Dysregulation of the Ubiquitin-Proteasome System in Human Carotid Atherosclerosis

Daniele Versari, Joerg Herrmann, Mario Gössl, Dallit Mannheim, Katherine Sattler, Fredric B. Meyer, Lilach O. Lerman, Amir Lerman

Objective—The ubiquitin-proteasome system is the principal degradation route of intracellular and oxidized proteins, thus regulating many cellular processes conceivably important for atherosclerosis. The aim of this study was to evaluate the activity of ubiquitin-proteasome system in human carotid artery plaques in relation to oxidative stress and clinical manifestation.

Methods and Results—In carotid endarterectomy specimens from 83 asymptomatic and 94 symptomatic patients, content of ubiquitin, ubiquitin conjugates, matrix metalloproteases, and NADPH-oxidase-p67 was evaluated by immunoblotting; proteolytic proteasome activity by fluorometric assay; single and double immunostaining for ubiquitin conjugates, 3-nitrotyrosine, apoptosis, smooth muscle α-actin, and macrophage CD-68, as well as Sirius Red staining for collagen were performed. Compared with asymptomatic patients, symptomatic patients showed a more unstable plaque phenotype, an increased degree of apoptosis, a significantly higher ubiquitin conjugates content (17.72 ± 1.36 versus 10.99 ± 1.04; \( P < 0.001 \)), and lower proteasome activity (5.01 ± 0.70 versus 9.41 ± 1.19 nmol AMC/mg protein/min; \( P < 0.01 \)). Ubiquitin conjugates content was directly correlated to NADPH-p67 and degree of apoptosis. Immunostaining revealed colocalization of ubiquitin conjugates and 3-nitrotyrosine, and accumulation of ubiquitin conjugates in smooth muscle cells and macrophages.

Conclusions—In human carotid plaques increased oxidative stress is associated with inhibition of the proteasome activity and accumulation of ubiquitin conjugates, particularly in symptomatic patients. These results suggest a possible role of the ubiquitin-proteasome system in influencing plaque stability. (Arterioscler Thromb Vasc Biol. 2006;26:2132-2139.)

Key Words: atherosclerosis ■ carotid plaque ■ endarterectomy ■ oxidative stress ■ proteasome ■ stroke ■ ubiquitin

See cover

A high rate of protein ubiquitination is associated with increased oxidative stress especially in neurological disorders and UPS has been demonstrated to be the principal system responsible for the degradation of oxidized proteins. However, high-level oxidative stress can also impair UPS by reducing proteasome activity. This could lead to intracellular accumulation of ubiquitinated substrates caused by an increased production and by a reduced degradation. As shown in the development of cataract, intracellular ubiquitinated damaged proteins may eventually accumulate, form aggre-
gates rich in carbonyl residues and be further enriched with ubiquitin moieties. These insoluble complexes cross-link and precipitate in the cell, inducing cytotoxicity and causing disruption of cell functions in several diseases. Inhibition of proteasome activity is also associated with increased apoptosis in several cell types, including smooth muscle cells, a crucial mechanism in determining atherosclerotic plaque instability.

It is an open question whether, in human atherosclerosis, an overactivation or an underactivation of the system is correlated with clinical manifestation of the disease. We previously demonstrated, in an autopsy-based study, an association between enhanced accumulation of ubiquitinated substrates and clinical manifestation of coronary artery disease. The study of autopsic specimens, however, allows just a semi-quantitative evaluation of tissue sections and does not allow the evaluation of oxidative stress parameters or proteasome enzymatic activity. Therefore, it is not clear, yet, whether the accumulation of ubiquitinated complexes in human plaques is related primarily to an increased ubiquitination of cell proteins or to a blockade of the proteasome degrading system.

