AGEs induce the expression of immunoproteasomal subunits. To elucidate a molecular basis underlying the observed effects we were able to demonstrate an activation of the Jak/STAT pathway. The Jak2 inhibitor AG-490 reduced AGE-induced activation of the Jak/STAT pathway and the induction of immunoproteasomal subunits. Furthermore, silencing of the receptor for AGEs (RAGE) revealed that AGE-induced upregulation of the immunoproteasome is mediated by a RAGE signalling process. Additionally, resveratrol, a polyphenol which is well known to control inflammatory responses, has an influence on the STAT1 activation. Thus, we described for the first time that resveratrol is able to inhibit the induction of the immunoproteasome by blocking the Jak/STAT pathway.

K**eywords:** advanced glycation end products; immunoproteasome; RAGE; STAT1; Jak2; resveratrol

The abbreviations used are: AGEs, advanced glycation end products; I-κB, Inhibitor of κB; IFN-γ, Interferon-γ; Jak, Janus kinase; LMP2, low molecular weight of protein 2; LMP7, low molecular weight of protein 7; MECL1, multicatalytic endopeptidase complex-like 1; NF-κB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; RAGE, receptor for AGEs; STAT, signal transducers and activators of transcription

INTRODUCTION

Accumulation of advanced glycation end products (AGEs) in the brain is a feature of aging and neurodegeneration. Additionally, AGEs accumulate in pathological settings such as diabetes, renal failure and inflammatory disorders [1]. AGEs are formed through a series of non-enzymatic reaction between reducing groups of sugars and aldehydes with amino groups in proteins, lipids and nucleic acids [1]. This post-translational modification, also termed glycation or glycosylation, leads via Schiff base adducts and Amadori products through subsequent oxidations and dehydrations to a broad range of heterogeneous products. The final products called AGEs are capable of forming cross-links between proteins leading to protein deposition and amyloidosis [1]. Other pathological features of AGEs include the induction of oxidative stress, production of cytokines and cell death [2].

Misfolded, damaged or undesired proteins and polypeptides have to be degraded to prevent the accumulation of non-functional and potentially toxic proteins. The proteasome is reported to be one of the major enzymes involved in the degradation of damaged proteins and additionally, it plays a role in antigen presentation, apoptosis and cellular proliferation [3]. Proteasomes, which exist in several different molecular forms, are large proteolytic complexes in the cytosol and nucleus of almost all cells. The 20S proteasome is the core particle and includes the multi-enzymatic activity. In eukaryotes it is formed by four stacked heptametrical rings, each comprising α- or β-type subunits arranged in an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ stoichiometry [3]. Three of the seven different β subunits are proteolytically active. These are β1 (Y), β2 (Z) and β5 (X) which have specific cleavage preferences known as peptidyl-glutamyl-peptide hydrolyzing activity, trypsin- and chymotrypsin-like activities, respectively [3]. The α-subunits are highly conserved and involved in the formation of the proteasome structure as they interact with a variety of regulatory complexes such as PA700/19S or PA28/11S [3]. The different regulators execute various tasks including recognition, unfolding and translocation of the substrates, as well as supporting the correct assembly of the different subunits to the entire proteasome. The PA700/19S regulator is involved in the ubiquitin-mediated pathway, which is responsible for the degradation of most intracellular proteins. PA700/19S forms a complex with the 20S core particle to generate the 26S proteasome [3]. Ubiquitin is tagged to redundant proteins forming chains of several ubiquitin molecules. These chains are recognized by the 19S regulator. In addition, proteasomes can degrade proteins by ubiquitin-independent mechanisms [3].

Interferon-γ (IFN-γ) is able to activate macrophages and induce alterations in the subunit composition of proteasomes [4]. In this respect, the constitutive catalytic β subunits (β1, β2 and β5) are replaced by IFN-γ-inducible catalytic subunits LMP2 (β1i), MECL1 (β2i) and LMP7 (β5i). They assemble together with the IFN-γ-inducible PA28/11S regulator to form the immunoproteasome, with a distinct subunit composition and altered catalytic specificity. This leads to the assumption that the immunoproteasome plays an important role in immune recognition of infected host cells. Immunoproteasomes are long thought to be responsible for the liberation of peptides that are presented by MHC class I molecules on the cell surface [5]. However, current reports claim that the overall effect of immunoproteasomes is to simply alter the repertoire of generated peptides [6]. In this aspect a recent study suggests that the immunoproteasome may remove oxidized proteins that accumulate after IFN-induced oxidative stress [4]. Studies also report that the proteasomal activity is decreased and the immunoproteasome is increased in the aged brain [7]. It is also reported that immunoproteasome expression is increased in brain regions where senile plaques are present [8]. As senile plaques often contain AGEs, we raised the question whether AGEs are able to induce the expression of immunoproteasomal subunits.

AGEs are known to increase oxidative stress and inflammation through binding to the receptor for advanced glycation end products (RAGE) [9]. RAGE belongs to the immunoglobulin superfamily, consisting of three immunoglobulin-like domains (one

variable type and two constant type) in the N-terminal extracellular part, one transmembrane domain and a short C-terminal cytoplasmic trail that is essential for RAGE-mediated signal transduction [10]. The variable domain (V-domain) of RAGE is critical for binding of various ligands, including advanced glycation end products (AGEs) [10] and others such as high-mobility group box-1 (HMBG1)/amphoterin [11], S100 [12], Mac-1 [13] and amyloid-β [14]. It is well investigated that RAGE activation leads to a signalling pathway which induces NF-κB liberation from I-κB and NF-κB translocation to the nucleus leading to the transcription of pro-inflammatory factors.
In this report we provide data to support the conclusion that AGEs trigger an increase in mRNA and protein expression of immunoproteasomal subunits. Here we report for the first time that AGEs are able to induce the expression of the immunoproteasome via the activation of RAGE and downstream activation of Jak2/STAT1. As resveratrol, a phytoalexin found in grapes, berries, red wine and peanuts, is able to prevent the activation of STAT1 we were able to measure an inhibition in the induction of the immunoproteasome in presence of this plant substance.

EXPERIMENTAL

Materials

RPMI medium 1640, penicillin (10 000 E) / streptomycin (10 000 µg/ml), foetal bovine serum and PBS (pH 7.4) were purchased from Biochrom (Berlin, Germany). Mouse-Interferon-γ (IFN-γ) 10^6 U/ml was from Miltenyi Biotec (Bergisch Gladbach, Germany). The Jak2 inhibitor AG-490 was obtained from Enzo Life Sciences (Loerrach, Germany). Other chemicals were of the best grade available from Sigma-Aldrich (Taufkirchen, Germany) or from Carl Roth (Karlsruhe, Germany). The murine macrophage cell line RAW 264.7 was used for cell culture experiments.

Preparation of AGE-modified bovine serum albumin

AGEs were produced as described before [15]. Briefly, 1 mM of fatty acid-free, endotoxin-free BSA was dissolved in PBS with 250 mM of D-glucose (Glc), D-fructose (Fru), D-ribose (Rib) and 20 mM of glyoxal (G) and methylglyoxal (MG). Additionally, 1 mM of fatty acid-free, endotoxin-free BSA was prepared without sugars and aldehydes. These preparations were sterilized by ultra filtration and incubated at 37°C for 6 weeks (sugars and pure BSA) and 1 week (aldehydes). Oxygen was present, but not added throughout the incubation phase. Metal ions were just present in a very small amount (under 10 ppm) in sugars, aldehydes, albumin and PBS according to the certifications of the producer. To remove unbound sugars or aldehydes the preparations were dialysed against PBS over 24 h followed by measurement of protein content with the Bradford assay in order to verify the BSA-concentration of 1 mM. To confirm AGE-formations, the preparations were determined for their optical density and fluorescence [15]. In this work we used the same advanced glycation end products that were characterized for their optical density, fluorescence intensity, protein cross-linking and protein oxidation as described before [15].

Cell culture

The RAW 264.7 cell line was grown in RPMI 1640 culture medium supplemented with 10 % foetal bovine serum and 1 % penicillin / streptomycin. Cells were kept at 37°C in 5 % CO_2 and 95 % humidity. Cells were passed twice weekly when reaching 80% confluence. For cell culture experiments cells were plated at a density of $2*10^5$ in RPMI culture medium with reduced foetal bovine serum (FBS) (2 %) for three days. After three days AGEs were delivered to the cells in reduced FBS-medium for 72 hours. The final concentrations of AGEs used in these studies were albumin-modified with 25 mM of the indicated sugar or 2 mM of the aldehyde. The final concentration of modified albumin was

100 µM. In order to apply the same protein concentration to each sample, we used 100 µM 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 before [15]. In some experiments IFN-γ was added to the cells in a final concentration of 250 U/ml for 72 hours as a positive control. Trans-resveratrol (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in DMSO in a concentration of 22 mM and diluted with medium to a final concentration of 5 µM and 10 µM (maximal concentration of DMSO was 0.05%). Indicated samples were treated with resveratrol 48 hours before and during AGE- or IFN-γ stimulation. The activity of Jak2 and thus the activation of STAT1 were inhibited by AG-490. AG-490 was dissolved in DMSO and further diluted in medium to a final DMSO concentration of <0.1%. Cells were treated with 10 µM or 30 µM of AG-490 1 h before they were stimulated with AGE-modified albumin or IFN-γ.

Preparation of cell lysates and measurement of proteasomal and immunoproteasomal activity

For the determination of proteasomal activity and the preparation of cell lysates for immunoblot, $2*10^6$ cells were harvested, centrifuged for 5 min at 900 x g and the cell pellet was resuspended in lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM magnesium chloride, 1 mM EDTA and 1.7 mM DTT). Cells were lysed using a syringe, followed by repeated freeze-thaw cycle. Cell lysates were used for the determination of protein content with Bradford assay, the measurement of proteasomal activity and the expression of proteins.

For proteasomal activity cell lysates were 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.

The substrates for the constitutively expressed proteasome and the immunoproteasome were chosen according to Blackburn et al. [16]. The substrates were obtained from AAT Bioquest (Sunnyvale, USA) to monitor the activity of the constitutive proteasome (c) and immunoproteasome (i): β1c, Z-LLE-R110; β2c/ β2i, Ac-KQL-R110; β5c, Ac-WLA-R110; β1i, Ac-PAL-R110; β5i, Ac-ANW-R110. R110 liberation was measured using a fluorescence reader at 485 nm excitation and 528 nm emission during 30 min at 37°C. Free R110 (rhodamine 110) was used as standards for quantification.

Immunoblot analysis

To detect the influence on protein expression $2*10^6$ control or treated cells were harvested, lysed and protein concentration was measured as described above. 20 to 50 µg protein extract was subjected in Laemmli buffer to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were transferred to nitrocellulose transfer membranes (Whatmann, Dassel, Germany). Blots were blocked in 5 % non-fat milk/PBS buffer at room temperature for one hour. Before detection of phosphorylated proteins, blots have been blocked in Odyssey® blocking buffer (LI-COR Biosciences, Bad Homburg, Germany) at 4 °C over night. Blocking was followed by incubation with primary antibodies that were diluted according to the recommendations of the manufacturers under constant agitation for another hour. The following primary antibodies were used: anti-20S proteasome (Enzo Life Sciences, Loerrach, Germany), anti-α subunits (Enzo Life Sciences, Loerrach, Germany), anti-β1 20S proteasome (Santa Cruz Biotechnology, Santa Cruz, USA), anti-β2 20S proteasome (Enzo Life Sciences, Loerrach, Germany), anti-β5 PSMB5 (Abnova, Taipei City, Taiwan), anti-β1i proteasome 20S LMP2 (Abcam, Cambridge, USA), anti-β2i MECL-1 (Santa Cruz Biotechnology, Santa Cruz, USA), anti-β5i proteasome 20S LMP7 (Abcam, Cambridge, USA), anti-11Sα PSME1 (Abcam, Cambridge, USA), anti-pSTAT1(Tyr701) (Cell Signaling Technology, Danver,

USA), anti-STAT1 (Cell Signaling Technology, Danver, USA), anti-pJak2(Tyr1007/1008) (Cell Signaling Technology, Danver, USA), anti-Jak2(D2E12) (Cell Signaling Technology, Danver, USA), anti-RAGE (Abcam, Cambridge, USA) and anti-glyceraldehyd-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, USA). After having washed three times, the blots have been incubated with the appropriate secondary antibody: goat anti-rabbit IRDye® 800CW conjugated or donkey anti-mouse IRDye® 680LT conjugated (LI-COR Biosciences, Bad Homburg, Germany) for one hour. Blots were washed and analyzed for their near-infrared fluorescence signals with Odyssey® infrared imaging system (LI-COR Biosciences, Bad Homburg, Germany).

Quantitative PCR

Total RNAs were extracted from cells using RNeasy Mini Kit as described by the manufacturer (Qiagen, Hilden, Germany) and RNA integrity was checked with Agilent RNA 6000 nano Kit (Agilent Technologies, Boeblingen, Germany). 2 µg of total RNA was transcribed into cDNA using oligo dT_{12-18} primer, superscript™ II reverse transcriptase and 10 mM dNTP-Mix from Invitrogen (Darmstadt, Germany) according to the manufacturer's recommendations. A standard real-time PCR with a total volume of 25 µl was performed with 1 µl of the reverse transcriptase reaction mixture in 35 cycles, each with 95°C for 30 s, followed by 55°C for 30 s, continued with 72°C for 30 s. The reaction mixture contained SYBR Green Supermix (Qiagen, Hilden, Germany) and 10 pmol of the specific primers. The thermal cycler for quantitative real-time PCR was the iCycler IQ Multicolor Real Time PCR Detection System (Biorad, Munich, Germany). The primers were synthesized by biomers.net (Ulm, Germany) according to the following sequences:
GAPDH 5’ AAC CTG CCA AGT ATG ATG ACA 3’ and 5’ CAT ACC AGG AAA TGA GCT TGA 3’, 18s rRNA 5’ ATT AAG TCC CTG CCC TTT GTA 3’ and 5’ AGT CAA GTT CGA CCG TCT TCT 3’, actin-β 5’ TTC CTT CTT GGG TAT GGA ATC 3’ and 5’ GCT AGG AGC CAG AGC AGT AAT 3’, RAGE 5’ CAC AGC CAG TGT CCC TAA TAA 3’ and 5’ CTC TGA CCG CAG TGT AAA GAG 3’, β1i (LMP2) 5’ GCT AAT TCG ACA GCC CTT TAC 3’ and 5’ ACT AGA GCC ATC TCG GTT CAT 3’, 11Sα (PA28) 5’ AAG CAA CAG GAG AAG GAA GAG 3’ and 5’GTA GCT GCA ACC AGG TAG TGA 3’.
The GAPDH PCR product was 192 bp, 18s rRNA was 134 bp and actin-β was 178 bp long. The RAGE PCR product was 175 bp, β1i (LMP2) was 154 bp and 11Sα (PA28) was 178 bp long. Quantification of gene expression was based on the cycle threshold (Ct) value for each sample considering PCR efficiency using the relative expression software tool REST 2008 (Corbett Life Science, Qiagen, Hilden, Germany). Three different housekeeping genes were used. All experiments included negative controls consisting of no cDNA for each primer pair.

RNA interference

Cells were transfected with Accell SMARTpool AGER(11596) or Accell non-targeting pool (Thermo Scientific/Dharmacon, Epsom, UK) utilizing the Accell siRNA delivery protocol. RAW 264.7 cells were plated at a density of $2*10^5$ cells in RPMI medium with 10 % foetal bovine serum. The next day medium was replaced with 3 ml of Accell delivery medium containing 1 µM siRNA and cells were incubated for 24 h, 48 h or 72 h at 37°C. After 72 h, cells were stimulated with AGEs or IFN-γ for another 72 h at 37°C.

Statistical analysis

All experiments were performed at least in triplicate. The statistical analysis of the data was performed using one-way ANOVAs followed by the post hoc Tukey´s significant difference test. Data are presented as mean ± SD and statistical analysis was performed using GraphPad Prism® version 5.

RESULTS

Proteasomal activity and the expression of proteasomal subunits

In order to determine the effect of AGEs on the proteasomal activity in RAW264.7 cells we used specific proteasomal substrates. The samples used in the following studies include control samples (with freshly dissolved BSA), BSA samples (with 6 weeks incubated BSA), glyoxal (G)-, methylglyoxal (MG)-, glucose (Glc)-, ribose (Rib)- and fructose (Fru)-modified albumin. As the incubated BSA showed some characteristics of modification and oxidation due to traces of oxygen, aldehydes, metals and sugars in SDS-PAGE and autofluorescence analysis, we calculated our results in comparison to fresh BSA and to our control samples. We included the incubated BSA probe (BSA) in our experiments to demonstrate that the observed effects were exerted largely due to advanced glycation end products. A significant decrease in the constitutive proteasomal activity was measured for all AGEs and all catalytic subunits after 72 hours compared to control and BSA samples (Fig. 1A). To investigate whether the decreased activity is due to a reduction in proteasomal subunit expression, we quantified the relative expression of the total 20S proteasome by a polyclonal antibody (Fig. 1B) and the expression of α-subunits (Fig. 1C). Interestingly, both analyses showed a significant increase in protein expression. It can be assumed that decreased proteasomal activity is compensated by increased immunoproteasomal activity [17]. Therefore, we studied immunoproteasomal activity after incubation with AGE-modified proteins (Fig. 1D). To date, specific substrates are available for β1 and β5 of the immunoproteasome. Both substrates demonstrated an increase in its catalytic activity.

In order to reveal if the influence on proteasomal activity is obvious on protein level, we studied the expression of the catalytic subunits by immunoblotting. AGE-modified proteins significantly reduce all three constitutive proteasomal subunits (β1, β2 and β5) (Fig. 2A-2C). On the other hand, immunoblotting of the same cell lysates displayed 20 to 40 % increase in the catalytic immunoproteasomal subunits β1i, β2i and β5i (Fig. 2D-2F). Additionally, the cell lysates demonstrated an increase (50 - 100%) in the regulator of the immunoproteasome 11Sα compared to untreated cells (Fig. 3). These results indicate that within 72 hours of macrophage-stimulation by AGEs, the immunoproteasome is strongly induced.

Effect of AGEs on the phosphorylation of STAT1 and Jak2

Next we decided to investigate the pathway of immunoproteasome induction. Activation of Jak/STAT has been associated with inflammatory-related stimulation and immunoproteasome induction [18]. The transcriptional activity of STAT1 requires phosphorylation at Tyr701 residue by Jak2 [19]. Thus, we examined whether AGEs activate the Jak/STAT pathway in RAW 264.7 cells. It is well characterized that Jak2 and STAT1 are involved in IFN-γ signalling, therefore, we used INF-γ as a positive control (demonstrated by the inserted diagram in both panels of Fig.4). Immunoblot analysis showed significantly enhanced phosphorylated STAT1(Tyr701) and Jak2(Tyr1007/1008) after 72 hours of AGE-stimulation compared to unstimulated cells (Fig. 4A and 4B). Equal amounts of STAT1 and Jak2 were measured in all conditions, indicating that differences detected with the phosphotyrosine-specific antibodies (pSTAT1 and pJak2) were not due to differences in the amount of total STAT1 and Jak2 loaded in each lane.

Expression and role of RAGE in AGE-treated macrophages

Consequently, this led to the following question: which proteins or receptors upstream of Jak/STAT are involved. One possible receptor is the receptor of advanced glycation end products (RAGE) that is believed to play a role in several signalling pathways [9]. Therefore, we tested the presence and the amount of RAGE after AGE stimulation.

RAW264.7 cells have been incubated with AGEs for 72 hours followed by quantitative real time-PCR (qPCR) as well as immunoblot analysis. PCR revealed that RAGE-mRNA was significantly increased after 72 hours of AGE-incubation (Fig. 5A). Similar results were found in immunoblotting assays showing a significantly higher RAGE protein expression (Fig. 5B) due to AGE stimulation. This raises the question on a mechanistically involvement of RAGE in immunoproteasome induction. Therefore, we decided to use siRNA for the reduction of the RAGE amount in RAW cells. A time dependent decline of RAGE expression was measured after 24, 48 and 72 hours incubation with siRNA (Fig. 5C). The samples were compared to untreated controls and after 72 hours only 5 % of RAGE-expression was left (Fig. 5C). Therefore, the use of the siRNAs resulted in markedly reduced RAGE expression. To control for off-target effects of siRNA, a separate sample was used with a scramble siRNA (non-target siRNA, NTC) as a negative control. No influence on RAGE expression was detected after 72 hours of incubation with the non-targeting siRNA-pool.

RAGE silencing was able to prevent Jak2 and STAT1 phosphorylation after glyoxal-modified albumin treatment as shown in Fig. 6A and 6B. Most interestingly, no difference upon RAGE siRNA was detected in the activation of these signalling molecules by IFN-γ. Additionally, no influence on Jak2 and STAT1 was determined in the treatment with no-target control siRNA (NTC) and siRNA itself. The differences detected with the phosphotyrosine-specific antibodies (pSTAT1 and pJak2) were not due to differences in the amount of total STAT1 and Jak2 in each lane as equal amounts of STAT1 and Jak2 were detected for all samples.

Concurring to these results, the expression of β1i and 11Sα after stimulation with glyoxal-modified albumin was significantly reduced in the RAGE siRNA treated samples (Fig. 6C and 6D). In contrast, IFN-γ-treated cells with or without siRNA pre-treatment displayed no differences on the protein expression of β1i and 11Sα. In both samples the protein expression of the immunoproteasomal subunits was significantly increased. Again, the NTC-siRNA and RAGE-siRNA showed no interference in the protein expressions of β1i and 11Sα. This let us to the conclusion that the AGE-mediated pathway of immunoproteasome induction and the IFN-γ-mediated pathway are different, at least upstream of Jak/STAT.

Effect of Jak2 inhibitor and resveratrol on the induction of immunoproteasome

It was established that AG-490 acts as a Jak-specific inhibitor [20]. Thus we used 10 μM and 30 μM of AG-490, as no effect on cell viability was observed (data not shown). Treatment with AG-490 significantly reduces STAT1 activation (Fig. 7A). As STAT1 is a transcription factor for the immunoproteasomal subunits [18], further experiments should reveal if the induction of the immunoproteasome is influenced due to Jak2/STAT1 inhibition. Indeed, β1i mRNA was significantly reduced in glyoxal-AGE- and IFN-γ treated cells in the presence of AG-490 (Fig. 8A). The same effect was observed in the β1i-protein expression rate (Fig. 8C). To confirm that AG-490 reduces the expression of immunoproteasomal subunits, we decided to investigate the regulator 11Sα in addition to β1i. In Fig. 3 we demonstrated that IFN-γ or AGEs stimulate the expression of 11Sα. Fig. 8B shows that IFN-γ and glyoxal- or glucose-modified albumin induce the mRNA of 11Sα whereas both concentrations of AG-490 reduced the increased mRNA-expression. Immunoblot analysis verified the enhanced 11Sα-expression due to IFN-γ and AGEs. Also in this case we measure an inhibition effect of AG-490 on the induction of 11Sα (Fig. 8D).

