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Linkage Between the Proteasome Pathway and Neurodegenerative Diseases and Aging

Sophie Vigouroux, Marièle Briand, and Yves Briand*

Laboratoire de Biochimie Appliquée, Associé INRA,
Université Blaise Pascal, 63 174 Aubière, France

Abstract

During aging, the production of free radicals increases. This can result in damage to protein, the accumulation of which is characteristic of the aging process. This questions the efficacy of proteolytic systems. Among these systems, the proteasome and the adenosine triphosphate-ubiquitin-dependent pathway have been shown to play an important role in the elimination of abnormal proteins. There are two major steps in the ubiquitin-proteasome pathway: the conjugation of a polyubiquitin degradation signal to the substrate and the subsequent degradation of the tagged protein by the 26S proteasome. The 26S proteasome is build-up from the 20S proteasome, which is a cylinder-shaped multimeric complex, and two additional 19S complexes. The 20S proteasome can also bind to 11S regulator and is then implicated in antigen presentation. These regulators confer a high adaptability on proteasome.

With advancing age, predisposition to neurodegenerative diseases increases. These diseases are also characterized by protein aggregation. Several findings such as the presence of ubiquinated proteins, usually broken down by proteasomes, and genetic anomalies involving the ubiquitin-proteasome system (parkin, UCH-L1) suggest a link between the ubiquitin-proteasome pathway and the genesis of these diseases.

Index Entries: Proteolysis; proteasome; aging; neurodegenerative diseases.

Introduction

Free radicals are frequently formed in organisms in the course of certain physiological mechanisms (e.g., cellular respiration). When excessive amounts of free radicals are present because of overproduction or an impairment of antioxidant defenses (e.g., superoxide dismutase, catalase, or glutathione peroxidase), oxidative stress occurs. This oxidative stress causes damage to biological molecules (proteins, lipids, nucleic acids, etc.).

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* Author to whom correspondence and reprint requests should be addressed. E-mail: yves.briand@univ-bpclermont.fr

Tissues have ranging sensitivities to oxidative stress. The brain is particularly sensitive because of its high oxygen uptake per unit of tissue mass; the presence of polyunsaturated fatty acids, which are major constituents of the easily peroxidizable cellular membranes; the relative weakness of antioxidant defenses, especially catalase; the presence of self-oxidizable neurotransmitters, such as dopamine; and the accumulation of iron in certain brain areas (globus pallidus and substantia nigra), which is responsible for the formation of cytotoxic radicals.

With age, oxidative stress increases along with predisposition to neurodegenerative diseases. Therefore, it has been suggested that oxidative stress may be implicated in the physiopathology of cerebral aging and several neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Aging and neurodegenerative diseases are characterized by the accumulation and aggregation of oxidized, mutated, or damaged proteins. This build-up of damaged proteins can result not only from increased formation but also from slower degradation. In the central nervous system, proteolysis mediated by the ubiquitin-proteasome system plays a major role in the elimination of these proteins. Recent studies have shown deficient ubiquitin-proteasome proteolysis during aging and in neurodegenerative diseases.

Thus, determining how the proteasome is involved in aging and neurodegenerative processes is an important issue. This article presents the structure of the proteasome and its various modes of regulation. We then focus on its role in the degradation of damaged proteins following normal and increased oxidative stress. Finally, we discuss the possible dysfunction of the ubiquitin-proteasome pathway in neurodegenerative diseases.

The Ubiquitin-Proteasome System

The ubiquitin-proteasome-dependent proteolytic pathway is a complex set of machinery controlled by several hundreds of genes. It is able to degrade most cellular proteins and is involved in the regulation of multiple cellular processes such as antigen processing, response to stress, apoptosis, differentiation, cell cycle, signal transduction, and the inflammatory process. This pathway also may be implicated in many pathological states such as cancer, neurodegenerative diseases, and other age-related disorders.

The pathway comprises two major steps. The first consists of the ubiquitination of target proteins by covalent attachment of a polyubiquitin chain. Ubiquitin is a small ubiquitous protein of 76 amino acids strongly preserved during evolution. In the second step, polyubiquitinated substrate is recognized and degraded by the 26S proteasome.

Ubiquitination of Target Proteins

A series of three complex reactions are necessary for the conjugation of ubiquitin on a target protein *(1,2)* (Fig. 1). Briefly, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an adenosine triphosphate (ATP)-dependent reaction through a high-energy thioester intermediary E1-*S*-ubiquitin. Ubiquitin is then transferred to an enzyme from the E2 family via a new thioester intermediary. These E2 enzymes, called ubiquitin-conjugating enzymes (UBCs), belong to a related protein superfamily with molecular weights ranging from 14 to 35 kDa *(3)*. Often, but not always, these enzymes require the intervention of a member of the E3 family called ubiquitin-protein ligase to transfer ubiquitin to a target substrate protein. Ubiquitin can also form a third thioester bond with an E3 enzyme before being transferred to the target protein. These ubiquitin-ligases bind to both an E2 protein and the substrate; the interaction with the substrate can either be direct or via an ancillary protein. The E3 enzymes catalyse the last step of the ubiquitin conjugation process and specifically recognize the substrate. It was recently shown that in certain cases, a fourth enzyme (E4), which is a factor of elongation of the ubiquitin chains,

