On to the road to degradation: atherosclerosis and the proteasome

Joerg Herrmann¹, Lilach O. Lerman², and Amir Lerman¹*

¹Department of Internal Medicine, Division of Cardiovascular Diseases, Mayo Clinic Rochester, 200 First Street SW, Rochester, MN 55905, USA; and ²Division of Nephrology and Hypertension, Mayo Clinic Rochester, Rochester, MN, USA

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Protein metabolism is a central element of every living cell. The ubiquitin-proteasome system (UPS) is an integral part of the protein metabolism machinery mediating post-transcriptional processing and degradation of the majority of intracellular proteins. Over the past few years, remarkable progress has been made in our understanding of the role of the UPS in vascular biology and pathobiology, particularly atherosclerosis. This review reflects on the recent developments from the effects on endothelial cells and the initial stage of atherosclerosis to the effects on vascular smooth muscle and the progression stage of atherosclerosis and finally to the effects on cell viability and the complication stage of atherosclerosis. It will conclude with the integration of the available information in a synoptic view of the involvement of the UPS in atherosclerosis.

Keywords
Atherosclerosis • Inflammation • Oxidative stress • Proteasome • Ubiquitin

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1. Introduction

The proteasome complex accounts for the degradation of ~90% of all intracellular proteins. Although some proteins can be degraded directly by this complex, a substantial number of proteins require ‘ escorting’ to the proteasome by modifier molecules (Figure 1). Ubiquitin was the first modifier molecule to be recognized, and the entire protein modification–degradation sequence has been referred to as the ‘ ubiquitin-proteasome system’ (UPS). For their pioneering work in this field, Aaron Ciechanover, Avram Hersko, and Irwin Rose were awarded the Nobel Prize in Chemistry in 2004.

Assembly of the proteasome complex is a highly dynamic process. One important modifying regulator is interferon (IFN)-γ, under the influence of which the cap structure is formed by two homologous subunits, PA28α and PA28β (i.e. 115 proteasome), and proteolytic core units are modified to compose the immunoproteasome with unique protein processing properties. An intermediate- or hybrid-type of the proteasome containing both constitutive and inducible subunits has also been identified; in fact, electron microscopic imaging confirms a total of six different assembly types of the proteasome (Figure 2).

Beyond these proteasome types, the proteasome has been found to be present and functionally active in the extracellular space such as the alveolar space and serum (so-called circulating proteasome). As can be assumed from these few introductory statements, proteasome composition and function can vary greatly between different tissues and cell compartments. For the purpose of this review, we will focus on the proteasome in the vascular system and primarily its impact on atherosclerosis. Details regarding the structure, regulation, and function of the proteasome and the related ubiquitin system were provided in the initial review in this journal.

2. The UPS in the initial stage of atherosclerosis

Atherosclerosis is initiated by the dysfunction and the activation of the endothelium with increased oxidative stress and inflammation as important pathophysiological elements. Endothelial dysfunction has been traditionally linked to impaired vasorelaxation as a diagnostic parameter with reduction of nitric oxide (NO) as the
Figure 1  Overview of protein modification by the ubiquitin and the proteasome system. Ubiquitin is activated, transferred, and conjugated to a target protein by the action of E1, E2, and E3 enzymes. This process is balanced by the action of de-conjugating enzymes. Modified proteins can undergo degradation via the endosome/multi-vesicular body (MVB)/lysosome pathway or via the 26S proteasome with and without adaptor proteins. Functional activation [e.g. IκB kinase (IKK) via ubiquitination of its regulatory subunit NEMO or associates] or translocation (e.g. poly-ubiquitination of p53) can be other consequences. Proteins, modified by ubiquitin or the ubiquitin-like (UBL) modifiers NEDD8 or FAT10, are ‘escorted’ to the 26S proteasome by ubiquitin-domain proteins. As for ubiquitinated proteins, the escort process can be supported by E4 enzymes, which extend the ubiquitin chain by a few more ubiquitin molecules. This allows binding of adaptor proteins such as Rad23, whose UBL domain is recognized by the leucine-rich repeat (LRR)-like domain of the Rpn1/S2 subunit of the 19S proteasome. Besides substrate binding, the 19S complex facilitates deubiquitination and unfolding of the target protein by virtue of its ATPase activity as well as opening of the proteolytic tunnel of the 20S proteasome, which is formed by four stacked rings (two outer α-rings and two inner β-rings), each with seven subunits. Conformational changes of the gating structures of the outer α-rings, namely the N-terminal ends of the α2-, α3-, and α4-subunits, allow this opening into the inner proteolytic chamber. The subunits of the regulatory α-rings are paired over their numerically corresponding β-subunits. On the other hand, the subunits on the inner two β-rings are aligned counter to each other such that the β1 subunit of one ring lies above the β7 unit of the other ring. This orientation allows a staggered spatial distribution of the N-terminal threonin residues of the active centres of the β1-, β2-, and β5-subunits for caspase-like, trypsin-like, and chymotrypsin-like activity, respectively (matches ascending order of significance for cell viability with the β5-subunit as the most important). These proteolytic activities degrade target proteins into oligopeptic products, which are 8–12 amino acids in length on average and are processed further by proteases. In addition to operating in conjunction with the 19S complex as the 26S proteasome, the 20S proteasome can function independently. In this mode, hydrophobic structures of misfolded or (oxidatively) damaged proteins rather than regulatory subunits induce conformational changes of the gatekeeper α-subunits and gain access into the proteolytic tunnel. Further details regarding the proteasomal system are provided elsewhere.99
central molecular element. On the other hand, endothelial cell activation has been associated with increased adhesion molecule expression and nuclear factor-kappa B (NF-kB) as the pivotal molecular mediator. Over the past years evidence has been accumulating for the involvement of the UPS in these two key aspects of the initiation phase of atherosclerosis.