Resveratrol is another substrate to inhibit the Jak/STAT signalling pathway. There exists multifold evidence that resveratrol (trans-3,4',5-trihydroxystilbene) reduces inflammatory responses, possibly also by blocking the Jak/STAT cascade [21]. Therefore, we decided to test the possible effect of resveratrol on immunoproteasome induction by AGEs. RAW264.7 cells were treated with increasing concentrations of resveratrol (0-50 μM) and

cell viability was tested (data not shown). Concentrations of 20 μM resveratrol or higher resulted in a reduced cell viability after 72 hours of incubation, therefore, we decided to use final concentrations of 5 μM and 10 μM resveratrol. For both concentrations we measured significant reduced phosphorylation of STAT1 in glyoxal-AGE, glucose-AGE and IFN-γ-stimulated cells (Fig. 7B). As resveratrol reduces the phosphorylation of STAT1, we also tested if this has an effect on the expression of the immunoproteasome. Quantitative PCR and immunoblot analysis verified reduce β1i- and 11Sα-expression in glyoxal-AGE-, glucose-AGE- and IFN-γ-treated cells in the presence of resveratrol (Fig. 9A-D).

DISCUSSION

In order to determine the effect of AGEs on the inflammation status of macrophages, we performed experiments using the macrophage cell line RAW264.7 and sugar- or aldehyde-modified albumin. First experiments were performed with different concentrations of AGEs and different incubation times (4-96 h) (data not shown). Cell viability is reduced by higher AGE concentrations and longer incubation times. As AGEs are known to be formed over a longer period of time the effect of chronic activation would be more interesting. Thus we incubated the cells for 72 hours where no effect on cell viability or proliferation was observed (data not shown).

RAW 264.7 macrophage activation by AGEs leads to reduced constitutive proteasomal activity and slightly reduced constitutive proteasomal subunits, but increased immunoproteasomal components (Fig.1 and 2). Moheimani et al. investigated the effect of AGE-modified proteins on the proteasomal function in cell extracts of J774A.1 macrophages [22]. Similar to our work they show that the proteasomal activity is decreased. A possible explanation for reduced proteasomal activity is reported by Rivett *et al.* [23]. They theorized that reduced proteolytic activity is due to reduced proteolytic capacity of the immunoproteasome for the degradation of ubiquitin-tagged and oxidized, aggregated proteins [23]. Impaired proteasomal activity has also been reported in various biological phenomena including aging. Chondrogianni *et al.* have recently shown that a down-regulation of β-subunits is responsible for the decreased proteasomal activity in senescent human fibroblasts [24]. Beyond this, there are unequivocal evidences that proteasomal activity is decreased in Alzheimer´s disease brain. For example, Gregori *et al.* indicated that amyloid-β inhibits proteasomal activity [25]. Therefore, normal and damaged proteins are less degraded as a consequence of reduced proteasomal activity. Moreover, inhibitors of proteasomal activity have been found to impede the degradation of signalling molecules such as STAT1, Jaks and cytokine receptors [26;27]. These studies have determined that signalling activity is prolonged due to less proteasomal activity. It has been reported by several laboratories that LMP2-overexpression (β1i) reduces the caspase-like activity (β1) of the proteasome. Additionally, incorporation of LMP2 (β1i) led to a slight increase in the chymotrypsin-like activity [28]. Thus the replacement of β1 with LMP2 leads to a shift in proteolytic activities. It is generally assumed that the induction of the immunoproteasome serves to generate a different spectrum of peptide products and to alter the kinetic behaviour of the proteasomal system. Transfection of the immunoproteasome regulator PA28α (11Sα) alone has been reported to enhance class-I presentation of some antigens [29].

IFN-γ increases the level of immunoproteasomes [30]. Thus we used IFN-γ as a positive control in our studies. Our results show for the first time that the immunoproteasome is induced due to AGEs via the RAGE signalling pathway. siRNA treatment revealed that AGEs induce the activation of Jak2 and STAT1 via RAGE, whereas we could not measure any inhibitory effect on IFN-γ-induced Jak2 and STAT1 activation. Subunits of the immunoproteasome have also been reported to be induced by heat stress [31], nitric oxide

[32] or in neurodegenerative diseases [33]. Consequently the expression of the immunoproteasome may be initiated due to diverse pathological situations, supporting more functions of the immunoproteasome apart from antigen processing.
The accumulation of RAGE ligands has been shown to amplify inflammation in diabetic animal models [34]. It is generally accepted that AGE-accumulation contributes to an extensive inflammation status due to RAGE stimulation [35]. Consequently, it is of great interest to find out how plant substances with anti-inflammatory potential interact with the different inflammation pathways. Marambaud *et al.* demonstrated that resveratrol promotes the proteolytic degradation of amyloid-β by a proteasome-involved mechanism in the two different cell lines HEK293 and N2a that were transfected with human amyloid-β precursor protein (APP_{695}) [36]. Amyloid-β is reported to be a RAGE-ligand, therefore, it can be assumed that amyloid-β has an influence on the immunoproteasome/proteasome ratio and, therefore, on the proteolytic capacity of cells. On the other hand, resveratrol can probably be involved in modified proteasomal composition in direction of inhibiting immunoproteasome-induction and supporting the build-up of the constitutive proteasome.
RAGE receptors are predominantly studied for their role in inflammation, atherosclerosis, diabetes, cancer and neurodegenerative diseases [37-41]. Binding of AGEs to RAGE initiates cellular signals that activate NF-κB, which results in the transcription of diverse proinflammatory cytokines. The RAGE promoter contains NF-κB binding sites that are involved in the regulation of RAGE expression [42]. Therefore, activation of NF-κB results in increased RAGE expression and prolonging NF-κB activation. We demonstrated that AGEs enhance the expression of RAGE (Fig. 5A and 5B). Thus, sustained RAGE expression leads to chronic activation of inflammation. Binding of ligands to RAGE activates ERK1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases in monocytes, macrophages and tumour cells [43-46]. Furthermore, rho-GTPases and phosphoinositol-3-kinase are implicated in RAGE signalling [47]. To date, the only pathway in which RAGE induces the activation of Jak/STAT is described by Huang et al. [48] and Shaw et al. [49]. Huang et al. revealed that AGEs induce collagen production in fibroblasts via binding to RAGE and activation of Jak/STAT [48]. Shaw et al. observed that incubation of vascular smooth muscle cells with the RAGE ligand S100B induces tyrosine phosphorylation of Jak2 which was still maintained after 24 h [49]. This study reveals that the activation of Jak2 via RAGE induces a longer lasting signalling response. The Jak/STAT pathway is known to be primarily involved in the regulation of growth and development as well as inflammation. Different external ligands bind on receptors and induce the activation of the Jak/STAT pathway. Ligands are mainly cytokines, such as interferon, but the pathway may also be activated by different receptor tyrosine kinases, e.g. epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), non-receptor tyrosine kinases and G-protein coupled receptors (GPCR). Cell-surface receptors act by phosphorylation of STATs following by their dissociation from the membrane and dimerization before entering the nucleus. STAT1 is reported to bind to gamma activating sequences (GAS) in order to up-regulate expression of diverse inflammatory proteins such as LMP2 [18]. The signalling cascades leading to the induction of the immunoproteasome that are involving the IFN-γ-receptor or RAGE are depicted in Fig. 10.
Resveratrol can attenuate the activation of immune cells and the synthesis of pro-inflammatory mediators through the inhibition of the transcription factors NF-κB and activator protein-1 (AP-1) [50]. Recently, it was found that resveratrol inhibits the activation of Jak2 and STAT1 [21]. To determine whether resveratrol affects the induction of the immunoproteasome, we monitored the levels of β1i and 11Sα by quantitative PCR and immunoblot. Resveratrol, as an inhibitor of STAT phosphorylation, can interrupt this investigated pathway, leading to decrease immunoproteasomal expression. It is very

interesting that also relatively low concentrations of resveratrol can have a very pronounced effect on the immunoproteasomal induction.
Summarizing, malfunction of the proteasome-ubiquitin system might lead to potentially pathogenic protein aggregation, a hallmark for a variety of neurodegenerative and other inflammatory disorders. These protein aggregates such as aggregated AGE-modified proteins trigger chronic inflammatory response that might affect expression of many molecules such as cytokines and e.g. immunoproteasomal subunits. Such response can ultimately influence the development and/or clinical severity of multiple neurodegenerative diseases. As AGEs are known to induce chronic inflammation it is of great relevance to find substances that are able to ameliorate tissue-associated diseases that are triggered by AGEs. Indeed, resveratrol treatment significantly reduces immunoproteasome expression. To sum up we were able to demonstrate a new role for the anti-inflammatory role of resveratrol: the inhibition of the immunoproteasome induction. These observations lead to the overall conclusion that resveratrol may reduce pathological progression in models of inflammatory diseases.

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FIGURE LEGENDS

FIGURE 1 Influence of AGE-modified proteins on the 20S proteasome

(A) Constitutive proteasomal activity in RAW264.7 macrophages after 72 hours of AGE-incubation (fresh protein (control, C), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, glucose (Glc)-, ribose (Rib)- or fructose (Fru)-modified albumin). Cells were harvested and lysates were determined for their peptidyl-glutamyl-peptide hydrolyzing (β1c) activity, for their trypsin-like (β2c/β2i) and chymotrypsin-like (β5c) activity. R110 liberation from fluorogenic substrates was calculated according to a R110 standard curve. Relative protein expression of the 20S proteasome (**B**) and α-subunits (**C**) was measured in cell lysates after 72 hours of incubation. One representative blot is displayed under each respective diagram. **(D)** Immunoproteasomal activity after 72 hours of AGE-incubation. The lysates were determined for their β1i and β5i activity from R110 liberation of the specific immunoproteasomal substrates.

Controls (C) were set to 100 % and the treated cells were counted referred to the control sample. The statistical significance between control and AGE-treated cells is indicated as *($p<0.05$), **($p<0.01$) and ***($p<0.001$) and the statistical significance between cells treated with incubated BSA and AGEs is demonstrated as #($p<0.05$), ##($p<0.01$) and ###($p<0.001$).

FIGURE 2 Proteasomal β-subunit expression after AGE treatment in RAW cells

Relative protein expression of β1 (**A**), β2 (**B**), β5 (**C**), β1i (**D**), β2i (**E**), β5i (**F**) was determined in cell lysates after 72 hours of incubation (fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, glucose (Glc)-, ribose (Rib)- or fructose (Fru)-modified albumin). One representative blot is displayed below each diagram. The inserted diagram in panel D displays the positive control IFN-γ for its induction of β1i compared to control cells. Controls were set to 100 % and the other cells were counted referred to the control sample. The statistical significance between control and AGE-treated cells is indicated as *($p<0.05$), **($p<0.01$) and ***($p<0.001$) and the statistical significance between cells treated with incubated BSA and AGEs is demonstrated as #($p<0.05$), ##($p<0.01$) and ###($p<0.001$).

FIGURE 3 Expression of 11Sα during AGE treatment

The relative amount of 11Sα was determined in cell lysates after stimulation with AGEs for 72 hours of incubation (fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, glucose (Glc)-, ribose (Rib)- or fructose (Fru)-modified albumin). One representative blot is displayed under the diagram. The inserted diagram demonstrates the positive control IFN-γ for its induction of 11Sα compared to control cells. The statistical significance between control and AGE- or IFN-γ-treated cells is indicated as **($p<0.01$) and ***($p<0.001$) and the statistical significance between cells treated with incubated BSA and AGEs is demonstrated as #($p<0.05$) and ###($p<0.001$).

FIGURE 4 AGE-induced activation of the Jak/STAT pathway

Phosphorylated STAT1 (**A**) and phosphorylated Jak2 (**B**) were quantified after stimulation with AGEs (fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, glucose (Glc)-, ribose (Rib)- or fructose (Fru)-modified albumin) for 72 hours. The inserted diagram in panel A demonstrates the phosphorylation of STAT1

after IFN-γ stimulation whereas the inserted graph in panel B shows the phosphorylation of Jak2 due to IFN-γ activation. The statistical significance between control and AGE- or IFN-γ-treated cells is indicated as *($p<0.05$), **($p<0.01$) and ***($p<0.001$) and the statistical significance between cells treated with incubated BSA and AGEs is demonstrated as #($p<0.05$), ##($p<0.01$) and ###($p<0.001$).

FIGURE 5 Expression of RAGE in RAW cells during AGE- and siRNA-treatment
Quantification of relative mRNA amounts of the RAGE receptor after 72 hours of AGE-incubation (fresh protein (control), incubated BSA (BSA), glyoxal (G)- or methylglyoxal (MG)-modified albumin, glucose (Glc)-, ribose (Rib)- or fructose (Fru)-modified albumin) was determined by qPCR (**A**) and relative protein expression of RAGE under the same conditions determined by immunoblot (**B**). Controls were set to 100 % and the other cells were counted referred to the control sample. The statistical significance between control and AGE-treated cells is indicated as **($p<0.01$) and ***($p<0.001$) and the statistical significance between cells treated with incubated BSA and AGEs is demonstrated as ##($p<0.01$) and ###($p<0.001$).
Time-dependent silencing of RAGE was determined by immunoblot (**C**). Relative protein expression after siRNA-treatment was calculated to each control (C, 100 %) and a non-target control siRNA (NTC) was used over a time period of 72 hours and calculated to the 72 hour-control sample.

FIGURE 6 Influence of RAGE siRNA on the Jak-STAT pathway and the induction of the immunoproteasome
Relative protein phosphorylation or protein expression after siRNA-treatment and stimulation with glyoxal-modified albumin (G) and IFN-γ was measured for pJak2 (**A**), pSTAT1 (**B**), β1i (**C**) and 11Sα (**D**). No-target siRNA control (NTC) was also tested in each experiment. Cells have been incubated with siRNA for 48 hours, followed by the addition of glyoxal-AGE (G) or IFN-γ for further 72 hours. Controls were set to 100 % and the other probes were counted referred to the control sample. The statistical significance between control and AGE-or IFN-γ-treated cells is indicated as *($p<0.05$), **($p<0.01$) and ***($p<0.001$). Furthermore, statistical significance between stimulated cells with and without siRNA is indicated as +++($p<0.001$).

FIGURE 7 Influence of the Jak2 inhibitor AG-490 and resveratrol on the phosphorylation of STAT1
RAW 264.7 macrophages have been incubated with AG-490 (10 µM or 30 µM) for one hour or resveratrol (Resv, 5 µM or 10 µM) for 48 hours, followed by the stimulation with glyoxal (G)-modified albumin, glucose (Glc)-modified albumin or IFN-γ. Untreated cells were set to 100 % and the other samples were calculated according to the 100 % control sample. The statistical significance between control and AGE- or IFN-γ-treated cells is indicated as *($p<0.05$), **($p<0.01$) and ***($p<0.001$) and the statistical significance between stimulated cells with and without AG-490 or resveratrol is demonstrated as ++($p<0.01$) and +++($p<0.001$).

FIGURE 8 AG-490 inhibits the activation of STAT1 and the induction of the immunoproteasome
The influence of AG-490 on the mRNA expression (**A**) and protein expression (**C**) of β1i and the mRNA expression (**B**) and protein expression (**D**) of 11Sα is demonstrated. Cells have been incubated with AG-490 for 1 hour. Subsequently, cells have been stimulated with glyoxal (G)-modified albumin, glucose (Glc)-modified albumin or IFN-γ. Untreated cells were set to 100 % and the other probes were calculated according to the untreated sample. The statistical significance between control and AGE- or IFN-γ-treated cells is

indicated as **($p<0.01$) and *** $p<0.001$) and the statistical significance between stimulated cells with and without AG-490 is demonstrated as ++($p<0.01$) and +++($p<0.001$).

FIGURE 9 Resveratrol inhibits AGE induced expression of the immunoproteasome
The influence of resveratrol (Resv) on the mRNA expression (**A**) and protein expression (**C**) of β1i and on the mRNA expression (**B**) and protein expression (**D**) of 11Sα is shown. Cells have been incubated with resveratrol for 48 hours. Subsequently, cells have been stimulated with glyoxal (G)-modified albumin, glucose (Glc)-modified albumin or IFN-γ. Untreated cells were set to 100 % and the other probes were calculated according to the untreated sample. The statistical significance between control and AGE- or IFN-γ-treated cells is indicated as **($p<0.01$) and ***($p<0.001$) and the statistical significance between stimulated cells with and without resveratrol is demonstrated as ++($p<0.01$) and +++($p<0.001$).

FIGURE 10 IFN-γ- and RAGE-signalling and immunoproteasome induction
Binding of IFN-γ results in interferon-γ-receptor (IFNGR 1 + 2) oligomerization followed by phosphorylation of Jak1 or Jak2. Phosphorylated, and therefore activated Jaks phosphorylate receptor tyrosine residues, which then serve as docking sites for STAT1. Once recruited to the receptor STAT1 become phosphorylated by Jaks. Phosphorylated STAT1 homodimerizes and binds together with interferon regulatory factor-1 (IRF-1) to members of the GAS (gamma activated sequence) family of enhancers and IRF-1 to interferon-stimulated response element (ISRE). Binding of AGEs on RAGE activates Jak2 which induces the phosphorylation of STAT1. In addition, the phosphorylation of STAT1 by Jak1/2 can be inhibited by resveratrol independent of the upstream signalling pathway.