The current study was designed to test the hypothesis that clinically unstable atherosclerotic plaques are characterized by an enhanced accumulation of ubiquitinated proteins and to evaluate if the presence of a reduced proteasomal proteolytic activity contributes to this accumulation. We therefore evaluated the relationship between the presence of ubiquitin conjugates in human carotid atherosclerotic plaques after endarterectomy and the activity of the 20S proteasome, the degree of endogenous oxidative stress, the phenotypic features of plaque instability and the clinical manifestation of the disease.

Methods

For detailed methodology, please see http://atvb.ahajournals.org

Patients

The study was approved by the Mayo Foundation Institutional Review Board, and procedures followed institutional guidelines. Written informed consent was obtained before surgery.

We studied 177 carotid plaques specimens from patients undergoing carotid endarterectomy, following a previously described procedure. Patients were defined “symptomatic” in the presence of a cerebral ischemic event (stroke, transient ischemic attack [TIA] or amaurosis fugax) within 6 months before surgery, ipsilateral to the collected plaque, or “asymptomatic” in the absence of previous events.

Plaque Specimens

After surgery, plaques were halved at the site of the maximum plaque diameter. One half was fixed in formalin and embedded in paraffin for histology; the other half was immediately frozen at −80°C for subsequent tissue analysis. Plaque stability was evaluated by the tissue expression of matrix-metalloproteases (matrix metalloproteinase [MMP]-2 and MMP-9), macrophage infiltration, content of fibrous tissue, and degree of apoptosis.

Western Blotting

Equal amount of proteins from carotid lysates were resolved in a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. After autoclaving for 30’ to increase the sensitivity of ubiquitin conjugates detection and blocking, membranes were probed to detect ubiquitin conjugates (polyclonal antibody, 1:1000; Sigma), ubiquitin (monoclonal antibody, 1:500; Covance), MMP-9 (polyclonal antibody, 1:5000, Chemicon), MMP-2 (monoclonal antibody, 1:7500; Chemicon), NAD(P)H-oxidase p67-phox, D, Matrix metalloprotease-9 (MMP-9), E, Matrix metalloprotease-2 (MMP-2), F, β-actin. As indicates asymptomatic; Af, amaurosis fugax; St, stroke; TIA, transient ischemic attack.

In Situ Detection of Apoptosis by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Assay

Apoptosis was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (TUNEL) method using a commercially available kit (Apoptag® Peroxidase In Situ Apoptosis Detection Kit; Chemicon) according to the vendor’s instructions.

Sirius Red Staining for Collagen Content

Sections were stained following standard Sirius red procedure (http://atvb.ahajournals.org). The content of collagen type I and III, identified by birefringence under polarized light,28 was evaluated as percent of the plaque area.

Statistics

For clinical data, Western blotting and proteasome activity variables were compared by use of the Student t test or the \( \chi^2 \) test. Comparison among multiple groups was performed by ANOVA followed by Tukey-Kramer post-hoc analysis. Correlation was calculated with Pearson product moment. Data are expressed as percentage or mean±SE for continuous variables and by percentage for qualitative variables. Statistical significance was assumed for \( P<0.05 \).

Results

The study population included 83 asymptomatic and 94 symptomatic patients. Clinical characteristics and current medications of the population are shown in the Table. The symptomatic group had a significantly lower level of high-density lipoprotein cholesterol, a higher percentage of diabetics, and lower percentage of patients taking antioxidant vitamins. No differences were observed for the other clinical parameters and medication (Table). Thirty-four symptomatic patients experienced stroke, 46 TIA, and 18 amaurosis fugax.

