Molecular Medicine

Chronic Proteasome Inhibition Contributes to Coronary Atherosclerosis

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Abstract—The proteasome is responsible for the degradation of oxidized proteins, and proteasome inhibition has been shown to generate oxidative stress in vitro. Atherosclerosis is thought to be initiated as a consequence of increased endogenous oxidative stress. The current study was designed to assess whether chronic proteasome inhibition is associated with early coronary atherosclerosis. Female pigs, 3 months of age, were randomized to a normal (N) or high-cholesterol (HC) diet (2% cholesterol, 15% lard) without or with twice weekly subcutaneous injections of the proteasome inhibitor (PSI) MLN-273 (0.08 mg/kg, N+PSI and HC+PSI) for a period of 12 weeks (n=5 per group). Coronary vasorelaxation to bradykinin (10^{-10.5} to 10^{-6.5} mol/L) and sodium nitroprusside (10^{-9} to 10^{-5} mol/L) was assessed by in vitro organ chamber experiments, intima–media ratio by morphometric analysis of Elastica–van Gieson–stained slides, and intima superoxide production by dihydroethidium fluorescence. Vasorelaxation to 10^{-6.5} mol/L bradykinin was reduced in HC compared with N (69±7 versus 90±2%, P<0.05) and further reduced in N+PSI and HC+PSI (57±6 and 48±13%, P<0.05 versus N and HC for each). Compared with N (0.03±0.01), intima–media ratio was higher in N+PSI (0.09±0.04, P<0.01) and HC+PSI (0.15±0.06, P<0.05). Compared with N (0.6±0.9% of intima area), dihydroethidium fluorescence was higher in HC, N+PSI, and HC+PSI (8.9±1.6, 6.0±3.5, and 7.2±3.9% of intima area, P<0.05 for all). Thus, chronic proteasome inhibition is associated with increased coronary artery oxidative stress and early atherosclerosis. These findings support the significance of the proteasome and related protein quality control for vascular biology and pathology. (Circ Res. 2007;101:865-874.)

Key Words: atherosclerosis ■ endothelial dysfunction ■ oxidative stress ■ proteasome ■ ubiquitin

Atherosclerotic cardiovascular disease (ASCVD) is considered to be initiated as a response to an injurious stimulus, or clinically, a cardiovascular risk factor. A common denominator of the pathophysiological mode of action of many cardiovascular risk factors is an increase in the generation of reactive oxygen species, especially superoxide anions, surpassing antioxidant capabilities and resulting in oxidative stress. Superoxide anions rapidly react with NO, generating, for instance, the highly cytotoxic product peroxynitrite and its footprint nitrotyrosine. In addition, modification of signaling pathways leads to the alteration of the activity and expression of transcription factors and growth factors. The functional and structural consequences of these molecular changes include impairment of endothelium-dependent vasorelaxation and intimal thickening, constituting the early stage of atherosclerosis.

Eighty to 90% of all intracellular proteins are degraded via the 20S proteasome, a barrel-shaped complex formed by 2 outer (α) rings and 2 inner (β) rings, each composed of 7 subunits. The β1, β2, and β5 subunits harbor caspase-like, trypsin-like, and chymotrypsin-like activities, respectively, with the latter being of utmost importance for overall proteasome function. The 20S proteasome is self-sufficient for the degradation of oxidized proteins. It also operates in conjunction with the ubiquitin system, which catalyzes the binding of ubiquitin molecules to target proteins, allowing their recognition by the 26S proteasome, ie, by the 19S subunits to either side of the 20S proteolytic complex. Possibly related to the impairment of the function of the proteolytic core, accumulation of ubiquitin/ubiquitinated proteins can be seen in human atherosclerotic plaques and particularly in more active lesions, characterized by increased oxidative stress. Indeed, there is in vitro evidence that high levels of oxidative stress can impair proteasome function with cytotoxic consequences. In turn, impairment in proteasome function can increase intracellular oxidative stress. Whereas these studies support a detrimental effect of impaired proteasome function, other reports suggest that proteasome inhibition may be of benefit for atherosclerotic plaque progression and complication. Hence, there has remained uncertainty with regard to the pathophysiological role of the proteasome in ASCVD.
Potent and specific pharmacological inhibitors of the proteasome have been tested extensively over the past years, such as the boronate-type inhibitor MLN-273. As a result, PS-341 (bortezomib [Velcade]), a proteasome inhibitor (PSI) very closely related to MLN-273, was approved for the treatment of therapy-refractory multiple myeloma in 2003. Beyond therapeutic applications, the in vivo use of these specific inhibitors is an attractive means to gain pathophysiological insight. Thus, in the current study, chronic administration of MLN-273 was used to define the pathophysiological significance of the proteasome for ASCVD. Our hypothesis was that by virtue of the reported stimulating effect on oxidative stress, chronic proteasome inhibition would impair the function and structure of coronary arteries in normal animals and would aggravate the vascular changes of early atherosclerosis in hypercholesterolemic animals.