FIGURE 1

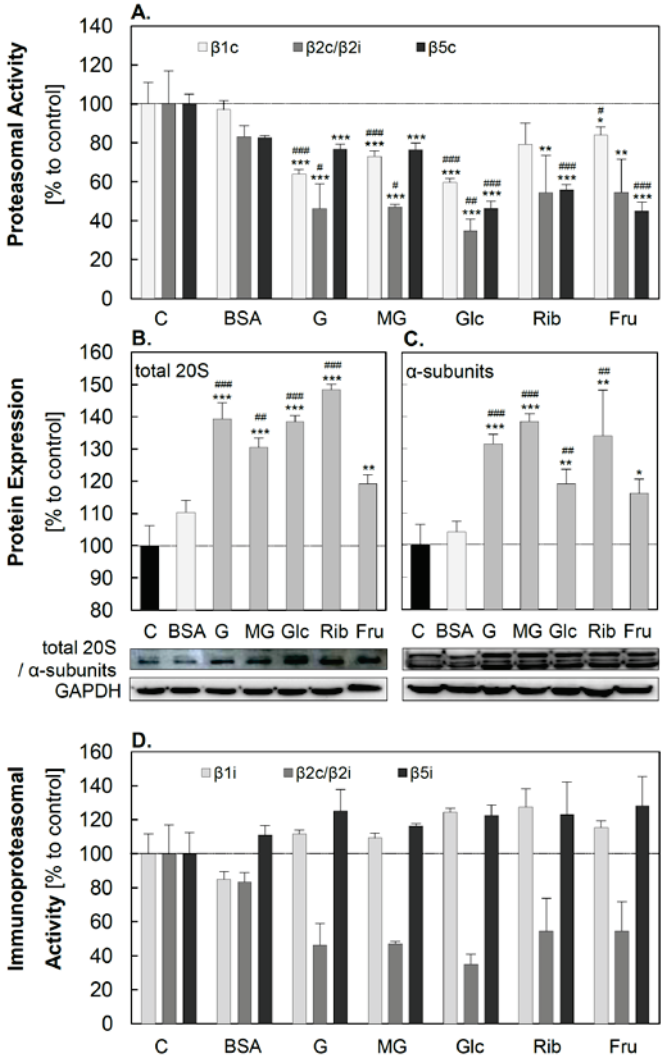


FIGURE 2

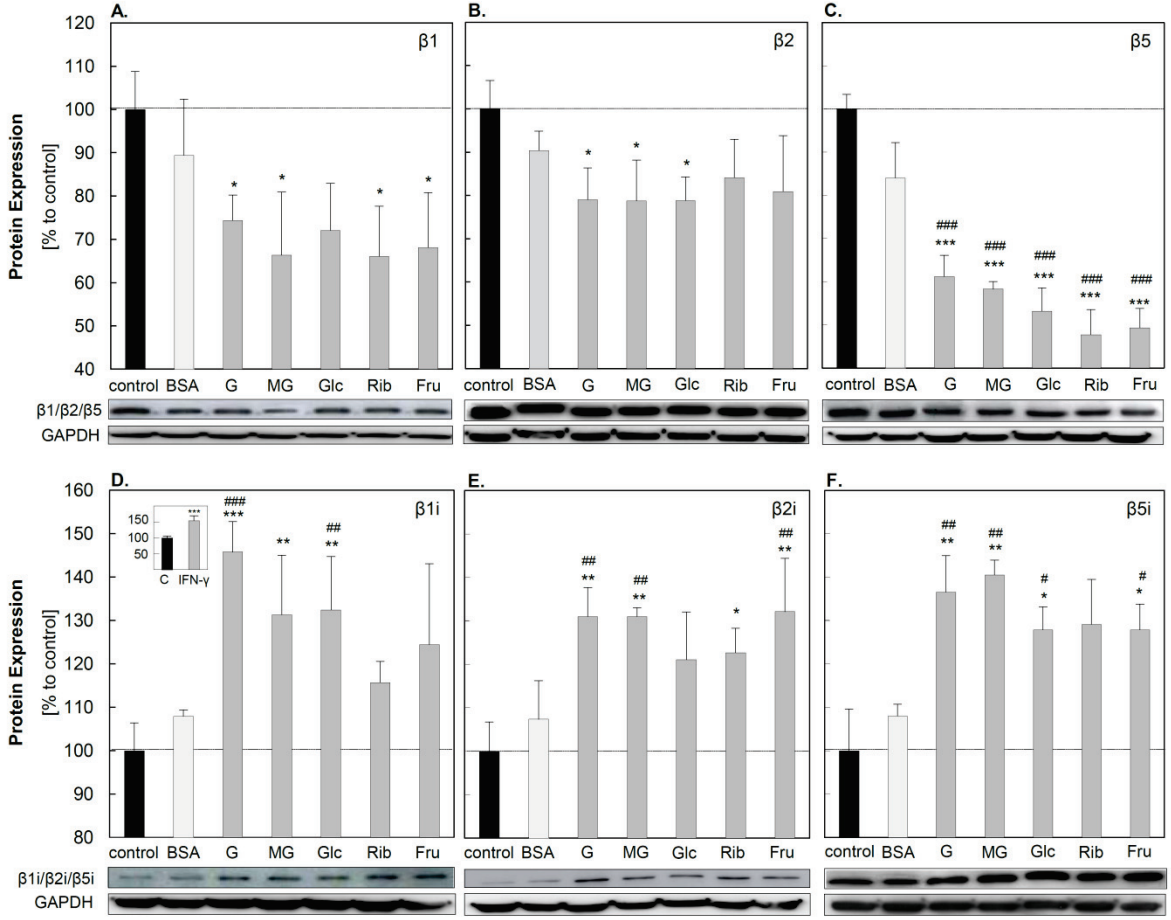


FIGURE 3

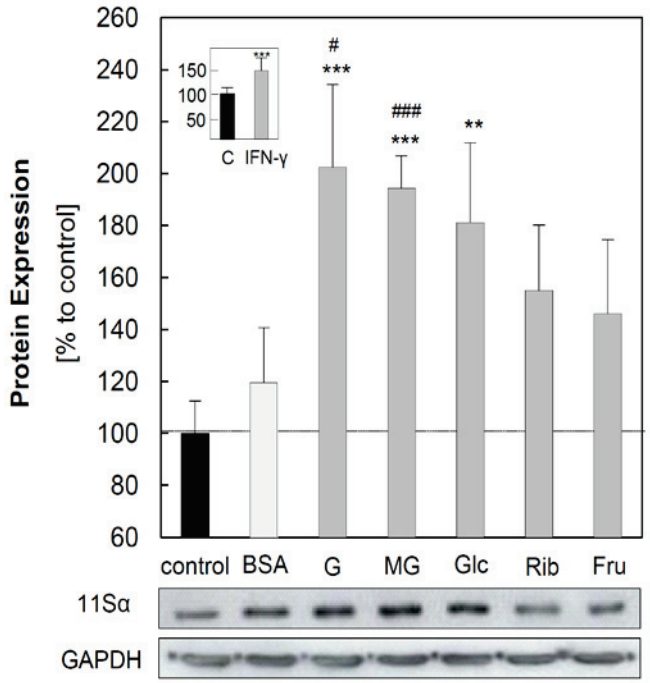


FIGURE 4

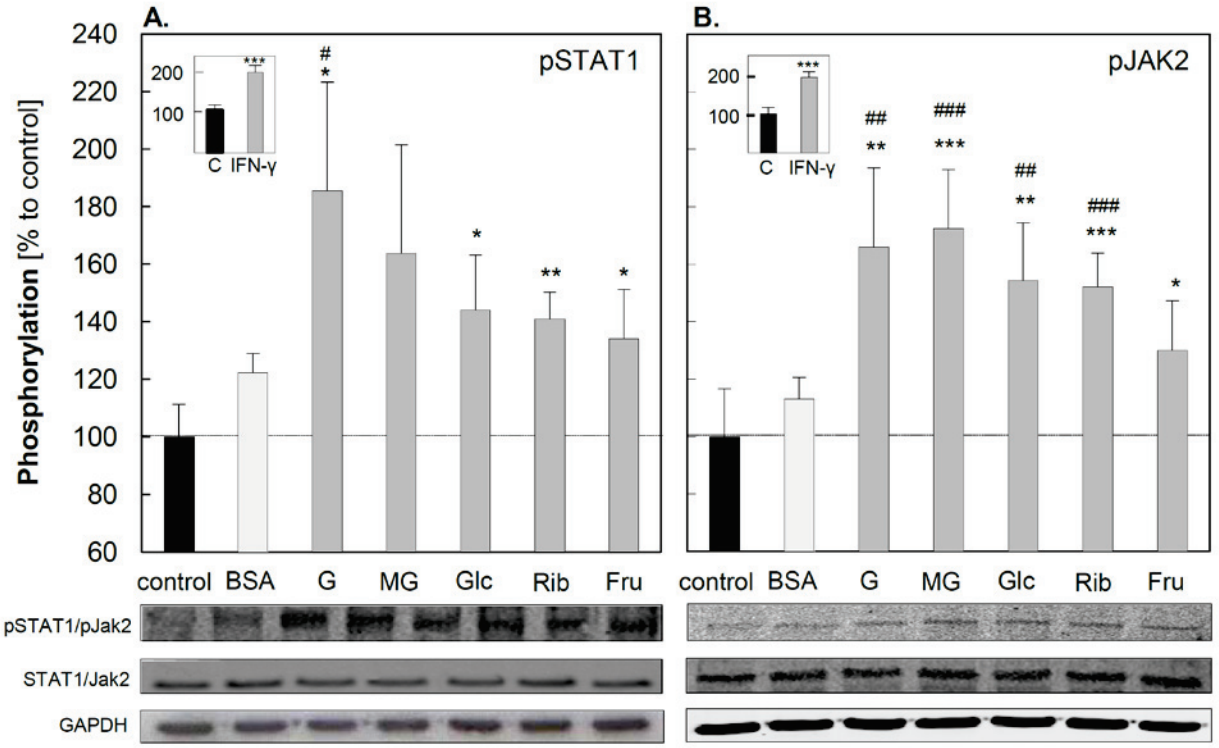


FIGURE 5

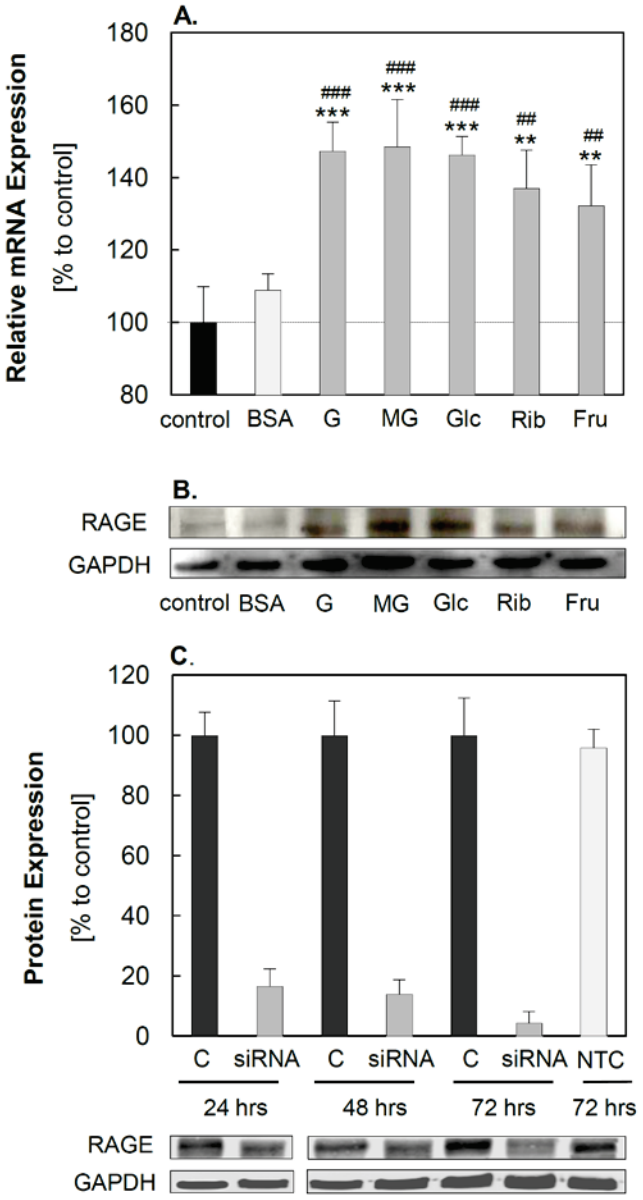


FIGURE 6

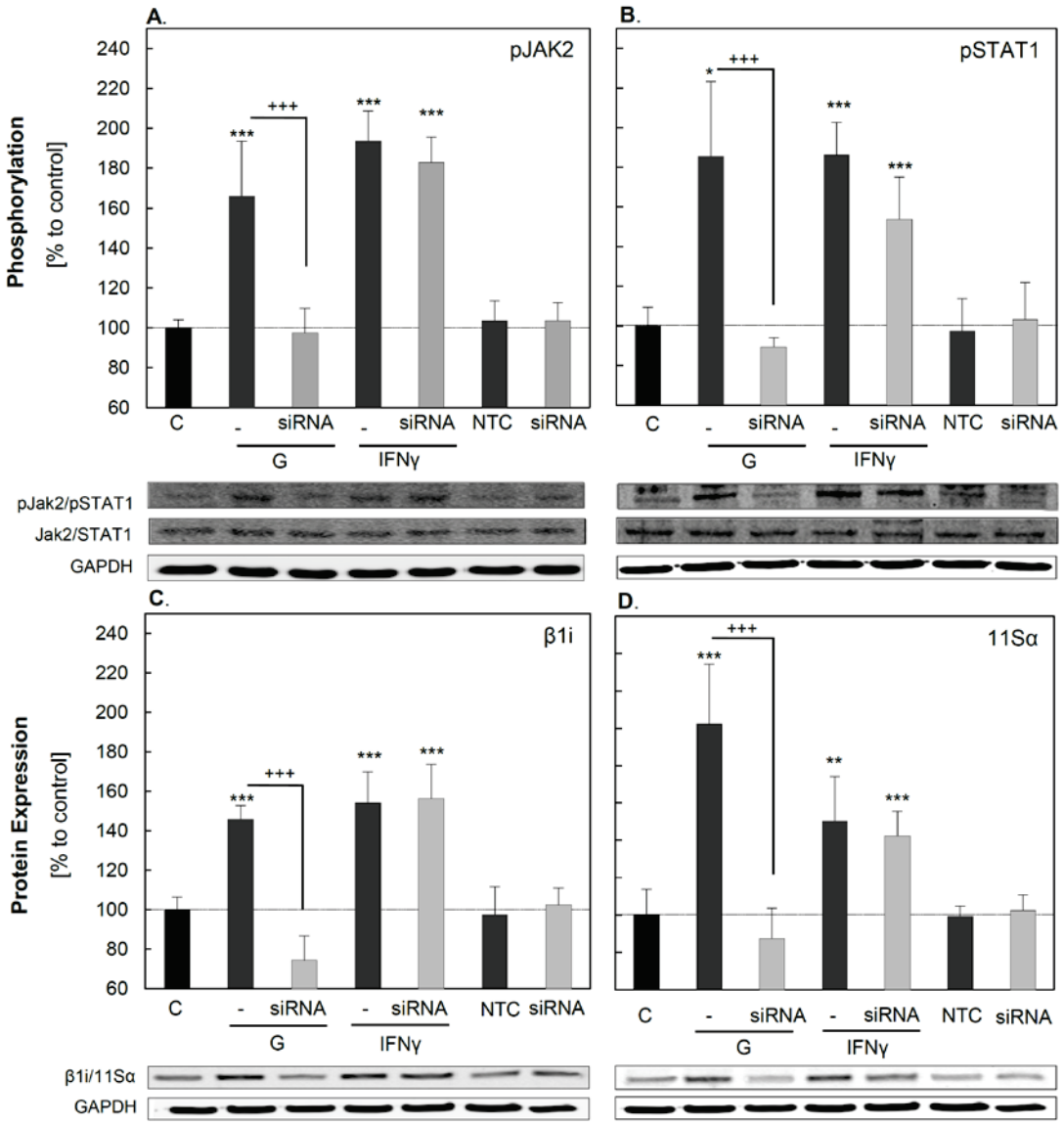


FIGURE 7

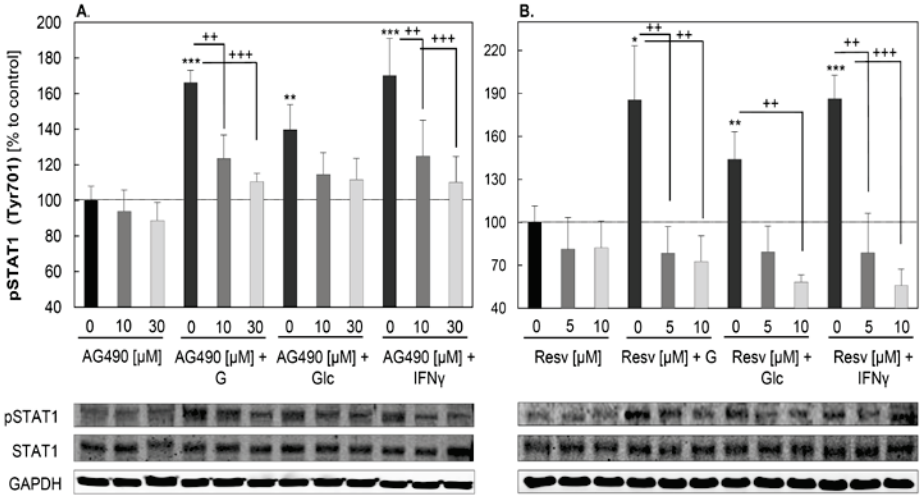


FIGURE 8

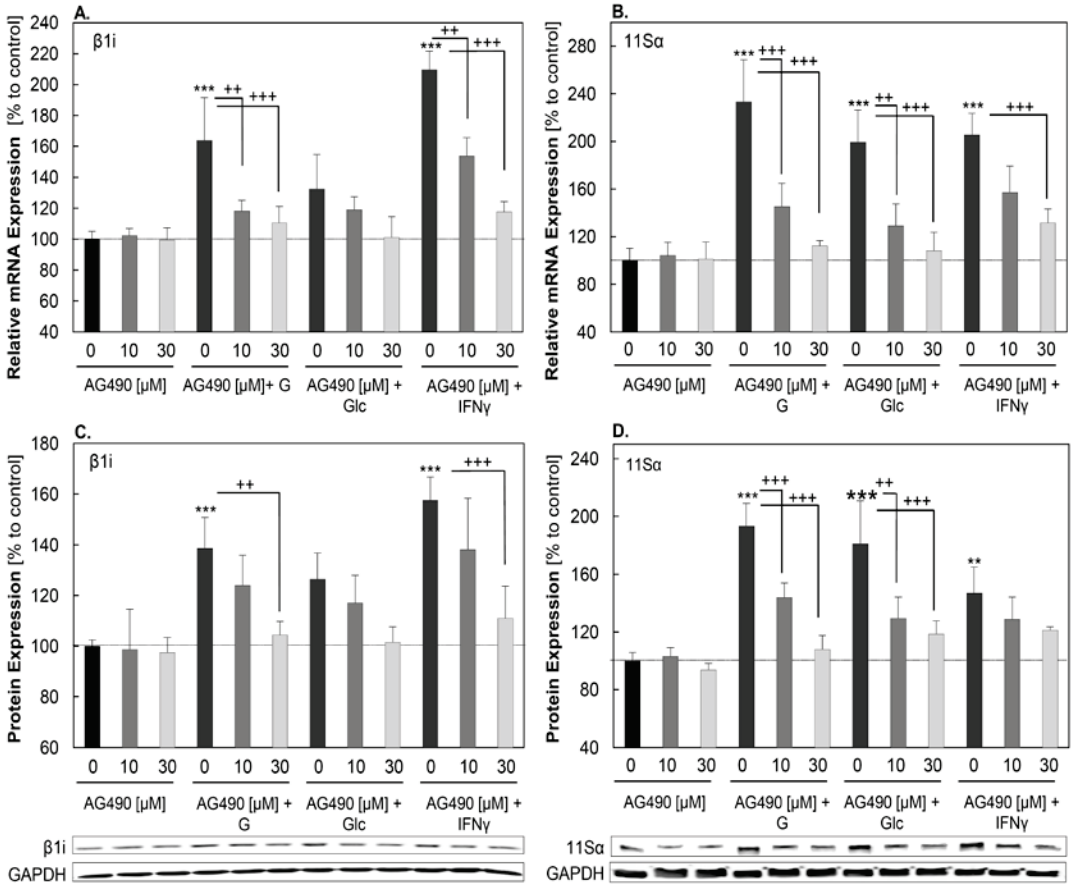


FIGURE 9

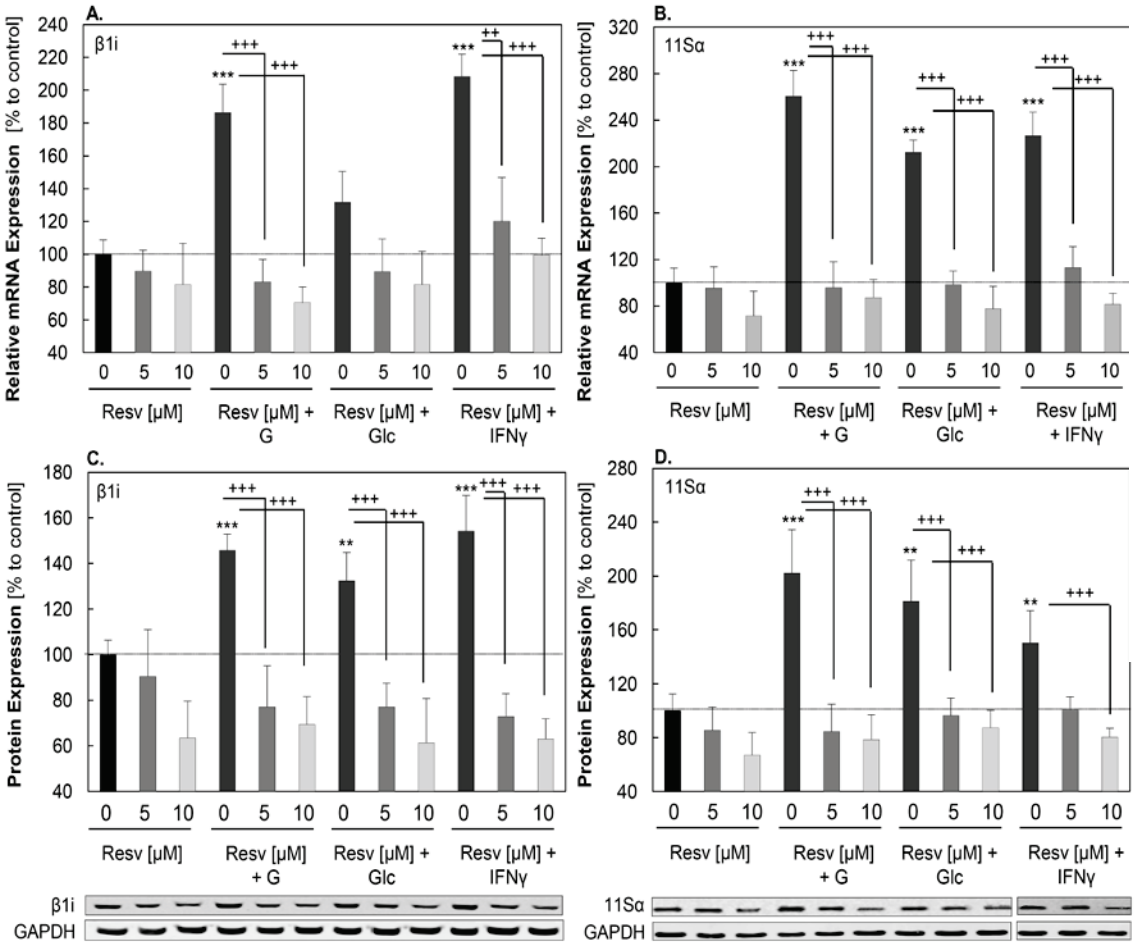
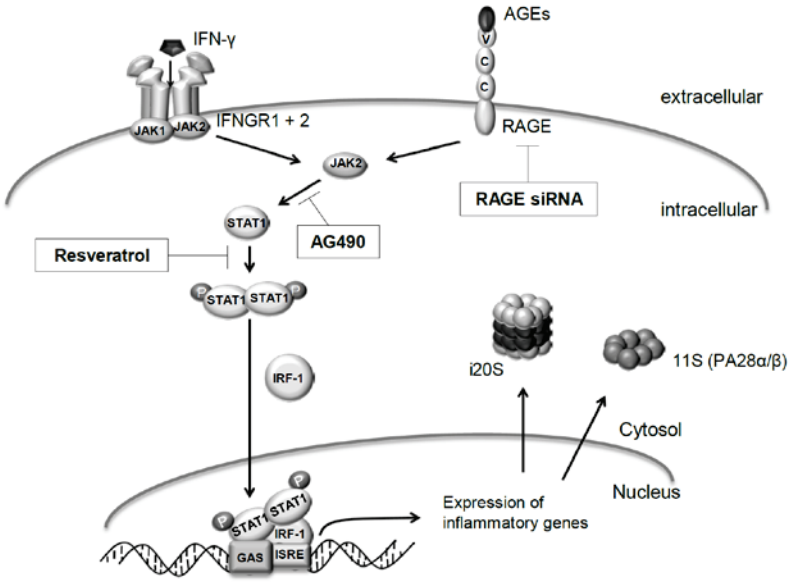


FIGURE 10



4 ZUSÄTZLICHE ERGEBNISSE

4.1 Altersabhängige lysosomale und proteasomale Aktivität

AGE-modifiziertes Material induziert eine signifikante Steigerung der lysosomalen Cathepsin D- und L-Aktivität und eine Reduktion aller konstitutiven katalytischen proteasomalen Aktivitäten in der RAW 264.7 Zelllinie (**Publikation III und IV**). Inwiefern diese Beobachtung auch für alte Zellen zutreffend ist, sollte nachfolgend gezeigt werden. Hierfür wurden Mikroglia aus alten Mäusen isoliert und die lysosomalen sowie die proteasomalen Aktivitäten mit jungen und adulten Zellen verglichen.