Markers of Plaque Stability

Atherosclerotic plaques from symptomatic patients were characterized by a more unstable phenotype (Figure 2). In particular, as compared with asymptomatic patients, they showed a significantly lower fibrous tissue content (9.8±1.5% versus 21.1±1.7% of plaque area; \( P<0.01 \); Figure 2A and 2B), higher apoptosis (20.5±2.2 versus 11.9±2.2% of nuclei; \( P<0.01 \); Figure 2C and 2D), compatible with previous findings,29 higher macrophage infiltration

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* \( P<0.05 \) vs asymptomatic.
†Generic multivitamins in 83% (asymptomatic) and 78% (symptomatic); in the other cases vitamin C and vitamin E, alone or in combination were taken.
HDL-c indicates high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; total-c, total cholesterol.
Values are expressed as mean±SE.
(8.8±1.2% versus 3.0±1.4% of area; P<0.01; Figure 2E and 2F), MMP-9 (2.01±0.41 versus 1.03±0.16; P<0.05), and, although not significantly, MMP-2 (1.37±0.25 versus 0.70±0.09; P=0.06) expression. Moreover, the percentage of plaque area positively stained for α-SMA was significantly lower in symptomatic (7.7±1.1%) than asymptomatic patients (12.4±1.3%; P<0.05; Figure 2E and 2F). Apoptotic nuclei were localized in cells expressing CD-68 (macrophages) and α-SMA (smooth muscle cells), as well as in cells negative for both markers.

Ubiquitin-Proteasome System

Immunoblotting for Ubiquitin and Ubiquitin Conjugates

The expression of free (8.6 KDa) ubiquitin was similar between the study groups (Figure 1B and 3A). On the contrary, there was a significantly higher content of ubiquitin conjugates in carotid plaques from symptomatic as compared with asymptomatic patients (17.7±1.36 versus 10.99±1.04, P<0.001, Figure 1A and 3B). As shown in Figure 3B, patients who experienced stroke (16.11±1.99), TIA (16.5±2.12), and amaurosis fugax (18.28±3.82) showed a significantly higher content of ubiquitin conjugates as compared with asymptomatic patients (P<0.05 versus asymptomatic for all groups).

Ubiquitin conjugates content was not significantly influenced by major stroke risk factors (age, sex, blood pressure, total, low-density lipoprotein and high-density lipoprotein cholesterol levels, smoke habit, diabetes mellitus) or principal cardiovascular drugs. Within the diabetic patients, a tendency to a reduced accumulation of ubiquitin conjugates was associated with the use of oral antidiabetic drugs (14.4±5.7) as compared with non treated patients (24.4±5.7), although not significant (P=0.09).

A direct correlation was observed between ubiquitin conjugates accumulation and percentage of cells undergoing apoptosis (r=0.56; P<0.01). Finally, a significant difference in the prevalence of past cerebrovascular clinical events was observed between the highest and the lowest quartile of ubiquitin conjugates expression, resulting in an odd ratio of 3.81 (95% CI, 1.36 to 10.88) for all events, 1.92 (0.62 to 6.66) for stroke, and 1.78 (95% CI, 0.69 to 4.82) for TIA.

Proteasome Activity Assay

Proteasome proteolytic activity was evaluated in 44 patients randomly selected among both asymptomatic (11 samples) and symptomatic patients (11 samples in each subgroup, ie, amaurosis fugax, TIA and stroke). Symptomatic patients showed a decreased proteasome proteolytic activity as compared with asymptomatic patients (5.01±0.70 versus 9.41±1.19 nmol AMC/mg protein/min, respectively; P<0.01; Figure 3C). Patients with a previous stroke showed the lowest proteasome activity (3.30±1.19 nmol AMC/mg protein/min; P<0.05 versus asymptomatic patients).

Immunohistochemistry for Ubiquitin Conjugates

Immunoperoxidase staining showed the presence of ubiquitin conjugates in human carotid plaques, with a clear prevalence in the regions surrounding the necrotic core, in the necrotic core and in the shoulder regions (Figure 4). Immunofluorescence demonstrated a co-localization of ubiquitinated proteins with both macrophage marker CD-68 (Figure 5A and 5B) and even to a greater level, with α-SMA (Figure 5C and 5D), suggesting accumulation of ubiquitin conjugates in smooth muscle cells and macrophages.