Materials and Methods

Animals

The current study was approved by the Mayo Foundation Institutional Animal Care and Use Committee, and all procedures were in accordance with NIH guidelines. Three-month-old female domestic pigs (Pork Partners, Stewartsville, Mn, and Larson Products, Sargeant, Mn) were randomized to a normal chow diet (n = 5) or a high-cholesterol diet (HC) (2% cholesterol, 15% lard; TD 93296, Harlan Teklad, Madison, Wisconsin; n = 5) without or with twice weekly subcutaneous injections of the boronate-type PSI MLN-273 (0.08 mg/kg; Millennium Pharmaceuticals, Cambridge, Mass; normal diet [N] + PSI [PSI] and HC + PSI, n = 5 each). Similar to PS-341, this drug specifically inhibits the chymotrypsin-like activities of the proteasome, and in vivo analysis of chymotrypsin-like proteasome function was performed in treated animals to ensure a maximum degree of 60% to 80% inhibition at 1 hour after drug administration. After 11 weeks, mean arterial blood pressure and heart rate were measured by an invasive signal transducer. After 12 weeks, blood was obtained for plasma analyses. Subsequently, animals were euthanized and the hearts were immediately harvested for coronary vasoreactivity testing and tissue fixation, either in formalin for microscopic analyses or in liquid nitrogen for storage at −80°C.

Determination of Plasma Lipid Profile and Oxidized Low-Density Lipoprotein Levels

As described previously, plasma concentrations of triglycerides and total, HDL, and LDL cholesterol were determined with a commercial agent. Circulating levels of oxidized LDL were measured in plasma using a spectrophotometric enzyme immunoassay kit (Merckodia, Uppsala, Sweden).

Analysis of Proteasome Activity

Proteasome activity in peripheral mononuclear cells was performed as described previously. Coronary artery proteasome activity was quantified as outlined previously but with substantial modifications (see the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org). In Vitro Analysis of Vascular Reactivity

Organ chamber experiments were performed according to an established protocol.

TUNEL Staining

Terminal deoxynucleotidyl transferase end-labeling (TUNEL) staining was performed by use of the ApopTag In Situ Apoptosis Detection Kit (Intergen Co, Purchase, NY), as described previously (see the online data supplement).

Histologic and Morphometric Analyses

Hematoxylin/eosin and Elastica–van Gieson staining was performed as previously reported. Using a digital image system, morphometric analyses on Elastica–van Gieson–stained slides were performed at a magnification of 10x (see the online data supplement).

Oil Red O Staining

Unfixed frozen coronary artery segments were cut into 10-μm-thick sections, placed on a glass slide, and stored at −80°C until the day of the experiment, which was performed as described previously.

Immunostaining and Immunoblotting

Immunostaining and immunoblotting were performed as outlined previously (see the online data supplement).

Fluorescent Microscopy

Dihydroethidium (DHE) (Sigma) was used to demonstrate in situ levels of superoxide production, as described previously. The percentage of intima area positive for immunofluorescence was quantified as outlined for immunostaining.

Lucigenin Chemiluminescence

Superoxide production was measured from aortic samples using lucigenin-enhanced chemiluminescence (5 μmol/L), as described previously, and validated previously.

Statistical Analysis

Continuous data were expressed as means ± SEM. Multiple group comparisons were made by 1-way ANOVA with Student–Newman–Keuls post hoc analysis. Two group comparisons were made by Student’s t test. Statistical significance was accepted at P < 0.05.