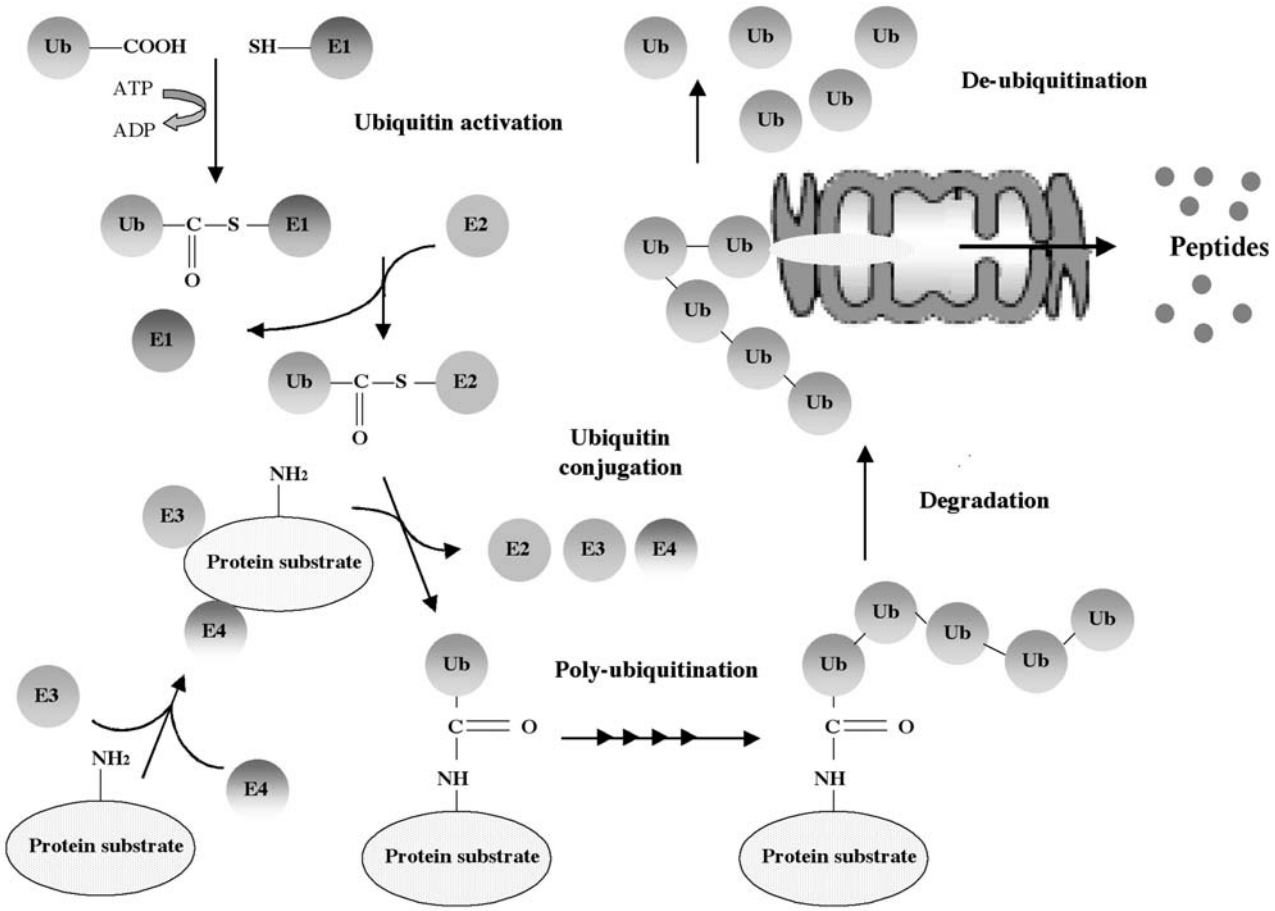

Fig. 1. The ubiquitin-proteasome pathway. This pathway comprises two major steps: the conjugation of a polyubiquitin degradation signal to the substrate and the subsequent degradation of the tagged protein by the 26S proteasome.

may also be necessary *(4)*. Because of the large number of enzymes in the E2 and E3 families, which can produce multiple combinations, and the presence or absence of the ancillary proteins, the system exhibits a wide diversity and ranging specificity for the controlled ubiquitination of many target proteins. This ubiquitination step thus marks proteins for destruction by the 26S proteasome. To be recognized by the 26S proteasome, the target proteins require a chain of at least four ubiquitin residues *(5)*.

The Proteasome: Its Structural and Catalytic Properties

The second step in this proteolytic pathway is the degradation of proteins, whether or not they have been ubiquitinated by the 20S proteasome, alone or bound to a regulating complex. The 20S proteasome forms the catalytic core of this system. It is described in a broad range of eukaryote cells, from yeast to humans. An ancestral protease is also found in archaebacteria *(6)*. The 20S proteasome from

eukaryotes is a multicatalytic complex of high molecular weight (700 kDa). Studies by X-ray crystallography indicate that the proteasome purified from yeast is composed of 28 subunits of low molecular weight (ranging from 21 to 32 kDa) arranged in four rings stacked to form a barrel-like structure *(7).* The proteasome isolated from *Thermoplasma acidophilum* is the simplest, made up of only two subunit subtypes, α and β, each of which is present in 14 copies *(8).* The composition of the proteasome in eukaryotes is more complex: 14 different subunits have been found for the yeast 20S proteasome. They are divided into two subfamilies and are classified into seven subunits of the α-type and seven subunits of the β-type, which are coded by distinct genes but belong to the same family. Each of these subunits is present in two copies and occupies a single position in each ring. The α-subunits form the outer rings of the cylinder, and the β-subunits form the two inner rings. The two α- and β-rings represented in duplicate are assembled in an $\alpha_7\beta_7\beta_7\alpha_7$-structure with rotational symmetry C2 *(7,9).* These rings form three internal cavities, two localized at the junction of the α- and β-rings and the third framed by the two β-rings. This central cavity contains the catalytic sites, which are thus accessible only through narrow pores in the center of the α-rings *(6,7,10).* These pores normally are closed by the N-terminal ends of five α-subunits, allowing control of the entry of the substrate-protein into the catalytic cavity and of its hydrolysis. By allowing the opening of the outer rings and acting as a support with the various regulators that can accompany the 20S proteasome, these α-subunits control the entry of the substrates. Two subunits (α3 and α7) can be phosphorylated; this phosphorylation is essential for association with the 19S regulator complex *(11,12).*

Only three of the seven β-subunits of the 20S proteasome are catalytically active, with a threonine residue in the N-terminal position serving as a catalytic nucleophile, whereas the function of the other β-subunits is still unknown. Initial studies on the proteolytic activity of the proteasome identified three endopeptidase activities *(13–16).* The β5-subunit (PRE2) preferentially cleaves at the C-terminal of the hydrophobic amino acids (chymotrypsin-like activity), the β2 (PUP1)-subunit cleaves at the C-terminal of basic amino acids (trypsin-like activity), and the β1 (PRE3)-subunit cleaves at the C-terminal of the acid amino acids (peptidylglutamyl-peptide hydrolase) *(17–19).* Two other peptidase activities were also highlighted: the branched-chain amino acid preferring (BrAAP) and small neutral amino acid preferring (SNAAP) activities, which hydrolyze at the C-terminal of amino acids with ramified sidechains and small neutral amino acids, respectively *(20).* No catalytic site specific to these two activities has been found. Although the catalytic components show a preference for certain peptide bonds, they also can break other peptide bonds, although probably less effectively. The proteins are degraded gradually to produce peptides of small size, from 3 to 23 residues *(21,22).* These small peptides do not accumulate and can be further degraded by other peptidases to produce free amino acids *(23).*