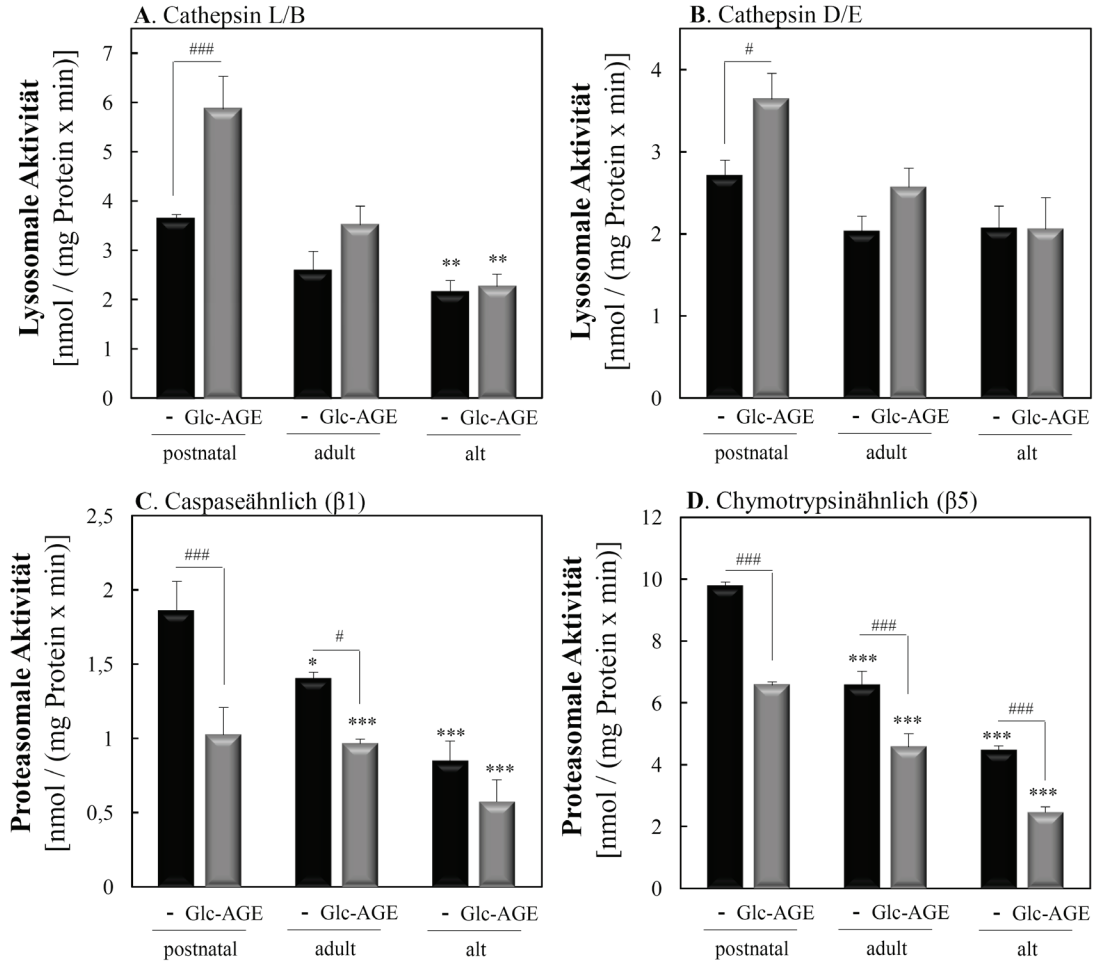


Abbildung 7: Lysosomale und proteasomale Aktivität in Abhängigkeit von Glucose-modifiziertem BSA in postnatalen, adulten und alten Mikroglia

Cathepsin L (A.), Cathepsin D (B.), caspaseähnliche Aktivität (C.) und chymotrypsinähnliche Aktivität (D.) wurden nach 72 h Inkubation mit Glucose-modifiziertem BSA (Glc-AGE) in mikroglialen Zellen, isoliert aus postnatalen, adulten und alten Mäusen, gemessen. Ein statistisch signifikanter Unterschied zu unbehandelten postnatalen Zellen ist durch die einfache Varianzanalyse (ANOVA, Post-Test: Tukey-Test), n=4, mit *($p<0.05$), **($p<0.01$) und ***($p<0.001$) dargestellt. Einen statistisch signifikanten Unterschied zwischen unbehandelten und mit Glc-AGE behandelten Mikroglia ist

durch die einfache Varianzanalyse (ANOVA, Post-Test: Tukey-Test), n=4, mit #($p<0.05$) und ###($p<0.001$) gekennzeichnet.

Eine signifikante Zunahme der Cathepsin D- und L-Aktivität ist nur in postnatalen Mikroglia zu erkennen (Abb. 7A/B). Adulte und alte Mikroglia weisen auf eine reduzierte lysosomale Aktivität hin, die in Anwesenheit der AGE-modifizierten Proteine nicht beeinflusst wird. Auffällig ist zudem eine signifikante Reduktion der proteasomalen caspaseähnlichen (β1) (Fig. 7C) und der chymotrypsinähnlichen (β5) Aktivität (Fig. 7D) in den adulten und alten Zellen im Vergleich zu postnatalen Mikroglia. Diese verringerte Aktivität kann in Anwesenheit der AGE-modifizierten Proteine noch stärker reduziert werden.

4.2 Altersabhängige Akkumulation von fluoreszierendem Material

Eine altersabhängige verringerte lysosomale und proteasomale Aktivität in unbehandelten Zellen lässt vermuten, dass vermehrt Proteinaggregate akkumulieren. Proteinaggregate weisen meist eine autofluoreszierende Eigenschaft auf. Aus diesem Grunde konnten postnatale, adulte und alte Mikroglia mittels Fluoreszenzmikroskopie auf ihre Eigenfluoreszenz hin untersucht werden.

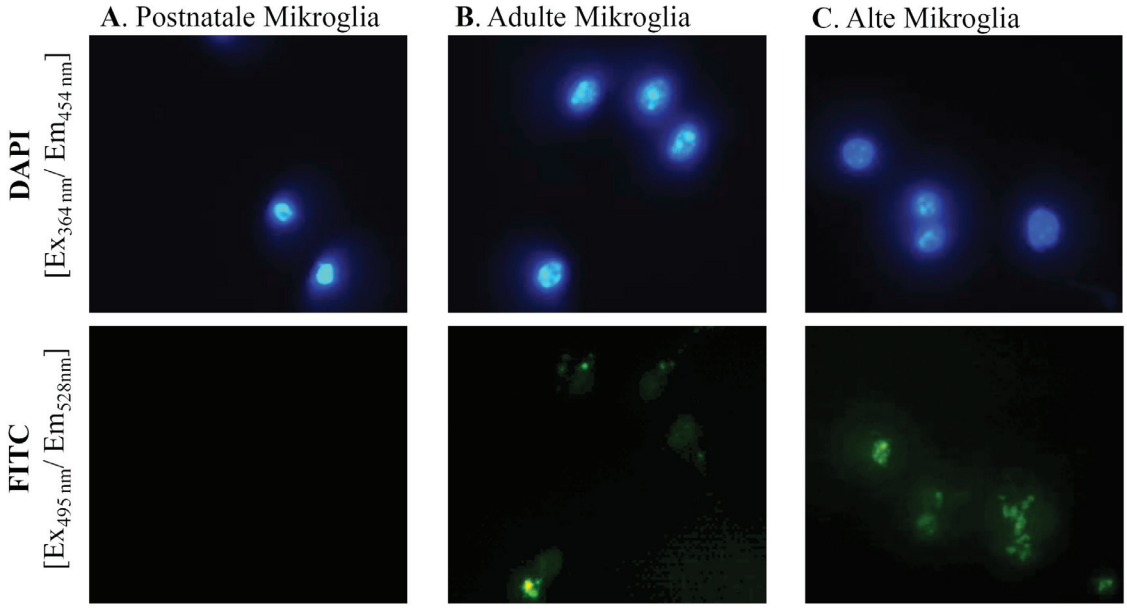


Abbildung 8: Autofluoreszenz postnataler, adulter und alter Mikroglia

Die DNA mikroglialer Zellen wurde mittels DAPI (4´,6´-Diamidin-2-phenylindol) gefärbt, um das Auffinden der Zellen im Fluoreszenzlicht zu erleichtern. Die Autofluoreszenz der Zellen wurde im FITC-Kanal bei einer Emission von 528 nm bestimmt.

Tatsächlich weisen alte Mikroglia eine für Proteinaggregate typische Autofluoreszenz im Wellenlängenbereich Ex_{495nm}/Em_{528nm} auf (Abb. 8).

Publikation III zeigt eine erhöhte ROS-Produktion durch AGE-modifizierte Proteine bei einer lysosomalen Dysfunktion. Hieraus kann vermutet werden, dass auch primäre Zellen, wie beispielsweise mikrogliale Zellen, aufgrund der verstärkten Akkumulation von aggregiertem Material vermehrt ROS freisetzen.

4.3 Altersabhängige Induktion der ROS-Produktion

Die Produktion reaktiver Sauerstoffspezies wurde mittels einer Lumineszenz-Methode unter Verwendung von Luminol bestimmt. Hierbei reagiert das Luminol mit Superoxid-Anionen unter Bildung einer instabilen Aminophthalat-Verbindung, die beim Übergang in einen energetisch stabileren Zustand Photonen emittiert [133].

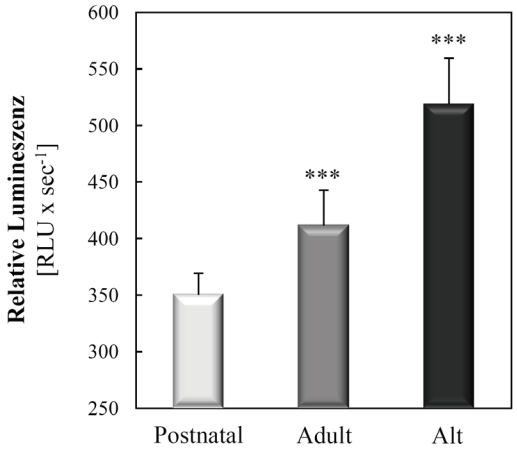


Abbildung 9: Produktion reaktiver Sauerstoffspezies in postnatalen, adulten und alten Mikroglia
Eine ROS-Freisetzung wurde mittels Chemilumineszenz in unbehandelten mikroglialen Zellen, isoliert aus postnatalen, adulten und alten Mäusen, bestimmt. Einen statistisch signifikanten Unterschied zwischen postnatalen und adulten bzw. alten Mikroglia ist durch die einfache Varianzanalyse (ANOVA, Post-Test: Tukey-Test), n=4, mit ***($p<0.001$) dargestellt.

Mikrogliale Zellen, isoliert aus adulten und alten Mäusen, zeigen eine signifikant erhöhte ROS-Freisetzung im Vergleich zu postnatalen Zellen. Hieraus stellt sich die Frage, ob die verstärkte ROS-Freisetzung mit dem Auftreten von oxidierten Proteinen korreliert. Um dieser Hypothese nachzugehen, wurden Proteincarbonyle, als Marker für oxidativ-modifizierte Proteine, in den unterschiedlich alten mikroglialen Zellen bestimmt. Proteingebundende Carbonylgruppen wurden mittels 2,4-Dinitrophenylhydrazin zu Hydrazonen derivatisiert, die über spezifische Antikörper nachweisbar sind. Entsprechende

fluoreszierende sekundäre Antikörper binden an die Hydrazon-gebundenen Antikörper und ermöglichen einen Nachweis in einem für das Fluorochrom spezifischen Wellenlängenbereich.

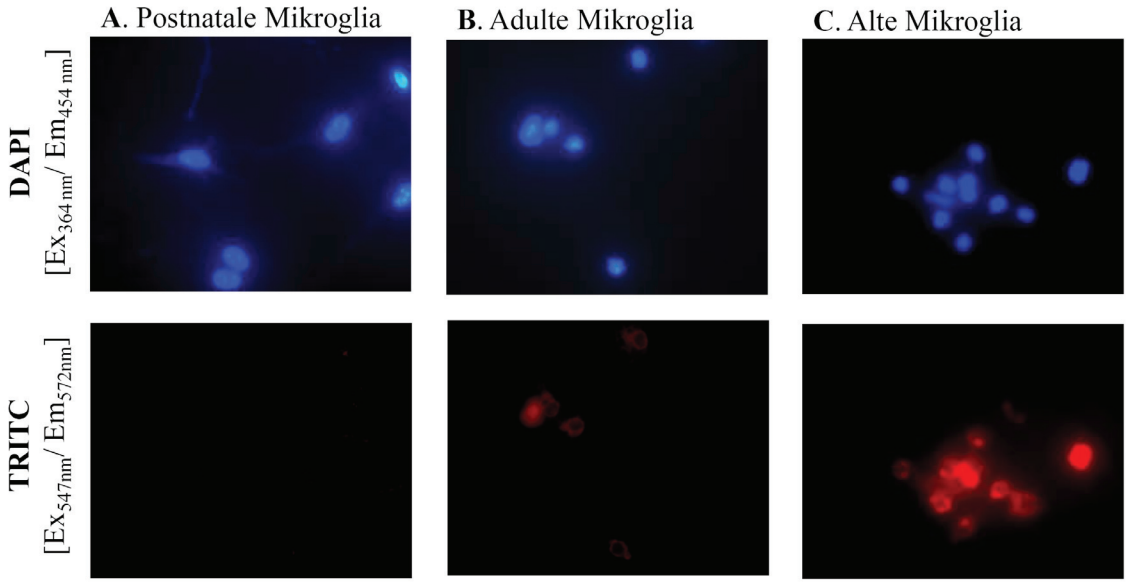


Abbildung 10: Oxidative Proteinmodifikationen in postnatalen, adulten und alten Mikroglia
Oxidative Proteinmodifikationen sind über Carbonylgruppen nachweisbar. Hierfür wurden die Carbonylgruppen mittels 2,4-Dinitrophenylhydrazin derivatisiert und anschließend über spezifische Antikörper detektiert. Fluoreszenzmarkierte sekundäre Antikörper ermöglichen eine Detektion mittels Fluoreszenzmikroskopie.

Eine Zunahme oxidierter Proteine am Beispiel der Proteincarbonyle kann in den alten mikroglialen Zellen im Vergleich zu postnatalen oder adulten Mikroglia beobachtet werden (Abb. 10). Zusammenfassend zeigen mikrogliale Zellen aus alten Mäusen eine signifikant erhöhte ROS-Freisetzung (Abb. 9) und Proteincarbonyle als Marker für oxidativ geschädigte Proteine (Abb. 10). Weitere Untersuchungen sollten zeigen, inwiefern diese alten Zellen noch ausreichend auf Stimulanzien reagieren. Hierfür wurden postnatale, adulte und alte Mikroglia mit Glucose-modifiziertem BSA oder Lipopolysaccharide (LPS) für 16 Stunden inkubiert. Anschließend erfolgte eine Aktivierung der Zellen mittels Phorbol-Myristat-Acetat (PMA). PMA durchdringt die Zellmembran und aktiviert direkt die Proteinkinase C, die wiederum für die Aktivierung der NADPH-Oxidase verantwortlich ist. Die aktivierte NADPH-Oxidase überträgt anschließend Elektronen von NADPH auf den Sauerstoff unter Bildung von Superoxid-Anionen. Diese Reaktion wird in Phagozyten als oxidativer oder respiratorischer Burst bezeichnet [134] und kann über eine Luminol-Lumineszenz-Methode detektiert werden.

Abbildung 11 zeigt, dass ältere Zellen nicht mehr ausreichend auf Stimulanzien reagieren. In postnatalen Mikroglia führt Glucose-modifiziertes BSA zu einer verstärkten ROS-Antwort nach PMA-Stimulation. Dieser Effekt zeigt sich in geringerem Ausmaße in adulten Mikroglia, während alte mikrogliale Zellen weder auf LPS- noch auf Glucose-modifiziertes BSA nach Aktivierung mit einem deutlich sichtbaren Burst reagieren.

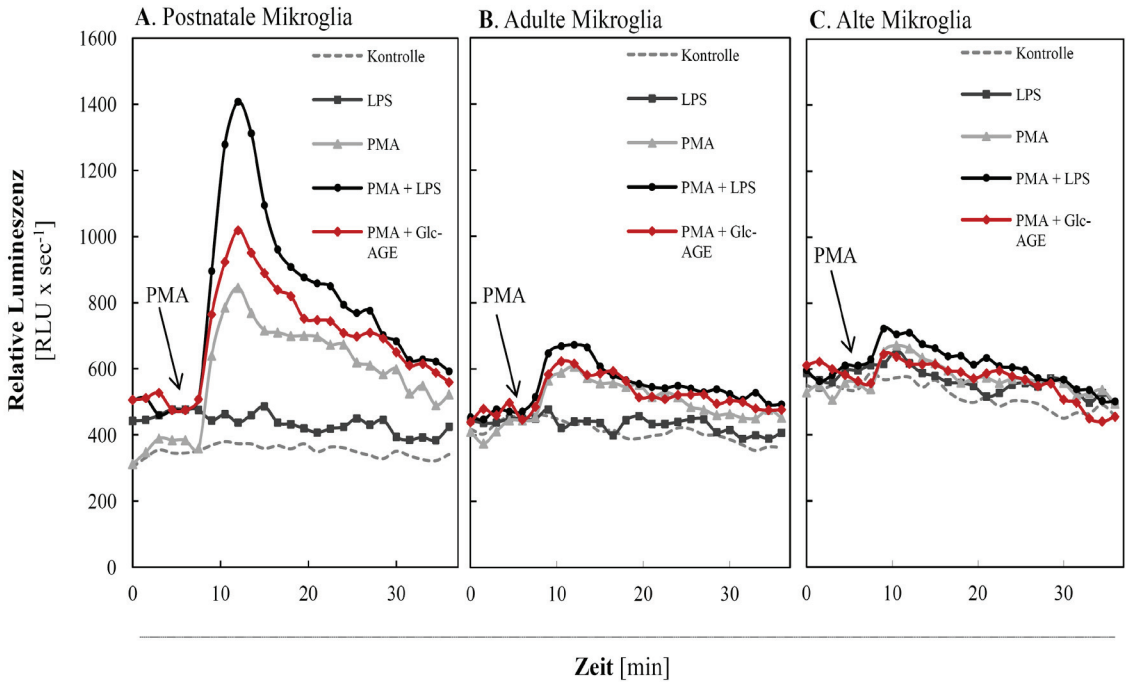


Abbildung 11: Oxidativer Burst in postnatalen, adulten und alten Mikroglia

Eine Aktivierung mikroglialer Zellen wurde mittels der Chemilumineszenz-Methode unter Verwendung von Luminol bestimmt. Die Zellen wurden hierfür mit LPS oder Glucose-modifiziertem BSA für 16 Stunden inkubiert. Anschließend wurde 50 µM Luminol zugegeben und für 5 Minuten die Basis-Lumineszenz gemessen. Die Zugabe von 2 µM Phorbol-Myristat-Acetat (PMA) erfolgte nach der Messung der Basis-Lumineszenz mittels Injektor am Lumineszenz-Reader, so dass die Lumineszenz unmittelbar im Anschluss über einen ausgewählten Zeitraum bestimmt werden konnte.

4.4 Altersabhängige Induktion des Immunoproteasoms

Eine Stimulierung der mikroglialen Zellen während der Inkubation mit Glucose-modifiziertem BSA kann vermutlich auf einen RAGE-induzierten Signalweg zurückzuführen sein. Über den RAGE werden zahlreiche Signalwege aktiviert, dazu zählt die in der vorliegenden Arbeit nachgewiesene Jak/STAT-induzierte Expression der immunoproteasomalen Untereinheiten (**Publikation IV**). Eine Stimulierung postnataler, adulter und alter Mikroglia sollte zusätzlich aufweisen, ob diese Induktion altersabhängig beeinträchtigt sein kann. Hierfür wurden die Zellen 72 Stunden mit Glucose-modifiziertem

BSA inkubiert und anschließend über einen spezifischen LMP7(β5i)-Antikörper und einem fluoreszenzmarkierten Sekundärantikörper im Fluoreszenzmikroskop untersucht.

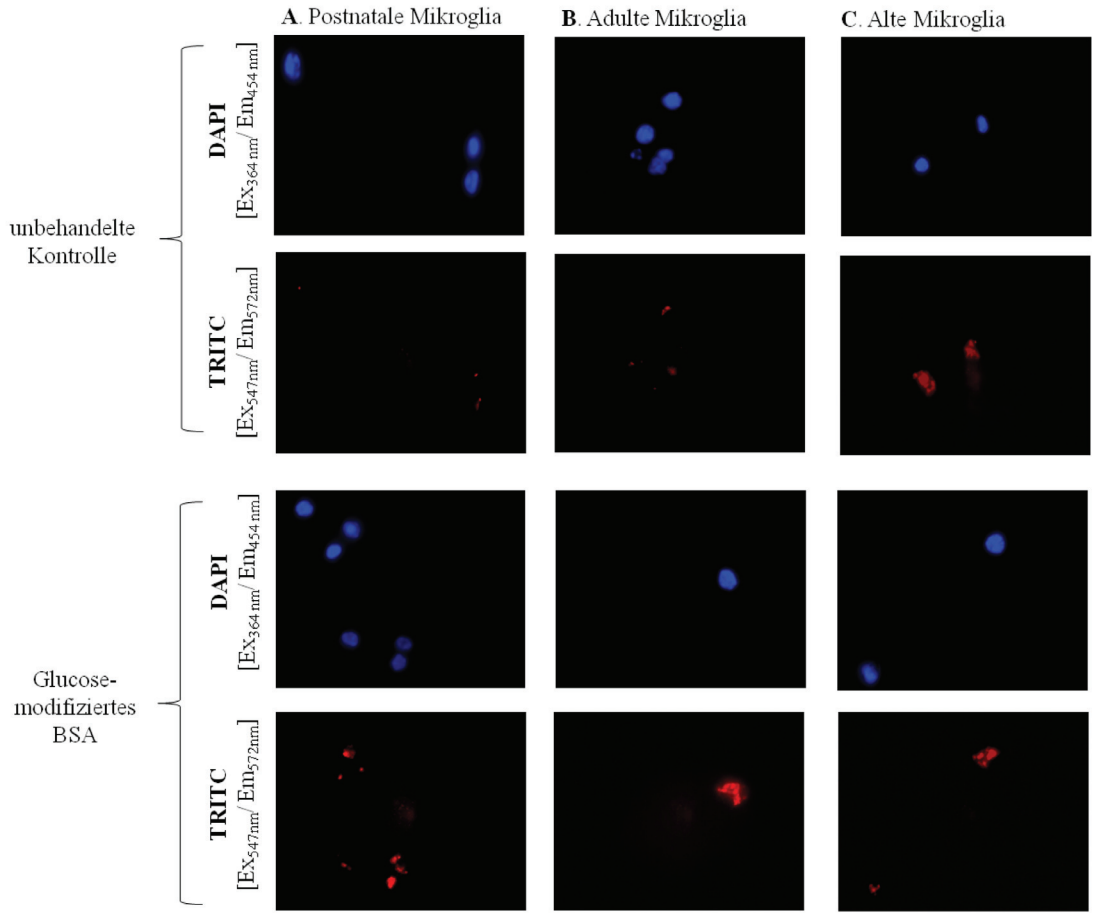


Abbildung 12: Induktion des Immunoproteasoms in postnatalen, adulten und alten Mikroglia

Eine Induktion des Immunoproteasoms in Mikroglia, isoliert aus postnatalen, adulten und alten Mäusen, wurde nach 72 Stunden Inkubation mit Glucose-modifiziertem BSA über einen LMP7(β5i)-spezifischen Antikörper mittels Immunofluoreszenz bestimmt.