Results

Hemodynamic and Laboratory Parameters

Animals treated with MLN-273 had similar body weight and mean arterial blood pressure but slower heart rate compared

### Table 1. Characteristics of the Experimental Groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N</th>
<th>HC</th>
<th>N + PSI</th>
<th>HC + PSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>51±3</td>
<td>64±6</td>
<td>58±3</td>
<td>53±5</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>113±6</td>
<td>117±5</td>
<td>121±6</td>
<td>122±6</td>
</tr>
<tr>
<td>Heart rate, beats per minute</td>
<td>64±4</td>
<td>62±6</td>
<td>60±6</td>
<td>56±3</td>
</tr>
<tr>
<td>Serum triglyceride, mg/dL</td>
<td>34±6</td>
<td>62±12</td>
<td>88±30</td>
<td>83±25</td>
</tr>
<tr>
<td>Serum total cholesterol, mg/dL</td>
<td>72±5</td>
<td>479±41</td>
<td>87±11</td>
<td>471±14*</td>
</tr>
<tr>
<td>Serum HDL, mg/dL</td>
<td>39±3</td>
<td>84±10</td>
<td>28±5</td>
<td>99±4*</td>
</tr>
<tr>
<td>Serum LDL, mg/dL</td>
<td>26±4</td>
<td>382±34*</td>
<td>42±9</td>
<td>356±12*</td>
</tr>
<tr>
<td>Oxidized LDL, U/mL</td>
<td>12.5±1.6</td>
<td>19.1±1.8*</td>
<td>11.1±0.8</td>
<td>27.9±5.3*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. *P < 0.05 vs N and N + PSI. †P < 0.05 vs N. ‡P < 0.05 vs HC.
with untreated animals (Table 1). Cholesterol plasma concentrations were higher in animals fed a HC but not significantly different between animals treated and not treated with MLN-273 (Table 1). A trend toward higher triglyceride plasma concentrations was noted in HC, HC/H11001 PSI, and even in N/H11001 PSI compared with N.

**Proteasome Activity**
A single dose of 0.08 mg/kg MLN-273 resulted in 68 ± 4% and 40 ± 9% inhibition of chymotrypsin-like proteasomal activity in circulating peripheral mononuclear cells 1 hour and 24 hours after subcutaneous injection, respectively. Chymotrypsin-like proteasome activity in coronary arteries was inhibited by 68 ± 4% in N+PSI and 72 ± 4% in HC+PSI on the day of euthanasia, which was 2 ± 1 days after the last injection (Figure 1A). As expected, there was no decrease in trypsin-like proteasome activity in animals treated with MLN-273. A significant increase in the extent of ubiquitinated proteins in the coronary artery wall was observed in HC+PSI (Figure 1B).

Coronary artery expression of the β5 subunit was higher in HC+PSI, whereas no difference in coronary artery expression of the β2 subunit of the proteasome was observed among the groups (Figure I in the online data supplement). The percentage of TUNEL-positive endothelial cells, indicating apoptosis, was not significantly higher in N+PSI than in N (1.36 ± 0.38 versus 0.39 ± 0.22%) and elevated to a similar degree in HC and HC+PSI (1.93 ± 0.41 and 1.60 ± 0.19%, P < 0.05 versus N; supplemental Figure I).

**Oxidative Stress Parameters**
Compared with N, plasma concentrations of oxidized LDL were elevated in HC and even more in HC+PSI as previously reported (Table 1).30 DHE fluorescence was higher in the area of the coronary intima in HC, HC+PSI, and N+PSI compared with N (8.9 ± 1.6, 6.0 ± 3.5, 7.2 ± 3.9 versus 0.6 ± 0.9% intimal area, P < 0.05 for all, Figure 2A). Nitrotyrosine immunoreactivity was highest in HC+PSI and higher in HC than in N (4.1 ± 1.4 and 2.7 ± 0.5 versus 0.4 ± 0.1, P < 0.05) and tended to be higher in N+PSI than in N (1.0 ± 0.4 versus 0.4 ± 0.1, P = 0.01; Figure 2A). Similarly, Western blotting showed an increased amount of 4-hydroxy-2-nonenal–modified proteins and most prominently in HC+PSI (Figure 2B). Additional lucigenin chemiluminescence studies, performed on aortic samples, demonstrated the highest values in HC+PSI and milder elevations in HC and N+PSI (Figure 2C). The expression of the NAD(P)H oxidase subunit p47phox but not of the gp91phox and p67phox subunits was higher in the intervention groups than in N (Figure 3).
In Vitro Vasoreactivity and Endothelial NO Synthase Expression

Compared with N, coronary arteries from HC, N+PSI, and HC+PSI displayed an attenuated response to the endothelium-dependent vasodilator bradykinin but not to the endothelium-independent vasodilator sodium nitroprusside (Figure 4). The degree of impairment in endothelium-dependent vasorelaxation was higher in PSI-treated animals, also reflected in the impaired vasorelaxation response to calcium ionophore (Figure 4). There was no difference in vasoreactivity between N+PSI and HC+PSI. Compared with their untreated counterparts, coronary artery expression of endothelial NO synthase (eNOS) was higher in PSI-treated animals (Figure 5).