The β-subunit composition of the proteasome can be modified. In the higher eukaryotes, but not in yeast, three constitutive β-subunits (β1, β2, β5) can be replaced by three homologous β-subunits (β1i, β2i, β5i). The biosynthesis of these subunits can be induced by cytokines such as interferon-γ or tumor necrosis factor-α *(24).* These subunits are assembled in neosynthesized proteasomes to make immunoproteasomes. The immunoproteasome has increased capacities to generate peptides bearing a basic or hydrophobic amino acid on their C-terminal end, which possess a greater affinity for major histocompatibility complex-I (MHC-I) for the stimulation of the cytotoxic T cells *(25),* whereas there is a reduction in the acid activity *(26).*

The purified 20S proteasome seems to occur in a latent form that is unable to hydrolyze native proteins. Peptidase activities of the 20S proteasome can be stimulated by many gentle

treatments such as exposure to low concentrations of detergents sodium dodecyl sulfate (SDS), fatty acids, heat treatments, or high ionic strengths *(14,27).* These treatments are believed to facilitate the accessibility of the substrates inside the catalytic chamber by opening up a channel in the center of the α-rings. In vivo, the 20S proteasome can occur in the form of free particles assumed to be weakly active. However, it seems that this catalytic core is not used solely as a reserve for association with the regulators detailed in the next section. Many studies show that the free 20S proteasome plays a major role in the degradation of oxidized and damaged proteins *(28–30).* An increase in protein degradation is observed after moderate oxidation, whereas proteins that have undergone severe oxidation form aggregates and are no longer degradable by the 20S proteasome. The unfolded or oxidized proteins expose hydrophobic residues that can be recognized by the 20S proteasome and then degraded *(28).* Kisselev *(31)* recently showed that the activity of the 20S proteasome was stimulated after hydrophobic peptides opened the channel in the α-rings. An increase in the production of damaged proteins is a consequence of aging. Their accumulation and aggregation as a result of partially or totally impaired capacity for degradation can be observed in certain age-related pathologies, particularly neurodegenerative disorders.

Proteasome Regulators

As stated in the previous section, the accessibility of the substrates to the catalytic cavity of the 20S proteasome can be facilitated by regulators that join onto the two α-rings and allow the unfolding of substrate proteins, the opening of the central channel, and the introduction of proteins into the catalytic chamber for peptide hydrolysis. Three main regulators have been highlighted: (a) the 19S regulator, responsible for the ATP-ubiquitin-dependent proteolysis; (b) the 11S regulator, involved in the antigen presentation and the immunizing response; and (c) the PA200, the function of which remains to be defined.

The 19S or PA700 regulator is a high-molecular-weight (700 kDa) complex composed of at least 18 different subunits with molecular weights between 25 and 110 kDa *(32–34).* It is composed of two subcomplexes: a lid and a base *(35).* The base is made up of nine subunits, the six ATPases of which are attached to the α-rings of the 20S proteasome in an ATP-dependent manner. This base acts to unfold the substrate proteins, open the central pore of the outer rings on which it is bound, and transport proteins inside the catalytic chamber of the 20S core *(7,10,36,37).* These ATPase subunits provide energy necessary for the assembly of the 20S proteasome, with the 19S regulator to produce the 26S proteasome. ATP is also required for the elimination of the ubiquitin residues and the introduction of the substrate into the catalytic chamber. The lid covers the base and is probably involved in the recognition and elimination of the polyubiquitinated chains that are fixed on the substrate before its transfer and degradation in the catalytic chamber. Recent studies showed that the recognition and transfer of the ubiquitinated substrate can take various pathways, bringing into play some substrate recruiting proteins involving molecular chaperones *(38).* This 19S complex stimulates the peptidase activity of the 20S proteasome *(39).* The 26S proteasome is mainly involved in the degradation of proteins by the ATP-ubiquitin-dependent pathway, but it can hydrolyze nonubiquitinated proteins independently of the ATP. This is the case for ornithine decarboxylase after association with a protein called antizyme *(40)* or various other proteins *(41).*

The 11S regulator (11S reg or PA28) is a hexameric or heptameric complex of two distinct subunits (molecular weight: 28 kDa) called PA28α and PA28β. Similarly to the exchangeable β-subunits of the 20S core, these subunits are inducible by the cytokines. Unlike the 19S particle, this complex (α3β4 or α4β3) is fixed on the external rings of the 20S core independently of the ATP *(42).* This fixation stimulates

the short peptide hydrolysis but not the proteolytic activity *(43,44).* This complex PA28-20S proteasome probably plays a role in the antigenic presentation in connection with the immunoproteasome *(25,45).* A strong increase is observed in the numbers of this type of proteasome during inflammatory processes. Like the 19S, this regulator may activate the 20S core by opening the pore located at the center of the external rings. Its inability to hydrolyze large proteins suggests that the PA28-20S proteasome may increase the efficacy of antigenic presentation by modifying the peptides produced by the 26S proteasome; this role could be played by the hybrid proteasome, which has a 11S complex at one end and a 19S regulator at the other. A third subunit, PA28γ (also called Ki antigen), near the PA28α- and -β-subunits, can form a homohexamer that binds to the 20S proteasome. The resulting complex has a mainly nuclear localization and may be involved in the control of cell multiplication.

A third regulatory complex was highlighted recently *(46).* This complex, called PA200, activates peptide hydrolysis, but not protein hydrolysis, by the 20S core. It is localized primarily in the nucleus and may be involved in DNA repair.

Diversity of the Proteasome Regulation

This short review of the proteasome and its regulators shows the great complexity of this proteolytic system. Regarding the 20S proteasome, a first level of regulation can be performed by phosphorylation of two α-subunits supporting association with the 19S regulator *(11).* A second level of regulation relates to the exchangeable subunits inducible by the cytokines. Merforth et al. *(47)* highlighted the presence of several populations of 20S proteasome made up of 20S proteasomes, immunoproteasomes, and their intermediary forms in the same tissue in the rat. Each of these subtypes presents different hydrolytic activities. This distribution into subtypes can be modified in physiological cases *(47).* This presence of subpopulations also was reported in various tissues in the rat *(48)* and in various cellular compartments *(49).* A third level of regulation is exerted on the level of the association of the 20S core with the various regulators (11S, 19S, or PA200), the quantity of which can range significantly from one tissue to another *(50–52).* It was reported recently that the various proteasomal complexes can coexist in the same cell. In cells, there is an excess of 20S proteasomes *(53,54).* Also, the existence of hybrid proteasomes PA28-20S-19S was demonstrated by Tanahashi et al. *(50)* and Koop et al. *(55).* In the rat, this difference in composition observed from one tissue to another results in characteristic peptidase and proteolytic activities *(48,50,52).* The various components of this proteolytic pathway undergo significant modifications during the muscle wasting induced by catabolic diseases *(56).*

Three levels of regulation defined above (phosphorylation, exchanges of subunits in the 20S core, and association with the various regulators) lend the cell high plasticity and adaptability and make the 20S proteasome a very dynamic waste removal system capable of undergoing significant modifications to adapt its peptidase activities to specific cellular needs. However, this great complexity can give rise to many dysfunctions implicated in various pathologies, from cancer to neurodegenerative diseases. This makes this proteolytic pathway a privileged focus for the discovery of new therapies.