The reduction in vascular NO bioavailability is the consequence of reduced NO production and increased consumption before NO reaches its physiological targets. Endothelial NO synthase (eNOS) is the central source of NO located in flask-shaped invaginations of the plasma membrane called caveolae where it remains inhibited by interaction with the caveolar coat protein caveolin-1. It has been shown that treatment of endothelial cells with proteasome inhibitors increases de novo synthesis and cellular eNOS levels without changing the expression of caveolin-1. The concomitant improvement in eNOS activity and endothelial function might therefore possibly be the consequence of a shift in the stoichiometry between eNOS and caveolin-1 with relatively less eNOS being bound in inhibitory restrain with caveolin-1.9 Under physiological circumstances, activation of eNOS occurs when its inhibitory conformation with caveolin-1 is reversed by excess Ca\textsuperscript{2+}/calmodulin and Akt-induced phosphorylation of eNOS.10 On the contrary, ubiquitination of protein phosphatase 2A leads to the translocation of this enzyme from the cytosol to the membrane where it associates with and dephosphorylates eNOS, leading to decreased eNOS activity.11 Besides phosphorylation, the normal function of eNOS requires dimerization, the substrate l-arginine, and the essential cofactor tetrahydro-L-biopterin (BH4). It has been recognized that cardiovascular risk factors deplete this co-factor and as a consequence eNOS produces superoxide instead of NO (referred to as ‘eNOS uncoupling’). Superoxide reacts avidly with any locally present NO reducing NO bioavailability further and generating peroxynitrite, which oxidizes BH4. It has been postulated that superoxide production by the stimulation of NADPH oxidases would be the primary process to reduce BH4.12 Recently, however, it was shown that hyperglycaemia stimulates proteasome activity in endothelial cells and increases ubiquitination and proteasome-dependent degradation of guanosine 5’-triphosphate cyclohydrolase I (GTPCH), which then leads to BH4 deficiency and eNOS uncoupling.13 This sequence seems to be operational even in vivo, and proteasome inhibition does reverse the reduction of GTPCH, BH4, and endothelial dysfunction in streptozotocin-induced diabetes mellitus. Oxidative stress products such as 4-hydroxynonenal (4-HNE) are also capable of decreasing GTPCH levels and activity in endothelial cells via the proteasomal pathway with the aforementioned consequences.14 Lastly, the co-chaperone/ubiquitin ligase