Oxidative Stress

Immunoblotting expression of p67, indicator of NADPH-oxidase content, was significantly increased in symptomatic
patients (2.31±0.29) as compared with asymptomatic patients (1.13±0.17; P<0.01). Moreover, a significant direct correlation between p67 expression and ubiquitin conjugates content was observed (r=0.72, P<0.001). The presence of hypertension was also associated with an increased expression of p67 (hypertensives 2.08±0.18 versus normotensives 1.03±0.43; P<0.05) and a trend toward increased expression of p67 was observed in hypercholesterolemic (1.94±0.21) as compared with normocholesterolemic patients (1.85±0.31; P=0.07).

A partial co-expression of ubiquitinated proteins and 3-nitrotyrosine was detected, mainly in shoulder regions and necrotic core of the plaque (Figure 5E and 5F).

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Figure 3. Quantification of free ubiquitin (A) and ubiquitin conjugates (B) immunoblots. Results are expressed as ratios between target signal and β-actin to correct for protein load. C, Proteasome activity assay. Forty-four carotid specimens were included, randomly selected from asymptomatic patients (n=11) and patients with a previous stroke (n=11), TIA (n=11), or amaurosis fugax (n=11). *P<0.01 vs asymptomatic; §P<0.05 vs asymptomatic.

Figure 4. Representative immunostaining for ubiquitin conjugates (brown) of a human carotid plaque (original magnification 2×). A and B, Detail of ubiquitin conjugates expression (brown) by cells (arrows) in shoulder region (A) and necrotic core (B) of the plaque (original magnification 40×). C, Recent intraplaque hemorrhage. Ubiquitin conjugates positive cells (arrows) among red blood cells.
Discussion

The current study shows that the content of ubiquitin conjugates in plaques from patients with symptomatic carotid atherosclerosis is significantly higher as compared with asymptomatic patients, and correlates with 3-nitrotyrosine and NADPH-oxidase p67 expression and with the percentage of cells undergoing apoptosis. Importantly, symptomatic patients, characterized by a more unstable plaque phenotype, showed an imbalance between the accumulation of ubiquitin conjugates and proteasome proteolytic activity, which was reduced. Although correlative, this study suggests a possible role of the endogenous UPS in human atherosclerosis, potentially participating in the regulation of plaque stability and clinical complication of the disease.

The balance of the UPS is dependent on the active process of proteins ubiquitination and their degradation by the proteasome system. An increased formation of ubiquitin conjugates has been demonstrated in the presence of increased oxidative stress especially in neurological disorders. Similarly, in the present study, we demonstrated increased accumulation of ubiquitin conjugates in human carotid plaques, associated with increased oxidative stress, underscored by the significant correlation between ubiquitin conjugates and NADPH-oxidase subunit p67 expression, one of the principal sources of superoxide anions in human atherosclerosis and by the partial co-expression of ubiquitinated proteins and 3-nitrotyrosine. Besides enhancing the oxidative damage of proteins and the subsequent ubiquitination, oxidative stress may lead to accumulation of ubiquitinated proteins via inhibition of proteasome enzymatic activity. Indeed, reduced 20S proteasome activity was observed in symptomatic patients, suggesting an imbalance between the load of ubiquitinated substrates and the ability of the proteasome to degrade them. It can be speculated that the oxidative protein damage is responsible for the activation of the UPS leading to accumulation of ubiquitinated proteins within the plaque. As long as the proteasome complex is able to efficiently clear the cells from ubiquitinated substrates, an equilibrium is maintained. In the presence of a higher level of oxidative stress, the proteasome degrading activity may become insufficient to clear the cell from damaged proteins. These in turn are progressively enriched with ubiquitin moieties, are further oxidized, and form hydrophobic and cross-linking aggregates. Experimental studies have demonstrated this process in human cells exposed to a proteasome inhibitor. Protein aggregates are difficult to unfold or degrade, precipitate in the hydrophobic fraction of the cell and eventually become virtually undegradable, leading to cytotoxicity. Several age-related degenerative diseases, such as Alzheimer disease and Parkinson disease, cataracts, and some cancers are characterized by protein aggregates formation and precipitation. In these degenerative processes the accumulation of protein deposits is frequently associated with oxidative modification and increased ubiquitination of proteins. Our study suggests that a similar process may play a role in human atherosclerotic plaques, which can be considered, along with inflammatory, a degenerative disease. Furthermore, in atherosclerosis these processes might also enhance the acute complications of the atherosclerotic plaque. Besides facilitating direct cytotoxicity through accumulation of protein aggre-
gates, inhibition of the proteasome can increase cell apoptotic rate, thus further contributing to plaque instability. According to the present study we found a direct correlation between the accumulation of ubiquitin conjugates and plaque apoptosis, suggesting a relation between the two phenomena.