Free Radicals and Aging

The damaged proteins that accumulate during aging result from the action of free radicals. With advancing age, proteins are the target of several types of modification (oxidation, glycation, glycoxidation, conjugation with lipid peroxidation products, etc.). The accumulation of oxidized proteins observed in numerous tissues *(57)* during aging raises the question of the efficacy of proteolytic systems, particularly the proteasome, which has been found to play an important role in the elimination of these proteins.

Production of Free Radicals

Free radicals are chemical species that contain an unpaired electron in their outermost orbital. They can extract an electron from a neighboring molecule to fill this orbital vacancy. Therefore, free radicals are very unstable and reactive, which accounts for their very short half-lives (approx 1 ms).

Oxygen is responsible for most of the free radicals found in the body. In its ground state, oxygen is a biradical with two electrons that react only with molecules that also have two outer electrons. The reduction of molecular oxygen involves redox reactions that produce intermediate free-radical species, such as superoxide anion, hydrogen peroxide, and the very reactive hydroxyl radical.

$$\underset{\text{oxygen}}{O_2} \xrightarrow{+e^-} \underset{\text{superoxide anion}}{O_2^{\circ -}} \xrightarrow{+e^- + 2H^+} \underset{\text{hydrogen peroxide}}{H_2O_2} \xrightarrow{+e^- + H^+} \underset{\text{hydroxyl radical}}{OH^\circ} \xrightarrow{+e^- + H^+} \underset{\text{water}}{H_2O}$$

These reactive oxygen species are formed in eukaryotic cells, particularly in the mitochondrial respiratory chain. The mitochondria are equipped with enzyme systems that use oxygen to form molecules of ATP by oxidative phosphorylation. The cellular respiratory cytochromes, particularly cytochrome oxidase, reduce molecular oxygen to water by supplying four electrons. This mechanism is the basis for the production of superoxide anion *(58).*

Free radicals can also be formed in the microsomes when oxygen is activated by cytochrome P450.

In the cytosol, various enzyme reactions can produce superoxide radicals and hydrogen peroxide. The most important enzyme is xanthine oxidase, which generates superoxide in the presence of xanthine or hypoxanthine. Other metabolic activities also generate free radicals in the body. Inflammatory processes induce the stimulation of phagocyte cells, which is accompanied by the production of oxygenated free radicals. During inflammation, activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane enzyme of phagocyte cells that can use oxygen, produces large quantities of superoxide anion.

Environmental factors such as ionizing radiation (IR), ultraviolet (UV) light, drugs, and pesticides increase the production of free radicals. Under the action of IR, H_2O_2 produces two OH^- radicals. In particular, aging of the skin results from UV exposure, which generates oxygenated free radicals that cause peroxidation of polyunsaturated fatty acids in cell membranes *(59).*

A moderate production of free radicals stimulates cell growth signals and induces proliferation and differentiation. Oxygenated radicals activate factors such as c-jun, c-fos, and, especially, nuclear factor-κB (NFκB) *(60).* Also, the redox potential of the cells controls the expression of a large number of genes via transcription factors sensitive to redox potential at their cysteine residues (NFκB, etc.) Therefore, the oxidative stress responsible for increased production of radical species induces a deregulation of these biological systems that depends on redox potential.

To combat the oxidizing action of free radicals, the body possesses antioxidant defenses designed to eliminate free radicals. These defenses are comprised of enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione transferase. Other substances are also able to neutralize a single free radical per molecule. This line of defense is formed by vitamins E and C, flavonoids, and carotenoids. The cell's defense mechanisms can be overloaded by free radicals. Because free radicals are highly reactive, they react with any molecules they encounter—particularly lipids, nucleic acids, and proteins—causing considerable damage.

Oxidative Damage Caused by Free Radicals

Damage to Nucleic Acids

The DNA molecule is an important cellular target for radical attacks. The oxidation of purine and pyrimidine bases in DNA, which cause strand breaks and base modifications,

can have marked effects on genome replication. The main DNA oxidation product is 8-hydroxydeoxyguanosine, formed by the attack of hydroxyl radicals on deoxyguanosine *(61).* The 8-hydroxydeoxyguanosine serves as a biomarker of DNA oxidation.

Peroxidation of Lipids

Oxygen and reactive oxygen species are involved in a set of lipid peroxidation reactions. Polyunsaturated fatty acids such as linolenoic and eicosapentaenoic acids, which contain several double bonds, are the most vulnerable to free radical attacks. Monounsaturated fatty acids such as oleic acid, which contains only one double bond, are less sensitive. The hydroxyl radical (OH°) extracts an atom of hydrogen from a methylene group of an unsaturated fatty acid. The carbon atom with a single electron captures a molecule of oxygen to produce a peroxide radical. In turn, this radical can react and propagate the peroxidation.

Lipid peroxidation alters membrane permeability and can even cause the membrane to disintegrate. The peroxidized lipids also create aldehydes such as malondialdehyde and 4-hydroxynonenal, which can attack protein amine or thiol functions. Unlike free radicals, these reaction products are fairly longlasting. Lipid peroxidation can be evaluated by measuring malonaldehyde, conjugated dienes, and lipid hydroperoxides.

Oxidation of Proteins

Like DNA and lipids, proteins are vulnerable to oxidative damage. Some amino acids are particularly sensitive to oxidation, including aromatic amino acids (tyrosine, tryptophan, and phenylalanine), sulphur-containing amino acids (methionine and cysteine), and basic amino acids (histidine, arginine, and lysine). Any modification of an amino acid by oxidation can bring about a structural and functional modification of the protein. The oxidation of amino acids can add hydroxyl and carbonyl groups to proteins, and proteins can undergo fragmentation or crosslinking via bityrosine bridges.

Proteins can also be modified by the products of lipid peroxidation such as malondialdehyde and 4-hydroxy-2-nonenal, which can react with the thiol and amine groups of proteins, respectively *(62,63).* Another mechanism, glycation, can contribute to the alteration of proteins and make them more resistant to degradation by proteasomes. The oxidation of glucose, which releases keto-aldehydes, H_2O_2, and OH^-, causes the cleavage of proteins and their glycation by attachment of the keto-aldehyde.