Figure 2 Electron micrographs of a negatively stained proteasome mixture showing the six different proteasome species: a 20S proteasome, proteasomes that are singly capped (19S–20S) and doubly capped (19S–20S–19S) with 19S complex, proteasomes that are singly capped (PA28–20S) and doubly capped (PA28–20S–PA28) with PA28 rings as well as a hybrid proteasome with a 19S complex and a PA28 ring bound to either end (19S–20S–PA28). The length of the side of the individual small frames corresponds to 80 nm. The magnified view shows a hybrid proteasome complex to highlight the differences in the cap structure of 19S vs. PA28. Modified and reproduced with permission from Nature Publishing Group.3
carboxyl terminus of Hsc70 interacting protein (CHIP) mediates ubiquitination and proteasomal degradation of the NO receptor soluble guanyl cyclase in smooth muscle cells (SMCs), thereby attenuating NO donor-induced relaxation of rat aortic rings.\(^{15}\) Increase in endogenous oxidative stress has been considered to be the underlying mechanisms for the reduction of NO bioavailability. The UPS has an important role in oxidative stress regulation in endothelial cells with the transcription factor nuclear erythroid 2-related factor 2 (Nrf2) as a central element.\(^{16}\) Transcription factors are proteins that control the transfer of genetic information from DNA to mRNA by binding to specific enhancer or promoter DNA sequences adjacent to the genes they regulate.\(^{17}\) They initiate a program of either increased or decreased gene expression by affecting the association of RNA polymerase and/or histones with the DNA and the recruitment of co-activators or co-repressors to a gene expression regulation complex. The activity level of these factors is regulated by their synthesis and degradation rate, ligand binding and modifications such as phosphorylation, nuclear translocation, and dimer formation, which facilitates binding into the groove of the DNA. In case of Nrf2, this transcription factor is negatively regulated by undergoing constitutive ubiquitination and degradation via the proteasome as long as the level of oxidative stress is low and Keap-1 serves as a substrate adaptor for a Cul3-dependent E3 ubiquitin ligase complex.\(^{18}\) Under circumstances of increased cellular oxidative stress, Keap-1 itself becomes a substrate for the ubiquitin system and undergoes proteasomal degradation. Consequently, Nrf2 is stabilized and binds to genome sequences with an antioxidant response element. This leads to expression of genes encoding for proteins which have been linked to the amelioration of oxidative stress.\(^{19}\) Of note, Nrf2 also leads to an upregulation of proteasome subunits, and their overexpression via the Nrf2 pathway has been found to increase cellular resistance even against toxic, misfolded proteins.\(^{20}\) This mechanism may compensate for the loss in stimulation with decreasing NO levels under circumstances of increased oxidative stress.\(^{21}\) Endogenous production of NO is particularly important in the basal regulation of the immunoproteasome.\(^{21}\) Recently, some reports suggest that NO mediates the alteration of the activity of the contiguous proteasome in response to hydrogen peroxide (increase with low levels, decrease with high levels).\(^{22}\) A similar dual effect on proteasome function has been shown for oxidative stress products such as oxidized low-density lipoprotein (oxLDL) and HNE.\(^{23,24}\)

Endothelial cell activation is mediated by NFκB and the UPS has a central role in the classical activation of pathway of this transcription factor.\(^{25}\) One of the gene sequences controlled by NFκB encodes for the pro-molecule of endothelin-1 (ET-1) as well as adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and E-selectin and chemotacticants such as monocyte chemoattractant protein-1. Related to the interference with the NFκB pathway, proteasome inhibition has been shown to decrease vascular ET-1 content and adhesion molecule expression.\(^{26}\) Most recently, not related to potent NFκB inhibition but reduction of oxidative stress, low-dose proteasome inhibitor treatment reduced adhesion molecular expression in the aorta of hypertensive rats.\(^{25}\) We contrarily noted an increase in the expression of VCAM-1 and E-selectin and in fact, an increase in macrophage accumulation and intimal thickening in coronary arteries of hypercholesterolemic pigs subjected to chronic, non-low-dose, in vivo proteasome inhibition (Figure 3).\(^{27}\) Even under otherwise normal conditions, chronic proteasome inhibition over 3 months led to impairment in endothelium-dependent vasorelaxation and increased oxidative stress despite upregulation of eNOS (Figure 4).

Taken together, while some studies support a contributing role of the proteasome to atherosclerosis as it mediates a decrease in eNOS activity and an increase in NFκB activity, it may also have a protective role as it eliminates damaged protein metabolites. Consistent with the dual implications, proteasome inhibitor studies have yielded opposite results. A central element for the understanding of this dualism is the interaction with oxidative stress. Low-level oxidative stress increases proteasome activity, whereas high-level oxidative stress exerts an inhibitory effect.\(^{28,29}\) Vice versa, low-level proteasome inhibition enhances the oxidative defence of endothelial cells, whereas high-level proteasome inhibition contributes to oxidative stress.\(^{30–33}\) The UPS, therefore, takes an important regulatory role in the interplay of oxidative stress with inflammation and endothelial function and even small deviations in the activity of the system can be of great and diverse consequences.

