

Protein carbonylation, cellular dysfunction, and disease progression

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- Introduction
- The impact of carbonylation on protein function
- The impact of carbonylation on protein folding
- The impact of carbonylation on proteolysis
- Is protein carbonylation an early event of cellular dysfunction and disease progression?
- Conclusion and perspectives

Abstract

Carbonylation of proteins is an irreversible oxidative damage, often leading to a loss of protein function, which is considered a widespread indicator of severe oxidative damage and disease-derived protein dysfunction. Whereas moderately carbonylated proteins are degraded by the proteasomal system, heavily carbonylated proteins tend to form high-molecular-weight aggregates that are resistant to degradation and accumulate as damaged or unfolded proteins. Such aggregates of carbonylated proteins can inhibit proteasome activity. A large number of neurodegenerative diseases are directly associated with the accumulation of proteolysis-resistant aggregates of carbonylated proteins in tissues. Identification of specific carbonylated protein(s) functionally impaired and development of selective carbonyl blockers should lead to the definitive assessment of the causative, correlative or consequential role of protein carbonylation in disease onset and/or progression, possibly providing new therapeutic approaches.

Keywords: protein carbonyls • reactive oxygen species • reactive carbonyl species • protein unfolding/misfolding • proteasome • aggregation diseases

Introduction

Reactive oxygen species (ROS) are constantly generated within cells at low concentrations under physiological conditions, playing a part in the cellular redox regulation. Cellular production of ROS occurs from both enzymatic and non-enzymatic sources (Fig. 1) [for reviews, see 1, 2]. ROS can also occur as the outcome of acute cell stresses and

may result in cell death *via* apoptosis or necrosis. Cellular oxidative damage develops when the balance between ROS-generating systems and ROS-scavenging ones tilts in favour of the former.

The primary cellular target of oxidative stress can vary depending on the cell type, the absolute level and duration of oxidant production, the species

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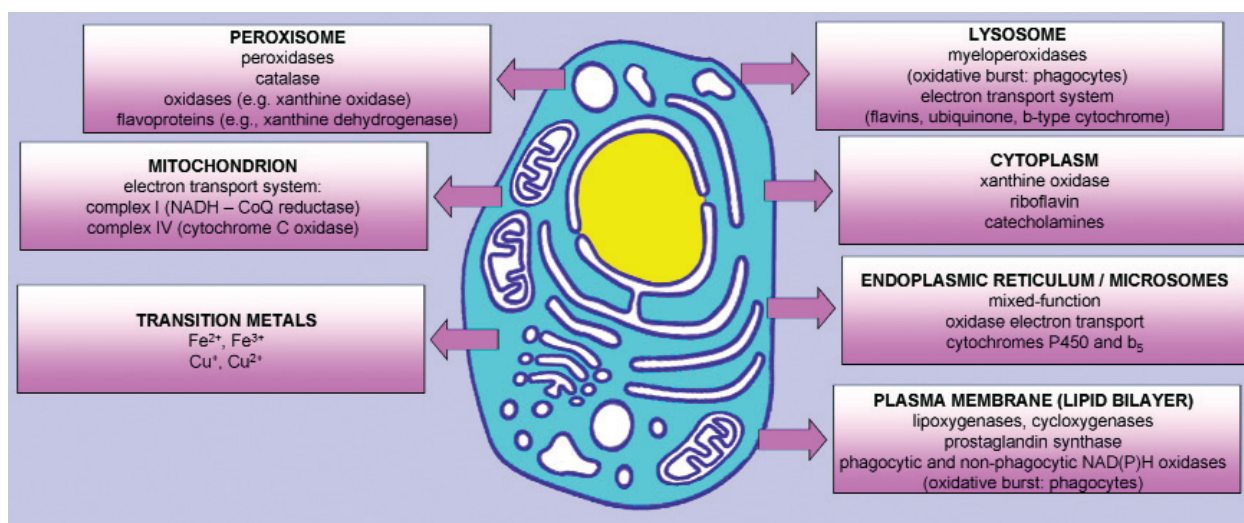


Fig. 1 Cellular sources of reactive oxygen species. Any electron-transferring protein or enzymatic system can result in the formation of ROS as "by-products" of electron transfer reactions. The mitochondrial electron transport chain is a significant source of ROS. Plasma membrane is a major source of ROS through NAD(P)H oxidases located on either side. Enzymes of the same class displaying low activity, as well as their components, are also present free in cytoplasm. Smooth endoplasmic reticulum (ER) contains enzymes, including cytochrome P-450 and b₅ families, which catalyse a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. Peroxisomes are an important source of total cellular H₂O₂ production. They contain a number of H₂O₂-generating enzymes of the oxidase family. Peroxisomal catalase utilises H₂O₂ produced by these oxidases to oxidise a variety of other substrates in peroxidative reactions, particularly in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules that enter the circulation. Another major function of the oxidative reactions carried out in peroxisomes is β-oxidation of fatty acids. In addition to intracellular membrane-associated oxidases, cytoplasmic soluble enzymes such as xanthine oxidase, aldehyde oxidase, flavoprotein dehydrogenase, and tryptophan dioxygenase can generate ROS during catalytic cycling. Autooxidation of small molecules such as dopamine, epinephrine, flavins, and hydroquinones can be an important source of intracellular ROS production. In most cases, the direct product of such autooxidation reactions is O₂^{-•}.

of ROS generated, its site of generation (intra- vs. extra-cellular), and the proximity of the oxidant to a specific cellular substrate. The extent of damage to particular targets depends on a number of factors (Fig. 2). Proteins are major targets for ROS and secondary by-products of oxidative stress when these are formed *in vivo* either in intra- or extracellular environments, as they are the major component of most biological systems and can scavenge 50–75% of reactive radicals such as ·OH [3].

Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events [4]. For example, protein reversible modifications, such as S-glutathionylation, S-nitrosation, and methionine sulfoxidation, may have a dual role of protection from irreversible oxidation and modulation of protein function (redox regulation) [5–7]. Differently,

irreversible protein modifications can lead to inactivation of various proteins and could have lasting detrimental cellular effects.

Although the overall biology of oxidative protein modifications remains complex and ill defined, protein carbonylation is quite well characterised. Carbonylation is an irreversible, non-enzymatic modification of proteins. The chemistry of the reactions that give rise to carbonyl groups is discussed in detail in other excellent reviews [8–11].