Abbildung 12 zeigt, dass in nicht-stimulierten postnatalen Mikroglia (unbehandelte Kontrolle) eine Expression der immunoproteasomalen Untereinheit LMP7(β5i) nicht detektierbar ist. Glucose-modifiziertes BSA induziert hingegen die Expression dieser Untereinheit. Adulte Mikroglia zeigen, im Vergleich zu alten Mikroglia, eine verringerte Expression des LMP7. Die Induktion durch Glucose-modifiziertes BSA ist nur in adulten Mikroglia, im Vergleich zu alten Mikroglia, zu beobachten. Alte Mikroglia weisen durch die Inkubation mit Glucose-modifiziertem BSA keine Änderung in der Fluoreszenzintensität zum nicht-stimulierten Zustand (unbehandelte Kontrolle) auf.

5 DISKUSSION

5.1 AGE-Modelle und deren Bezug auf *in vivo*

Diabetes Mellitus, Arteriosklerose, renale Dysfunktionen und neurodegenerative Erkrankungen werden mit dem Auftreten von AGE-modifizierten Proteinen in Verbindung gebracht. Darüber hinaus treten AGE-modifizierte Proteine auch während des Alterungsprozesses zunehmend auf. Die Charakterisierung der AGEs und ihre Rolle bei der Entstehung dieser Erkrankungen sind von besonderem Interesse, um mögliche Interventionen ableiten zu können, damit ein gesundes Altern unterstützt werden kann.

Die *in vitro* Herstellung einer adäquaten AGE-Konzentration, die einer *in vivo* Konzentration entspricht, ist bis dato nicht möglich. Da es sich bei den AGEs um eine heterogene Substanzklasse handelt und es enorm schwierig ist alle Strukturen zu identifizieren, konnten *in vivo* AGEs noch nicht vollständig quantifiziert werden. Die Entstehung der AGEs *in vivo* ist ein Prozess, der über mehrere Jahre stattfindet und durch komplexe individuelle Unterschiede beeinflusst wird. Es ist zudem noch sehr umstritten, welche Rolle exogen zugeführte AGEs im Stoffwechsel innehaben und wie diese den endogenen AGE-Spiegel beeinträchtigen. Auch die exogen zugeführte AGE-Menge kann individuell sehr unterschiedlich ausfallen und hängt beispielsweise von der Ernährungsweise und der Zubereitung der Lebensmittel ab. Zudem ist die Absorptionsrate der einzelnen bis heute identifizierten AGEs sehr variabel [43]. Der AGE-Serumspiegel ist somit abhängig von der endogenen AGE-Produktion, der exogenen Aufnahme sowie der renalen und enzymatischen Elimination.

Die in den Studien verwendeten AGE-Konzentrationen dienen vielmehr als Modell, um den Einfluss der durch chronische Prozesse gebildeten AGEs in einem verkürzten zeitlichen Rahmen darzustellen und Mechanismen aufzuweisen, die durch AGEs beeinträchtigt werden können. Als Ziel hierbei sollen Ansätze gefunden werden, wie in diese Mechanismen eingegriffen werden kann. Um signifikante zelluläre Effekte detektieren zu können wird beispielsweise Albumin mit sehr hohen Glucosekonzentrationen (bis zu 1 M), und somit weit über den physiologischen (5 mM) und pathologischen (25 mM) Konzentrationen, modifiziert [135]. Diese hohen Konzentrationen induzieren AGE-Strukturen in weitaus höherem Ausmaße als in Geweben und Körperflüssigkeiten zu finden sind. Thornalley *et al.* berechneten 1 mmol AGEs pro 1 mol Lysin in physiologisch relevanten Proteinen [135]. Xie *et al*. verwendeten bei der Herstellung der AGEs Zucker- und BSA-Konzentrationen, sowie Inkubationsbedingungen,

die häufig in der Literatur beschrieben werden [57]. Hierbei wurden ca. 0,5 mol AGE pro mol Lysin gebildet. Der Bezug der *in vitro*-AGE Modelle auf *in vivo* wird kontrovers diskutiert. In Untersuchungen zu den strukturellen Voraussetzungen für die Bindung von AGEs an den RAGE konnte festgestellt werden, dass die V-Domäne des Rezeptors AGE Strukturen unabhängig von ihrem Ausmaß der Proteinmodifikationen bindet [136]. Thornalley *et al.,* zeigte jedoch, dass nur hochgradig modifizierte AGEs, wie sie in *in vitro* Studien verwendet werden, die Fähigkeit aufweisen, den RAGE zu aktivieren [135]. Demzufolge ist für eine ausreichende AGE-RAGE-Bindung ein hohes Maß an Glykierung notwendig und deren Bezug auf *in vivo* ist eher fraglich [137]. Im Gegensatz dazu wird beschrieben, dass AGE-modifizierte Proteine in nanomolaren Konzentrationen für die Liganden-Rezeptor-Interaktionen ausreichen [138]. Eine weitere Studie mit derselben Schlussfolgerung konnte mittels aufgereinigten AGEs, isoliert aus Diabetespatienten, zeigen, dass diese *in vivo* vorkommenden AGEs die Aktivierung von RAGE in gleicher Weise wie die *in vitro* produzierten AGEs induzieren [139]. Um hierfür Klarheit zu schaffen sind weitere Studien notwendig.

Die in der vorliegenden Arbeit artifiziell hergestellten AGE-modifizierten Proteine wurden entsprechend den Konzentrations- und Inkubationszeiten, wie in der Literatur beschrieben, hergestellt [140,141]. Dies ermöglicht einen Vergleich mit dem aktuellen Stand der Wissenschaft.

5.2 Herstellung und Charakterisierung der AGE-modifizierten Proteine

Die hergestellten AGE-modifizierten Proteine wurden in den vorliegenden Arbeiten als Modell für die Untersuchungen zum Abbau, zur Akkumulation und zu den zellulären Reaktionen verwendet. Hierfür wurde endotoxinfreies BSA mit unterschiedlichen Konzentrationen der reduzierenden Zucker Glucose, Fructose und Ribose, sowie den reaktiven α-Dicarbonylen Glyoxal und Methylglyoxal unter sterilen Voraussetzungen und physiologischen Bedingungen (pH 7,4 und 37°C) inkubiert. Aufgrund der hohen Sequenzhomologie (ca. 80 %) mit humanem Serumalbumin wird BSA in zahlreichen Studien verwendet und fand demzufolge in den vorliegenden Experimenten als Proteinquelle Anwendung [142].

Untersuchungen der letzten Jahre kritisieren die Glaubwürdigkeit der AGE-induzierten zellulären Effekte [143]. Als Ursache hierfür werden AGE-Präparate, die mit Endotoxinen kontaminiert sind, berichtet. Die proinflammatorischen zellulären Effekte, die nach AGE-

Stimulation beobachtet werden, entsprechen häufig den Reaktionen auf Endotoxine. AGEs und Endotoxine aktivieren unter anderem folgende proinflammatorische Signale über den Transkriptionsfaktor NF-κB: (a) TNF-α, (b) induzierbare Stickstoffmonoxid Synthase (*inducible nitric oxide synthase,* iNOS) und somit Stickstoffmonoxid (NO) und (c) Interleukin-6 (IL-6) [144-146]. Diesbezüglich muss eine endotoxinfreie und sterile AGE-Herstellung garantiert werden, um Effekte, die durch Kontaminationen ausgelöst werden könnten, auszuschließen.

5.2.1 Verwendung von Methylglyoxal und Glyoxal

Glyoxal und Methylglyoxal können durch oxidative Spaltung von Amadori-Produkten und Schiffschen Basen, durch Fragmentierung (Retroaldolspaltung) von Glucose, infolge von Autooxidationen ungesättigter Fettsäuren oder in der Glykolyse entstehen [147,148]. Methylglyoxal wird beispielsweise intrazellulär durch eine nicht-enzymatische Fragmentierung von Triosephosphaten aus Intermediaten der Glykolyse und des Polyol-Stoffwechselweges gebildet [149,150]. In physiologischen Systemen sind Glyoxal und Methylglyoxal potente Glykierungsreagenzien, sowohl intrazellulär als auch extrazellulär. Beide reagieren vorrangig mit Arginin-Seitenketten, die sich besonders häufig in aktiven Zentren von Enzymen und Liganden-Bindungsstellen in Rezeptoren befinden, und beeinträchtigen somit deren biologische Funktionen [151]. Für die zytosolische Entgiftung von α-Oxoaldehyden (z.B. Methylglyoxal oder Glyoxal) ist das Glyoxalase System verantwortlich. Das Glyoxalase System beinhaltet die Glyoxalase I ((R)-S-Lactoylglutathion-Methylglyoxal-Lyase, EC 4.4.1.5), die eine Isomerisierung von Hemithioacetal-Verbindungen, die zuvor aus einer spontanen Reaktion zwischen 2-Oxoaldehyden und reduziertem Glutathion (GSH) hervorgegangen sind, katalysiert. Als Produkt entsteht das S-2-Hydroxyacylglutathion, das mittels der Glyoxalase II (S-2-Hydroxyacylglutathion Hydrolase, EC 3.1.2.6] zu den entsprechenden 2-(R)-Hydroxysäuren und GSH hydrolysiert wird [152]. Eine verringerte Expression und folglich eine reduzierte Aktivität wurde für die Glyoxalase I mit zunehmendem Alter bestimmt [153]. Methylglyoxal und Glyoxal können somit nicht mehr ausreichend detoxifiziert werden und dementsprechend steigt die Glykierungsrate. Aufgrund ihrer physiologischen Bedeutung sind Methylglyoxal und Glyoxal für die Herstellung der AGE-modifizierten Proteine als Modellsubstanzen relevant.

5.2.2 Verwendung von Glucose, Fructose und Ribose

D-Glucose ist in hohen Konzentrationen im Plasma zu finden und spielt somit eine große Rolle in der *in vivo* Glykierung von Proteinen. Gleichzeitig ist es unter den verschiedenen reduzierenden Zucker am wenigstens reaktiv [154]. D-Fructose ist ungefähr achtmal reaktiver als D-Glucose. In der Augenlinse von diabeteserkrankten Ratten und in peripheren Nervenzellen von Diabetikern wurden erhöhte Fructosespiegel gemessen [155,156]. D-Fructose-modifiziertes BSA ist in den letzten Jahren in zahlreichen Arbeiten gut charakterisiert worden und findet weit verbreitet Anwendung [157]. Neben D-Glucose und D-Fructose ist D-Ribose ein effizientes Glykierungsreagenz. Im Vergleich zu D-Glucose besteht D-Ribose aus einem instabilen Aldofuranose-Ring, welcher wesentlich reaktiver mit Aminogruppen reagiert [158]. Aufgrund ihrer physiologischen Bedeutungen im Hinblick auf Glykierungsreaktionen wurden diese drei Zucker als Substrate für die AGE-Herstellung verwendet. Der Erfolg der AGE-Bildung wurde nach Inkubation mit verschiedenen Methoden verifiziert.

5.2.3 Charakterisierung von AGE-modifiziertem BSA

5.2.3.1 Optische Dichte

Als wesentlicher Marker für das Vorhandensein von AGEs dient die Eigenschaft der optischen Dichte [140]. Während der Bräunungsreaktion verändert sich das Absorptionsverhalten der AGEs in einem bestimmten Wellenlängenbereich verglichen zu nicht-modifiziertem BSA. Ein signifikanter Absorptionsanstieg wurde bei 360 nm in den Fructose-, Ribose- und Methylglyoxal-modifizierten Proben beobachtet (**Publikation II**). Dies legt den Schluss nahe, dass diese Proben am stärksten modifiziert wurden.

5.2.3.2 Fluoreszenz

Wie bei der optischen Dichte verhält sich der Anstieg der Fluoreszenzintensität in Abhängigkeit zum Modifikationsgrad des Proteins [159]. Eine AGE-spezifische Fluoreszenz wurde in einigen Publikationen beschrieben [160,161] und ist auf Protein-Protein-Vernetzungsstrukturen zurückzuführen. Trotz allem ist bekannt, dass nicht alle identifizierten AGE-Strukturen fluoreszieren [26]. Dennoch wird die Fluorezenzmessung als einfache und schnelle Methode zur Detektion von AGEs *in vivo* und *in vitro* angewendet [26]. Schmitt *el al.* ermittelte eine AGE-spezifische Fluoreszenz bei λ_{Ex} 485 nm/ λ_{Em} 530 nm [2]. Mit dieser Wellenlängenkombination konnte die allgemeingültige These, dass die Fluoreszenzintensität mit zunehmender Modifikation ansteigt, in der

vorliegenden Arbeit bestätigt werden. Analog zur optischen Dichte konnte gezeigt werden, dass Ribose- und Methylglyoxal-BSA am stärksten modifiziert wurden (**Publikation II**).

5.2.3.3 Aggregatbildung

Mithilfe der SDS-PAGE kann untersucht werden, inwiefern während der AGE-Herstellung Aggregate ausgebildet werden. Diese Methode wurde von Milkulikova *et al.* beschrieben [141]. Glykierungsreaktionen an Arginin, Lysin und Cystein-Seitenketten des Albumins führen zu unterschiedlichen Strukturmodifikationen und zur Erhöhung des Molekulargewichtes [2]. Eine SDS-PAGE trennt Proteingemische aufgrund der unterschiedlichen Molekulargewichte auf, indem größere Molekülstrukturen langsamer zur Anode wandern als kleinere Moleküle. Bei allen AGEs konnte eine Aggregatbildung beobachtet werden. Ribose-, Fructose- und Methylglyoxal-BSA zeigten auch mittels SDS-PAGE die stärksten Modifikationen im Vergleich zu reinem BSA (**Publikation II**).

5.2.3.4 Proteinoxidation

Die durch irreversible Oxidationsprozessen in Proteinen gebildeten Carbonylgruppen dienen als Biomarker zur Bestimmung des oxidativen Stresses [162]. Diese Carbonylverbindungen lassen sich mit Hilfe des Carbonyl-ELISAs (*enzyme-linked immunosorbent assays*) untersuchen [163]. Es konnten tendenziell mehr Carbonylgruppen in modifiziertem BSA im Vergleich zu unmodifiziertem BSA bestimmt werden (**Publikation II**). Die erhöhte Konzentration an Carbonylgruppen ist nicht durch das Vorhandensein freier Zucker- und Aldehydmoleküle begründet. Zur Vermeidung dieses Artefakts wurden die AGE-Präparate nach Herstellung einer gründlichen Dialyse unterzogen, um ungebundene Zucker und Aldehyde zu entfernen. Die Carbonylkonzentration in den AGE-Präparaten korrelierte jedoch nicht mit dem Grad der Modifikation. Vermutlich führt eine hohe Quervernetzung dazu, dass Carbonylgruppen für das 2,4-Dinitrophenylhydrazin, welches Hydrazone mit den Carbonylgruppen bildet und über Antikörper detektiert werden kann, schlechter zugänglich wurden. Andererseits ist es denkbar, dass die entstandenen Proteincarbonyle weitere kovalente Bindungen eingehen und somit nicht mehr nachweisbar sind. Die Bestimmung der Carbonylgruppen eignet sich hierbei nur bedingt zur Charakterisierung der AGE-modifizierten Proteine. Es können einerseits Modifikationen nachgewiesen werden, die auf Oxidationsprozesse beruhen, andererseits lässt sich hiermit keine Aussage über das Ausmaß der Modifikation treffen.

5.2.3.5 Zusammenfassung der AGE-Charakterisierung

Zusammenfassend lässt sich sagen, dass Glucose im Vergleich zu den anderen Zucker und Aldehyde die geringste Modifikation induziert. Ribose und Methylglyoxal modifizieren BSA am stärksten. Aldehyde sind bis zu 20.000fach reaktiver als beispielsweise Glucose [164]. Dies ist begründet in der Tatsache, dass Aldehyde die Notwendigkeit zur Bildung von Fructosamine, einem Amadori-Produkt, umgehen und direkt in der Reaktion mit Proteinen AGEs ausbilden können. Somit ist es auch nicht verwunderlich, dass mit den Aldehyden nach kurzer Inkubationszeit (1 Woche) im Vergleich zu Zucker-BSA-Lösungen (Inkubationszeit 6 Wochen) AGE-Modifikationen auftreten.

Eine geringe Modifikation wurde auch in der BSA-Kontrollprobe nach sechswöchiger Inkubation bei 37°C in PBS (pH 7,4) beobachtet (**Anhang 9.1, Abbildung I**). Dies beruht zum einen in einer moderaten Aggregatbildung, zum anderen in einem leichten Anstieg der Fluoreszenz. In Übereinstimmung mit unseren Untersuchungen zur Charakterisierung der BSA-Kontrolllösung wurden CML und CEL in BSA-Lösungen ohne Zugabe von Zucker oder reaktiven Aldehyden im Vergleich zu einer frisch hergestellten BSA-Lösung von Waanders *et al*. detektiert [143]. Die BSA-Kontrolllösung wurde in die Untersuchungen miteinbezogen (**Publikation II, III und IV**). Entscheidend für die Beurteilung der AGE-Wirkung war dabei, dass mit den AGE-modifizierten Proben ein höherer Effekt im Vergleich zur BSA-Kontrolllösung zu verzeichnen war.

5.3 Makrophagiale Zellen und murine embryonale Fibroblasten als Modellsysteme

Für die vorliegenden Untersuchungen wurden die Makrophagen-Zelllinie RAW 264.7 und embryonale murine Fibroblasten, mit Cathepsin D knock-out und Cathepsin L knock-out, verwendet. Ergänzend wurden Untersuchungen mit den primären mikroglialen Zellen durchgeführt. Der Vorteil in der Verwendung primärer Zellen, die direkt aus einem Organismus isoliert werden, besteht darin, dass es sich um keine transformierten Zellen handelt. Bekanntlich weisen transformierte Zellen weniger *in vivo* Charakteristika auf [165]. Die murine Makrophagen-Zelllinie RAW 264.7 wurde aus einem Tumor gewonnen, der im Vorfeld durch intraperitoneale Injektion eines Abselon Leukämie-Virus induziert wurde [166]. Aus Gründen der Praktikabilität wurden die RAW 264.7 Zellen für die Untersuchungen der AGE-induzierten Effekte herangezogen (**Publikationen III und IV**), denn Makrophagen sind bekannt als professionelle Phagozyten und können somit große

Mengen an modifiziertem Material aufnehmen. Zudem lassen sich Makrophagen durch zahlreiche Stimulanzien aktivieren. Daneben wurden primäre mikrogliale Zellen, die Makrophagen-ähnlichen Zellen im zentralen Nervensystem, für weitere Untersuchungen aus den oben genannten Gründen bezüglich Transformation und *in vivo* Charakteristika herangezogen (**zusätzliche Ergebnisse**). Mikrogliale Zellen sind insbesondere im Hinblick auf dysfunktionelle Proteine, die mit dem Autreten neurodegenerativer Erkrankungen korrelieren, interessant. Diese Zellen wurden aus postnatalen Mäuse (< 5 Tage alt), adulten Mäuse (2-3 Monate alt) und alten Mäuse (> 18 Monate) mittels einer MACS (*magnetic cell sorting*)-Methode isoliert. Der Vorteil dieser Methode beruht in der spezifischen Bindung von Antikörpern an das CD11b-Protein, einem für Mikroglia typischen zellulären Oberflächenmarker. Diese Antikörper sind gekoppelt mit magnetischen Beads, die in einem Magnetfeld retiniert werden. Mittels dieser Isolation werden sehr reine Mikroglia-Kulturen ermöglicht (> 95% mikrogliale Zellen). Die Mäuse (Stamm: C57BL/6) wurden unter Standardbedingungen (Luftfeuchte: ~ 55%, Temperatur: 22°C, Tag-Nacht-Zyklus: 12 Stunden) gehalten. Wasser und Futter standen den Mäusen *ad libitum* zur Verfügung.

Als weiteres Zellmodell wurden Fibroblasten verwendet (**Publikation II und III**). Fibroblasten sind befähigt modifiziertes Material aufzunehmen [167]. Sitte *et al*. zeigten eine zeitabhängige Akkumulation nach Lipofuszin-Exposition [167]. Lipofuszin ist als Alterungspigment bekannt. Es entsteht aus oxidierten Proteinen und Lipiden unter deren Quervernetzung [168]. Da die physikochemischen Eigenschaften von Lipofuszin mit denen der AGEs sehr ähnlich sind und zudem die AGEs Pentosidin und CML in neuronalen Perikaryon in Alzheimer Patienten und im alternden Gehirn mit Lipofuszin kolokalisieren, sind Lipofuszin und AGEs als modifiziertes Material vergleichbar [169]. Fibroblasten wurden des Weiteren auch in der Literatur für Untersuchungen zum AGE-Abbau durch das Proteasom verwendet [170].

Die Herstellung der AGEs wurde in **Publikation II** ausführlich beschrieben. Für die Anwendung der AGEs an Zellen mussten im Vorfeld die AGE-Konzentrationen auf Vitalität getestet werden (**Publikation II und Anhang 9.2, Tab. I-V**). Die Toxizität glykierter Proteine korreliert mit dem Ausmaß der Proteinglykierungen in verschiedenen Zelltypen [171]. Sowohl in den Fibroblasten als auch in den RAW 264.7 Zellen konnte kein signifikanter zeit- oder konzentrationsabhängiger Effekt der AGEs auf die Zellvitalität bestimmt werden (**Publikation II und Anhang 9.2, Tab. I-V**). Mikrogliale Zellen aus postnatalen Mäusen isoliert, zeigten eine Reaktion auf höhere AGE-Konzentrationen nach längerer Inkubationsdauer (**Anhang 9.2, Tab. III-V**). Um einen toxizitätsbedingten

Einfluss auf die Ergebnisse zu vermeiden, wurden einheitlich für alle Zelltypen die AGE-Konzentrationen gewählt, die auch in den primären Zellen keine verringerte Vitalität verursacht haben. Mittels Trypanblau-Assay konnte zusätzlich kein Proliferationseffekt in den verwendeten Zellen durch AGE-Applikation festgestellt werden (diskutiert in **Publikation III**).

Die Aufnahme der AGE-modifizierten Proteine wurde mittels Fluoreszenz in RAW 264.7 und Fibroblasten bestätigt (**Publikation II und III**). Eine alleinige Bestimmung über Fluoreszenz ist jedoch nicht ausreichend, da bekanntlich nicht alle AGE-Strukturen fluoreszieren [26]. Hierfür sind weitere Methoden unerlässlich. Demzufolge wurde radioaktives ^{14}C-Glucose-modifiziertes Albumin hergestellt und die Aufnahme mit einer Szintillationsmessung verifiziert (**Publikation III**). Nach bestätigter AGE-Aufnahme konnte im weiteren Verlauf das intrazelluläre Schicksal dieser modifizierten Proteine bestimmt werden.