### 3. The UPS in the progression stage of atherosclerosis

The initial disease process matures as macrophages turn into foam cells with intracellular lipid accumulation and populate the subendothelial intimal space to produce so-called fatty streaks.\(^8\) SMCs invade these areas from the media and transform into a proliferating and metabolically active cell population. With additional extracellular lipid accumulation, an atheromatous core develops that is capped by a collagen-rich matrix. Neovascularization contributes to plaque growth that eventually leads to a gradual compromise of luminal dimensions. As work over the past years has highlighted, the UPS exerts important pathophysiological function in this progression phase of atherosclerosis.

A mechanism that may contribute to foam cell formation is the suppression of apoptosis of lipid-bearing macrophages by aggregated LDL (agLDL). Importantly, one of the central genes induced by agLDL but not native LDL is termed LDL-inducible gene and encodes a human homologue of bovine ubiquitin-conjugating enzyme E2-25K.\(^{34}\) Expression of this enzyme leads to increased ubiquitination and subsequent degradation of p53. Mediating preferentially the reduction in the bioavailability of this pro-apoptotic molecule, the UPS may contribute significantly to foam cell formation. Another pathway by which the UPS may contribute to foam cell maintenance is via adipose differentiation-related protein. This protein is associated with lipid droplets in various types of cells including foam cells, and a functional UPS is required for the regression of these cells.\(^{35}\)

As summarized before, proteasome inhibition prevents conversion of SMCs from a contractile to a metabolic phenotype.\(^6\) Another mechanism that was recently discovered to possibly contribute to the effects of proteasome inhibition on SMC transformation has been linked to the inhibition of the degradation of
myocardin. This transcriptional co-activator favours the expression of genes for a contractile SMC phenotype but is targeted for proteasomal degradation by the aforementioned ubiquitin ligase CHIP.36

In agreement with previous studies,37 it was found that proteasome inhibition reduced the S-phase entry of vascular SMCs, the cell count of cultured vascular SMCs as well as the migration of vascular SMCs in a modified Boyden chamber assay. Treatment of a local balloon injury site with the relatively specific proteasome inhibitor lactacystin resulted in a potent reduction of neointimal formation along with an upregulation of the p21 cyclin-dependent kinase inhibitor.38 Another study suggested that an intracellular accumulation of the pro-apoptotic molecule Bad contributed to the induction of apoptosis of vascular SMCs by proteasome inhibitors and could be prevented by endothelial growth factor (EGF) receptor signalling.39 Furthermore, inhibition of proteasome activity by S-nitrosylation of proteolytic core proteins and modification of proteasome composition may be the very mechanism by which NO inhibits SMC proliferation.40

One possible mechanistic pathway linked to these findings is NFκB. Originally identified as a deubiquitinating enzyme, ubiquitin C-terminal hydrolase L1 (UCHL1) hydrolyzes protein bonds between ubiquitin and small adducts or unfolded polypeptides, yielding free ubiquitin. In the vascular wall, UCHL1 is expressed in endothelial cells and SMCs as well as the migration of vascular SMCs in a modified Boyden chamber assay. Treatment of a local balloon injury site with the relatively specific proteasome inhibitor lactacystin resulted in a potent reduction of neointimal formation along with an upregulation of the p21 cyclin-dependent kinase inhibitor.40

In vitro, UCHL1 overexpression inhibited NFκB activation as potently as proteasome inhibitors. Despite some debate on the in vivo substrates of UCHL1, the concomitant decrease in IκBa ubiquitination is somewhat suggestive.41 For cylindromatosis (CYLD), another deubiquitinating enzyme, the substrate has been identified as TRAF2, a central molecule in the intracellular TNFα-receptor signalling cascade upstream from IκBs. Similar to UCHL1, CYLD attenuates NFκB activity and neointima formation.42 Contrary to UCHL1, however, CYLD is expressed primarily in vascular SMCs including those in human carotid plaques. Its expression increases in response to mechanical vascular injury but in a delayed manner suggesting its involvement in the down-regulation of the inflammatory response. Taken together, these studies confirm the significance of ubiquitination, de-ubiquitination, and the UPS as a whole in the regulation of NFκB activity and its impact on vascular biology and atherosclerosis.