Our study demonstrates an increased presence of ubiquitin conjugates both in smooth muscle cells and macrophages and an increased apoptosis, which was undergoing in both cell types; we can speculate that the resulting increased rate of cell loss might contribute to the weakening of the fibrous cap and the enlargement of the necrotic core, respectively.

This scenario suggests that the active and high rate of ubiquitination, in the presence of a functioning proteasome complex, seems to have a protective role against the complication of atherosclerosis, via the degradation of oxidized and damaged proteins. On the contrary, when the proteasome-dependent degradation of its substrates is impaired, the accumulation of oxidized and ubiquitinated protein aggregates may enhance the damage and foster plaque instability. So far, proteasome inhibitors have been used for the treatment of several human cancers; however, because of the short duration of the treatment in such conditions, it is impossible to evaluate their effect on cardiovascular events. Interestingly, in patients with HIV the use of protease inhibitors, which have been demonstrated to significantly inhibit the proteasome system, is associated with an increased cardiovascular risk independently from associated risk factors. It is intriguing to hypothesize that such an increased risk might be partly related to their anti-proteasome activity. It is noteworthy that aging per se has been associated with an impairment of the proteasome activity in human fibroblasts and induction of proteasome expression reduces cell senescence. In the present study, however, we did not find any association between age and proteasome activity or ubiquitin conjugates expression. This is likely due to the relatively homogeneous elderly population of our study which does not allow detection of age-related differences.

The association found in the present work, between ubiquitin conjugates content and oxidative stress, partly through proteasome inhibition, suggests an additional mechanism through which oxidative stress could favor not only the development but also the complication of human atherosclerosis. An accumulation of damaged, oxidized and ubiquitinated proteins can lead to proteasome inhibition by inducing a clogging of the 20S proteasome core. Therefore, either directly or through the formation of big proteins aggregates, high levels of oxidative stress can eventually lead to a net reduction of the clearance of damaged proteins. Finally, because the inhibition of the proteasome activity can increase oxidative stress, a vicious cycle between decreased enzymatic activity of the proteasome and increased oxidative stress might onset.

Study Limitations and Clinical Implications
Considering the relative small population, it was not possible to consider all the possible confounding factors, including specific drugs, duration, and combination of treatments. However, the principal drug classes did not appear to influence per se the UPS. The associative nature of the present work does not allow drawing definitive conclusions on the role of a dysregulation of the UPS in the pathophysiology of atherosclerosis. However, considering the amount of literature demonstrating the importance of the UPS in regulating the principal processes of atherosclerosis and our results, although it cannot be completely ruled out a role of bystander, it is probable that the modulation of the system plays a major role in the disease and may become one of the future targets for the treatment of atherosclerosis. So far, UPS has been clinically studied mainly in cancer research and in a recent trial the proteasome inhibitor bortezomib was found effective in the treatment of relapsed multiple myeloma. However, UPS regulates also many processes that are fundamental in atherosclerosis development and complication. It has been hypothesized that the inhibition of the proteasome might have a favorable effect on atherosclerosis through, among other mechanisms, the inhibition of the NF-κB system and the induction of apoptosis. This is likely in the early stages of atherosclerosis, when the inhibition of the inflammatory process and cell proliferation are crucial to reduce the onset of the disease. Moreover, an inhibitor of proteasome activity has been found to prevent neointimal proliferation and restenosis after arterial injury, highlighting the possible application of this drug class for the prevention of restenosis after angio-plasty. However, an untargeted and nonspecific inhibition of the system does not seem to be desirable in the treatment of atherosclerosis. Therefore, it is crucial to develop UPS-modulating drugs, which are able to target the specific degradation of key regulators in the atherosclerotic process, in relation to the stage of the disease. In this view, similarly to cancer research, the goal of future cardiovascular pharmaceutical engineering should be the development of drugs interfering with the regulators of UPS substrate-specificity, particularly the different E3 ubiquitin substrate-conjugating enzymes or the ancillary proteins.