Histology and Morphometry

Histological review showed no abnormality other than intimal thickening, which was of significant degree in HC+PSI (Table 2 and supplemental Figure II). Immunostaining for smooth muscle α-actin was positive in areas of intimal thickening in HC and minimally in PSI-treated animals (supplemental Figure II). Compared with N, oil red O staining was observed in areas of intimal thickening mainly in HC+PSI and HC+PSI but also in N+PSI animals (0.5±0.07 versus 6.8±2.7 and 5.6±2.9 and 1.7±0.5% intimal area, P<0.05 for all; Figure 6). Macrophage/foam cell accumulation by macrophage scavenger receptor A immunoreactivity was noted in HC, less prominently in N+PSI, and most prominently in HC+PSI rather than in N (0.015±0.012, 0.004±0.004, and 0.056±0.034 versus 0.001±0.001% intimal area, P<0.01 HC+PSI versus N; Figure 6). Macrophage accumulation correlated with a significantly higher expression of vascular cell adhesion molecule-1 and E-selectin in HC+PSI (supplemental Figure III). The increase in these 2 nuclear factor κB–regulated cell adhesion molecules was observed in the presence of an increased level of its endogenous inhibitor IκBα, whose expression and degradation is controlled by nuclear factor κB and the ubiquitin–proteasome system (UPS), respectively (supplemental Figure III).

Discussion

The current study demonstrates in a large animal model that chronic proteasome inhibition increases oxidative stress and...
impairs coronary endothelium-dependent vasodilatation under otherwise normal conditions and aggravates functional and structural changes of coronary arteries in hypercholesterolemia. Collectively, these findings support the significance of the proteasome and related protein quality control in the pathophysiology of vascular degenerative diseases such as atherosclerosis.

Proteasome Inhibition and Oxidative Stress

In line with our current studies on coronary arteries in hypercholesterolemic pigs, other investigators recently reported on an increase in proteasome activity in the aorta of hypercholesterolemic rabbits. Of further note, previous in vitro studies showed that low-dose oxidative stress stimulates proteasome proteolytic activity, namely its chymotrypsin-like activity. The increase in vascular proteasome activity may therefore be a reflection of the increase in vascular oxidative stress in hypercholesterolemia and may, in fact, represent a compensatory response to the increased production of proteasome substrates, including oxidatively damaged proteins. Indeed, inhibition of the chymotrypsin-like activity of the proteasome under these circumstances resulted in a prominent accumulation of ubiquitinated proteins and oxidatively modified proteins, eg, by 4-hydroxy-2-nonenal. Furthermore, serum concentrations of oxidized LDL were highest in these animals, even higher than in the untreated hypercholesterolemic animals, underscoring aggravation of oxidative stress by proteasome inhibition. In normocholesterolemia, chronic in vivo proteasome inhibition did not lead to a higher circulating level of oxidized LDL but to slightly higher LDL levels and a trend toward higher triglyceride plasma concentrations. These changes in the lipid profile may relate to an alteration of apolipoprotein B metabolism. Even in normocholesterolemic animals, chronic proteasome inhibition increased oxidative stress in the intima in vivo, as reflected by DHE fluorescence for superoxide anion production and nitrotyrosine staining for peroxynitrite formation. Culture-based studies confirm these findings by demonstrating that higher yet nontoxic degrees of proteasome inhibition increase endogenous oxidative stress and render cells more susceptible to oxidative stress–related injury. Taken together, the current results extend in vitro findings on the interaction between oxidative stress and proteasome activity. Coronary proteasome activity is increased in hypercholesterolemia in association with enhanced oxidative stress, and oxidative stress is increased by chronic in vivo proteasome inhibition.