Briefly, carbonyl groups are introduced into proteins by a variety of oxidative pathways (Fig. 3). ROS can react directly with the protein or they can react with molecules such as sugars and lipids, generating products (reactive carbonyl species, RCS) that then react with the protein. Direct oxidation of proteins by ROS yields highly reactive carbonyl derivatives resulting either from oxidation of the side chains of

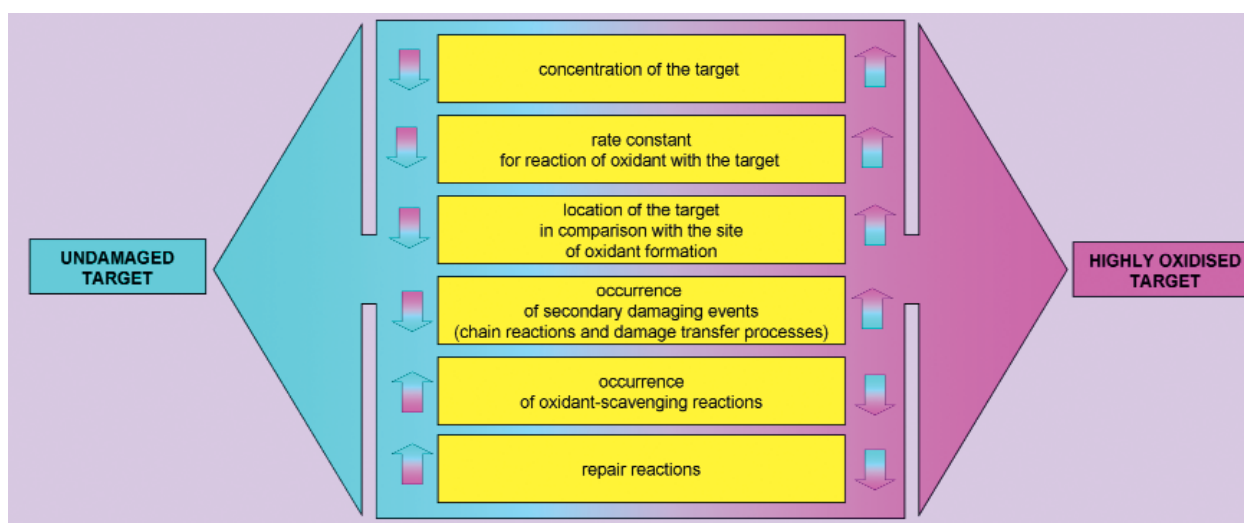


Fig. 2 Major factors determining the extent of oxidative damage to specific cellular targets. The first two of these determine the rate at which any particular reaction is likely to occur; the others modulate the extent of the reaction that occurs.

lysine, arginine, proline, and threonine residues - particularly *via* metal-catalysed oxidation [8] - or from the cleavage of peptide bonds by the α -amidation pathway or by oxidation of glutamyl residues. Glutamic semialdehyde, a product of oxidation of arginine and proline, and amino adipic semialdehyde, a product of oxidation of lysine, are the main carbonyl products of metal-catalysed oxidation of proteins, this reaction being a major route leading to the generation of protein carbonyls in biological samples [12]. Carbonyl groups can also be generated by secondary reaction of the primary amino group of lysine residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation/glycooxidation reactions), eventually leading to the formation of advanced glycation end-products (AGEs). Finally, carbonyl groups may be introduced into proteins by adduction of carbonyl-containing oxidized lipids derived from the metal-catalysed oxidation of polyunsaturated fatty acids [13–15]. These include malondialdehyde (MDA), which reacts with lysine residues, and α,β -unsaturated aldehydes [4-hydroxy-2-nonenal (HNE), acrolein], which can undergo Michael-addition reactions at their C=C double bond with the sulfhydryl group of cysteine, the ϵ -amino group of lysine or the imidazole group of histidine residues, forming advanced lipoxidation end-products (ALEs).

Protein carbonylation is the most widely used biomarker for oxidative damage to proteins, and reflects cellular damage induced by multiple forms of ROS [7, 10, 16–19].

The impact of carbonylation on protein function

Increases in carbonylated proteins during ageing and in response to oxidative stress are not random, some proteins being more susceptible than others. However, the set of proteins that become carbonylated differs in different species. For example, mouse plasma ageing-associated protein carbonylation was only seen in two proteins, albumin and transferrin [20]. But, in the rat plasma, only albumin and α 1-macroglobulin showed significant age-dependent accrual of carbonylation [20]. Human brain copper-zinc superoxide dismutases (SOD1) is one of the major targets of oxidative damage in brains of subjects afflicted with Alzheimer's disease (AD) and Parkinson's disease (PD); however, only one out of four human brain SOD1 isoforms is heavily carbonylated [21]. The selectivity of protein carbonylation is clearly demonstrated by the fact that the relative amount of a protein is not a factor in determining the degree of carbonylation [20, 22]. Similar specificity of protein carbonyla-