The formation of carbonyl groups and nitrotyrosines are the main biomarkers of protein oxidation. Peroxynitrite produced by the reaction of nitric oxide and superoxide anion is responsible for the formation of nitrotyrosine groups.

20S Proteasome Removes Damaged Proteins

With aging, the production of free radicals overloads the cell's defense mechanisms, resulting in a gradual accumulation of damaged biomolecules, especially proteins. *(64–66).* These damaged proteins are normally eliminated by the proteolytic systems, particularly by the 20S proteasome in an ATP- and ubiquitin-independent manner.

Slight oxidative damage (two to nine amino acids oxidized in a protein) causes a modification of the three-dimensional structures of proteins. This partial denaturing of oxidized proteins exposes hydrophobic residues at the protein surface *(63,67).* The proteasome is known to have a preference for hydrophobic residues. Grune et al. *(68)* and Davies *(28)* proposed that the 20S proteasome selectively recognizes exposed hydrophobic patches of denatured oxidized proteins. Actually, ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome *(69).* In addition, oxidized proteins appear to be relatively poor substrates for ubiquitination *(68).* Furthermore, Shringarpure et al. *(69)* also demonstrated that rates of oxidized protein degradation by cell lysates are not significantly altered by addition of ATP. Interestingly, their

work suggested that oxidized proteins are degraded without ubiquitin conjugation or ATP hydrolysis. It appears that the 20S core proteasome conducts the recognition and elimination of oxidized proteins in an ATP- and ubiquitin-independent pathway *(70).* Gomes-Marcombes and Tisdale *(71)* reported an increase in the activity of 20S proteasome in response to mild oxidative stress. The 20S proteasome degrades the oxidized proteins, thereby preventing their accumulation. This makes the 20S proteasome an important means of antioxidant defense.

Polyunsaturated fatty acids are easily peroxidizable. The products of this peroxidation are toxic for the cell, producing damaged proteins. An increase in the activity and expression of the proteasome was observed in the muscles of rats receiving a diet enriched in polyunsaturated fatty acids *(72).* This increase helped to eliminate the abnormal proteins.

In severe oxidative stress, the oxidized proteins can undergo chemical fragmentation or form large aggregates. Fragmentation generates fragments bearing modified terminal amino acids *(73).* These modified proteins can aggregate covalently or noncovalently. Aggregates are formed by covalent crosslinks involving the recombination carbon-centered radicals of amino acid sidechains. The formation of a 2,2′-biphenyl crosslink by two tyrosyl radicals and disulphide crosslinks are reported as common covalent crosslinks *(74).* Noncovalent crosslinks also are formed. Oxidative stress results in partial denaturing of the proteins, exposing hydrophobic residues that can interact with neighboring proteins to form large aggregates. These aggregates are poor proteasome substrates *(63)* and, therefore, resist proteolytic degradation. They can inhibit proteasome activity by binding to the active site and blocking the entry of substrates. Highly oxidized proteins that are not broken down then accumulate in the cells and are responsible for cell toxicity and cell death.

The elimination of proteins oxidized by the proteasome is not the only means to rid the cell of proteins damaged by free radicals. Oxidative stress induces various chaperone molecules, which, combined with the ubiquitin-proteasome pathway, can cause the appearance of aggresomes that improve proteolysis *(74).* The formation of these aggresomes could represent a response to compartmentalize and eliminate abnormal proteins that are potentially cytotoxic *(75).* This mechanism is believed to underlie the formation of proteinaceous inclusion bodies under both normal and pathological conditions. The production and accumulation of abnormal proteins in neurodegenerative disorders could overwhelm a putative protective aggresomal response, resulting in the extensive aggregation of proteins to form insoluble inclusions as Lewy bodies.

Proteasome Activity During Aging

Several models have been used to study the evolution of proteasome activity with age, including *Caenorhabditis elegans,* rodents, and cells in culture. In general, a decline of proteasomal activity and oxidized protein degradation capability is observed in cell cultures *(76,77).* The results are less consistent in experiments on tissues. Carney et al. *(57)* and Starke-Reed and Oliver *(78)* reported a diminution of proteasomal activity during aging in the brains of gerbils and in the hepatocytes of rats. Bulteau et al. *(79)* showed lowered activities of the chymotrypsin and peptidylglutamyl peptide hydrolase types in purified proteasomes from aged epidermis. Hayashi and Goto *(80)* reported an age-related decline in activities of the trypsin-type (17%) and of the peptidyl glutamyl peptide hydrolase-type (60%) in proteasomes 20S and 26S in the livers of aged Fisher rats. To explain this lowered activity, Bulteau et al. *(79)* and Anselmi et al. *(81)* carried out an analysis of proteasome subunits by two-dimensional electrophoresis. The results showed a modification of the structure of the proteasome and/or the replacement of certain subunits, which could account for reduced activity. Keller et al. *(82)* showed proteasome inhibition and loss of proteasome expression during aging and oxidative injury and suggested that it may induce cell death.

However, other groups found no modification in the activity of the 20S proteasome during aging. In work by Agarwal and Sohal *(83)*, a comparison between Sprague-Dawley rats ages 3, 13, and 23 mo indicated no lowering of proteasome activity in the brain. Similarly, in flies, activity did not fall with age. In contrast, the same experiment found falls of 50 and 20% in the liver and heart, respectively, from 13 to 22 mo. Results of these analyses suggest that a decline in proteolytic activity, which could help to explain the accumulation of oxidized proteins with age, is not a reliable criterion of aging. Studies carried out in the brain, heart, kidney, liver, and testicles of gerbils ages 3–6, 15, and 23–25 mo, found no age-linked change in proteasome activity, which preferentially degraded oxidized proteins *(84)*. A study performed by Keller et al. *(85)* indicated that change in proteasome activity is cell-type specific. The spinal cord, hippocampus, and cerebral cortex demonstrated age-dependent decreases in proteasome activity, but at any timepoint, the proteasome activity was decreased in either the brainstem or cerebellum.