Figure 3. A and B, Quantification of gp91phox and p67phox expression in coronary arteries from animals on a N or HC for 12 weeks without (N and HC) or with (N+PSI and HC+PSI) additional proteasome inhibition (n=4 for all groups). C, Quantification of p47phox expression in the 4 study groups by immunoblotting (N, HC, and HC+PSI [n=3 each]; N+PSI [n=4]). *P<0.05 vs HC and N+PSI. D, Immunoreactivity for p47phox in coronary arteries from the 4 study groups; note the focal positive staining in N+PSI (arrow) and HC and the very prominent staining in HC+PSI (original magnifications, ×75).
The current study suggests eNOS uncoupling as a potential mechanism for the increase in vascular oxidative stress in PSI-treated animals, given the constellation of increased eNOS expression, yet signs of reduced functional NO availability in vasoreactivity studies. Superoxide production has been shown to contribute to increased eNOS expression via formation of the dismutation product H2O2 and to reduce NO bioavailability via formation of the reaction product peroxynitrite. Peroxynitrite can oxidize tetrahydrobiopterin, an essential cofactor for eNOS, leading to the uncoupling of the enzyme from normal NO production. The initial increase in superoxide production might have been instigated by NAD(P)H oxidase, given the increased expression of p47phox, which plays an important role in the sequence of events leading to increased vascular oxidative stress.

Whether proteasome inhibition stimulates the gene expression of this particular NAD(P)H oxidase subunit or contributes to its posttranscriptional stabilization remains to be determined.

In endothelial cell culture experiments, Meiners et al noted an increase in the expression of genes encoding for antioxidant enzymes and a decrease in the expression of the NAD(P)H oxidase family member NOX4 by incubation with PSIs for 24 hours. These findings were translated into a reduction of the formation of reactive oxygen species when endothelial cells were treated with PSIs before H2O2 challenge. Then again, other studies indicate a reciprocal interaction between proteasome and mitochondrial function as well. Based on the current study, chronic proteasome inhibition is associated with increased oxidative stress on the level of the coronary intima and especially the endothelial cell layer. In association with these findings, impairment in endothelial function and intimal thickening were observed in the epicardial coronary circulation, consistent with the oxidative stress and response-to-injury theory of atherosclerosis.

Proteasome Inhibition and Endothelial Function

Clearly, pigs treated with the PSI MLN-273 for 12 weeks developed impaired coronary vasorelaxation in response to bradykinin and calcium ionophore. The normal vasorelaxation response to sodium nitroprusside in these animals underscores selective impairment of endothelium-dependent vasorelaxation rather than a functional impairment on the level of the vascular smooth muscle cell layer. Importantly, the degree of in vivo proteasome inhibition on the level of the coronary artery was in a range defined previously as nontoxic. Also, there was no general accumulation of ubiquitinated proteins in animals chronically treated with MLN-273 unless combined with hypercholesterolemia. These findings highlight the fact that the current mode of proteasome inhibition was not to such an extensive (and then potentially toxic) degree that the generation of ubiquitinated products would surpass deubiquitination and degradation capacity under otherwise normal conditions. Furthermore, proteasome inhibition did not lead to an increase in TUNEL (apoptotic) cells in the coronary artery wall beyond the extent that can be seen in hypercholesterolemia. Nevertheless, chronic inhibition of the proteasome impaired coronary endothelium-dependent vasorelaxation to a degree that resembled the additive effect of systemic hypertension and hypercholesterolemia. Possibly because of the already maximally impaired endothelium-dependent vasorelaxation with chronic proteasome inhibition alone, no further aggravation could be observed with concomitant hypercholesterolemia. Finally, this impairment was observed even in the presence of increased eNOS expression. This latter finding is reminiscent of the increase in the expression and activity of eNOS in cultured bovine pulmonary arterial endothelial cells following 1- to 2-day incubation with the peptide aldehyde-type PSI MG-132 at doses that correlate with reversible, acutely >80% inhibition of the chymotrypsin-like activity of the proteasome. Interestingly, in the second part of that study, pretreatment of rat aortic rings with this drug–dose combination for 48 hours was found to increase their vasorelaxation response to acetylcho-
Hence, the current study extends previous in vitro reports by showing that nontoxic doses of proteasome inhibition can increase eNOS expression in vivo but nevertheless impair endothelial function profoundly when applied over a prolonged period of time.

Proteasome Inhibition and Intimal Thickening

As highlighted by the current results, chronic proteasome inhibition favors intimal thickening in animals on a N diet and significantly adds to its development in hypercholesterolemia. Furthermore, lipid deposition in areas of intimal thickening even in animals on a N diet supports increased permeability of the endothelial monolayer. Similarly, the degree of lipid accumulation was enhanced in hypercholesterolemic animals in the presence of comparable circulating lipid levels. In association with these findings, accumulation of the macrophages by staining for CD172a and CD204/scavenger receptor A could be detected. In the most severely affected areas, the distribution pattern of this surface marker became diffuse, likely reflecting the clustering of macrophages. Thus, chronic proteasome inhibition exaggerated the development of early atherosclerotic lesions, as defined by intimal thickening, lipid deposition, and macrophage accumulation.