5.4 Proteolytischer Abbau von AGE-modifiziertem Albumin

Interessanterweise sind weniger AGEs im Organismus zu finden als aufgrund von *in vitro* Studien zu erwarten wären [172]. Diesbezüglich werden Mechanismen vermutet, die diese Strukturen selektiv abbauen und ausscheiden können [172]. Auch wenn Indizien darauf hindeuten, dass verschiedene Rezeptoren eine AGE-Endozytose ermöglichen können, sind der genaue Mechanismus und das intrazelluläre Schicksal der AGEs bislang nicht vollständig erforscht. Es gibt nur wenige Hinweise, die auf einen proteolytischen Abbauprozess hindeuten [53]. Vlassara *et al.* stellten während ihrer Untersuchungen zu den pathogenen Folgen der AGEs fest, dass Makrophagen AGE-Strukturen spezifisch erkennen, aufnehmen und abbauen können [173]. Daraufhin wurde vermutet, dass ein Mechanismus für die Entgiftung von AGE-modifizierten Proteinen in Geweben existieren muss. Allerdings liegen bislang keine Arbeiten vor, in denen einzelne intrazelluläre Proteasen genauer untersucht wurden. Des Weiteren sind mögliche Proteasen aus dem Gastrointestinaltrakt, die über die Nahrung aufgenommenen AGEs abbauen könnten, noch nicht ausreichend bekannt.

5.4.1 Proteasen des Gastrointestinaltraktes

Die Verdauung von Nahrungsproteinen kann teilweise bereits im Magen durch das Pepsin beginnen und im Duodenum durch die Pankreasproteasen fortgeführt werden. In der

Publikation II wurden Pepsin (EC 3.4.23.1), eine Endopeptidase, und die beiden Pankreasproteasen Trypsin (EC 3.4.21.4) und Chymotrypsin (EC 3.4.21.1), die zur Kategorie der Serinproteasen gehören, als Enzyme des Gastrointestinaltrakts verwendet. Dabei zeigte Pepsin gegenüber den beiden anderen Proteasen eine verstärkte Proteolyse der AGE-modifizierten Proteine (**Publikation II**). Pepsin weist ein saures pH-Optimum auf [174]. Säure denaturiert wiederum Proteine und erleichtert somit vermutlich den katalytischen Angriff der Enzyme. Zudem konnte in der **Publikation II** gezeigt werden, dass die Verdauungsenzyme Trypsin und Chymotrypsin in der Lage sind AGE-modifiziertes BSA abzubauen. Der Abbau fand jedoch im Vergleich zu unmodifiziertem BSA in geringerem Ausmaße statt. Auch das Referenzenzym Proteinase K (EC 3.4.21.64) zeigte ein ähnliches Abbauverhalten. Proteinase K, ebenfalls eine Serinprotease, gehört zu den aktivsten Endopeptidasen [175]. Eine verringerte Verdaubarkeit von AGE-modifiziertem BSA im Vergleich zu unmodifiziertem BSA konnte von Lapolla *et al.* bestätigt werden [176]. Eine verminderte Proteolyse ist vermutlich das Resultat von hitzeinduzierten Veränderungen an den spezifischen Spaltstellen im Protein während der Maillard-Reaktion [177].

5.4.2 Das lysosomale System

Das lysosomale System ist mit einer Vielzahl von Proteasen ausgestattet. Dazu zählen diverse Cathepsine, unter denen die Cathepsine B, D und L als die Hauptproteasen in humanen Lysosomen aufgeführt werden [125]. Cathepsin B zeigte in den Zucker-modifizierten BSA-Proben ein ähnliches Proteolyseverhalten verglichen zu Cathepsin D. Im Abbau von Glyoxal- und Methylglyoxal-modifiziertem Albumin war Cathepsin B weniger effektiv (**Publikation II**). Für weitere Charakterisierungsstudien wurde somit Cathepsin D näher betrachtet, da es zudem für intrazelluläre Proteolysen aufgrund seiner hohen Konzentration und ubiquitären Verteilung von besonderer Bedeutung ist [178]. In **Publikation II** konnte gezeigt werden, dass AGE-modifizierte Proteine in Cathepsin D-defizienten Zellen verstärkt in den Lysosomen akkumulieren. Dies konnte in Wildtyp-Zellen mit einer intakten Cathepsin D Funktion nicht beobachtet werden. Es muss jedoch erwähnt werden, dass in Cathepsin D knock-out Zellen auch die Cathepsin B- und L-Aktivität signifikant um ca. 25 % reduziert ist (**Publikation II**). Einen Einfluss auf den Proteinabbau konnte von Tiwari *et al.* in Cathepsin D knock-out Fibroblasten ausgeschlossen werden [179]. Cathepsin D trägt somit als intrazelluläre Protease zum Abbau von AGE-modifiziertem Material bei. Zusätzlich wurde beschrieben, dass

Cathepsin D das α-Synuclein, das mit dem Auftreten der Parkinson Erkrankung korreliert, proteolytisch abbauen kann [180]. Die Lysosomen weisen, wie bereits erwähnt, einen sauren pH-Wert auf. Dieser pH-Bereich denaturiert Proteine und somit auch Proteinaggregate und AGE-modifizierte Proteine. Peptidbindungen von denaturierten Proteinen sind im Allgemeinen für Proteasen leichter zugänglich.

Frühere Arbeiten zur AGE-Induktion durch Glyoxal in fetalen epithelialen Lungenzellen zeigten eine Reduktion der Cathepsin D Aktivität und der Cathepsin D mRNA Expression [181]. Folglich scheint Cathepsin D in seiner Aktivität negativ durch AGEs beeinflussbar zu sein. Im Gegensatz dazu zeigte sich in der vorliegenden Arbeit, dass AGEs in RAW 264.7 Makrophagen und in Fibroblasten die Aktivität des Cathepsin D und die Expression dieser mRNA signifikant steigern kann (**Publikation III**). Zusätzlich konnte eine erhöhte Aktivität von Cathepsin L und dessen Expression nach AGE-Inkubation festgestellt werden (**Publikation III**). Weitere Ergebnisse in den primären postnatalen Mikroglia zeigten eine signifikant erhöhte Cathepsin D und L Aktivität, induziert durch Glucose-modifiziertes BSA (**zusätzliche Ergebnisse, Abb. 7**). Eine verringerte Cathepsin D und L Aktivität konnte in alten Zellen nachgewiesen werden (**zusätzliche Ergebnisse, Abb. 7**). Interessanterweise kann diese Reduktion durch AGE-modifiziertes Material nicht beeinflusst werden. In Übereinstimmung zu den vorliegenden zusätzlichen Ergebnissen konnte beispielsweise eine reduzierte Cathepsin L Aktivität in Gehirnproben aus alten Ratten festgestellt werden [182].

Erste *in vivo* Untersuchungen bestätigen einen lysosomalen AGE-Abbau. So konnte gezeigt werden, dass Pentosidin in den Zellen des proximalen Tubulus abgebaut werden kann. Pentosidin wurde hierfür in Ratten injiziert, die teilweise mit Gentamycin behandelt wurden. Die Gentamycin-behandelten Ratten waren nicht in der Lage Pentosidin abzubauen, da Gentamycin vermutlich die lysosomale Aktivität in den Zellen des proximalen Tubulus reduziert [183].

5.4.3 Das proteasomale System

Zusätzlich zu den Lysosomen trägt das proteasomale System im Zytosol und im Nukleus zur proteolytischen Aktivität in Zellen bei. In der vorliegenden Arbeit zeigte sich das proteasomale System nicht geeignet für den Abbau der AGE-modifizierten Proteine (**Publikation II**). Dies bestätigte die Untersuchung von Bulteau *et al.,* die in Glyoxal-behandelten Fibroblasten die Bildung von CML-modifizierten Proteine induziert hatten

[170]. CML-modifizierte Proteine zeigten sich hierbei resistent gegenüber einer Proteasomproteolyse [170].

Bekannt ist, dass Proteinaggregate die proteasomale Funktion direkt beeinflussen können (**Publikation I**). Die Auswirkung von oxidierten Proteinen und deren Aggregate auf das proteasomale System wurden ausführlich in **Publikation I** zusammengefasst. Während der AGE-Bildung treten verstärkt oxidative Modifikationen der proteinhaltigen Komponenten auf, die eine Aggregatbildung begünstigen (**Publikation II**). Vermutlich beeinflussen AGEs das proteasomale System in ähnlicher Weise wie die in Publikation I beschriebenen oxidierten Proteine.

Ergebnisse aus **Publikation IV** und **zusätzliche Ergebnisse** (**Abb. 7**) zeigen, dass AGE-modifizierte Proteine die proteasomale Aktivität beeinträchtigen. Die signifikante Reduktion der Aktivität lässt sich vermutlich aufgrund sterischer Interaktionen mit dem Proteasom entweder auf katalytischer Ebene oder bei der Erkennung und Entfaltung der Substrate für den Zugang zum aktiven Zentrum begründen. Vergleichsweise hemmt α-Synuclein das 20S Proteasom [184]. Hierbei wird von den Autoren vermutet, dass die Hemmung über die Blockierung des Zugangs zum aktiven Zentrum durch α-Synuclein erfolgt [184].

In anderen Fällen wurde berichtet, dass die Proteolysefähigkeit in Anwesenheit von Proteinaggregaten gesteigert werden kann. Die Induktion der proteasomalen Aktivität wurde beispielsweise in der Huntington-Erkrankung beobachtet [185]. Das Verhältnis zwischen Proteinaggregation und proteasomaler Funktion ist komplex, da es von zahlreichen Faktoren abhängig ist, die zur Induktion oder Depression verschiedener proteasomaler Untereinheiten führen können. Letztendlich spielt das Substrat auch eine wesentliche Rolle. Hierbei ist es auch relevant in welcher Form und Struktur (z.B. Aggregate, ungefaltete Peptidketten) die Proteine vorliegen.

Die Methode zur Messung der proteasomalen Aktivität mittels fluoreszenzgekoppelten Substraten ist weit verbreitet [186]. Die Fluoresezenz, beispielsweise 7-Amino-4-Methylcoumarin (AMC) oder Rhodamin 110 (R110), wird initial im Substrat stillgelegt und nach erfolgreicher Proteolyse freigesetzt. Demzufolge ist die Erhöhung der Fluoreszenz proportional zur proteasomalen Aktivität. Eine verringerte Aktivität der konstitutiv exprimierten proteasomalen Untereinheiten (**Publikation IV und zusätzliche Ergebnisse Abb. 7**) nach AGE-Inkubation könnte eine abnormale Zellphysiologie und einen veränderten Stoffwechsel der regulatorischen Proteine, die für den normalen Zellstoffwechsel essentiell sind, nach sich ziehen.

5.5 Akkumulation der AGE-modifizierten Proteine

Eine altersabhängige Abnahme der proteasomalen Aktivität begründet das verstärkte Auftreten von oxidizierten und aggregierten Proteinen [187], die ihrerseits wieder die proteasomale Funktion beeinträchtigen können. Die altersabhängige Abnahme konnte in den **zusätzlichen Ergebnissen** bestätigt werden (**Abb. 7**). Zudem konnte eine verstärkte Akkumulation von fluoreszierenden Proteinaggregaten und Proteincarbonylen als Marker für oxidativ geschädigte Proteine bestätigt werden (**zusätzlichen Ergebnisse, Abb. 8 und Abb. 10**).

Neben dem proteasomalen System wird eine verringerte lysosomale Aktivität mit dem Auftreten von Proteinaggregaten bei neurodegenerativen Erkrankungen assoziiert [129,188]. Es ist bis heute nicht bekannt, inwiefern diese reduzierte Aktivität Ursache oder Folge der neurodegenerativen Erkrankungen ist. Eine Akkumulation von AGE-modifizierten Proteinen konnte in Cathepsin D knock-out Fibroblasten (**Publikation II**) und Cathepsin L defizienten Fibroblasten (**Publikation III**) beobachtet werden. Einen synergistischen Effekt in der AGE-Akkumulation wurde in Cathepsin L knock-out Zellen nach gleichzeitiger Inhibierung von Cathepsin D deutlich (**Publikation III**). Diese Ergebnisse weisen auf die bedeutende Rolle der Cathepsin D und L Aktivität im Abbau und somit in der Verringerung der AGE-Akkumulation in zellulären Systemen hin.

Es gibt zahlreiche Faktoren, die die Akkumulation glykierter Proteine beeinflussen. Der wichtigste Faktor ist hierbei die Balance zwischen Proteinsynthese und Proteinabbau. Zudem trägt die Konzentration an Proteincarbonylen wesentlich zur Entstehung glykierter Proteine bei [189]. Zu den weiteren Faktoren zählen die Art der Carbonylsubstrate, die Verfügbarkeit und die Reaktivität von Aminogruppen in den Proteinen, sowie die chemische und enzymatische Degradierung glykierter Proteine und ihre renale Ausscheidung [189].

Es ist denkbar, dass in der Peripherie auftretende AGEs über die Blut-Hirn-Schranke transportiert werden können und im zentralen Nervensystem akkumulieren. Hinweise hierfür liefert die Studie von Deane *et al* [190]. In einem Alzheimer-erkrankten Mausmodell konnte gezeigt werden, dass Amyloid-β an den RAGE bindet und dieser das Amyloid-β über die Blut-Hirn-Schranke transportieren kann [190]. Da AGE-modifizierte Proteine ebenso Liganden für den RAGE darstellen, wäre dieser Mechanismus denkbar. Es muss hierfür jedoch geklärt werden, ob der RAGE an der Endozytose beteiligt ist bzw. eine Aufnahme der unterschiedlichen Liganden ermöglicht wird oder ob dieser Effekt durch

andere AGE-Rezeptoren an den epithelialen Zellen der Blut-Hirn-Schranke ermöglicht werden kann.

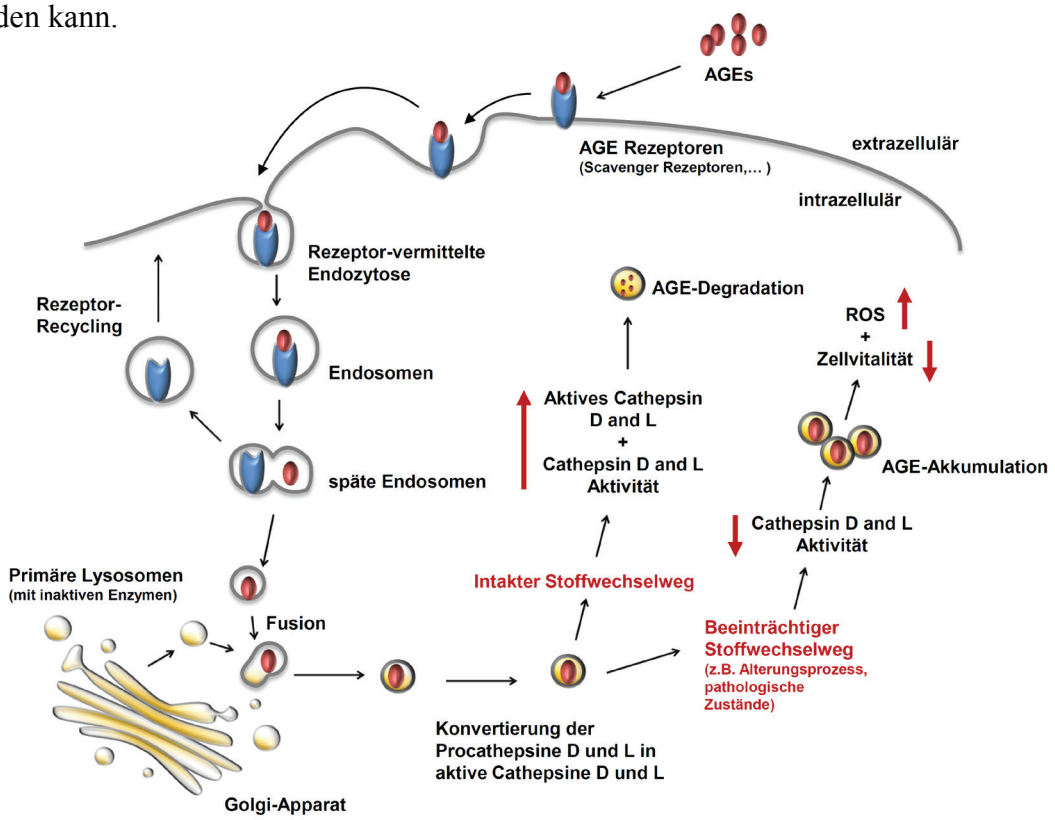


Abbildung 13: Akkumulation der AGE-modifizierten Proteine in Cathepsin D und L-defizienten Zellen

Die Aufnahme der AGEs über die Interaktion mit den AGE-Rezeptoren erfolgt über das endosomale-lysosomale System. Während der Lysosomenreifung werden die Cathepsine aktiviert und degradieren folglich AGE-modifizierte Proteine. Eine verringerte Cathepsin D- und L-Aktivität resultiert in einer AGE-Akkumulation, die nachfolgend für eine gesteigerte ROS-Produktion und eine reduzierte Zellvitalität verantwortlich ist (**Zusammenfassung aus Publikation III**).

5.6 AGE-induzierte zelluläre Reaktionen

5.6.1 AGE-induzierte zelluläre Signalkaskaden

AGEs induzieren die Freisetzung zahlreicher Zytokine und tragen somit zum proinflammatorischen Status bei [191]. Diese Reaktionen sind häufig auf Interaktionen mit dem RAGE zurückzuführen. Obwohl die ligandenspezifische Aktivierung des Rezeptors intensiv erforscht wird, gibt es immer noch wenige Hinweise über den Mechanismus der RAGE-Aktivierung und welche Signalmoleküle nachfolgend die zellulären Reaktionen induzieren. Bekannt ist, dass RAGE multimere Strukturen innerhalb der Plasmamembran ausbildet. *In vitro* Daten deuten darauf hin, dass diese Multimere aus mindestens vier RAGE-Molekülen bestehen und größere Einheiten ausbilden können [57]. Die Geometrie der Liganden und die Anordnung der RAGE-Moleküle ist eine wichtige Voraussetzung für die Aktivität des Rezeptors. Die intrazellulären Domänen müssen hierfür in eine direkte Nähe mit spezifischer Orientierung zueinander gebracht werden [57]. Dieses Modell ist auch bekannt für die Rezeptor-Tyrosinkinasen [192] bei denen eine bestimmte Nähe und Orientierung der intrazellulären Domänen die intrinsische Kinaseaktivität und somit die Initiierung der Signalkaskaden hervorruft. Im Falle des RAGE lässt sich vermuten, dass durch Bindung der multimeren Liganden, wie beispielsweise S100 Proteine oder AGEs, ein Zusammenführen der Rezeptoreinheiten ermöglicht wird. Diese Vermutung könnte auch die erhöhte Expression des RAGE nach dessen Aktivierung erklären, da erhöhte Rezeptorkonzentrationen in der Zellmembran weitere Rezeptorvereinigungen unterstützen könnten. Eine erhöhte RAGE-Expression konnte in den RAW 264.7 Zellen nach AGE-Inkubation, sowohl auf mRNA-Ebene als auch auf Proteinebene, bestätigt werden (**Publikation IV**).

Es gibt jedoch bis dato keine ausreichenden Informationen über die intrazelluläre Domäne des RAGE. Zwei Adapterproteine wurden bisher mit einer RAGE Signalkaskade in Verbindung gebracht: Diaphanous 1 [193] und ERK [194]. Außerdem muss berücksichtigt werden, dass glykierte Aminosäuren, wie CML und CEL, nicht an RAGE binden können, wobei zahlreiche AGE-modifizierten Proteine in nanomolaren Konzentrationen für die Liganden-Rezeptor-Interaktionen ausreichen [138]. Des Weiteren kann ein proteolytischer Abbau der AGE-Komplexe die Bindungsfähigkeit an Rezeptoren wie den RAGE reduzieren [195].

Eine Aktivierung des RAGE induziert zudem eine verringerte Expression der Glyoxalase-1, einem Enzym, dass eine Proteinglykierung durch Methylglyoxal und Glyoxal verhindern

kann [196]. Eine verringerte Glyoxalase-1-Expression führt zu einer gesteigerten Proteinglykierung und somit zu einer erhöhten Konzentration an intrazellulären AGE-modifizierten Proteinen [196]. Es lässt sich insofern nicht ausschließen, dass eine Kolokalization von RAGE und AGEs auf eine RAGE-Aktivierung durch andere Liganden, wie beispielsweise S100 Proteinen, zurückzuführen ist. Diese Aktivierung würde somit eine verringerte Expression der Glyoxalase-1 und eine erhöhte lokale AGE-Konzentration bewirken. Inwiefern diese intrazellulären AGE-modifizierten Proteine als Substrate für das lysosomale oder proteasomale System dienen bedarf zur Aufklärung weitere Studien.
Neben endogen-gebildeten AGEs können die über die Nahrung zugeführten AGEs als RAGE Liganden wirken. Diese aktivieren *in vitro* sämtliche Signaltransduktionswege, die durch eine Aktivierung von RAGE ausgelöst werden können [55,197]. Es ist denkbar, dass diätetische AGEs zusammen mit endogen-gebildeten AGE-modifizierten Proteinen zur Zellaktivierung und zum oxidativen Stress beitragen können, sowie allgemein die glykoxidative Belastung unterstützen, die wiederum vielfältige Schäden nach sich ziehen kann.