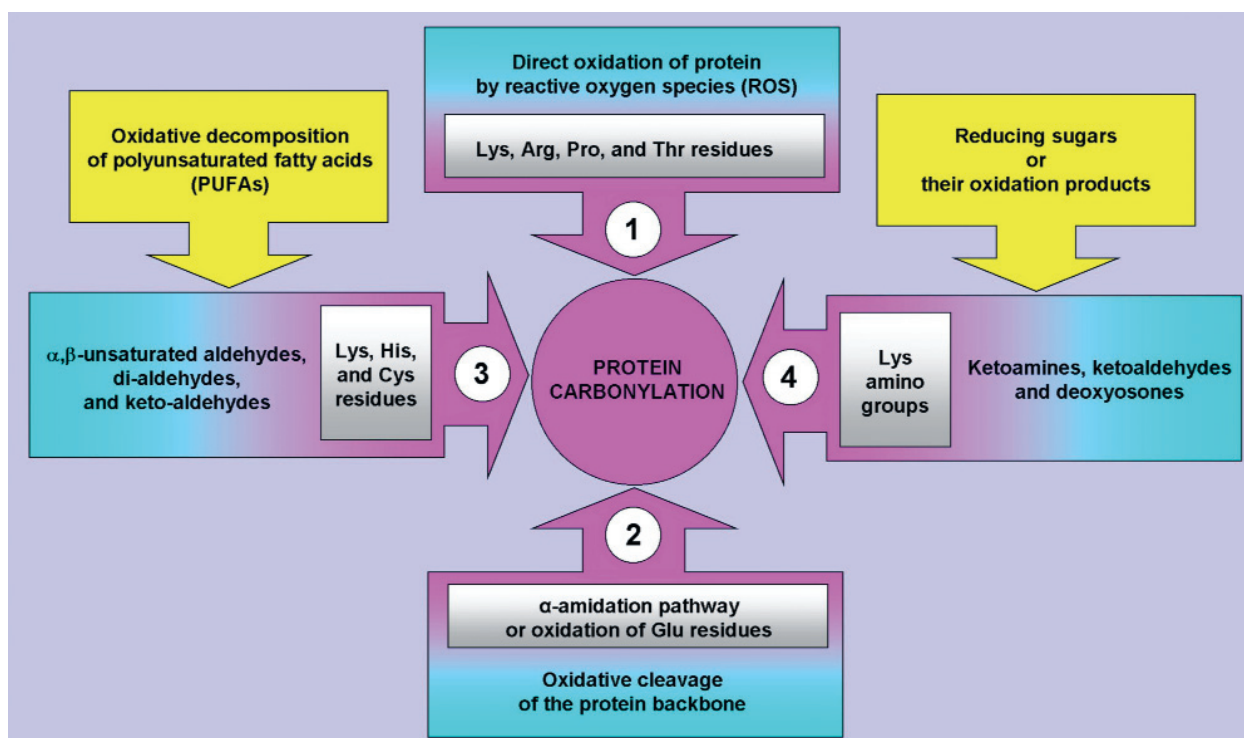


Fig. 3 Origins of carbonylated proteins. Protein carbonyl derivatives can be produced by a variety of oxidative pathways. Direct oxidation of the Lys, Arg, Pro, and Thr side chains - particularly *via* metal-catalysed oxidation - results in α -amino adipic semialdehyde from lysyl residue, glutamic semialdehyde from arginyl and prolyl residue, 2-pyrrolidone from prolyl residue, and 2-amino-3-ketobutyric acid from threonyl residue (pathway 1). Direct oxidation of proteins by ROS can also yield highly reactive carbonyl derivatives resulting from the cleavage of peptide bonds by the α -amidation pathway or by oxidation of glutamyl residues (pathway 2). Carbonyl groups may be introduced into proteins by adduction of reactive aldehydes derived from the metal-catalysed oxidation of polyunsaturated fatty acids (pathway 3). These lipoxidation products include di-aldehydes such as malondialdehyde (MDA), which reacts with Lys residues, α,β -unsaturated aldehydes [4-hydroxy-2-nonenal (HNE), acrolein], which can undergo Michael-addition reactions between their electrophilic C=C double bond and the sulfhydryl group of Cys, the ϵ -amino group of Lys or the imidazole group of His residues, and γ -ketoaldehydes (levuglandins, isoketals, neuroketals), which react with Lys residues. The chemical modification of protein by reactive carbonyl compounds derived from lipid peroxidation reactions results in the formation of advanced lipoxidation end-products (ALEs) (pathway 3). Finally, carbonyl groups can also be generated by secondary reaction of the primary amino group of Lys residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation/glycoxidation reactions), eventually leading to the formation of advanced glycation end-products (AGEs) (pathway 4).

tion was previously noted in the mitochondria of the flight muscles of the flies, where only aconitase [23–25] and adenine nucleotide translocase [24] were found to exhibit an age-associated increase in carbonylation and a corresponding loss in functional activity. Cytochrome *c*, a relatively abundant mitochondrial protein, did not show detectable carbonylation at any age [26].

An obvious question arising from such studies is why this selectivity? There is not an easy way to

answer this question. It could be merely a consequence of protein structure.

Pioneering studies by Stadtman [8, 27] have suggested that the presence of a transition metal-binding site in a protein is a key feature to predict its susceptibility to undergo carbonylation *via* metal-catalysed oxidation. Protein-bound transition metals are sources of free radicals that initiate a cascade of reactions, which result in the addition of carbonyl groups to the side chains of certain sur-

rounding amino acid residues [9]. Other factors, such as molecular conformation, rate of turnover and the relative abundance of amino acid residues susceptible to metal-catalysed oxidation, have also been suggested to be involved in the selectivity of protein carbonylation [28, 29]. In addition, some proteins (*e.g.*, enzymes of Krebs cycle and electron transport chain) may be carbonylated mainly because they are located near sites generating ROS.

Concerning selective oxidation of proteins, results obtained by Ros and colleagues [22] indicate that prokaryotic and eukaryotic cells display some homologies. These homologies can be a consequence of their structural and/or functional relationship. In this context, sequence homologies of pyruvate dehydrogenase and α -ketoglutarate dehydrogenase from *Saccharomyces cerevisiae* with respect to those from *Escherichia coli* were 40 and 59%, respectively. The homologies are even greater when their active sites or lipoic acid binding signatures are compared. This analysis would tend to suggest the importance of the protein structure on the selectivity of oxidative targets. Nevertheless, in the case of heat-shock protein (Hsp) 60 and the Hsp70 chaperone DnaK, they share no significant similarity, indicating that, in this case, chaperoning function could be a reason for their selective damage. The question of whether such proteins have been selected as targets during evolution to better preserve the integrity of the cell after a stress situation remains to be determined. Further studies by the same research group on the two types of yeast ageing models, replicative and chronological, showed that, although in both ageing models metabolic differences are important, major targets are almost the same [30]. Common targets include stress resistance proteins (Hsp60 and Hsp70) and enzymes involved in glucose metabolism. Interestingly, carbonylated proteins accumulating with replicative age are not inherited by daughter cells during cytokinesis [31].

Nyström and co-workers provided an attractive hypothesis, which supports an increased susceptibility of proteins to oxidation [32, 33]. Their elegant studies in *E. coli* established that transcriptional or translational errors produce aberrant proteins that are more susceptible to carbonylation (see also section "The impact of carbonylation on protein folding"). It is not completely clear why aberrant proteins are more susceptible to carbonylation, but it is

possible that a slight misfolding of the partly aberrant polypeptide exposes oxidation-sensitive amino acid residues that are normally hidden during the coupled translation-folding process. Introduction of carbonyl groups on those amino acids may result in further loss of the proteins' integrity. This, in turn, results in an increased exposure of hydrophobic surfaces increasing the target sites for the DnaJ/K/GrpE chaperone system [34].

The introduction of carbonyl derivatives (aldehydes and ketones) may alter the conformation of the polypeptide chain, thus determining the partial or total inactivation of proteins. A key question when addressing the significance of protein carbonyls is whether they are simply markers for the presence of oxidative stress, or have some substantive consequence on protein function that impacts on cell injury. Protein carbonylation is selective in inactivating particular proteins preferentially and it is likely to be deleterious, since cells are unable to repair protein carbonyls. In the flight muscle mitochondria of flies, aconitase and adenine nucleotide translocase were found to lose activity in association with the increase in carbonylation [23–25]. Endoplasmic reticulum (ER) proteins are readily carbonylated in response to peroxide treatment of HL-60 cells [35] and are preferentially carbonylated in the aged mouse liver [36]. Carbonylation of specific ER chaperone proteins may induce dysfunction of the protein folding processes [35, 36]. Hence, cells that have large amounts of protein carbonyls may be expected to have proteasomes and chaperones unable to keep up with the rate of production of unfolded or oxidatively damaged proteins and, therefore, an impaired cellular protein turnover, likely resulting in cellular impaired function (see below).