Figure 3 Histological evaluation of porcine coronary arteries as detailed. Left panel: DHE fluorescence, a marker of superoxide production, in coronary arteries from pigs on a normal or high-cholesterol diet for 12 weeks without (N and HC) or with proteasome inhibitor treatment (N + PSI and HC + PSI); notice the fluorescence in the intima of coronary arteries from all groups but N (arrows, Adv, adventitia); original magnification ×50. Mid left panel: nitrotyrosine immunostaining, a marker of peroxynitrite formation, in coronary arteries showing positive results in the intima in N + PSI (arrows) and even more prominently in HC and HC + PSI animals; original magnification ×75. Mid panel: immunoexpression for CD204/macrophage scavenger receptor A in coronary arteries; notice the focal positive staining in N + PSI (arrow), more intense staining in HC, and clustering in HC + PSI; original magnification ×75. Mid right panel: oil red O staining of coronary arteries demonstrating focal areas of staining in N + PSI, more prominently in HC and most intensely in HC + PSI; original magnifications ×50. Right panel: Elastica van Gieson (EvG) staining of coronary arteries shows intimal thickening in HC and N + PSI but most prominently in HC + PSI; original magnification ×50. Modified and reproduced with permission from Lippincott, Williams & Wilkins.27
Indeed, an increase in NFκB activity was noted with the progression to atheromatous changes in the aortic walls of rabbits fed a high-cholesterol diet for 3 months.51 This was related to a concomitant increase in the activity of the 20S but not of the 26S proteasome in the aortic wall. Aspirin reduced this increase in 20S proteasome activity only in the high-cholesterol group with additional proteasome inhibition (N + PSI and HC + PSI) compared with animals on a normal diet alone (N) (minimum of six rings per group). Modified and reproduced with permission from Lippincott, Williams & Wilkins.27

4. The UPS in the complication stage of atherosclerosis

Destabilization of extracellular matrix and cell viability leads to plaque disintegration in the form of plaque erosion or rupture, thrombus formation, and complete or near complete vascular occlusion as the substrate of acute clinical presentations.8 Destructive inflammatory activity and cell death are central pathophysiological elements for the development of complicated atherosclerotic plaques. The UPS has important molecular regulatory roles in both these processes.

In addition to macrophages, T cells are an important component of the inflammatory cell population in the atherosclerotic plaque.54 As reviewed before,55 macrophages are the epitome of the innate immune system. They provide the rapid first line of defence based on the recognition of common foreign molecular patterns by receptors that include scavenger and Toll-like receptors. T cells on the other hand are part of the adaptive immune system and recognize and target-specific molecular structures by specific antigen receptors, which are called T-cell receptors (TCRs) and...
are generated in great numbers by somatic rearrangement in blast cells. In order to avoid targeting of self-antigens, T-cell activity is highly and often times complexly regulated. For instance, the ubiquitination state of the regulatory protein Malt1 is crucial for TCR signalling. Upon T-cell stimulation, covalent attachment of regulatory ubiquitin chains to Malt1 allows interaction with the IκB kinase (IKK) complex and its paracaspase activity cleaves A20, its endogenous inhibitor. A20 catalyzes the removal of K63-linked ubiquitin chains from Malt1 and the balance between these two proteins regulates the strength and duration of the IKK/NFκB response upon TCR/CD28 costimulation.56 Another negative feedback loop in these cells involves Bcl10, which promotes the activation of the IKK complex, which phosphorylates Bcl10 after T-cell antigen receptor stimulation and causes its proteolysis via the beta-TrCP ubiquitin ligase/proteasome pathway.57 As T cells accumulating in atherosclerotic plaques often lack the co-stimulatory receptor CD28,58 the significance of this activation mode for atherosclerotic cardiovascular disease remains questionable.

