Misfolded or damaged proteins are recognized intracellularly by protein quality mechanisms. These include chaperones and the ubiquitin-proteasome system, which aim at restoration of protein function and protein removal, respectively. A number of studies have outlined the functional significance of the ubiquitin-proteasome system for the heart and, as of recently, for the vascular system. This review summarizes these recent findings with a focus on atherosclerosis. In particular, this paper reflects on the viewpoint of atherosclerosis as a protein quality disease.

Protein Quality Mechanisms

The quality of a protein and its function relies on its 3-dimensional structure and folding dynamics. The loss of proper 3-dimensional arrangement, referred to as unfolding, impairs not only protein function but eventually also cellular function and viability. To prevent these detrimental consequences, each cell is equipped with 2 operational systems: chaperones and proteases.

Chaperones recognize protein substrates based on hydrophobic protein regions, which are usually buried within the tertiary and quaternary structures but surface as the protein structure unfolds after translational or post-translational damage. Variations in the protein sequences that flank these hydrophobic sequences determine the large number and various types of chaperones across the different cellular compartments (1). Their main function is to hold misfolded proteins, to fold them back into their native structure, and to promote the degradation of proteins resistant to this process by making them more accessible to proteases. This latter group of enzymes cleaves proteins based on amino acid sequence patterns. Up to 90% of all intracellular proteins are degraded, both in a chaperone-dependent and in a chaperone-independent manner, by a specialized protease system, called the ubiquitin-proteasome system (UPS) (2). This system operates mainly in the cytoplasm and recognizes protein substrates, like chaperones, on the basis of bulky hydrophobic residues but also based on basic residues at the NH2-terminus (so-called N-end rule). Thus, numerous chaperones and the UPS work independently and in synergy to assure the quality of intracellular proteins.

The kinetics of the interactions between chaperones, the UPS, and damaged proteins were summarized in a triage model (Fig. 1) (3). One particular subtype of protein quality control mechanisms is the unfolded protein response (4). This response is triggered by the accumulation of unfolded or misfolded proteins in the lumen of the endoplasmic reticulum (ER), which is also referred to as ER stress and leads to the modification of transducer proteins in the ER membrane and the activation of their related pathways. These pathways include the inositol-requiring protein 1, the activating transcription factor-6, and the protein kinase ribonucleic acid-like kinase signaling pathways. Collectively, these pathways orchestrate the down-regulation of the synthesis and the translocation of proteins into the ER, which leads to a reduction in protein load. They also mediate the expression of chaperones and protein-modifying enzymes, which facilitates protein refolding. Furthermore, they engage the endoplasmic reticulum-associated protein degradation pathway, which entails the
retrotranslocation of unfolded proteins into the cytosol and their proteolysis by the proteasome. In case these mechanisms fail to resolve the mismatch between protein load and handling capacity over a period of time, the unfolded protein response induces autophagy as an alternative coping mechanism. This process involves the sequestration of the ER into membrane-bounded compartments (autophagosomes), its fusion with lysosomes, and the degradation of its content. The ER-specific autophagy is seemingly essential for cells to survive severe ER stress (5). However, with overwhelming ER stress cell death pathways are nevertheless activated.

In summary, cells are equipped with a robust apparatus to repair or remove damaged proteins. This includes chaperones, proteases, and the ER-related unfolded protein response along the general concept of protein triaging. The competitive balance between the activity of protein quality control mechanisms, on the one hand, and the generation of low-quality proteins, on the other hand, is the hallmark of this concept.