The introduction of carbonyl groups into proteins can be triggered by different ROS or secondary by-products of oxidative stress, and can arise at different sites and by different mechanisms (Fig. 3) [10, 11]. Hence, carbonylation can result in several different protein modifications, every one of which may produce (or not) a specific effect on the biological activity of different proteins. For example, HOCl-induced *in vitro* carbonylation of monomeric actin causes severe inhibition of actin filament formation [37], whereas actin carbonylation resulting from the adduction of HNE through Michael addition to Cys374 [38]

negligibly affects actin polymerisation [Dalle-Donne *et al.*, in preparation].

Due to their abundance in mammalian cells, cytoskeletal proteins are common targets for a variety of ROS and low-molecular-weight RCS. For instance, HNE forms Michael adducts with tubulin and disrupts microtubule assembly in neuroblastoma cells, blocking neurite outgrowth [39]; it also targets neurofilament heavy chains [40]. Actin isoforms are carbonylated both *in vitro* [37, 38] and *in vivo*, *e.g.*, in the skeletal muscles of a diabetes model Otsuka Long-Evans Tokushima Fatty (OLETF) rat [41], in macrophages exposed to hyperoxia [42], in the septic diaphragm [43], and in synaptosomes oxidized by treatment with the 42-amino acid peptide, amyloid β -peptide (1-42) [A β (1-42)] [44]. Actin carbonylation has been determined in human intestinal cells exposed to H₂O₂ or HOCl and in colonic mucosa from Crohn's disease patients, where it is associated with the disruption of the actin cytoskeleton and the loss of the monolayer barrier function [45, 46], as well as during reperfusion of the ischaemic rat heart [47] and in AD subjects [48].

Actin and a number of glycolytic enzymes (α -enolase, triose phosphate isomerase, phosphoglycerate mutase, and fructose bis-phosphate aldolase) were among the carbonylated proteins detected during etoposide (VP16)-induced apoptosis of HL60 cells. Consistently, glucose utilisation was reduced dramatically, likely due to carbonylation-mediated reduction in activity of glycolytic enzymes [49].

Protein carbonylation has been shown to exert a negative effect on creatine kinase and aldolase activity in the septic rat diaphragm [43]. Varying degrees of activity loss were detected in a number of peroxisomal enzymes following metal-catalysed-induced carbonylation [50]. The formation of protein carbonyls by HNE adducts has been shown to have significant effects on protein function and is frequently associated with their cross-linking. A number of mitochondrial enzymes have been shown to be inactivated following HNE binding, including Na⁺-K⁺-ATPase [51], adenine nucleotide translocator [52], and cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) in the ischaemic/reperfused rat heart [53], as well as the glial glutamate transporter, GLT-1 (EAAT2), in the brain of AD subjects [54].

Carbonylation and formation of HNE-adducts have been observed, under normal basal conditions, in specific protein subunits of the respiratory chain complexes I–V of adult bovine heart submitochondrial particles, as well as in two proteins that are part of a complex that forms the mitochondrial permeability transition pore, heart-specific T1 isotype of adenine nucleotide translocator and voltage-dependent anion channel 1 [55]. However, authors did not investigate the impact of these modifications on mitochondrial complex function. Substantial carbonylation of specific subunits of mitochondrial respiratory complexes I, III, and V has also been shown in chagasic murine hearts infected by *Trypanosoma cruzi*, which are characterised by deficiencies in the activities of the respiratory chain complexes and reduced mitochondrial ATP generation capacity during the course of infection and disease development [56]. The extent of protein carbonylation of specific subunits directly correlated with the loss in catalytic activities of the respiratory complexes in the infected myocardium [56]. In addition, the oxidative damage of complex I and III may potentiate oxidative stress in the mitochondria, as a decline in the activities of these complexes is likely to result in the increased release of electrons to molecular oxygen and ROS formation.

MDA-induced carbonylation of aconitase, very long chain acyl coenzyme A dehydrogenase, the β -polypeptide of the mitochondrial F1 complex of ATP synthase (or complex V), and the E2 component of α -ketoglutarate dehydrogenase complex was identified in both heart and skeletal muscle mitochondria from mice of three different ages [57]. While the amount of MDA-modified proteins did not appear to change during ageing, only aconitase and ATP synthase from heart exhibited an age-related decrease in activity, whereas very long chain acyl coenzyme A dehydrogenase and α -ketoglutarate dehydrogenase activities remained unchanged during ageing in both heart and skeletal muscle [57].

Inhibition of mitochondrial complex I reduces profoundly the activity of the proteasome system degrading oxidized proteins by oxidative modification of the 20S β subunit with acrolein, to which other acrolein-modified proteins were found to bind, resulting in dopamine neuron degeneration [58]. Current evidence resulting from studies in human post-mortem material suggests that mitochondrial complex I inhibition may be the central

cause of sporadic PD and that derangements in complex I cause α -synuclein aggregation, which binds directly to the proteasome and inhibits ubiquitin-dependent proteasomal function. Inhibition of the proteasome would lead to failure to clear protein targeted for degradation by the ubiquitin-proteasome system (UPS), ultimately resulting in the demise of dopamine neurons [59].

The impact of carbonylation on protein folding

Many of the proteins that are synthesised in a cell are destined for secretion to the extracellular environment. These proteins are translocated into the ER, where folding and post-translational modifications take place before secretion through the Golgi apparatus. Thus, any ROS effect on the structure and function of ER chaperone proteins could affect protein processing efficiency ("quality control") and could result in a decline in cell/tissue function. The ER contains a wide range of molecular chaperones and folding catalysts, and the proteins that fold here must satisfy a "quality-control" check before being exported. Such a process is particularly important because there seem to be few molecular chaperones outside the cell, although at least one (clusterin) has been discovered [60]. This quality-control mechanism, involving a remarkable series of glycosylation/deglycosylation reactions, enables correctly folded proteins to be distinguished from misfolded ones: incorrectly folded proteins are detected by the quality-control mechanism and sent along another pathway (the "unfolded protein response") in which they are ubiquitinated and then degraded in the cytoplasm by proteasomes [61]. Failure to fold correctly, or to remain correctly folded, will give rise to the malfunctioning of living systems and hence to disease [62, 63]. Some of these diseases, such as cystic fibrosis and some types of cancer, result from proteins folding incorrectly and not being able to exercise their proper function, ultimately being degraded by the proteasome. In other cases, proteins with a high propensity to misfold escape all the protective mechanisms and form insoluble aggregates within cells or, more commonly, such as in the amyloidoses, in extracellular space. An increasing number of disorders,

including AD, PD, the spongiform encephalopathies, and types II diabetes, are directly associated with the deposition of protein aggregates in tissues [63]. Just as the aberrant behaviour of enzymes can cause metabolic diseases, the aberrant behaviour of the chaperones and other machinery regulating polypeptide conformations can contribute to misfolding and aggregation diseases [64–66]. Such a process would explain why most of the amyloid diseases are associated with old age, when there is likely to be an increased tendency for proteins to become misfolded and/or damaged, for instance by increased oxidative stress, coupled with a decreased efficiency of the molecular chaperones and unfolded protein responses.