As it might be expected, incubation of alphaCD3/CD28-costimulated T-cells from healthy volunteers and patients with rheumatoid arthritis with the proteasome inhibitor bortezomib downregulates the release of several NFκB-inducible cytokines [including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10] within the first 24 h. With proteasome inhibition beyond this time frame, a reduction of T-cell activation and induction of T-cell apoptosis is noticeable.59 These findings were confirmed in CD4(+) T cells activated by allogenic dendritic cells (DCs).58 Proteasome inhibition suppresses activation and nuclear translocation of nuclear factor of activated T cells and thereby the expression of the activation-associated cell surface receptors CD25, CD28, CD102, and CD134 as well as production of IFN-γ, TNF-α, IL-4, and IL-5. Owing to accumulation of cyclin-dependent kinase inhibitors p21(WAF1/CIP1) and p27(KIP1) and the disappearance of cyclin A, cyclin D2 and proliferating cell nuclear antigen, G(1) phase arrest is induced in T cells. However, most T cells eventually undergo apoptosis along with accumulation and stabilization of the tumour suppressor protein p53. Of utmost significance, proteasome inhibition of T cells may occur in atherosclerotic plaques. As least in vitro, oxLDL induced changes outlined above, most notably apoptosis of CD4+ / CD25+ regulatory T cells in a time- and concentration-dependent manner.60 CD4+ /CD25+ regulatory T cells isolated from patients with end-stage renal disease who have a high prevalence of atherosclerotic cardiovascular disease fail to suppress cell proliferation, exhibit cell-cycle arrest, and enter apoptosis by altering proteasome activity. In murine models of atherosclerosis, this regulatory T-cell population, which down-regulates T-cell responses to foreign and self-antigens, has been shown to exert otherprotective effects.61 Natural inhibition of the proteasome in these T cells may therefore contribute to atherogenesis.

T cells are potentiated by interaction with DCs, which are present in atherosclerotic plaques, primarily in the shoulder regions.62 They are particularly prevalent in complicated and symptomatic carotid plaques supporting a pathophysiological link.63 This functional aspect was recently supported by the finding that DCs remain functional antigen-presenting cells and maintain their ability to prime CD4(+) T cells even under hypercholesterolemic conditions.64 Importantly, the proteasome is crucial for maturation and function of immature and mature DCs. As a consequence of proteasome inhibition, DCs fail to stimulate allogeneic CD4(+) and CD8(+) T cells and autologous CD4(+) T cells sufficiently and DCs lose their ability to regulate innate and adaptive anti-tumour immunity.65,66 In part, this effect is mediated by NFκB.66 In addition, proteasome inhibition will also induce apoptosis of DCs via the mitochondrial pathway.67 The sensitivity of DCs to this effect, however, seems to be related to the activity of the NFκB pro-survival pathway.68 NFκB is also important for the upregulation of the expression of the PA28β unit of the immunoproteasome in DCs, which is the limiting factor for proper PA28αβ complex formation.69 Whereas expression of the immunoproteasome is under the influence of Th1 cells in most cell types,70 expression of the 11S proteasome units in DCs is differentially regulated and not solely dependent on IFN-γ, possibly due to the significance of the immunoproteasome for the antigen-presenting function of DCs, which are the most potent antigen-presenting cells for naive T cells.

An important new discovery is the identification of the immunoproteasome as a potential link between inflammation and apoptosis of plaque cells.71 IFN-γ sensitizes cells isolated from the fibrous cap of carotid artery plaques to apoptosis via the Fas/Fas ligand pathway. This sensitivity is abolished by interference with the induction of the inducible β5 subunit of the immunoproteasome and related proteolytic processing of myeloid cell leukaemia (Mcl)-1. The longer isoform of Mcl-1 exerts potent pro-survival effects by sequestering pro-apoptotic molecules of the additional members of the Bcl-2 family of proteins to which it belongs itself.72 Intriguingly, this mode of regulation is in addition to the possible degradation of Mcl-1 via the conventional UPS.73,74 As incorporation of immunoproteasome subunits alters proteasome function,75 even subtle nuances in proteasome composition and activity may be important for the phenotype of biological and pathobiological processes. Indeed, this has been demonstrated in patients with Crohn’s disease, in which the colonic expression of the immunoproteasome subunits β1i and β2i was markedly higher and correlated with a considerably higher chymotrypsin-like activity and an impressively faster processing rate of the NFκB/p50 precursor p105 and IκBα.76 In mouse models of rheumatoid arthritis, selective inhibition of low-molecular mass polypeptide-7, the chymotrypsin-like subunit of the immunoproteasome, reversed signs of disease along with reductions in cellular infiltration, cytokine production, and autoantibody levels.77 It remains to be seen if a pathological role of the immunoproteasome will be confirmed for atherosclerosis as well.