### Protein Quality Diseases

Protein quality diseases, also called protein misfolding, precipitation, or conformational diseases, are a group of disorders that are associated with and occasionally caused by the conformational change, aggregation, and deposition of 1 or more proteins (6). Neurodegenerative diseases were the first prominent example for this entity. Mutation analyses in familial counterparts to sporadic neurologic disorders and experimental studies highlighted the pathogenetic role of the accumulation of misfolded proteins in these disease processes (7,8). Research efforts also showed that the accumulation of these proteins is due to their increased production, abnormal processing, and/or decreased elimination via chaperones, the UPS, or the unfolded protein response (8,9). The “amyloid hypothesis” of neurodegenerative diseases links the aggregation of misfolded proteins into ordered protease-resistant structures, called amyloid fibrils, to aberrant protein interactions that culminate in neuronal dysfunction and ultimately neurodegeneration (10). Indeed, the pathological momentum seems to be with the very process of amyloid formation.
Amyloid formation is an ordered process and begins when misfolded proteins start to assume a beta-pleated sheet structure and self-associate to form soluble pre-amyloid oligomers. As these soluble preamyloid oligomers start to form protofibrils and coalesce, classic amyloid fibrils, plaques, and tangles are formed, which then exhibit Congo red-positive staining. Classic amyloid plaques are therefore a late and final reflection of a process that remains undetected in its peak toxicity. Consistent with this view, neurologic disease precedes the histologic appearance of classic amyloid plaques. As another implication, the focus of attention has shifted from the large aggregates and microscopic hallmarks to the small aggregates and molecular processes of the pre-amyloid stage both extracellularly and intracellularly (11). Moreover, there has been the realization that the process of aggregation is not inherent to only a few proteins but can be observed with various proteins once misfolding remains unresolved (12).

In summary, unfolded/misfolded proteins can accumulate in cells and tissues when they are produced in large amounts and/or not cleared sufficiently. These proteins then form aggregates, which can remove amino acids from the recycling pool, tie up chaperones and proteases, serve as foci for the aggregation of other unrelated proteins, and finally disrupt cellular and tissue functions. Over time, the accumulation of low-quality proteins can therefore result in disease, a so-called protein quality disease. Precipitates such as amyloid are characteristic late-stage histologic fingerprints.

### Protein Quality Control in Atherosclerosis

Besides inflammation, excess generation of reactive oxygen species (ROS), so-called oxidative stress, is considered to be an important element in the pathophysiology of atherosclerosis (13). Among the various effects, oxidative stress has been shown to increase the expression of chaperones, including those of the family of heat shock proteins (HSPs). The HSPs most closely associated with atherosclerosis include HSP60 and HSP70. The expression of HSP60 in the vascular wall correlates with the severity of atherosclerosis and is particularly prominent in the shoulder regions and around necrotic cores of atherosclerotic plaques (14). The HSP70 is mainly expressed in more advanced atheromas around sites of necrosis and lipid accumulation (15). Importantly, these findings can be reproduced experimentally in hypercholesterolemic apolipoprotein E<sup>−/−</sup> mice, in which the expression of both HSPs increases in the early and progressive disease stages (Fig. 2) (16).

**Figure 2** Chaperone Expression in Early and Advanced Experimental Atherosclerosis

Aortic samples from apolipoprotein E<sup>−/−</sup> mice fed a Western diet for up to 40 weeks and a chow diet for 69 weeks. As presented in the upper panels, there is an increase in the expression of heat shock protein (HSP) 60 and HSP70 by immunohistochemistry from 3 to 20 weeks. Immunoblotting, as presented in the lower panels, confirms these findings and shows that this increase in expression is specific for lesion sites and decreases in chronic lesions of aged mice. Images used with permission of the American Heart Association (16).
There is also evidence for stimulation of the UPS in atherosclerosis. Coronary artery plaques associated with fatal acute myocardial infarction are characterized by increased expression of ubiquitin/ubiquitinated proteins, especially in the shoulder and cap regions (Fig. 3) (17). Similarly, carotid artery plaques from patients with symptoms of focal cerebral ischemia display a higher level of ubiquitinated proteins than those obtained from asymptomatic patients (Fig. 3) (18,19). In experimental models of atherosclerosis, we and others demonstrated that levels of ubiquitinated proteins start to increase at early disease stages (20,21). Furthermore, there is evidence of a concomitant increase in proteasome activity in experimental hypercholesterolemia and hyperglycemia, probably to compensate for the increased amount of substrates for the system, including misfolded proteins, under those circumstances (21–23).