In conclusion, the current study highlights the association between oxidative stress and UPS imbalance in advanced stages of atherosclerosis. The correlated progressive accumulation of ubiquitinated proteins, which may eventually become cytotoxic, might play a role in the regulation of plaque stability and eventually clinical manifestation of the disease.

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Disclosures
None.

References


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METHODS

Patients

The study was approved by the Mayo Foundation Institutional Review Board, and procedures followed institutional guidelines. Written informed consent was obtained before surgery. We studied 177 carotid plaques specimens from patients undergoing carotid endarterectomy, following a previously described procedure\textsuperscript{1}. The decision for the surgical intervention was based, following current guidelines, on neurological and neurosurgical examination, carotid ultrasound and/or MRI, both in symptomatic or progressive asymptomatic patients with extracranial carotid artery disease. Demographic data and detailed clinical history were obtained for each patient by chart review (Mayo Clinic Documents Browser and Mayo Integrated Clinical Systems (MICS) Last Word), with particular attention to carotid territory ischemic events. Carotid atherosclerosis clinical instability was defined subdividing the patients into symptomatic, in the presence of a cerebral ischemic event within six months prior to surgery, ipsilateral to the collected plaque, and asymptomatic, in the absence of ischemic events prior to surgery. Eligible clinical events to include patients in the symptomatic group were ischemic stroke, transient ischemic attack (TIA) and amaurosis fugax.

Plaque specimens

After surgery, plaques were halved at the site of the maximum plaque diameter. One half was fixed in formalin and embedded in paraffin for histology; the other half was immediately frozen at –80°C for subsequent tissue analysis. Plaque stability was evaluated by the tissue expression of matrix-metalloproteases (MMP)-2 and 9, macrophage infiltration, content of fibrous tissue and apoptotic rate.

Western Blotting
Samples were prepared and separated by electrophoresis as previously described\textsuperscript{2}. Frozen samples were homogenized in lysis buffer and protein concentration of lysates calculated by Bradford assay (Bio-Rad, CA). Equal amount of proteins were diluted in lysis buffer and reducing SDS loading buffer and resolved in a 10% SDS-Page gel. Subsequently, electrottransfer to nitrocellulose membrane was performed and membranes were autoclaved for 30’ to increase the sensitivity of ubiquitin-conjugates detection\textsuperscript{3} and blocked in 5% fat-free dry milk. Membranes were then immunoblotted to detect ubiquitin-conjugates (rabbit polyclonal antibody, 1:1000, Sigma, St Louis, MO), ubiquitin (mouse monoclonal antibody, 1:500, Covance, Princeton, NJ), MMP-9 (rabbit polyclonal antibody, 1:5000, Chemicon International, Temecula, CA), MMP-2 (mouse monoclonal antibody, 1:7500, Chemicon International), NADPH-oxidase p67phox (rabbit polyclonal, 1:500, Santa Cruz Biotechnology Inc, Santa Cruz, CA), one of the principal sources of oxidative stress in human atherosclerosis\textsuperscript{4}; protein loading control was evaluated using anti- ß-Actin antibody (Sigma, 1:1000). Anti-rabbit (1:1000-1:5000, Amersham Life Sciences, IL ) or anti-mouse (1:1000, Amersham Life Sciences) antibodies conjugated to horseradish peroxidase were used as secondary antibodies, as appropriate. After developing with chemiluminescence (Pierce, IL)\textsuperscript{2} and exposing to X-ray film (Kodak, NY), signals were analyzed using ImageJ software (National Institutes of Health) and expressed as integrated density. For quantification of large ubiquitin-conjugates, the density of the membrane corresponding column was analyzed in each sample (see manuscript Figure 1A). To avoid the influence of slight differences in membranes conditions –e.g. different backgrounds- and to obtain comparable data among different runs, one same sample was repeated in every membrane and used to normalize the signal. On the basis of the signal obtained, we divided the whole population into quartiles of ubiquitin-conjugates content. Immunoblotting results are expressed as ratios to actin signal.