Areas of intimal thickening in animals receiving proteasome inhibition were characterized by a relative paucity of smooth muscle cells (SMCs) compared with those in untreated hypercholesterolemic animals. This may agree with the initial in vitro study by Thyberg and Blomgren highlighting an inhibitory effect of MG-132 on vascular SMC proliferation and transformation. Meiners et al extended these observations by showing a 75% reduction in neointima formation with a 1-time local application of MG-132 (1 mol/L) directly after balloon injury to carotid arteries. These findings suggested a proproliferative role for the ubiquitin--

Table 2. Morphometric Data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N</th>
<th>HC</th>
<th>N+PSI</th>
<th>HC+PSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumen area (mm²)</td>
<td>1.56±0.06</td>
<td>1.31±0.07</td>
<td>1.43±0.22</td>
<td>1.42±0.26</td>
</tr>
<tr>
<td>IEL area (mm²)</td>
<td>1.60±0.05</td>
<td>1.37±0.07</td>
<td>1.57±0.16</td>
<td>1.68±0.34</td>
</tr>
<tr>
<td>EEL area (mm²)</td>
<td>2.77±0.15</td>
<td>2.52±0.10</td>
<td>3.04±0.04</td>
<td>3.32±0.45</td>
</tr>
<tr>
<td>Intima thickness (mm²)</td>
<td>0.04±0.01</td>
<td>0.06±0.01</td>
<td>0.15±0.07†</td>
<td>0.26±0.10*</td>
</tr>
<tr>
<td>Media thickness (mm²)</td>
<td>1.18±0.13</td>
<td>1.16±0.12</td>
<td>1.47±0.17</td>
<td>1.64±0.11</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>0.03±0.01</td>
<td>0.05±0.01</td>
<td>0.09±0.04†</td>
<td>0.15±0.06*</td>
</tr>
</tbody>
</table>

Values are means±SEM. N indicates N; IEL, internal elastic lamina; EEL, external elastic lamina. *P<0.05 vs N, †P<0.1 vs N.
proteasome system and a potential benefit for the induction of vascular SMC apoptosis by proteasome inhibition at least in the vascular response to mechanical injury. On the contrary, Stone et al demonstrated a downregulation of genes encoding for components of the ubiquitin–proteasome system in areas of intimal hyperplasia at the anastomosis sites of polytetrafluoroethylene grafts after their implantation into canine carotid arteries. Thus, in line with these previous studies, the current results underscore that even though chronic proteasome inhibition may prevent vascular SMC transformation and proliferation, it nevertheless leads to intimal thickening. The effects of proteasome inhibition on primary atherosclerosis at a level that induces vascular SMC apoptosis remain to be explored.

Clinical Implications
The possible contribution of chronic impairment in proteasome proteolytic function to ASCVD agrees with the decreased proteasome proteolytic activity recently observed in carotid artery plaques of symptomatic patients undergoing carotid endarterectomy. In vitro experiments substantiate the view of a decrease in proteasome function by high levels of oxidative stress and its products such as oxidized LDL. In this context, the induction of endoplasmic reticulum stress by reactive nitrogen species must be mentioned as well, which can overload the ubiquitin–proteasome system, and, vice versa, inhibition of the proteasome and the unfolded protein response can lead to endoplasmic reticulum stress. Hence, increasing oxidative stress in the vascular wall may eventually impair proteasome function, which in turn further increases oxidative stress, thereby initiating a self-propagating cycle that decreases cell and tissue viability, ultimately of profound clinical significance for ASCVD. Intriguingly, a similar cycle was suggested for protein precipitation diseases, in which an etiologic role has been ascribed to the accumulation of ubiquitinated and oxidatively modified proteins. Taken together, these considerations eventually point to atherosclerosis not only as a “degenerative” but also as a protein quality-control disease.