Oxidative stress increases with age. Recent studies showed deficient ubiquitin-proteasome-dependent proteolysis during aging, which could explain the accumulation and aggregation of damaged proteins observed in aging. These findings suggest that the damage caused by free radicals may be an important factor in the degeneration of nerve cells in the encephalon observed in Parkinson's disease *(86)*. The brain is particularly sensitive to oxidative stress. Because of its high oxygen consumption and elevated metabolic activity, it generates many oxygenated free radicals *(87,88)*. The oxidation of dopamine leads to the formation of hydrogen peroxide. In addition, polyunsaturated fatty acids, which are major constituents of membranes, are readily peroxidized *(89,90)*, and iron, which is responsible for the formation of cytotoxic radicals, builds up in certain areas of the brain. The antioxidant defense mechanisms (especially catalase) are relatively weak in the brain. The presence of lipids *(91)*, proteins *(92)*, and nucleic acids *(93)* damaged by oxidation in the postmortem brains of patients with Parkinson's disease attests an oxidative stress associated with the disease. A fall in the activity of complex I of the mitochondrial respiratory chain *(94,95)*, a state considered to generate free radicals, is observed in Parkinson's disease.

The Proteasome in Neurodegenerative Diseases

Protein aggregation is a common feature of all the chronic human neurodegenerative diseases. Numerous studies point to a causal role of aggregated proteins in the development of Alzheimer's disease, Parkinson's disease, and polyglutamine disorders. Biochemical and immunocytochemical studies have reported that many intraneuronal inclusions, which are the main neuropathological feature in these disorders, are ubiquitinated or associated with ubiquitin *(96)*. These include Lewy bodies in Parkinson's disease, amyloid plaques and neurofibrillary tangles in Alzheimer's disease, and nuclear inclusions in polyglutamine extension disorders *(97)*. Ubiquitin-conjugated proteins usually are efficiently degraded by the 26S proteasome; thus, the presence of intracellular deposits of ubiquitinated and aggregated protein suggests a linkage between dysfunction of the ubiquitin-proteasome pathway and pathogenesis. Furthermore, proteasome subunits have been identified in Lewy bodies *(98)*. This accumulation of proteins can be explained by a dysfunction of proteasomes at different levels. First, the ubiquitin-proteasome system can be overloaded by a greater production of mutated or damaged proteins. The efficiency of this system of degradation can also be reduced as a result of an inhibition by altered proteins, mutations in genes coding for some components of the ubiquitin-proteasome system, or by modifications in the proteasome itself. The toxic nature of modified proteins also may not be identified by the ubiquitin-proteasome system. This article focuses on mutations in pro-

teins that are proteasome substrates and in the ubiquitin-proteasome itself.

Mutations in Substrate Proteins

Parkinson's disease is a common progressive neurodegenerative disease caused by the loss of dopaminergic neurons in the substantia nigra pars compacta and is characterized by the presence of intraneuronal cytoplasmic inclusions known as Lewy bodies, the fibrous portion of which contains the protein α-synuclein. These α-synuclein immunoreactive Lewy bodies also are immunoreactive for ubiquitin *(99).* The name "α-synuclein" derives from its apparent localization in presynaptic terminals and nerve cell nuclei *(100).* By exposing hydrophobic residues that can interact with each other, wild-type α-synuclein is naturally prone to forming insoluble amyloid fibril aggregates *(101).* It has been reported that α-synuclein is degraded by proteasomes *(102).* This finding was strengthened by the results of Ghee et al. *(103),* who indicated that α-synuclein directly affects the proteasome complex by interacting specifically with Tat binding protein 1, a subunit of proteasome regulatory complexes. Two missense mutations occurring at either Ala 53 Thr *(104)* or Ala 30 Pro *(105)* in human α-synuclein were associated with the familial autosomal dominant form of early-onset Parkinson's disease. Both mutant α-synucleins are natively unfolded, which increases protein–protein interactions. The formation of fibrils appears to be accelerated in the presence of mutant α-synuclein *(106).* Mutant α-synuclein is less efficiently degraded than wild-type, which may explain the accumulation and aggregation of the mutant protein *(102).* Once aggregated, it is more resistant to proteolytic degradation. Mutant α-synuclein decreases proteasome activity *(107).* The suggestion by Ghee et al. *(103)* that mutant α-synuclein directly affects the proteasome complex by interacting specifically with Tat binding protein 1, a subunit of the proteasome regulatory complex, supports the results obtained by Bennett et al. *(102),* who presented pharmacological evidence that α-synuclein is degraded by the 26S proteasome. The catabolism of both wild-type and mutant α-synuclein is blocked by specific inhibition of the ubiquitin-proteasome pathway (40 μ*M* of lactacystin), indicating that they are both degraded by the ubiquitin-proteasome pathway *(102).* On the other hand, Ancolio et al. *(108)* found that metabolism of wild-type and mutant α-synuclein in human embryonic kidney (HEK) cells or murine neurons overexpressing α-synuclein was not altered by treatment with 5 μM of lactacystin. However, Tofaris et al. *(109)* reported that inhibition of the proteasome with 10 μM of lactacystin led to an increase in aggregated α-synuclein, but neither α-synuclein nor α-synuclein-positive inclusions were ubiquitinated before or after the proteasome. These findings suggest that degradation of α-synuclein can be performed by the proteasome without ubiquitylation.

Another neurodegenerative disease, early-onset familial Alzheimer's disease, is also caused by genetic mutations. These mutations in genes, coding for β-amyloid precursor protein (β-APP), presenilin (PS)-1 and -2, lead to increased amyloid-β $A\beta_{42}$ production. The formation of Aβ deposits, which are insoluble fibrils of Aβ peptides 1-40 and 1-42, is an early event in Alzheimer's disease. Aβ is a principal constituent of senile plaque and derives from proteolytic cleavage of the β-APP *(110).* Cleavage of β-APP by α-secretase followed by γ-secretase releases a fragment called α-APP and forms the nonamyloidogenic pathway. Because α-secretase cleaves within the Aβ domain, this pathway does not yield Aβ, preventing deposition of the amyloidogenic peptide. Cleavage of β-APP by β-secretase at the N-terminus of the Aβ peptide and by one or more γ-secretases at the C-terminus forms the amyloidogenic pathway for processing of β-APP. The position of the γ-secretase cleavage is central to the production of long Aβ. If the cleavage event occurs between residues 712-713, then short Aβ called $A\beta_{40}$ results; however, if it occurs after residue 714, then there is

generation of long Aβ known as $A\beta_{42}$. The production of $A\beta_{42}$ is of particular importance because it has been shown to be the earliest and most abundant species of Aβ in neuritic plaques.