Along these lines, activation of the unfolded protein response has been reported as a very early phenomenon in atherosclerosis (24). Cholesterol accumulation and oxidative stress products such as peroxynitrite seem to be the most potent inducers for the unfolded protein response in macrophages and endothelial cells, respectively (25–27). In these cells, activation of the unfolded protein response has been linked to cytotoxicity but might not be sufficient to trigger cell death by itself (24,28). How the unfolded protein response directs to life or death of a cell is currently incompletely understood. Nevertheless, there is consistency in the view that cell death pathways are activated under circumstances of unmitigated or overwhelming ER stress.

Taken together, there is up-regulation of the expression and activity of chaperones, the UPS, and the unfolded protein response with overlapping features in atherosclerosis. These findings point to an increased demand of protein quality

**Figure 3** Ubiquitin Expression and Proteasome Activity in Advanced Atherosclerosis

Increase in ubiquitin immunoreactivity in complicated plaques of fatal acute myocardial infarction-related coronary arteries compared with advanced plaques in the non-infarction-related coronary arteries of the same patient, relating to differences in the shoulder and fibrous cap areas (left). Images on the left and data for the bottom left graph adapted, with permission, from Herrmann et al. (17). In carotid artery plaques of patients with symptoms of transient ischemic attack (TIA), stroke, or amaurosis (Am.) fugax, the level of ubiquitin-protein conjugates, but not of free ubiquitin, is higher and proteasome function is lower than in carotid plaques from asymptomatic patients (right). Graphs on the right used with permission of the American Heart Association (19).
mechanisms to prevent the vascular accumulation of misfolded proteins increasingly generated by oxidative stress and other factors. The compensatory efforts might be successful in the beginning but might not suffice over a long period of time.

**Protein Quality Disease Aspects of Atherosclerosis**

Autopsy-based studies highlighted lower-level expression of HSPs in complicated, acellular, and collagenous plaques in both human disease and experimental models (15,29). Furthermore, in hypercholesterolemic apolipoprotein E-deficient mice, HSP expression decreases in the more advanced plaques before the development of complications (Fig. 2) (16). These findings indicate that chaperone function may become relatively insufficient in the aged and advanced disease stages.

Although the activity of the ubiquitin system remains seemingly unimpaired even in advanced atherosclerotic plaques, increase as well as decrease in proteasome activity has been observed in symptomatic carotid artery plaques (18,19). In a large animal model, we have recently demonstrated that chronic inhibition of the proteasome contributes to endogenous oxidative stress and atherogenesis (22). In contrast, other groups have noted that in vivo or ex vivo proteasome inhibition for a relatively short period of time yields beneficial vascular effects (23,30,31). The complexity and diversity of the data is recaptured in the in vitro finding that high concentrations of oxidized low-density lipoprotein (LDL) lead to an initial transient activation of proteasome function followed by a sustained decay of proteasome activity and that proteasome inhibition potentiates the cytotoxic effects of oxidized LDL (32). Intensity and duration of the modulation of proteasome function and its specific environment are seemingly key variables for a number of biological processes, and their interaction determines the ultimate consequences for cells and tissues.

If protein quality mechanisms decreased in their activity over time, one would expect accumulation and aggregation of misfolded proteins in “aged” arteries. If causally related to atherogenesis, these changes might also be particularly prominent in atherosclerotic lesions. As recently shown in experimental hypercholesterolemia, oxidatively modified and ubiquitinated proteins accumulate in the vascular wall, especially with inhibition of the compensatory increase in proteasome activity (22). Under those circumstances, prominent intimal thickening can be observed as well (22). With progression of disease, deposition of amyloid can be found in the intima adjacent to atherosclerotic plaques in up to one-half of the cases and to a similar extent within the plaque area, mainly in the necrotic core regions (Fig. 4) (33,34). Accumulation of intracellular proteins, modified by oxidation, nitration, phosphorylation, or (as particularly prominent in diabetes) glycation, may represent part of the amyloidogenic burden. Apolipoproteins contribute the most important extracellular source for amyloid fibrils in atherosclerotic plaques. This relates to mutations as well as their limited conformational stability in the absence of lipids, which can result from enzymatic or oxidative particle modification, dissociation, or displacement (35,36). Furthermore, members of the cathepsin family of proteases may cleave apolipoproteins to generate amyloid-prone fragments (37). Apolipoprotein amyloidosis may therefore be simply a reflection of lipoprotein metabolism. However, fibrillar amyloid-like proteins have been shown to stimulate CD36 signaling in atherosclerotic plaques, thereby assuming a more active role in tissue inflammation and ROS production (38). Finally, the load of amyloidogenic particles in atherosclerotic plaques might be increased by platelet-derived and locally expressed amyloid precursor protein (APP), which has been linked to a number of vascular changes.