So far only one study has examined the expression of the PA28β/11S proteasome in normal human vessels, saphenous venous bypass grafts (SVG) and atherosclerotic lesions from various vascular regions.78 Similar to the 20S proteasome, the 11S proteasome was identified predominately within the cytoplasm of vascular SMCs and endothelial cells in all types of vessels. PA28 was more intensely expressed in normal vessels than in areas of SVG intimal hyperplasia and atherosclerosis implying that it may be necessary to maintain normal vascular homeostasis. However, this remains to be confirmed.
As examined in several studies, plaques of patients at high risk for or already with symptomatic carotid artery disease have a higher level of oxidative stress, NFκB expression and likely NFκB activity, more inflammatory cells, less collagen content, and greater necrotic burden.\textsuperscript{46,47,79,80} It has also been noted that the ubiquitin level is higher in these advanced and complicated plaques, an observation that was originally made in coronary arteries of patients with acute myocardial infarction.\textsuperscript{81} As pointed out in one of our studies, this is due to an increase in the level of ubiquitinated proteins but not free ubiquitin and possibly relates to a decrease in proteasome function.\textsuperscript{79} Conversely, Marfella et al. found an increase in proteasome activity in high-risk plaques, macrophages extracted from these plaques, and even peripheral monocytes.\textsuperscript{46,47} These apparently discrepant findings could be explained at least in part by differences in the composition of the study population. Indeed, Marfella et al.\textsuperscript{82} very recently showed that proteasome activity was lower in atherosclerotic plaques of patients > 60 years of age than in those from younger adult patients. A similar decrease of proteasome function with ageing has been shown in other organs including the heart.\textsuperscript{83} Collectively, these data raise the question if it is, in fact, a decrease in proteasome function that contributes to the senescence of the vascular system, which includes atherosclerotic changes. Then again the question is how a decrease in proteasome activity could contribute to plaque destabilization, if unimpaired UPS activity relates to inflammation. It may be speculated that the predominant effect of chronic impairment in proteasome function is on apoptosis of plaque cells, thereby contributing to plaque destabilization (Figure 5). Indeed, experimental support for this theory has been provided very recently.\textsuperscript{84} Carotid artery plaques were induced by placement of a constricting collar for 6 weeks in ApoE-deficient mice maintained on a high-cholesterol diet. Subsequent treatment with Bortezomib for 4 weeks changed their composition to a ‘rupture-prone’ phenotype, evident in a significant decrease in collagen content and a significant increase in necrotic core size regardless of a low-dose or high-dose proteasome inhibitor approach. Concomitantly, it was found that SMCs and activated macrophages were highly sensitive to Bortezomib, relating to the activity level of protein synthesis. In these cells, proteasome inhibition led to the accumulation of protein substrates, induction of endoplasmic reticulum stress and activation of related apoptotic pathways. Detrimental effects on cell viability with plaque destabilizing consequences may therefore prevail with decreasing proteasome function in advanced atherosclerotic plaques over time.

In addition to apoptosis, there has been increasing recognition of autophagic cell death in atherosclerotic plaques with potentially detrimental consequences.\textsuperscript{85} Importantly, autophagy is activated under circumstances of impaired proteasome function likely as a consequence of endoplasmic reticulum stress caused by misfolded proteins and as a compensatory effort to remove polyubiquitinated protein aggregates.\textsuperscript{86} However, there are other pathomechanisms by which autophagy can be induced in the atherosclerotic plaque, which are not necessarily related to impairment of proteasome function.\textsuperscript{85} Moreover, autophagy can also have cytoprotective

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\caption{Double immunohistochemistry for ubiquitin (brown) and TUNEL (red) in coronary artery plaques showing colocalization in both cap and lipid core regions (upper panel, reproduced with permission from Elsevier\textsuperscript{81}). Similarly, there is association between the content of apoptotic nuclei and ubiquitin-conjugates in carotid artery plaques (lower panel, reproduced with permission from Lippincott, Williams \& Wilkins\textsuperscript{79}).}
\end{figure}
effects and the detrimental effects prevail only with excessive stimulation of autophagic activity.85

Taken together, preserved or even increased proteasome activity in the atherosclerotic plaque favours plaque inflammation but also cell survival, whereas a decrease in proteasome activity would be of opposite consequences. Ultimately, the decay of plaque cells may be the overwhelming mechanism as it diminishes the matrix producing pool of cells, contributes to rupture of plaque neovessels, and triggers secondary injury response with the attraction of blood-borne inflammatory cells that have not been exposed to the plaque environment. The UPS therefore exerts an important influence on the inflammatory activity and cell viability that determine plaque stability.