The PS genes have been identified in chromosomes 14 and 1 and encode the proteins PS-1 and -2, respectively. Mutations in PS-1 appear to account for most cases of early-onset Alzheimer's disease, whereas PS-2 mutations account for much fewer cases. These mutations lead to altered processing of β-APP and, subsequently, increased production of $A\beta_{42}$ *(111).* Weidemann et al. *(112)* reported that PS-1 interacted with β-APP noncovalently. Overexpression of PS-2 caused a decrease in β-APP secretion, suggesting a role for PS-2 in APP proteolytic processing. Many studies *(113–116)* demonstrated that both wild-type and mutant PS-1 and -2 are ubiquitinated and degraded by the ubiquitin-proteasome pathway. This observation suggests that the proteasome may act as a regulator in β-APP maturation by regulating intracellular concentrations in presenilins. Actually, in the presence of proteasome inhibitors, α-APP production is increased by wild-type presenilins *(117),* whereas an increased production in $A\beta_{42}$ is observed in cells expressing mutated PS-1.

Other neurodegenerative diseases, including Huntington's disease, have also been thought to result from mutant proteins that aggregate in intracellular bodies and fail to be degraded because of expanded CAG/polyglutamine repeats *(118,119).* In prion diseases, pathology results from accumulation of a conformationally altered prion proteins, which form aggregates *(120).* The normal cellular protein prion (PrP) is converted into infectious protein prion (PrP^{SC}) through a process in which the α-helical content diminishes and the amount of β-sheet increases *(121).*

Mutations in Genes Coding for Some Components of the Ubiquitin-Proteasome System

Genetic disorders have also been reported in some components of the ubiquitin-proteasome system in certain hereditary forms of Parkinson's disease. Mutations in two genes that code for parkin and ubiquitin C-terminal hydrolase L1 (UCH-L1), which are involved in regulation of the ubiquitin-proteasome pathway, cause familial Parkinson's disease and juvenile Parkinsonism. This suggests that dysregulation of the ubiquitin-proteasome pathway is involved in the mechanism by which these mutations cause Parkinson's disease *(122).*

Parkin, the causal gene product for autosomal recessive juvenile Parkinsonism (AR-JP), acts as an E3 ubiquitin-protein ligase *(123)* and is contained in Lewy bodies *(124).* E3 enzymes are responsible for substrate recognition for the ubiquitin pathway and, therefore, for the selection of target proteins for ubiquitylation and degradation. Imai et al. *(125)* suggested that parkin is involved in the ubiquitination pathway, which degrades misfolded endoplasmic reticulum proteins, and its normal activity may be to protect the cell from the neurotoxicity that can be induced by unfolded protein stress. Disease-associated parkin mutations demonstrate decreased ubiquitin-ligase activity *(123).* Because of disruption of parkin's ubiquitination function, accumulation of target proteins normally degraded by the ubiquitin-proteasome pathway may contribute to the development of AR-JP. This accumulation of proteins that are substrates of parkin causes selective neuronal death without formation of Lewy bodies *(126).* Absence of Lewy bodies in patients with mutated parkin suggests that parkin plays an essential role in the formation of these ubiquitinated inclusions.

In addition, a missense mutation (Ile 93 Met) of exon 4 in the UCH-L1 has been identified in rare cases of autosomal dominant Parkinson's disease *(122).* UCH-L1 belongs to a family of deubiquitinating enzymes and is selectively present in Lewy bodies characteristic of human neurodegenerative diseases *(127).* UCH-L1 is believed to release ubiquitin from small adducts to generate ubiquitin monomers necessary to label abnormal proteins for 26S proteasomal degradation. This mutation results in a partial loss of the catalytic activity of the thiol protease, which may result in

reduced labeling, impaired clearance of abnormal proteins, aggregation of proteins, and consequent neurodegeneration *(122).*

Another dysregulation of the ubiquitin system has been characterized that may be significant in aging brains and those with Alzheimer's disease. Misreading of the ubiquitin B gene transcript results in the formation of a mutant ubiquitin polypeptide known as ubiquitin^{+1}. This entity may contribute directly to Alzheimer's disease pathogenesis, because since ubiquitin^{+1} immunoreactivity was found to be associated with brain lesions such as neurofibrillary tangles *(128).* Ubiquitin^{+1} contains the first 75 residues of the 76 amino acid ubiquitin sequence. Ubiquitin^{+1} can be polyubiquitinated and incorporated in an unanchored polyubiquitin chain by the E2^{25k} enzyme *(129).* The unanchored chain has the ability to bind to the 26S proteasome, leading to an inhibition in proteasome activity and, therefore, neuronal toxicity *(130).* These unanchored chains are normally disassembled by the isopeptidase T deubiquitinating enzyme, restoring proteasome activity *(131).* To function efficiently, the isopeptidase-T requires a free Gly^{76} in the proximal ubiquitin. However, chains with ubiquitin^{+1} do not exhibit a Gly^{76} at their proximal end and, therefore, are poor substrates for isopeptidase-T *(129).* This inefficient deubiquitination results in proteins that are targeted by ubiquitin for degradation being unable to enter the proteasome and accumulate *(132).* Altered disassembly of unanchored chains with ubiquitin^{+1} causes depletion of the free ubiquitin pool, leading to a dysregulation of the proteolysis. Recent observations of ubiquitin^{+1} expression in diseases other than Alzheimer's disease, including progressive supranuclear palsy *(133),* and in non-neuronal cells *(134)* suggest that proteasomal inhibition by ubiquitin^{n}-ubiquitin^{+1} may play a more generalized role in the pathogenesis of human diseases.

Changes in Proteasome Activity

Each of the genetic defects discussed in the previous section compromises ubiquitin-proteasome pathway-mediated protein degradation in the brain. Altered proteolysis leads to abnormal protein aggregation and subsequent neurodegeneration. These defects do not challenge the function of the proteasome itself. However, proteasome inhibition occurs in a wide array of neurodegenerative disorders *(135).* Decreased proteasome activity has been reported in Alzheimer's *(136)* and Parkinson's diseases *(137).* An inhibition of proteasome is sufficient to induce neuronal death in primary neuronal cultures and neural cell lines *(138–140).* Not all neuron populations are affected by neuronal cell death following proteasome inhibition *(141),* suggesting that neuronal death induced by proteasome inhibition may be cell-type specific. McNaught et al. *(142)* reported that dopaminergic neurons are more sensitive to decreased proteasome function than GABAergic neurons, possibly as a result of the oxidative metabolism of dopamine.