Study Limitations
The molecular mechanisms underlying the increase in endogenous oxidative stress must be explored in greater detail in future studies. This includes specific inhibitor and cofactor studies for the primary potential sources, including upregulation of NAD(P)H oxidase, eNOS uncoupling, and the mitochondria. Also, future studies must take into consideration that the ultimate vascular response to proteasome inhibition is dose and time related and determined by multiple factors. This is true even more so when studies extend beyond cell culture systems.
In contrast to our previous study, we performed more rapid tissue homogenization with a glass grinder and simultaneous, plate reader–based measurements of all probes and used epoxomicin, the most selective PSI currently available, to calculate the proteasome-attributable degradation of the fluorogenic substrate. It is very likely, for this reason, that the current results differ from our previous findings in the identification of an increase in chymotrypsin-like activity of the proteasome in coronary arteries of hypercholesterolemic animals. Indeed, as indicated by others, the outcome of proteasome activity studies is subject to and hence limited by the methodology.

Conclusions
Chronic proteasome inhibition is associated with increased oxidative stress, impairment in coronary endothelium-dependent vasorelaxation and intimal thickening, resembling and aggravating the vascular effects of traditional cardiovascular risk factors such as hypercholesterolemia. These findings support the significance of the proteasome and related protein quality-control mechanisms for vascular biology and pathology.

Acknowledgments
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Disclosures
None.

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Online Materials and Methods

Analysis of coronary proteasome activity

Frozen coronary arteries were homogenized by a motorized tissue grinder at 550 RPM while keeping the samples at ice-cold temperatures and using a lysis buffer of the following composition: 50 nM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100. Subsequently, the homogenate was centrifuged at 10,000x g at 4 °C for 15 minutes. The supernatant was obtained, its protein content was determined by Bradford assay, and 30 µg were used for the individual reaction set-up. The reaction buffer was of the following composition: 25 mM HEPES (pH 7.5), 5 mM EDTA, 0.5% NP-40 with and without 0.01% SDS and the substrates 25 µM LLVY-AMC and 50 µM VGA-AMC (BioMol, Plymouth Meeting, PA) for the determination of chymotrypsin-like and trypsin-like activity, respectively. The final reaction volume in the 96-well plate set-up was 100 µL. Increase in the concentration of the fluorogenic compound 7-amino-4-methylcoumarin (AMC) after an incubation period of 60 minutes at 37 °C was measured by use of a 380/460 nm filtered fluorometric plate reader (SpectraMax Gemini XPS, Global Medical Instrumentation Inc., Ramsey, MN). Measurements were performed in duplicates and in the presence and absence of epoxomicin (25 µM, A.G. Scientific Inc., San Diego, CA). Individual proteasome proteolytic activity was calculated as the difference between non-inhibited and inhibited activity and expressed as nanomoles per minute and mg tissue protein.
Histologic and morphometric analyses

Intima thickness was calculated as IEL area minus lumen area and media thickness as EEL area minus IEL area, allowing calculation of the intima-media ratio (IMR). IMRs were not different between proximal and distal segments (0.071±0.045 vs. 0.072±0.048) and between two different coronary arteries of the same pig (0.084±0.12 vs. 0.085±0.10) with all values falling within two standard deviations of the mean difference on Bland-Altman plots. Hence, one cross-section was taken per animal for the morphometric analyses in this study.

TUNEL staining

Apoptotic cells were defined as TUNEL positive cells with condensed, pyknotic nuclei. Female rodent mammary gland tissue, 3-5 days after weaning, was used as a positive control with a rate of apoptotic cells of 4-7%, underscoring a high sensitivity assay setup (standard sensitivity 1-2%). Absence of staining was confirmed on slides prepared with omission of the TdT enzyme from the labeling (negative control).

Immunostaining

Porcine coronary artery slides were deparaffinized and rehydrated, followed by antigen retrieval with steaming in citrate acid (1M, pH 6.0) and quenching of endogenous tissue peroxidase activity by incubation with 3% H$_2$O$_2$ solution. For CD172a/macrophage staining, 10 µm frozen slides were fixed in acetone at 4°C for 10 minutes prior to the incubation with 3% H$_2$O$_2$ solution. Primary antibodies, including anti-ubiquitin (Covance, Berkeley, CA, dilution 1:200), anti-nitrotyrosine (Zymed, San Francisco, dilution 1:500),
anti-p47phox (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:50), anti-eNOS (Assay Designs, Ann Arbor, MI, dilution 1:100), anti-alpha smooth muscle cell (α-SMC) actin (Dako Corp., Carpinteria, CA, dilution 1:1000), anti-CD172a/macrophage (VMRD Inc., Pullman, WA, 1:2000) and anti-CD204/macrophage scavenger receptor-A (Cosmo Bio, Carlsbad, CA, dilution 1:25), were applied at 4°C overnight and were detected with the EnVision kit (Dako) in peroxidase-labeling technique with 3,3-diaminobenzidine tetra-hydrochloride or NovaRed as chromogens (Vector Laboratories, Inc., Burlingame, CA). Incubations with unspecific isotype antibodies served as specificity controls. All sections were counterstained with Gill No. 2 hematoxylin. Immunoreactivity was quantified by use of a computer-aided image analysis program (MetaMorph 4.6, Molecular Devices Corp., Sunnyvale, CA).