A number of human diseases are now known to result, directly or indirectly, from aberrant folding [66, 67]. There are various mechanisms by which the accumulation of misfolded proteins may cause cellular dysfunction, and often a combination of these appears to be responsible for the disease. Misfolded proteins not only lose their normal function, they may also form toxic species, including oligomers or larger aggregates [*e.g.*, amyloid precursor protein in AD and other insoluble fibrillar aggregates in PD, Huntington's disease, amyotrophic lateral sclerosis (ALS), and transmissible spongiform encephalopathies], they may be prevented from reaching their proper cellular localisation due to retention and/or degradation (*e.g.*, cystic fibrosis transmembrane conductance regulator in cystic fibrosis), or they may prevent the function of interacting partners (*e.g.*, myosin in hypertrophic cardiomyopathy) [67, 68]. Importantly, the association of aggregating disease proteins with the quality-control machinery may itself contribute to cellular toxicity.

Chaperones normally act to render misfolded proteins harmless by shielding interactive surfaces, assisting in refolding or triggering degradation. However, in aggregation diseases, the accumulation of toxic misfolded proteins may overload the cellular chaperone protective capacity, thus giving rise to disease phenotypes, which increase with age. Misfolded proteins may sequester components of the chaperone and degradation systems, reducing their activity in the cell. Both these systems are functionally linked [69]; indeed, several components are known to function in both the folding and the degradation of substrate proteins. Furthermore,

aggregates of misfolded proteins in AD, PD, polyglutamine-expansion diseases, such as Huntington's disease and the spinocerebellar ataxias, and prion disease models also include ubiquitin and the 20S and 19S proteasomal components [69], suggesting that the UPS targets disease aggregates for degradation, in an attempt to clear proteins from failing proteasomes. However, this attempt is made vain by the degradation resistance of the aggregated disease proteins as compared with their non-aggregated wild-type counterparts [69–71].

Nyström and colleagues have proposed a possible role for protein carbonylation in protein quality control [72]. Studies of protein carbonylation in prokaryotes [32, 33] showed that misfolded proteins are more susceptible to carbonylation than native ones, suggesting that carbonylation, being an irreversible protein modification, could signal that a protein is irreparable and, hence, act as a tagging system for the degradation pathway. Protein carbonylation targets the modified (and generally dysfunctional) protein to degradation by the proteasomal system in oxidatively stressed mammalian cells [70]. The same studies in prokaryotes [32, 33] have raised the possibility that, in AD and other diseases, some proteins are more susceptible to carbonylation because they are misfolded (and, consequently, dysfunctional), rather than being dysfunctional because carbonylation has made them misfolded [16].

Actually, some normal, regulated cellular processes utilise the carbonylation of specific proteins as a mechanism for triggering their degradation. For example, iron regulatory protein 2 (IRP2) is selectively but very rapidly degraded in iron-sufficient cells. It is stable and therefore functional only in iron-depleted cells [73, 74]. A series of experiments established, both *in vitro* and *in vivo*, that IRP2 binds iron and undergoes metal-catalysed oxidative modification in the presence of oxygen, with introduction of carbonyl groups; the carbonylated IRP2 is ubiquitinated and then degraded by the proteasome [75]. When iron is deficient, IRP2 is stable and active. When iron becomes sufficient, it (or perhaps heme) binds to the protein and catalyses an oxidative modification that suffices to trigger IRP2 degradation by the proteasome. Thus, oxidative modification of proteins need not arise only as an undesirable by-product of an oxygen-based metabolism; it can also function as a mechanism for cellular regulation.

The impact of carbonylation on proteolysis

Proteins of reduced or lost function can be harmful to cells if accumulated. Oxidized proteins are either repaired, removed by proteolytic degradation or accumulate as damaged or unfolded proteins (Fig. 4). Major intracellular proteolytic systems include lysosomal proteases (cathepsins), calcium-dependent proteases (calpains), and multicatalytic proteases (20S proteasome, which has all the three catalytic activities - chymotrypsin-like, trypsin-like, and peptidyl glutamyl peptide hydrolase, or caspase-like - and 26S proteasome, with higher catalytic activity than 20S proteasome). Proteasome can degrade proteins by either ubiquitin-dependent or ubiquitin-independent non-lysosomal pathways. In cells, most proteins destined for degradation are labelled first by ubiquitin in an ATP-dependent process and then digested to small peptides by the 26S proteasome. However, oxidized proteins are mostly degraded by the 20S proteasome that, in contrast to the 26S proteasome, does not require tagging by ubiquitin of target proteins and ATP for the activity [76, 77]. Davies and colleagues have proposed that the 20S proteasome selectively recognises exposed hydrophobic patches of partially unfolded (or denatured) oxidized proteins, since oxidation may cause protein partial unfolding or denaturation with a concomitant increase in surface hydrophobicity [70, 76].

Although mild progressive oxidation of a protein increases its degradation by the proteasome, excessive oxidation and cross-linking of proteins render them resistant to proteolytic degradation by the proteasome, probably because the structural constraint does not allow aggregated or too structurally altered proteins to reach the catalytic sites located inside the cylinder of the enzyme complex [71]. Therefore, heavily oxidized and cross-linked protein aggregates accumulate in cells because they inhibit the proteasome and actually cause a progressive further increase in protein aggregation and cross-linking in nondividing (post-mitotic) cells, and may eventually induce apoptosis, as demonstrated in cardiomyocytes [70, 71, 78, 79]. This possibility may have particular importance in post-mitotic tissues such as brain, heart, and skeletal muscles, where accumulation of oxidized and cross-linked protein aggregates is most marked.