McNaught and Jenner *(137)* demonstrated that proteasome activity is reduced in the substantia nigra of idiopathic Parkinson's disease. Work by McNaught et al. *(143)* indicated a loss of α-subunits, but not β-subunits, in dopaminergic neurons of the substantia nigra or other brain regions in sporadic Parkinson's disease. Because the activity measurements were performed in substantia nigra, where dopaminergic loss takes place, it may be that the observed decrease in proteasome activity is a secondary event related to a neurodegenerative process. Furthermore, proteasome activity, which has been reported by different groups *(137,138,144)* to decline in Alzheimer's and Parkinson's diseases, has been measured in tissue homogenate or crude extract samples from morphologically affected brain regions. However, if these human brain samples contain other proteases with some peptidase activities similar to those of proteasome, the low peptidase activities previously reported in Alzheimer's and Parkinson's diseases may not be simply attributable to proteasome dysfunction. Vigouroux et al. *(145)* reported a protease (molecular weight approx 105 kDa) that has trypsin- and chymotrypsin-like activities but no peptidylglutamyl peptide

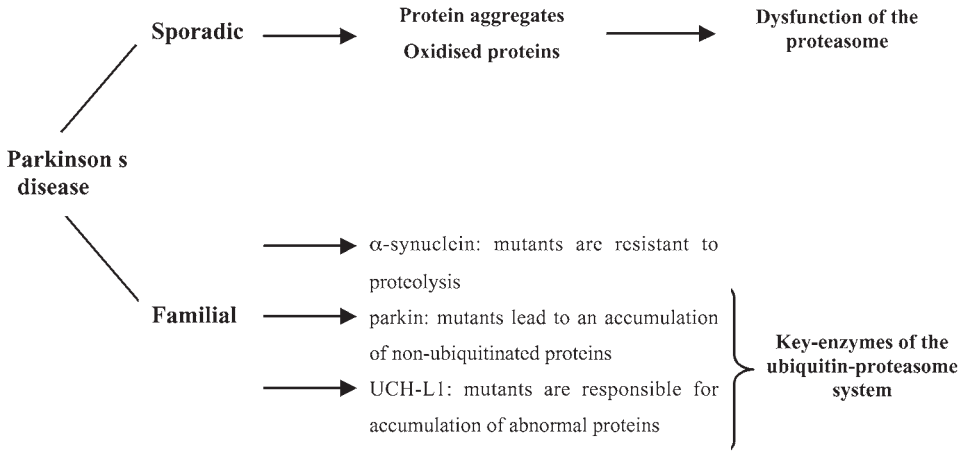

Fig. 2. Linkage between the ubiquitin-proteasome pathway and Parkinson's disease.

hydrolase activity in the human brain *(145).* Thus, human brain trypsin- and chymotrypsin-like activities shown in previous reports *(137,138,144),* in which the activities were determined in tissue homogenate or crude extract samples, may include those of the 105-kDa protease. Tests performed with lactacystin often are used to confirm function or activity of the proteasome *(137).* However, other proteases can also be inhibited by lactacystin *(146).* Chymotrypsin- and trypsin-like activities of the 105-kDa protease are inhibited by lactacystin and MG132 *(145).* This suggests that a brain fraction specific to the proteasome should be used to evaluate the actual status of the proteasome in neurodegenerative diseases, because there is another protease with trypsin- and chymotrypsin-like activities in the human brain. Vigouroux et al. *(145)* developed a method to separate the proteasome from the new protease. Using this method, Furukawa et al. *(147)* found that proteasome peptidase activities were preserved in the striatum and cortices of Parkinson's disease, multisystemic atrophy, and progressive supranuclear palsy and that protein levels of two proteasome subunits (an α-1-subunit and an ATPase subunit of the 19S complex) were normal in patients with Parkinson's disease compared with control patients. This finding is consistent with the results obtained by Keller et al. *(138),* who found no alteration in the levels of proteasome α- and β-subunits in Alzheimer's disease brains. These data suggest that brain proteasomal function (catalytic activity and degradation ability) is preserved in sporadic Parkinson's disease. Oxidative stress occurs in a number of neurodegenerative conditions. It has been demonstrated that nontoxic levels of oxidative stress do not result in impairment of proteasome activity but increase proteasome subunit expression in neural cells *(148).*

Accumulation of proteins observed in neurodegenerative diseases and the aging process suggest inefficient proteolysis. It has been widely demonstrated that the proteasome pathway plays an important role in the degradation of altered proteins. Therefore, dysfunction in this proteolytic system at different levels might account for protein accumulation (Fig. 2). The capacity of the ubiquitin-proteasome for protein degradation can be overloaded by an increased production of altered proteins by genetic or oxidative modifications, which escape degradation, accumulate, and aggregate. The ubiquitin-proteasome system may become less efficient because of mutations in genes coding for proteins involved in the ubiquitin-proteasome pathway (parkin, UCH-

L1, and ubiquitin^{+1}). Altered proteins may also bind to proteasomes, preventing other proteins from entering them and being degraded. Once aggregated, proteins can become resistant to degradation. Furthermore, modifications can occur in the proteasome itself. Finally, the toxic nature of some altered proteins can go unrecognized by the proteasome, resulting in their accumulation.

Conclusion

Many neurodegenerative diseases are associated with the aggregation of proteins. This accumulation of protein raises the question of the efficacy of proteolytic systems. The recent discovery of genetic anomalies in certain components of the ubiquitin-proteasome system has shown that there is a relation between this breakdown pathway and the pathogenicity of neurodegenerative diseases. These disorders are linked to aging, and the predisposition to them increases with age. During aging, there is an increase in the production of free radicals, which results in the build-up of damaged proteins. Because the proteasome plays an important role in the elimination of abnormal proteins, a lowered efficiency of this system can be suspected. Other major cellular proteolytic mechanisms mediated by the calpains and the lysosomal cathepsins also have been implicated with neurodegenrative and aging diseases *(149).* The strongest evidence for a direct role of calpains in neurodegeneration exists in acute brain insults such as ischemia and neurotroma *(150).* Activation of μ-calpain takes place immediately after an ischemic insult. Karlsson et al. *(151)* also observed an increase in Ca^{2+}-dependent proteolytic activity in patients with early onset of Alzheimer's disease. In Alzheimer's disease, toxicity of amyloid β-peptides could lead to intracellular calcium elevation in neurons and, therefore, calpains activation *(152).* Gafni and Ellerby *(153)* also reported that activated calpain was detected in the caudate of human tissue with Huntington's disease. In addition, an aging-dependent activation of the calpain/calpastatin system was demonstrated *(154).* Other recent work performed in human fibroblast cultures revealed a reduction of the lysosomal cathepsin activity during aging *(155).*

There are many possible causes for the aggregation of proteins implicated in the onset of neurodegenerative diseases. To achieve a better understanding of neurodegenerative pathologies and aging, more thorough study of proteolytic systems is necessary (e.g., using proteomic methods).

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