5.6.2 AGE-induzierte Produktion freier Radikale

Eine Aktivierung der NAPDH-Oxidase (NOX) führt in phagozytotischen Zellen zur Produktion von reaktiven Sauerstoffspezies (ROS). Dieser Mechanismus dient vorwiegend der Abtötung von Pathogenen und wird als oxidativer oder respiratorischer Burst bezeichnet. Der oxidative Burst definiert einen verstärkten Sauerstoffverbrauch mit gleichzeitigem Anstieg der ROS-Produktion aufgrund von Mikroorganismen oder anderen inflammatorischen Mediatoren. Die regulierenden Proteine der NOX-Familie werden beispielsweise durch den für hämatopoetischen Zellen spezifischen Transkriptionsfaktor PU.1 und den Transkriptionsfaktor *interferon regulatory factor-1/2* (IRF-1/2) als Antwort auf eine IFN-γ Aktivierung beeinflusst. Eine Stimulation mit LPS führt zur Aktivierung des Transkritptionsfaktors NF-κB, der die Expression des $gp91^{phox}$ induziert und nach Aktivierung der NADPH-Oxidase eine verstärkten ROS-Produktion bewirkt [198]. Bekannt ist, dass AGEs durch die Aktivierung der NADPH-Oxidase ROS induzieren können [86]. Dies konnte in den postnatalen Mikroglia bestätigt werden (**zusätzliche Ergebnisse, Abb. 12**). Eine verstärkte ROS-Produktion der NADPH-Oxidase in AGE-stimulierten Zellen kann mit einer gesteigerte $gp91^{phox}$ Expression über die Aktivierung des NF-κB begründet werden. Neben der Proteinkinase C können auch andere Kinasen in der Aktivierung der NADPH-Oxidase eine Rolle spielen, beispielsweise ERK 1/2 und p38

MAPK [199], welche auch durch AGE-modifizierte Proteine aktivieren werden können. Dies erklärt die Aktivierung der NADPH-Oxidase und folglich eine Messung des oxidativen Burst infolge einer direkter Aktivierung der PKC über PMA nach AGE-Exposition.

Des Weiteren konnte in Fibroblasten mit lysosomaler Dysfunktion eine erhöhte ROS-Produktion detektiert werden (**Publikation III**). Eine verringerte Aktivität dieser lysosomalen Enzyme korrelierte somit mit einer gesteigerten Toxizität der AGE-modifizierten Proteine.

Diese unterschiedlichen Zellmodelle zeigen, dass eine Akkumulation AGE-modifizierter Proteine über verschiedene Reaktionen zur verstärkten oxidativen Belastung der Zellen beitragen kann.

5.6.3 Die Induktion des Immunoproteasoms

Protein-Expressionsstudien zeigen einen Einfluss der AGE-modifizierten Proteine auf das proteasomale System. Nach Untersuchungen zur Aktivität konnte gezeigt werden, dass AGE-modifizierte Proteine die Expression der konstitutiven proteasomalen Untereinheiten verringern und dafür die Expression der induzierbaren proteasomalen Untereinheiten verstärken (**Publikation IV**). Die induzierbare Form des Proteasomes wird vorwiegend mit einer verbesserten Antigen-Prozessierung assoziiert [109,121]. Säugerzellen können je nach Bedarf die konstitutiven und induzierbaren proteasomalen Untereinheiten mit den verschiedensten Regulatoren in unterschiedlichen Verhältnissen aufweisen [121]. In Übereinstimmung mit den Daten von Gavilán *et al.,* die eine altersabhängige Steigerung der immunoproteasomalen Expression im Hippocampus zeigten [200], wurde eine erhöhte Proteinexpression der induzierten katalytischen Untereinheit β5i in alten Mikroglia ohne vorherige Stimulation bestimmt (**zusätzliche Ergebnisse, Abb. 12**).

Um die Funktion der immunoproteasomalen katalytischen Einheiten genauer untersuchen zu können, wurde aufgereinigtes Immunoproteasom im Hinblick auf seine Hydrolyseaktivitäten hin untersucht [201-203]. Insgesamt wurde eine eindeutige Reduktion der caspaseähnlichen- bzw. der peptidyl-glutamyl-peptid-hydrolase-Aktivität nach Inkorporation der immunoproteasomalen Untereinheit β1i nachgewiesen. Widersprüchliche Modifikationen wurden für die weiteren zwei Untereinheiten, dem β2i und β5i, gefunden [201-203].

Es wird der Präsenz von 11Sα eine wesentliche Rolle bei der Aktivierung des Immunoproteasoms zugesprochen [114,115]. Die Affinität der 11Sα-Untereinheiten zu den

α-Ringen des 20S Proteasoms ist dabei um ein Vielfaches höher als die Affinität der 11Sβ-Untereinheiten. In Übereinstimmung zu den induzierbaren katalytischen Untereinheiten wurde auch der 11Sα-Regulator verstärkt in AGE-behandelten RAW 264.7 exprimiert (**Publikation IV**).

Der Begriff „Immunoproteasom“ wurde aufgrund der Lokalisation von β1i/LMP2- und β5i/LMP7-Genen, die innerhalb der MHC-Klasse II Region kodiert werden, eingeführt [204]. Durch die zytokinabhängige Induktion des Immunproteasoms wird die immunrelevante Funktion des Proteasoms verbessert [109]. Zudem induzieren Interferone die unterschiedlichsten Komponenten des Ubiquitin-konjugierenden Systems, wie E1, E2 und E3 Enzyme [121]. Diese Induktion könnte auf eine allgemeine Erhöhung der Proteaseaktivität, ausgelöst durch Begleitreaktionen der Stimulation, hinweisen.

Die funktionelle Bedeutung des Immunoproteasoms in der Bewältigung des oxidativen Stresses wird in Immunoproteasom-defizienten Zellen und Geweben deutlich. In diesen speziellen Zellen akkumulieren oxidativ geschädigte polyubiquitinierte Proteine [205]. Insgesamt lässt die Datenlage aktueller Studien vermuten, dass eine Inkorporation immunoproteasomaler Untereinheiten, sowie die Verwendung der 11S-Regulatoren, mit oder ohne 19S-Regulatoren, strukturelle Veränderungen innerhalb des 20S Proteasoms hervorrufen, die einen verbesserten Zugang der Substrate zum aktiven Zentrum ermöglichen. Derartige Vermutungen müssen allerdings mit Studien zu strukturellen Veränderungen des proteasomalen Komplexes nachgewiesen werden.

Aktuelle Untersuchungen zeigen, dass β5i/LMP7 eine wichtige Rolle in der Zytokinproduktion spielt und Inhibitoren dieser Untereinheit weisen therapeutische Wirkungen in Mausmodellen mit Autoimmunerkrankungen und Entzündungen auf [206-208]. Der spezifische Inhibitor für β5i/LMP7, PR-957 (ein Epoxyketon), resultiert in einer verringerten Zytokinkonzentration, einer angepassten T-Zell-Antwort und verhindert die Progressionen der rheumatischen Arthritis, der experimentellen Kolitis und der systemischen Lupus erythematosus [206-208].

Infolge der signifikanten Induktion des Immunoproteasoms in AGE-behandelten Zellen, ist die Aktivität des konstitutiven Proteasoms im Vergleich zur Aktivität des Immunoproteasoms reduziert (**Publikation IV und zusätzliche Ergebnisse, Abb. 7**). Vermutlich ist die Induktion des Immunoproteasoms eine Antwort auf die allgemeine zelluläre AGE-Aktivierung. Arbeiten von Huber *et al*. konnten kürzlich strukturelle Bedingungen für spezifische Inhibitoren von β1 und β1i sowie β5 und β5i aufweisen [209]. Anhand dieser Datenlage können Inhibitoren gegen die einzelnen katalytischen

Untereinheiten entwickelt werden, die anschließend zu Untersuchungen der Aktivitäten und somit zur Aufklärung der exakten Funktionen eingesetzt werden können. Auch die Studie von Huber *et al*. konnte belegen, dass PR-957 ein spezifischer Inhibitor für β5i im Gegensatz zu β5 ist [209]. In **Publikation IV** konnte gezeigt werden, dass die Induktion des Immunoproteasoms durch Inhibitoren der Jak2-STAT1-Phosphorylierung, beispielsweise AG-490 und Resveratrol, verhindert werden kann. Damit kann eine überschießende Immunantwort auf AGE-modifizierte Proteine reduziert werden.

5.7 Altern

5.7.1 Theorien des Alterns

Es existieren mehr als 300 Theorien über das Altern [210]. Trotz der Komplexität der biochemischen Prozesse, die unvermeidbar zum Altern beitragen, klassifizieren Wissenschaftler die Theorien wie folgt ein: Alterstheorien am gesamtem Lebewesen, Alterstheorien auf Ebene der Organen und auf zellulärer als auch molekularer Ebene [211]. Ein Großteil der Theorien basiert auf spontane chemische Reaktionen, die willkürlich Proteine und andere biologische Makromoleküle schädigen. Diese Theorien stützen sich auf zahlreiche Beobachtungen die beschreiben, dass chemisch modifizierte Proteine während des Alterungsprozesses akkumulieren. Eine dieser Theorien ist die „Free radical theory of aging“, die besagt, dass reaktive Sauerstoff- und Stickstoffspezies die primären Quellen physiologischer und pathophysiologischer Schäden an Biomolekülen sind. Weitere Modifikationen, die hauptsächlich an Proteinen zu beobachten sind, umfassen die sogenannten nicht-enzymatischen Modifikationen, wie beispielsweise die Glykierungsreaktionen. Diese Reaktionen zählen zu den diversen Alterungshypothesen und werden als „Glykierungshypothese des Alterns“ bezeichnet [212].

Die meisten AGE-Strukturen entstehen während der Alterung an langlebigen Proteinen, wie beispielsweise an Kristallin in der Augenlinse und an Kollagen in diversen Geweben [213]. Zahlreiche Studien deuten darauf hin, dass die Rate der AGE-Akkumulationen in Proteinen mit der Lebensspanne und dem Abbau dieser Proteine korreliert [214]. So konnte gezeigt werden, dass Kollagen in Gelenkknorpeln, welche außergewöhnlich lange erhalten bleiben (> 100 Jahre), im Vergleich zu Kollagen in der Haut (Lebensdauer ca. 15 Jahre) mehr AGE-Strukturen beinhaltet [215]. Hinweise deuten darauf hin, dass Proteolyseprozesse während der Alterung reduziert sind und somit Proteinschäden akkumulieren [216]. Zudem wird die Hypothese aufgestellt, dass die Beseitigung von

AGE-Vorstufen in älteren Organismen verringert ist, was eine AGE-Zunahme begründet [217]. Darüber hinaus tragen Glykierungen an proteolytischen Enzymen zu deren Funktionsverlust und somit zur Zunahme der AGEs bei [213].
Eine weitere Theorie, die mit dem Vorgang des Alterns in Verbindung gebracht wird, ist die altersbedingte Seneszenz.

5.7.2 Altersbedingte Seneszenz

Zelluläre Seneszenz beschreibt ein Phänomen bei dem diploide Zellen die Fähigkeit verlieren sich zu teilen. Zudem wird die zelluläre Seneszenz mit Veränderungen der Morphologie und Funktionsverlust der Zellen assoziiert [218,219]. Die zelluläre Seneszenz wurde zum ersten Mal in humanen Fibroblasten im Jahre 1961 beschrieben [218]. Es gibt zahlreiche Faktoren, die zur Seneszenz beitragen können, dazu zählen (1) Telomer-verkürzungen, (2) oxidativer Stress, (3) mechanische Verletzungen und (4) Strahlungen [220-222]. Auch die mikroglialen Zellen sind von einer altersbedingten Seneszenz betroffen [223]. In jungen Jahren verfügen Mikroglia hauptsächlich über neuroprotektive Eigenschaften. Diese beinhalten die Beseitigung von toten Zellen und die Produktion von neurotrophen Faktoren, antiinflammatorischen Zytokinen, sowie antioxidativen Enzymen [224]. Mit zunehmendem Alter nehmen die Eigenschaften der Mikroglia einen neurotoxischen Stellenwert ein [224]. Aktuelle Studien gehen der Frage nach, ob Entzündungen ursächlich an der pathologischen Seneszenz beteiligt sind. Yu Hong-Mei *et al.* stimulierten dazu an mehreren aufeinanderfolgenden Tagen die mikrogliale Zelllinie BV-2 mit 10 ng/ml Lipopolysacchariden (LPS) und beobachteten hierbei eine verringerte Proliferation und einen Zellzyklus-Arrest in der G0/G1 Phase, welcher auf eine zelluläre Seneszenz hindeutet [225]. Zusätzlich beobachteten Yu *et al.* eine Zunahme der altersabhängigen Marker p53 und β-Galaktosidase in LPS-stimulierten Mikroglia [225]. Der Alterungsprozess wird häufig von einer verstärkten Entzündung begleitet [226]. Demnach konnten kürzlich zwei Studien berichten, dass inflammatorische Zytokine wichtige Mediatoren der Seneszenz sind [227,228]. In der Seneszenzstudie von Yu *et al.* wurde festgestellt, dass die Induktion der zellulären Seneszenz nicht nur auf eine Entzündung zurückzuführen ist, sondern sich diese vielmehr auf die Akkumulierung diverser inflammatorischer Signale innerhalb der Zelle bezieht [225]. Diese Beobachtung kann auch die Tatsache erklären, dass der Alterungsprozess vielmehr durch eine chronische Entzündung verstärkt wird, als durch einmalige oder akute Entzündungen. Eine Vielzahl von Hinweisen deuten darauf hin, dass periphere Entzündungen eine

Mikrogliaaktivierung im zentralen Nervensystem auslösen können und dass während der Lebensdauer mikrogliale Zellen sämtlichen Stimulanzien aus der Peripherie ausgesetzt sind [229]. Es lässt sich somit vermuten, dass eine wiederholte Aktivierung mikroglialer Zellen durch inflammatorische Stimulanzien zur zellulären Seneszenz beitragen und somit deren Funktionalität beeinträchtigt wird. Mikrogliale Zellen üben dann vermehrt neurotoxische Aktivitäten aus, die zum langen Prozess der Neurodegeneration beitragen. Diese Tatsache kann auch anhand des oxidativen Bursts vermutet werden. In primären Mikroglia konnte in Laufe des Älterwerdens eine Zunahme der ROS-Produktion ohne vorangegangene Stimulation beobachtet werden (**zusätzliche Ergebnisse, Abb. 9**). Eine altersabhängige Dysfunktion der mikroglialen Zellen konnte anhand des oxidativen Bursts nach Stimulation mit LPS, Glucose-modifiziertem BSA und PMA gezeigt werden (**zusätzliche Ergebnisse, Abb. 11**). Desweiteren lässt sich die Induktion des Immunoproteasoms nach AGE-Exposition in den alten Zellen nicht mehr beobachten (**zusätzliche Ergebnisse, Abb. 12**). Zusammengefasst konnte gezeigt werden, dass ältere Zellen in ihrer Funktionalität beeinträchtigt sind, ein Phänomen das häufig als Immunoseneszenz bezeichnet wird [230]. Eine Induktion des Immunoproteasoms in primären Mikrogliazellen wurde von Strohwasser *et al.* bestimmt. Diese Zellen, isoliert aus neugeborenen Mäusen, wurden mit IFN-γ und LPS für 7 Stunden aktiviert. Beide Stimulanzien induzieren die Expression der immunoproteasomalen katalytischen Untereinheiten [231]. Eine Induktion des Immunoproteasoms konnte auch in postnatalen Mikroglia nach 72 Stunden Inkubation mit Glucose-modifiziertem BSA nachgewiesen werden (**zusätzliche Daten, Abb. 12**). Es wird allgemein vermutet, dass die immunoproteasomale Expression im gesunden und jungen Gehirn sehr gering ist. Gehirnproben aus Alzheimer-Patienten (durchschnittliches Alter: 70.3 Jahre) und gesunden älteren Kontrollen (durchschnittliches Alter: 70.2 Jahre) zeigten eine Induktion des Immunoproteasoms [232]. Zudem wurde in der Alzheimer-Gruppe eine verringerte trypsinähnliche Aktivität (β2/β2i) gemessen [232]. Die zusätzlichen Ergebnisse bestätigen eine kaum messbare immunoproteasomale Expression in jungen Zellen, während ältere Mikroglia eine erhöhte Expression aufweisen, die jedoch nach Stimulation der Zellen nicht verstärkt werden konnte (**zusätzliche Daten, Abb. 12**).

Es kann vermutet werden, dass die Induktion des Immunoproteasoms als zelluläre Reaktion auf erhöhten oxidativen Stress dazu führt, dass oxidativ geschädigte Proteine in stärkerem Ausmaß abgebaut werden. Eine erhöhte proteolytische Aktivität gegenüber oxidativen geschädigten Proteinen wird dem Immunoproteasom zugesprochen [121]. Diese

Behauptung steht jedoch im Wiederspruch zu zahlreichen Studien, die eine altersabhängige Abnahme der proteasomalen Aktivität bei gleichzeitiger Akkumulation oxidativen geschädigten Proteinen beschreiben [187]. Zukünftige Studien, die die Funktionen des Immunoproteasoms näher charakterisieren, sollen hierzu Klarheit verschaffen.

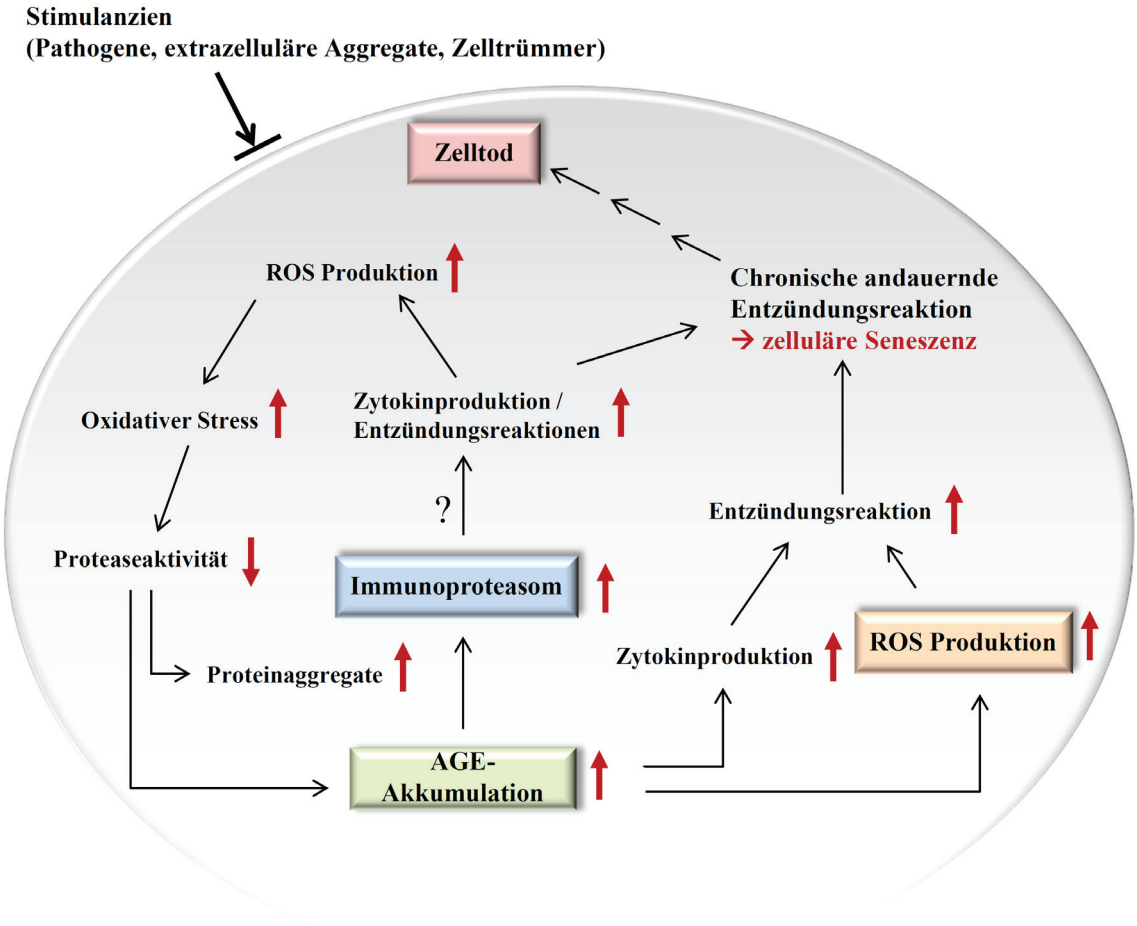


Abbildung 14: Zelluläre Seneszenz

Übersicht über die Faktoren, die zur zellulären Seneszenz beitragen können.

5.8 Ausblick

In dieser Arbeit wurde ein Abbau AGE-modifizierter Proteine durch die lysosomalen Cathepsine D und L aufgezeigt. Ihre essentielle Funktion konnte des Weiteren in der verringerten AGE-induzierten Toxizität bestätigt werden. Für die Untersuchungen wurden die Makrophagen-Zelllinie RAW 264.7 und murine embryonale Mausfibroblasten verwendet. Im Vordergrund weiterer Studien sollten vergleichende Untersuchungen an alternden Zellen durchgeführt werden, da gezeigt werden konnte, dass diese sich in ihrer Funktionalität von jungen Zellen deutlich unterscheiden.

Im Hinblick auf den Mechanismus zur Induktion des Immunoproteasoms gilt es herauszustellen, ob eine verminderte Synthese von immunoproteasomalen Untereinheiten zur erhöhten Quantität der konstitutiven β-Untereinheiten führen und unter welchen Bedingungen deren Einbau in die 20S Kerneinheit erfolgt. Mit spezifischen Inhibitoren gegen immunoproteasomale und konstitutive katalytische Untereinheiten des Proteasoms können zukünftig die Unterschiede dieser Subtypen dargestellt werden.

Ferner bleiben weitere Fragen im Hinblick auf die Funktionen und die Mechanismen des RAGE offen. Dazu zählen die Bestimmung der normalen physiologischen Funktionen und die Charakterisierung weiterer Signalwege. Zusätzlich muss der genaue Mechanismus der RAGE Aktivierung über mögliche Konformationsänderungen, Bindungsaffinitäten der Liganden und Signalmolekülen bestimmt werden. Weitere Untersuchungen sollten eine mögliche Interaktion des RAGE mit anderen Rezeptoren und daraus resultierenden Mechanismen aufzeigen.

Studien zur Bioverfügbarkeit der AGEs und deren exogener Aufnahme sollten in Zukunft Klarheit über deren Beitrag zum endogenen AGE-Spiegel verschaffen und der Fragestellung nachgehen, ob eine erhöhte Einnahme der AGEs einen verstärkten Einfluss auf Entzündungen und oxidativen Stress ausüben können. Darüber hinaus sollten Untersuchungen zeigen, inwiefern AGEs aus der Peripherie die Blut-Hirn-Schranke überwinden können und im zentralen Nervensystem zu einer inflammatorischen Reaktion beitragen. In diesem Zusammenhang sollten weitere Studien zur Bedeutung der AGEs im Hinblick auf die Entstehung neurodegenerativer Erkrankungen durchgeführt werden.

Die Tatsache, dass bis heute keine endgültigen Aussagen über die gesundheitlichen Auswirkungen der AGEs *in vivo* gemacht werden können, zeigt die Notwendigkeit weiterer Untersuchungen.

