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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

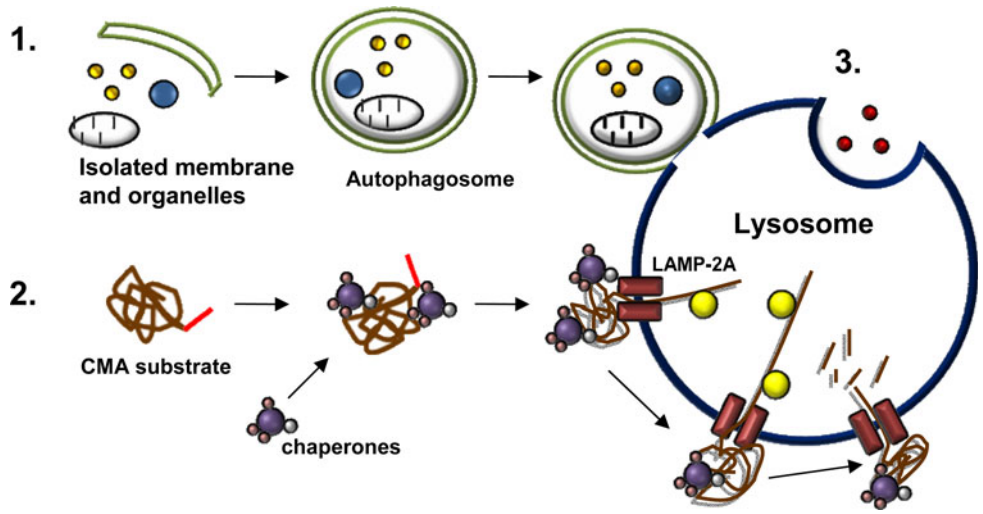

Fig. 1 Lysosome-linked autophagy system. The lysosome-linked autophagy system consists of three different types: (1) macroautophagy, (2) microautophagy and (3) chaperon-mediated autophagy (CMA). Substrates bearing a targeting motif that is recognized by a chaperone complex delivers them to the lysosomes. 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 $\beta 1$, $\beta 2$, and $\beta 5$ are responsible for the catalytic activity. These subunits exhibit the peptidyl-glutamyl-peptide-hydrolyzing ($\beta 1$), trypsin-like ($\beta 2$) and chymotrypsin-like ($\beta 5$) 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 $\beta 1$i, $\beta 2$i and $\beta 5$i ("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

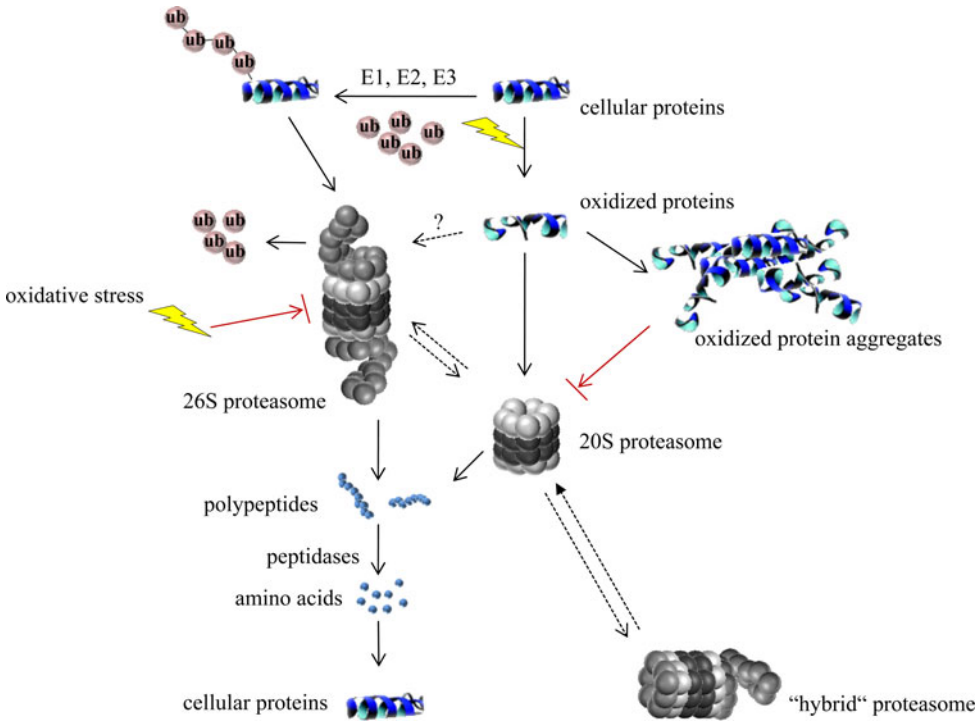

Fig. 2 Degradation by the proteasome. Many substrates of the 26S proteasomes are short-lived, undamaged proteins. They need an external signal in form of a polyubiquitin chain to ensure their recognition by the 19S regulators of the 26S proteasomes. The ubiquitin pathway includes ubiquitin activation (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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