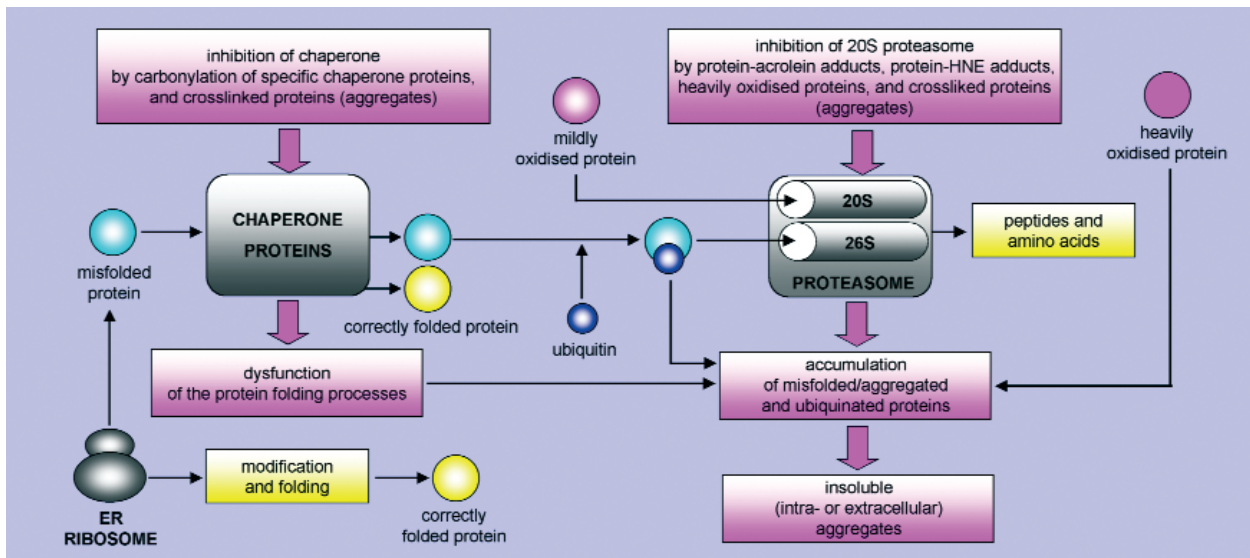


Fig. 4 Degradation and accumulation of oxidized and/or misfolded proteins. Many newly synthesised proteins are translocated into the ER, where they fold into their three-dimensional structures with the help of a series of molecular chaperones and folding catalysts. Correctly folded proteins are then transported to the Golgi complex and then delivered to the extracellular environment. However, incorrectly folded proteins are detected by a "quality-control" mechanism and sent along another pathway (the "unfolded protein response") in which they are ubiquitinated and then degraded in the cytoplasm by the 26S proteasome. Proteins can also be mildly or heavily damaged by oxidative stress. Mildly oxidized proteins are degraded essentially (if not only) by the 20S proteasome. Cross-linkers like HNE or acrolein further enhance the process of aggregation. The heavily oxidized proteins aggregate and these aggregates cannot be degraded by the 20S proteasome, thus they accumulate and build up larger and larger aggregates. More importantly, they even inhibit the removal of oxidatively damaged proteins by the proteasomal system. Cross-linkers like HNE or acrolein further enhance the process of aggregation as well as the inhibition of the proteasome.

Proteasome activity declines during ageing, as the protease is progressively inhibited by binding to ever increasing levels of oxidized and cross-linked protein aggregates [71, 76]. Conversely, healthy centenarians, and rodents placed on a dietary restriction regiment, exhibit a marked amelioration of age-related increases in protein oxidation and proteasome alterations [80]. On the other hand, direct inhibition of the proteasome in NT-2 (human teratocarcinoma) and SK-N-MC (human neuroblastoma) cells led to increased oxidative damage, NO formation, elevated protein nitration [81], and the formation of protein aggregates [82]. Furthermore, interference with polyubiquitination in the same cell lines led to increased levels of protein carbonylation and nitration, lipid peroxidation, and NO production [83]. Disruption of the UPS may form a common mechanism underlying a number of neurodegenerative diseases associated with the accumulation of misfolded and/or oxidized proteins.

Proteasomal dysfunction occurs in neurodegenerative disorders [66, 80, 84–86] and, consistently, the observation of ubiquitinated-protein inclusion bodies in neurons is one of the hallmarks of neurodegeneration [66, 85, 87, 88]. In PD, which is currently the only neurodegenerative disease known to be caused by mutations in proteins within the UPS such as parkin [66], there is genetic evidence for a contribution of UCH-L1 (ubiquitin C-terminal hydrolase-L1), a crucial enzyme for proteasomal protein degradation that generates free monomeric ubiquitin [89]. HNE-cross-linked amyloid β -peptide, which forms the senile plaques in AD, is able to inhibit the proteasome, whereas neither the amyloid β -peptide nor free HNE alone at moderate concentrations inhibit proteasome activity [90]. High concentrations of HNE are unlikely to accumulate in tissues due to very active HNE metabolism. Therefore, a direct inhibition of the proteasome by HNE *in vivo* seems to be very unlikely, even under pathological conditions.

Much more probable is a decline in the protein turnover or the proteasomal activity due to the formation of inhibitory HNE-modified protein aggregates. Indeed, an accumulation of proteins modified by HNE occurs in PD and ALS patients [91–93] and both HNE and HNE-modified proteins conjugate with 20S proteasome during oxidative stress, which may contribute to an impairment of proteasomal function [70, 71, 90, 94, 95].

Oxidative modification and inactivation of the 20S proteasome has been demonstrated in the ischaemic heart [96]. Proteasome plays a significant role in removal of proteins oxidized (carbonylated) during myocardial ischaemia. A recent study demonstrated an inverse correlation between post-ischaemic proteasome activity and levels of carbonylated and ubiquitinated proteins. In particular, inhibition of the 20S proteasome correlates with increases in protein carbonylation, whereas post-ischaemic inhibition of the 26S proteasome leads to accumulation of ubiquitinated proteins [79, 97]. A more recent study [98] has shown that inhibition of the proteasome results in enhanced accumulation of carbonylated proteins in the post-ischaemic rat heart. Furthermore, actin degradation is increased in the post-ischaemic heart, a process that is partially blocked by a proteasome inhibitor, and there appears to be no formation of ubiquitinated homologues of actin, suggesting proteolysis by the 20S proteasome. These observations provide the first evidence that proteasome mediates removal of some of the proteins oxidized during myocardial ischaemia/reperfusion, and that at least carbonylated actin is removed by the 20S proteasome.

Proteasome subunits may be themselves the target of carbonylation. Treatment with an endogenous inducer of ROS production, a prostaglandin D₂ metabolite, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), on human neuroblastoma SH-SY5Y cells resulted in the accumulation of protein carbonyls. Proteomic analysis of oxidation-sensitive proteins showed that the major intracellular target of protein carbonylation was one of the regulatory subunits in 26S proteasome, S6 ATPase, which was associated with (i) a dramatic increase in protein carbonyls within S6 ATPase, (ii) a significant decrease in the S6 ATPase activities, and (iii) a decreased ability of 26S proteasome to degrade substrates [99].

Is protein carbonylation an early event of cellular dysfunction and disease progression?