**Immunoblotting**

Protein content in coronary homogenates was analyzed by a Bradford assay (Bio-Rad, Hercules, CA), and under reducing conditions (unless indicated otherwise) equal amounts of protein (at least 30 µg) from coronary artery homogenates were dissolved in SDS-polyacrylamide gels and electrophoretically transferred on polyvinylidene difluoride or nitrocellulose membranes. These membranes were blocked in 1xPBS/3% non-fat milk overnight and incubated for 1 hours at room temperature with the following primary antibodies: anti-ubiquitin (Covance, Berkeley, CA, dilution 1:1000), anti-proteasome subunit β5 and β2 (BioMol, dilution 1:500), anti-eNOS (Transduction Laboratories, Lexington, KY, dilution 1:200), anti-vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz Biotechnology, dilution 1:100), anti-E-selectin (Abcam, Cambridge, MA, dilution
1:100, non-reducing conditions), anti-4-hydroxynonenal (4-HNE, Abcam, dilution 1:1000, non-reducing conditions), anti-gp91phox, anti-p67phox and anti-p47phox (Santa Cruz Biotechnology, dilution 1:100, 1:100, and 1:200, respectively). Primary antibody-labelled membranes were then rinsed with 1xPBS/Tween20, incubated with horseradish-linked secondary antibodies for 1 hour, and subjected to enhanced chemiluminescence (Pierce, Rockford, IL). Following membrane exposure to an X-ray film (Kodak, Rochester, NY), optical density of immunoblots was determined by use of NIH Image and expressed relative to the expression of β-actin (Sigma, dilution 1:2000). Human endothelial cell lysate (Transduction Laboratories, 1 mg/mL) was used as a positive control for eNOS immunoblotting.
Online Figure Legends

Online Figure I.

Panels A and B: Quantification of the expression of the β5- and β2-subunits of the proteasome in coronary arteries from animals on a normal or high-cholesterol diet for 12 weeks without (N and HC) or with additional proteasome inhibition (N+PSI and HC+PSI, n=4 for all groups); *p<0.05 vs. N.

Panel C: TUNEL staining of 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). Original magnification: 100x.

Panel D: Bar graphs illustrating the percentage of TUNEL-positive endothelial cells in the four study groups (n=5 for N and HC+PSI, n=4 for HC and N+PSI); * p<0.05 vs. HC and N+PSI.

Online Figure II.

Left panels: Elastica-van-Gieson (EvG) staining of 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) with black-white display to enhance contrast; intimal thickening can be seen of minor degree in HC and N+PSI and of major degree in HC+PSI; original magnifications 50x.

Right panels: alpha-smooth muscle cell (α-SMC) actin immunostaining of coronary arteries from the four study groups; notice the relative paucity of α-SMC actin-positive cells in the area of intimal thickening in proteasome inhibitor-treated animals; original magnifications 150x.
Online Figure III.

Panels A-C: Quantification of the expression of VCAM-1, ELAM-1 (E-selectin), and \( \text{IκB}_\alpha \) in coronary arteries from animals on a normal or high-cholesterol diet for 12 weeks without (N and HC) or with additional proteasome inhibition (N+PSI and HC+PSI, \( n=4 \) for all groups); *\( p<0.05 \) vs. N.
Online Figure I

A

Densitometric ratio
(β5-subunit / β-actin)

N HC N+PSI HC+PSI

B

Densitometric ratio
(β2-subunit / β-actin)

N HC N+PSI HC+PSI

C

TUNEL+ cells
[% of endothelial cells per cross-section]

N HC N+PSI HC+PSI

D

TUNEL+ cells
[% of endothelial cells per cross-section]
Online Figure II

Elastica-van-Gieson

\(\alpha\)-SMC actin

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<th>N+PSI</th>
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Online Figure III

A

Densitometric ratio
(VCAM-1/\(\beta\)-actin)

B

Densitometric ratio
(ELAM-1/\(\beta\)-actin)

C

Densitometric ratio
(IkB\(\alpha\)/\(\beta\)-actin)