The APP processing leads to formation of amyloid beta protein (Abeta), causally related to Alzheimer’s disease. Intriguingly, overexpression of a mutant form of APP in
mice (Tg-APP23) leads to progressive flow abnormalities within the cerebral circulation on magnetic resonance angiography, relating to vessel constriction, deformation, and closure (Fig. 5) (39). Moreover, impairment of endothelium-dependent vasorelaxation of carotid arteries and the aorta can be found in related mice (Tg2576) and is attenuated by endothelin-receptor antagonism (40). Likewise in a mouse model, systemic infusion of soluble Abeta leads to an increase in vascular resistance and a decrease in cerebral perfusion (41). These findings correspond to the provasoconstricting/antivasorelaxing properties of Abeta, which were documented in in vitro studies and ultimately attributed to Abeta-induced inhibitory signaling on endothelial nitric oxide synthase activity (42). Of further note, histologic studies confirm the capacity of Abeta to cause structural damage of endothelial cells and to induce tissue inflammation (43,44). Intracellular accumulation of Abeta can also lead to mitochondrial dysfunction, with release of free radicals and its important implications (11). It can also impair proteasome function, resulting in further Abeta accumulation (11). As amyloid deposition progresses, so do the degenerative changes of the vascular wall. These include aneurysmal dilation, vascular rupture, and hemorrhage on the one end and narrowing and occlusion of vessels on the other end of the spectrum. In its entirety, this vascular process has become known as cerebral amyloid angiopathy and is frequently seen in patients with Alzheimer’s disease (45). In fact, the neuropsychologic deficits of Alzheimer’s disease seem to correlate better with the degree of amyloid deposition in the cerebral vessels than in the brain. Last but not least, learning disabilities and cerebral Abeta deposits are greater in atherosclerosis-susceptible APP-overexpressing Tg2576 mice fed an atherogenic diet, and the extent of aortic atherosclerosis is enhanced by the APP-transgenic trait and correlates with the cerebral Abeta burden in these mice (46).

In summary, nonfibrillar and fibrillar forms of the classic amyloidogenic protein Abeta have been shown to cause endothelial injury and dysfunction as well as progressive structural changes of the entire vascular wall in the context of cerebral amyloid angiopathy over time. Similar degenerative changes can be observed in atherosclerosis, and synergism has been suggested in the pathophysiology of Alzheimer’s disease and atherosclerosis. Within the framework of protein quality diseases, the accumulation of damaged intracellular proteins and the deposition of amyloidogenic extracellular proteins may be of significance for the atherosclerotic disease process (Fig. 6).
Future Cardiovascular Perspective

Certainly, one of the central remaining questions is whether the accumulation of dysfunctional proteins is a causal factor, a contributing factor, or simply an epiphenomenon in atherosclerosis, similar to the current discussion in heart failure (47,48). Mice expressing an anchorless prion protein that is insoluble and resistant to proteolysis develop cardiomyopathy, which constitutes the strongest experimental evidence so far for the detrimental consequences of protein precipitation on cardiac function (49). Intriguingly, in this model, amyloid deposition can be seen primarily within and around endothelial cells (49,50). Further studies on the vasofunctional and vasostructural consequences of these findings would be very helpful in attesting that protein precipitation by itself exerts a pathophysiological and notably atherogenic momentum. Positive results would prompt further studies on how proteins can escape protein quality control mechanisms and perturb vascular homeostasis in the human vasculature. These studies should be complemented by gain-of-function and loss-of-function studies on central components of the 2 arms of the protein quality control system. Clinical studies evaluating the effect of selective positive or negative modulation of the systems in relation to the stage of disease will give final answers from a human disease and therapy standpoint. With this perspective, the viewpoint of atherosclerosis as a protein quality disease may contribute to current theory and future treatment of atherosclerotic cardiovascular disease.

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