Proteasome inhibitors can induce several hallmarks of apoptosis, including caspase activation, cytochrome C release, elevated p53 expression, chromatin fragmentation, and DNA laddering, in both neuronal and glial cells [80]. It has been suggested that protein carbonylation is an early event in NO-induced apoptosis in insulin-secreting RINm5F cells [100, 101]. NO-triggered carbonylation of Bcl-2, adenine nucleotide translocator, and GAPDH precedes DNA fragmentation, and inhibitors of ALEs block NO-dependent carbonylation, prevent NO-induced GAPDH inhibition, DNA fragmentation [100], and caspase activation [101]. In addition, NO-induced carbonylation of poly (ADP-ribose) polymerase (PARP) protein precedes its apoptotic degradation and inhibitors of ALE formation prevented both events [100], thus suggesting that carbonylation of PARP could be mechanistically involved in its degradation during apoptosis. Accumulation of aggregates of heavily oxidized proteins (lipofuscin-like materials) induce apoptosis of cardiomyocytes through inhibition of both 20S- and 26S-proteasome activity, accompanied by large increases in ubiquitinated proteins and dysregulation of pro-apoptotic proteins [79]. In addition to directly mediating neurotoxicity, proteasome inhibitors increase neural vulnerability to subsequent oxidative injury [102]. The ability of mild, non-toxic, proteasome inhibition to increase vulnerability to oxidative stress may be particularly important in ageing, AD, and PD, in which proteasome inhibition would be expected to occur gradually, and not directly induce cell death within the central nervous system. However, once a certain level of proteasome inhibition is achieved, it could then serve as a trigger, and increase the toxicity of subsequent stressors.

A key question is whether protein carbonylation occurs at an early stage of disease, contributing to its development, or whether it is merely a consequence of the oxidative tissue damage, reflecting the presence of disease. Basically, the answer to this question requires identification of a specific carbonylated protein and a positive correlation between altered function of this protein and development of diseases. Increased levels of protein car-

bonyls have been detected in a large variety of pathological states occurring in humans, thus suggesting their potential causative role in disease pathogenesis [7, 18, 19, 89, 103–105].

The plasma protein carbonyl content of children with juvenile rheumatoid arthritis is much higher than in healthy children and, notably, grows with the activity of the inflammatory process [106]. Therefore, carbonyl groups of plasma proteins seem to be a good link of inflammatory process activity and disease progression. Increased protein carbonyls have also been observed in tracheal aspirates from premature infants undergoing ventilation therapy, and a correlation between the protein carbonyl content and myeloperoxidase activity (index of pulmonary inflammation) was established [107]. Severe sepsis and major trauma patients had elevated protein carbonyl concentrations in both plasma and bronchoalveolar lavage fluid, which correlated well with ALE measurements and indices of neutrophilia and neutrophil activation [108]. Moreover, patients with acute pancreatitis had significantly increased concentration of protein carbonyls in plasma, which were related to disease severity [109]. Elevated levels of protein carbonyls were observed in the brain of persons with mild cognitive impairment, a condition that often precedes AD, suggesting that oxidative damage may be one of the earliest events in the onset and progression of AD [110].

Increased oxidative stress in newly diagnosed child and young diabetic patients with no complications [111, 112] suggests that the increase in oxidative stress may not be due to complications, but rather may contribute to their development. Studies on young type 1 diabetic patients showed that the content of carbonyl groups in plasma proteins was much higher than in their healthy peers, and that plasma protein carbonyl levels were even higher in diabetic patients with microvascular complications [113]. Although the primary pathophysiological mechanisms by which diabetic complications develop remain to be conclusively determined, results showing that the increased protein oxidative damage and reduced antioxidative defences were greater in young diabetic patients with microvascular complications than in those without suggest that protein carbonylation could be an important early event in the pathogenesis of complications secondary to diabetes [113]. These findings may also indicate that underlying subclinical pathology

(oxidative stress and vascular dysfunction) may be present despite the apparently good glycemic control and outcome in the majority of these young diabetic patients [113].

The increase in glycooxidation and lipoxidation products in plasma and tissue proteins suggests that oxidative stress is increased in diabetes. However, some of these products are formed independent on oxidation chemistry and may also result from elevated levels of substrates prone to oxidation. Moreover, there is also an increase in products of reaction of proteins with dicarbonyl compounds formed by non-oxidative mechanisms [114]. The increased chemical modification of proteins by carbohydrates and lipids in diabetes and other diseases such as uremia and atherosclerosis may therefore be viewed as the result of increased "carbonyl stress" (carbonyl overload), which is caused by a generalised increase in the steady-state concentration of reactive carbonyl precursors of AGEs/ALEs, glycooxidation and lipoxidation products. Carbonyl stress may result from an increase in substrate stress and/or a decrease in the efficiency of detoxification of RCS, *i.e.*, an imbalance between the rates of production and detoxification of reactive carbonyls. Compared with oxidative stress (a condition in which carbonyls are derived exclusively from oxidative reactions), carbonyl stress is a more comprehensive term, since it includes increases in carbonyls derived from both oxidative and non-oxidative reactions [114–117].

Thus, the consequent loss of function of carbonylated proteins may be the cause of subsequent cellular dysfunction and tissue damage (Fig. 5). Some studies discussed in this review suggest a positive correlation between increases in protein carbonylation and disease progression. Since carbonylation can alter protein structure and function and cause the formation of protein aggregates, the "carbonyl stress" hypothesis emphasises the role of RCS, derived from different sources through both oxidative and non-oxidative reactions, and resulting from decreased renal detoxification and/or excretion of reactive carbonyl precursors of AGEs/ALEs from plasma, in the induction of pathogenic protein modifications [114, 115, 118, 119].

Although the importance of protein carbonyls in the pathogenic processes responsible for the development of several diseases remains to be decisively determined, experimental evidences suggest a