6 ZUSAMMENFASSUNG

Einleitung: „Advanced glycation end products“ (AGEs) werden mit diversen Krankheiten in Zusammenhang gebracht. Ihre Entstehung kann bis dato nicht gänzlich verhindert werden. Im Hinblick auf die Beeinflussung der durch AGE-modifizierten Proteine beteiligten Erkrankungen sind die Kenntnisse zu den zelluläre Effekten und mögliche Abbausysteme unerlässlich. Da das endogene bzw. zelluläre Schicksal der AGE-modifizierten Proteine bisher nur unzureichend untersucht wurde, war dies Gegenstand der vorliegenden Arbeit.

Zielsetzung: Der Schwerpunkt dieser Untersuchungen beinhaltete die Charakterisierung der proteolytischen Systeme, die die artifiziell hergestellten AGEs abbauen können. Zudem wurde in speziellen Zellmodellen mit enzymaler Defizienz die Bedeutung der jeweiligen Proteasen aufgeführt. Die Darstellung eines AGE-induzierten zellulären Signalweges sollte dessen Regulation erstmalig aufweisen. Die Verwendung primärer mikroglialer Zellen verschiedenen Alters diente der Verifizierung der vorangegangen Untersuchungen. Hierbei sollten weitere Eigenschaften der zellulären Seneszenz, die eine bedeutende Rolle im Alterungsprozess und bei der Entstehung neurodegenerativen Erkrankungen innehalten, dargelegt werden.

Methoden: Artifiziell hergestelltes AGE-modifiziertes BSA wurde *in vitro* mit unterschiedlichen proteolytischen Enyzmen inkubiert und die Proteolyse mittels Fluorescamin-Assay untersucht. Unter Verwendung der Fluoreszenzmikroskopie und Herstellung radioaktiver Glucose-modifizierter Proteine erfolgte die Bestimmung der zellulären AGE-Aufnahme. Zur Verifizierung der Cathepsin D und L-Funktionen in AGE-exponierten Zellen wurden Cathepsin D- und L-defiziente Zellen verwendet. Zelluläre Reaktionen, ausgelöst durch AGE-modifizierte Proteine wurden größtenteils mittels Protein- und Genexpressionsstudien bestimmt. Zudem dienten Aktivitätsassays für die lysosomalen Enzyme und das proteasomale System nach AGE-Inkubation zur weiteren Charakterisierung der AGE-modifizierten Proteine und ihren ausgelösten zellulären Reaktionen. Für die Beurteilung des RAGE-induzierten Signalweges im Hinblick auf die Induktion des Immunoproteasoms wurde eine *small-interfering RNA* (siRNA) gegen RAGE verwendet.

Ergebnisse: *In vitro* Untersuchungen lassen auf einen lysosomalen AGE-Abbau schließen. Das in den Lysosomen lokalisierte Cathepsin D kann im Vergleich zu Cathepsin B AGE-modifizierte Proteine effizienter abbauen. Eine zeitabhängige Akkumulation AGE-modifizierter Proteine konnte in Cathepsin D- und L-defizienten Fibroblasten bestimmt werden. Des Weiteren konnten AGE-modifizierte Proteine die proteasomale Aktivität reduzieren und eine verringerte Expression der konstitutiven proteasomalen katalytischen Untereinheiten bewirken. Andererseits zeigten AGE-modifizierte Proteine eine Steigerung der Expression immunproteasomaler Untereinheiten, sowohl auf mRNA- als auch auf Proteinebene. Die Induktion wird über eine Aktivierung des RAGE und nachfolgender Phosphorylierung der Signalmolekülen Jak2/STAT1 vermittelt.
Studien in primären postnatalen mikroglialen Zellen zeigten eine erhöhte Cathepsin D und L Aktivität, sowie eine Reduktion der proteasomalen Aktivität nach AGE-Inkubation. Weniger effizient reagieren adulte und alte Zellen auf die AGE-modifizierten Proteine.

Schlussfolgerung: In der vorliegenden Arbeit konnte insbesondere Cathepsin D den Abbau AGE-modifizierter Proteine bewerkstelligen und einer Akkumulation dieser Aggregate entgegenwirken. Cathepsin D und L sind im Wesentlichen für die Reduzierung der zellulären AGE-induzierten Toxizität verantwortlich. Alternde Zellen reagieren jedoch auf AGE-modifiziertes Material unzureichend. Eine möglicherweise damit verbundene erhöhte Akkumulation dieser Proteine und verstärkte chronische Antwort der zellulären Reaktionen ist denkbar. Die vorliegenden Arbeiten ergänzen die bisherigen Studien zu der Verminderung der AGE-induzierten pathologischen Eigenschaften.

7 ABSTRACT

Introduction: "Advanced glycation end products" (AGEs) are associated with various pathological conditions. Until today the formation of AGEs cannot be prevented completely. With regard to the influence of AGE-modified proteins involved in diseases, the knowledge of their cellular effects and possible degradation pathways is essential. Since the cellular fate of AGE-modified proteins has been inadequately investigated, the object of the present study was to analyze their cellular reactions.

Aims: The focus of these studies was the characterization of the proteolytic systems that are able to degrade artificially produced AGE-modified proteins. The importance of proteases should further be affirmed by specific cellular models with enzymatic deficiencies. The regulation of an AGE-induced cellular signalling pathway should be shown for the first time. Furthermore, a comparison with primary microglial cells of different ages was used to verify the previous results. Further properties of cellular senescence, playing an important role in aging and development of neurodegenerative diseases, should be reported.

Methods: Artificially produced AGE-modified BSA was incubated *in vitro* with different proteolytic enzymes and examined for the degradation capability by the fluorescamine assay. The cellular uptake of AGEs was determined by fluorescence microscopy and radioactive glucose-modified proteins. In order to verify the functions of cathepsin D and L in AGE-exposed cells, specific cathepsin D- and L-deficient cells were used. Cellular responses triggered by AGE-modified proteins were largely determined by protein and gene expression studies. In addition, activity assays were used for the characterization of lysosomal and proteasomal enzymes due to AGE-incubation. A small-interfering RNA (siRNA) against RAGE was used to assess the RAGE-induced signalling pathway leading to the induction of the immunoproteasome.

Results: *In vitro* studies demonstrate a lysosomal degradation of AGEs, primarily by cathepsin D. The 20S proteasome was completely unable to degrade AGE-modified proteins. A time-dependent accumulation of AGE-modified proteins could be determined in cathepsin D- and L-deficient fibroblasts. Furthermore, AGE-modified proteins could reduce proteasomal activity and expression of the constitutive catalytic proteasomal subunits. On the other hand, a significant increase in the mRNA- and protein-expression of

immunoproteasomal subunits was detected in AGE-exposed cells. The induction of the immunoproteasome is initiated due to the activation of the Jak2/STAT1 pathway by a RAGE signalling process. Studies in postnatal primary microglial cells confirmed the previous experiments. Thus, increased activity of cathepsin D and L and reduced proteasomal activity was detected. The response of adult and old microglial cells to AGE-modified proteins is less efficient.

Conclusion: The present work reveals the important role of cathepsin D in AGE-exposed cells. Cathepsin D was able to reduce the accumulation of AGE-modified proteins. Furthermore, cathepsin D and L are responsible for the reduction of cellular AGE-induced toxicity. Aging cells show insufficient reaction to AGE-modified materials. A potentially associated increased accumulation of these proteins and therefore enhanced chronic cellular responses is conceivable. These studies contribute to the general understanding how the pathological features of AGE-modified proteins may be affected.

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9 ANHANG

9.1 Charakterisierung der BSA-Kontrolle

Die Herstellung der AGEs erfolgte wie bereits beschrieben (**Publikation II, III und IV**). Als zusätzliche Kontrolle zu modifiziertem Albumin wurde bovines Serumalbumin (BSA) ohne Zugabe von Zucker und Aldehyde für sechs Wochen bei 37°C in phosphatgepufferter Salzlösung (PBS) inkubiert.

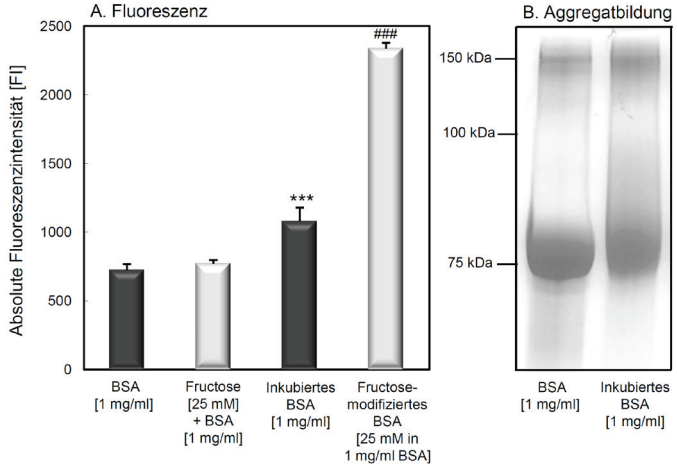

Abbildung I: Charakterisierung der BSA-Kontrolllösung ohne Zugabe von Zucker und Aldehyde

Zur Charakterisierung der BSA-Kontrolllösung wurde 1 mg BSA in 1 ml PBS gelöst und entsprechend den Zucker-BSA-Lösungen für 6 Wochen bei 37°C inkubiert. Anschließend wurde die Modifizierung der BSA-Kontrolllösung mittels Fluoreszenz (A.) und SDS-PAGE (B.) überprüft und mit einer frisch hergestellten BSA-PBS-Lösung verglichen. Zusätzlich wurde Fructose in BSA-PBS gelöst und sowohl frisch, als auch nach 6 Wochen Inkubation mittels Fluoreszenz untersucht. Ein statistisch signifikanter Unterschied zu der frisch hergestellten BSA-Lösung ist durch den Student´s t-test, n=4, mit ***($p < 0.001$) gekennzeichnet. Zusätzlich ist ein statistisch signifikanter Unterschied zwischen der Fructose-BSA-Lösung vor und nach 6 Wochen Inkubation bei 37°C durch den Student´s t-test, n=4, ###($p < 0.001$) dargestellt.

Untersuchungen zeigen eine signifikante Zunahme der Fluoreszenz (λ_{Ex} 485 nm / λ_{Em} 530 nm) in der BSA-PBS-Kontrolllösung nach sechswöchiger Inkubation bei 37°C (inkubiertes BSA [1 mg/ml]) im Vergleich zur frisch hergestellten BSA-PBS-Lösung (BSA [1 mg/ml] (Abb. 7A). Fructose (25 mM), angesetzt in 1 mg/ml BSA-PBS, zeigt nach sechswöchiger Inkubation eine signifikante Erhöhung der Fluoreszenz verglichen zu frisch angesetzter Fructose-BSA-Lösung (Fructose [25 mM] + BSA [1 mg/ml]) (Abb. 7A). Die BSA-Modifikation nach dieser Inkubationsdauer konnte des Weiteren mittels einer SDS-PAGE nachgewiesen werden (Abb. 7B). Eine frisch hergestellte BSA-PBS-Lösung ist in der linken Laufbahn zu erkennen. Die rechte Laufbahn hingegen zeigt eine inkubierte BSA-PBS-Lösung mit modifiziertem

Molekulargewicht. Diese Ergebnisse zeigen eindeutig eine Modifizierung von reinem BSA nach entsprechender Inkubationszeit.

9.2 Zeitabhängige Vitalität nach Inkubation mit AGE-modifiziertem BSA

Unterschiedliche Konzentrationen von AGE-modifiziertem BSA wurden zu Beginn der experimentellen Untersuchungen mittels Vitalitätsassays an den Zellen erprobt. Hierfür wurde der MTT (3-[4,5-Dimethylthiazol-2yl]-2,5-Diphenyltetrazolium-Bromid)-Assay sowie der Trypanblau-Assay verwendet (**Publikation II, diskutiert in Publikation III**). Die Methode des MTT-Tests basiert auf der Aktivität mitochondrialer Dehydrogenasen. Das gelbliche MTT wird von den Dehydrogenasen in lebenden Zellen zu einem violetten Formazanfarbstoff konvertiert. Die daraus erhaltene Farbintensität ist proportional zur Anzahl lebender Zellen [233]. Der Trypanblau-Assay hingegen beruht auf der Anfärbung toter Zellen. Lebende Zellen schließen mit ihrer intakten Zellmembran das Trypanblau aus.
Infolge einer Auszählung der Zellen mittels Neubauer Zählkammer kann zwischen toten und lebenden Zellen unterschieden und zudem die Gesamtzellzahl bestimmt werden.

9.2.1 Zeitabhängige Vitalität nach Inkubation von AGE-modifizierten Proteinen in Fibroblasten

Für die Bestimmung der Vitalitäten wurden die Fibroblasten für 24, 48 und 72 Stunden mit AGE-modifiziertem BSA inkubiert und im Anschluss der MTT-Assay durchgeführt (n=3).

AGE-BSA (mM)	Glucose-AGE			Ribose-AGE			Fructose-AGE		
	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-
0	100,0 3,5	92,3 5,7	96,3 2,3	100,0 3,1	95,6 2,3	98,7 2,4	100,0 5,3	99,1 2,7	97,6 3,6
5	93,6 4,8	91,6 6,4	89,6 7,1	87,4 7,6	96,6 8,1	94,6 5,2	94,6 5,3	96,3 1,6	88,6 5,6
25	85,6 8,1	87,5 5,9	85,6 1,3	84,6 5,9	85,6 3,6	82,6 1,3	93,6 5,7	89,3 5,4	82,7 8,4
100	81,3 7,4	75,3 2,6	71,6 1,6	84,7 4,8	72,4 6,9	76,5 7,7	84,3 2,3	70,2 3,6	76,3 5,6

AGE-BSA (mM)	Glyoxal-AGE			Methylglyoxal-AGE		
	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-
0	100,0 3,0	97,0 3,6	96,3 2,6	100,0 3,9	98,4 2,0	92,3 4,6
2	79,3 6,4	89,6 3,1	89,1 6,5	90,6 5,6	96,3 2,3	94,6 5,6
10	79,3 8,1	72,3 5,6	70,3 2,7	84,6 7,1	77,6 1,3	79,6 6,3

Tabelle I: Vitalität nach 48 h Inkubation mit AGE-BSA in Cathepsin D (D-/-) und L-defizienten (L-/-) Fibroblasten

Prozentuale Vitalität wurde nach 48 Stunden Inkubation mit AGE-modifiziertem BSA mittels MTT-Assay bestimmt und auf die unbehandelte Wildtyp-Kontrolle (100 % vital) bezogen.

AGE-BSA (mM)	Glucose-AGE			Ribose-AGE			Fructose-AGE		
	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-
0	100,0 1,5	95,6 2,9	97,2 4,5	100,0 3,5	96,5 4,5	97,6 3,9	100,0 2,5	99,5 3,6	96,3 2,3
5	96,6 2,6	89,9 8,9	93,6 8,4	95,3 9,4	92,6 4,7	90,9 2,4	96,3 7,0	95,3 0,9	91,6 5,0
25	88,6 5,6	82,6 6,9	85,1 6,8	93,6 8,5	86,3 5,6	89,6 4,4	96,3 5,1	81,6 8,8	88,3 4,1
100	**80,6 1,8**	**61,6 3,3**	**63,6 5,9**	**86,6 8,1**	**64,1 8,4**	**68,1 6,1**	**83,6 2,3**	**58,3 4,2**	**63,6 4,1**

AGE-BSA (mM)	Glyoxal-AGE			Methylglyoxal-AGE		
	Wildtyp	D -/-	L -/-	Wildtyp	D -/-	L -/-
0	100,0 2,5	98,6 2,1	96,3 3,0	100,0 4,2	95,6 3,3	90,6 5,7
2	91,6 5,6	86,3 8,6	87,6 5,1	96,3 2,5	79,6 5,3	78,6 8,1
10	**79,6 2,3**	**52,3 5,9**	**59,6 6,2**	**69,3 4,5**	**54,6 5,5**	**55,6 3,3**

Tabelle II: Vitalität nach 72 h Inkubation mit AGE-BSA in Cathepsin D (D-/-) und L-defizienten (L-/-) Fibroblasten

Die Vitalität wurde nach 72 Stunden Inkubation mit AGE-modifiziertem BSA mittels MTT-Assay bestimmt und entsprechend der unbehandelten Wildtyp-Kontrolle (100 % vital) berechnet.

Innerhalb 24 Stunden kann kein Effekt der AGE-modifizierten BSA-Proben auf die Vitalität in den embryonalen Fibroblasten registriert werden (**Publikation II**). Auch innerhalb 48 Stunden ist kein zeit- und dosisabhängiger Effekt auf die Vitalität in den verschiedenen Zelltypen zu verzeichnen (Tab. 1). Nach 72 Stunden zeigt sich jedoch eine konzentrationsabhängige Reduktion der Zellvitalität und somit einen toxischen Effekt der AGE-modifizierten BSA-Proben. Im Vergleich zu den Wildtyp-Fibroblasten ist diese Toxizität in den knock-out Zellen stärker ausgeprägt (Tab. 2).

9.2.2 Zeitabhängige Vitalität nach Inkubation mit AGE-modifiziertem BSA in makrophagialen Zellen

Zur Untersuchung der AGE-induzierten zellulären Effekten wurde vorrangig die RAW 264.7 Makrophagen-Zelllinie verwendet. Zeitweise wurden Versuche an nicht-transformierten, primären Mikroglia durchgeführt. Folglich wurde in Vorversuchen die Auswirkung der AGE-modifizierten Proteine auf die Vitalität auch in den primären Zellen getestet. Hierbei wurden

aus tierschutzrechtlichen Gründen nur postnatale Mäuse (< 5 Tage alt) verwendet, da sich die Ausbeute an isolierbaren mikroglialen Zellen mit dem Alter der Tiere verringert.

AGE-BSA (mM)	Glucose-AGE		Ribose-AGE		Fructose-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 3,3	100,0 3,6	100,0 2,3	100,00 5,3	100,0 0,3	100,00 4,1
5	98,6 2,6	99,6 1,1	97,6 3,6	98,6 5,1	99,3 2,3	96,9 6,7
25	97,6 3,6	93,6 3,6	95,6 7,4	95,4 1,2	96,0 3,6	89,6 4,4
100	**95,6 2,3**	**85,6 3,2**	**92,3 6,5**	**87,6 6,6**	**91,6 3,4**	**84,6 3,9**

AGE-BSA (mM)	Glyoxal-AGE		Methylglyoxal-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 1,5	100,0 4,5	100,0 1,4	100,0 1,4
2	97,8 5,5	97,6 5,6	98,6 5,4	96,6 7,1
10	**95,6 1,9**	**90,3 4,9**	**93,6 4,9**	**90,6 6,0**

Tabelle III: Vitalität nach 24 h Inkubation mit AGE-BSA in makrophagialen Zellen

Eine prozentuale Vitalität nach 24 Stunden Inkubation mit AGE-modifiziertem BSA wurde in den RAW 264.7, einer Makrophagenzelllinie, und in primären postnatalen Mikroglia bestimmt. Die Ergebnisse wurden auf die unbehandelten Zellen (100 % vital) bezogen (n=3).

AGE-BSA (mM)	Glucose-AGE		Ribose-AGE		Fructose-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 6,9	100,0 5,0	100,0 5,6	100,00 5,1	100,0 2,3	100,00 6,1
5	99,6 5,1	96,3 6,3	96,9 4,1	97,6 6,1	97,6 2,3	98,3 4,1
25	95,1 3,6	92,3 5,1	92,6 7,8	87,2 4,7	91,6 4,6	89,6 2,5
100	**84,9 5,5**	**70,1 5,3**	**88,6 6,9**	**73,6 2,6**	**89,3 7,9**	**64,3 5,1**

AGE-BSA (mM)	Glyoxal-AGE		Methylglyoxal-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 2,6	100,0 3,5	100,0 0,6	100,0 3,3
2	94,5 6,9	86,3 5,1	94,1 6,3	84,9 7,8
10	**83,6 4,8**	**72,3 6,1**	**80,3 5,9**	**69,3 3,5**

Tabelle IV: Vitalität nach 48 h Inkubation mit AGE-BSA in makrophagialen Zellen

Die prozentuale Vitalität nach 48 Stunden wurde auf die unbehandelten Zellen (100 % vital) bezogen (n=3).

AGE-BSA (mM)	Glucose-AGE		Ribose-AGE		Fructose-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 8,1	100,0 2,1	100,0 3,6	100,00 2,1	100,0 3,6	100,00 3,4
5	97,1 2,6	98,6 3,6	95,6 2,1	96,3 3,8	94,5 8,3	95,6 4,7
25	94,6 5,1	82,6 8,1	93,1 4,7	84,3 3,6	96,0 0,6	85,0 6,8
100	70,3 5,4	**55,3** **5,6**	70,3 6,3	**55,8** **2,6**	69,6 5,8	**49,6** **3,7**

AGE-BSA (mM)	Glyoxal-AGE		Methylglyoxal-AGE	
	RAW 264.7	Mikroglia	RAW 264.7	Mikroglia
0	100,0 3,6	100,0 1,2	100,0 0,3	100,0 5,9
2	91,6 7,3	82,3 6,4	99,6 4,3	81,3 8,3
10	77,6 3,5	**52,1** **3,6**	76,9 3,6	**46,6** **9,1**

Tabelle V: Vitalität nach 72 h Inkubation mit AGE-BSA in makrophagialen Zellen

Nach 72 Stunden Inkubation mit AGE-modifiziertem BSA wurde die zelluläre Vitalität bestimmt und in Prozent zu den unbehandelten Zellen (100 % vital) angegeben (n=3).

Einen zeitabhängigen Effekt der AGEs kann in den RAW 264.7 Makrophagen nur in geringem Ausmaß detektiert werden. Stärker modifiziertes BSA (mit 100 mM Zucker oder 10 mM Aldehyde) zeigt jedoch einen Einfluss auf die zelluläre Vitalität in den RAW 264.7 Zellen (Tab. 3-5). Postnatale Mikroglia reagieren sensitiver auf eine AGE-Inkubation im Vergleich zu dem RAW 264.7. Sowohl zeitabhängig als auch konzentrationsabhängig ist die Vitalität signifikant reduziert und die Toxizität der AGEs kann somit begründet werden (Tab. 3-5).

Für die in den vorliegenden Arbeiten durchgeführten Untersuchungen wurden für alle Zelltypen eine einheitliche AGE-Konzentration gewählt: 25 mM Zucker-modifiziertes BSA und 2 mM Aldehyd-modifiziertes BSA.

EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmitteln und Quellen angefertigt habe.

Die Arbeit wurde in keiner Form einer anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Jena, 29.08.2012

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