5. Genome-based studies—identifying the vulnerable patient from an UPS standpoint

Additional important developments in the prediction of plaque stability relating to the UPS over the past years include genomic studies. A single nuclear polymorphism (SNP), specifically -8C/G in the 5′-untranslated region of exon 1 of the proteasome subunit alpha type 6 gene (PSMA6), was found to differ among Japanese patients with and without acute myocardial infarction.87 It was postulated that the risk-conferring G allele could lead to an increase in the expression of PSMA6 which could then lead to increased NFκB activation and inflammation. However, the results of this case–control study could not be confirmed in another study in a Japanese patient population.88 Likewise, initial studies in Caucasian populations could not provide strong confirmation.89 Moreover, the most recent case–control study in a Caucasian population did not find an overall difference in the allele frequency and genotype distribution of various PSMA6 gene polymorphisms between patients who did and those who did not experience an AMI.90 Yet when the analysis was restricted to diabetic patients, it was noted that the G allele and CG genotype frequency was higher in diabetics with AMI history than in diabetics without AMI history. Finally, an analysis of the Chinese subgroup of the INTERHEART study showed that the presence of the outlined G allele increased the risk for MI by 22%.91 A meta-analysis of available data found overall supporting evidence for a mildly increased risk of MI in patients with a G-allele SNP in the PSMA6 gene. However, the frequency of the risk gene variant is several times lower in the Caucasian than in the Asian population. Therefore, the overall MI risk that can be attributed to this rare gene variant in the Western world is unlikely to be high. Moreover, the pathophysiology underlying this risk remains a matter of debate. Initial data suggest that the activity of the 20S proteasome and extent of ubiquitination were higher in patients with the risk

![Figure 6](image_url) The potential role of the proteasome system in atherosclerosis is conceptually outlined. The initial stage of atherosclerosis is characterized by an increase in oxidative stress, an ensuing inflammatory response and an upregulation of proteasome activity to compensate for the increase in the generation of proteasomal substrates. The progression stage is marked by proliferation and transmigration of smooth muscle cells, foam cell and then atheroma formation and sprouting of neovessels into the developing plaque. The proteasome system is still permissive to these processes. The complication stage of atherosclerosis is highlighted by a decrease in cell viability, a destructive inflammatory response, and tissue instability. Impairment of cell viability has been related to impairment in proteasome function which can be mediated by the accumulation of oxidative stress products over time.
gene variant. However, further experimental studies, expressing this gene mutation in a very controlled in vitro environment, need to be performed to delineate the exact consequences on proteasome function. Subsequently, it needs to be defined how the resulting alteration in proteasome function affects cardiovascular biology and predisposes to MI.

6. Summary—atherosclerosis as a consequence of too much or too little degradation?

The UPS, and particularly the proteasome, is a vital part of protein quality-control mechanisms preventing the accumulation of dysfunctional proteins which can be toxic to the cell and its environment. As reviewed in detail before, a reduction in the function of the vascular proteasome could lead to a degenerative disease process that shares similarities with neurodegenerative disorders and bears characteristics of protein quality diseases. Various lines of evidence suggest that possibly related to low-level increase in oxidative stress, the activity of the UPS is increased upon cardiovascular risk factor exposure such as hyperglycaemia and hypercholesterolemia. Yet controversy exists, if this exerts a pathophysiological mechanism or constitutes a compensatory mechanism with opposite implications for proteasome inhibition. This discussion extends from the initial to the complication phase of atherosclerosis, and a dual role of the UPS has been suggested as well (Figure 6). Clearly, depending on the environment, dose and duration, and cell type and organ studied, proteasome inhibitors can have quite diverse and broad effects and can act as ‘poisons or remedies’ as elegantly phrased before. Hence, this class of drugs is far from being used as general therapeudic approach in atherosclerosis other than possibly the progression stage and neointima formation. While second generation proteasome inhibitors have become available, targeting the immunoproteasome might allow differential upregulation of immunoproteasomes by nitric oxide: potential antioxidative mechanism in endothelial cells. Free Radic Biol Med 2009; 47:100–101.

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