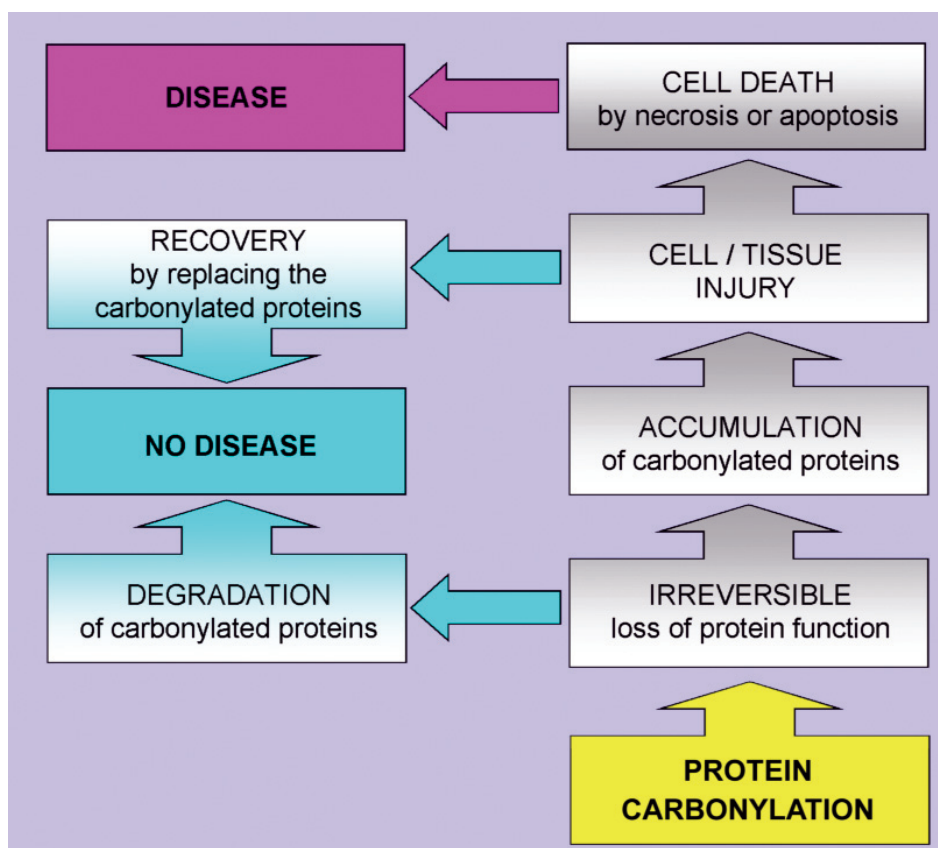


Fig. 5 Possible fates of carbonylated proteins. Protein carbonylation is usually associated with permanent loss of function and can lead to elimination or accumulation of the carbonylated proteins, often leading to tissue injury, and eventually cell death.

causative role of protein carbonylation in the development of long-term complications of diabetes, as well as in ageing-related diseases. For instance, the increased oxidative/carbonyl stress as well as the accumulation of AGEs/ALEs in tissue proteins are in fact thought to contribute to the development of diabetic complications such as atherosclerosis, vascular and neural dysfunction, and retinopathy [114–117, 119–121]. Cumulative modifications by AGEs occur predominantly (but non exclusively) on long-lived proteins such as collagen, neural myelin, and lens crystallins, resulting in insoluble and dysfunctional aggregates that accumulate progressively with time. The formation of inter- and intramolecular cross-links following the glycation of collagen leads to structural alterations, *i.e.*, increased stiffness and resistance to proteolytic digestion [122, 123].

The causative role of protein carbonylation in tissue injury in diabetic complications is substantiated by the pharmacological effects elicited by a variety of novel therapeutic agents able to reduce the accumulation of AGEs/ALEs in diabetes, which have also gained interest as potential cardioprotective

approaches. These agents include aminoguanidine, AVE7688, pyridoxamine, carnosine, benfotiamine, OPB-9195, LR-90, and the so-called cross-link breakers such as ALT-946 and thiazolium salts (*e.g.*, *N*-phenacylthiazolium bromide and alagebrium chloride) [116, 117, 124–128]. In addition, it has been demonstrated that a number of established therapies have the ability to reduce the accumulation of AGEs/ALEs in diabetes, including angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, metformin, peroxisome proliferators receptor agonists, metal chelators and some antioxidants [116, 117, 119, 124–127]. These compounds have been shown to inhibit several diabetic complications such as nephropathy, retinopathy, neuropathy, diabetes-accelerated atherosclerosis, and vascular diseases [116, 117, 129–133] as well as the increased blood pressure, decline in glomerular filtration rate, glomerulosclerosis, nephron loss, proteinuria, cardiac hypertrophy, and aorta stiffness in aged rats [129, 134]. The fact that many of these inhibitors of AGEs/ALEs are effective in experimental models, despite their disparate mechanisms of action, supports the keystone role of AGEs/ALEs in diabetic tissue

damage. Nonetheless, the clinical utility of AGE/ALE inhibition remains to be firmly established.

Conclusions and perspectives

Most pharmacological approaches of the "anti-AGEs/ALEs strategy" are strictly related to inhibition of protein carbonylation and AGE/ALE formation. AGE/ALE inhibitors, even if belonging to different chemical classes, have a common chemical feature: a strong nucleophilic centre able to react with glucose- or lipid-derived RCS at a faster rate than do cell macromolecules. This chemical feature suggests that trapping of RCS and inhibition of protein modification is essential to restrain the pathological events. A key role for lipoxidative modification of proteins in the development of chronic complications of diabetes was clearly demonstrated by investigating the mechanism of action of pyridoxamine [116]. As elevated levels of pyridoxamine adducts with the intermediates of lipid peroxidation have been determined in the urine of diabetic and hyperlipidemic rats, the protective effect of pyridoxamine is consistent with its ability to trap RCS.

However, it must be highlighted that AGE/ALE inhibitors can not be considered as optimal pharmacological tools, because all of them have promiscuous effects. For example, aminoguanidine is a potent inhibitor of inducible nitric oxide synthase, while carnosine and pyridoxamine are also quenchers of ROS. Thus, the real challenge for future research will be, besides to identify target proteins for RCS and to gain a deeper insight into the molecular mechanisms of carbonylation reactions, to develop more specific pharmacological tools, *i.e.*, selective carbonyl blockers (without any antioxidant/metal chelation effect) for definitive assessment of the possible causative role of protein carbonylation in diseases. Significant advancement on this issue should also contribute to suitable updating of pharmacological intervention in human diseases associated with protein oxidation. To achieve successfully this aim, investigations should focus to the unequivocal identification of specifically carbonylated proteins in pathological tissues and fluids.

Proteomic tools now available represent a promising way to elucidate disease mechanism(s) at the protein level [135], because identification of

sites of carbonyl modification should help understanding the factors affecting protein function. A subsequent goal will be to evaluate the impact of carbonylation on protein function. This point is crucial to establish whether carbonylation of specific proteins is causative, correlative or consequential of oxidative stress-associated conditions, because carbonylation does not necessarily result in protein function alteration. Furthermore, it is essential to compare the *in vitro* with the *in vivo* settings when assessing the extent of carbonylation and the consequences to protein activity. For instance, it has been demonstrated that the age-related increase in HNE adduction to rat heart α -ketoglutarate dehydrogenase does not cause loss of its catalytic activity, contrarily to what observed *in vitro*, suggesting that the extent of HNE binding remains low *in vivo*, possibly because the cellular HNE concentrations are manyfold lower than those used to inhibit the mitochondrial enzyme *in vitro* [136]. Hence, if carbonylation leads to protein dysfunction, the use of an appropriate pharmacological tool, able to inhibit/prevent protein carbonylation, would unequivocally indicate a causative role of protein carbonylation in disease development and/or progression. Otherwise, if carbonylation does not lead to functional consequences of the oxidized protein, its causative role in disease onset and/or progression would be excluded.

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