**Proteasome activity assay**
Chymotrypsin-like activity of the proteasome was assayed using a commonly available fluorimetric kit (APT280, Chemicon International), following company instructions. Briefly, frozen samples were homogenized in lysis buffer and protein concentration of lysates calculated by Bradford assay (Bio-Rad). Progressive dilutions of 20S purified proteasome were analyzed for standard activity curve. One hundred µg of proteins from each carotid plaque lysate and standard proteasome dilutions were incubated with the fluorogenic proteasome substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC) for 60 minutes at 37°C, in the presence or absence of the proteasome inhibitor lactacystin. Generated fluorescence was read with a 380/460 nm filtered fluorometer (SpectraMax Gemini XPS, Global Medical Instrumentation Inc., Ramsey, MN). The proteasome activity was calculated subtracting the inhibited from the non-inhibited activity and expressed as generated nanomoles of AMC/milligram of protein/minute.

**Immunostaining for ubiquitin-conjugates, nitrotyrosine, macrophages and smooth muscle cells**

After deparaffinizing, hydrating, quenching endogenous peroxidase and blocking, carotid plaque sections were incubated overnight at 4°C with primary antibody and then for 1 h with anti-rabbit IgG secondary antibody (Dako A/S, Glostrup, Denmark). Diaminobenzidine was used as chromogen. For double staining procedures, after staining with the first primary antibody, slides were blocked (Doublestain block, Dako) and incubated with the second primary antibody (1 hour), followed by alkaline phosphatase conjugated secondary antibody (Dako) and Vector Blue (Dako, blue label) or Fast Red (Dako, red label) substrate. All sections were counterstained either with Hematoxylin or Nuclear Fast Red (Sigma). Normal rabbit or mouse immunoglobulin fraction was substituted to primary antibodies as negative immunostaining control.

Primary antibodies used: anti-ubiquitin-(conjugates) (1:100, Sigma), anti-3-nitrotyrosine (1:250, Sigma), anti-α-SMA (1:500, Dako), and anti-CD68(1:500, Dako).
Stained sections were then mounted and visualized under microscope (Olympus, Leeds Precision Instruments) and pictures were taken with an imaging program (SPOT Advanced 3.3, Diagnostic Instruments Inc). For the quantification of smooth muscle cells and macrophage, the percent area of the specimen stained with the corresponding color was calculated by the use of a image analysis computer software (MetaMorph, Meta Imaging Series 4.6).

**Expression of ubiquitin-conjugates in smooth muscle cells and macrophages**

Representative slices of whole plaques were prepared for double immunofluorescence by deparaffinizing and hydrating. Double immunofluorescence was also applied to single cells after dispersion and adherence of cells to glass coverslip. Briefly, after removing fat, adventitia, and blood residues from carotid plaque specimens, the remaining tissue was incubated with a collagenase II dissociation solution, at 37°C in a shaker bath. After repeated trituration, sieving and washing, cells were plated in Hanks’s solution on glass coverslips and fixated with methanol:acetone 1:1.

After overnight incubation with the two primary antibodies, a mixture of secondary donkey anti-rabbit FITC and anti-mouse Texas Red conjugated antibodies (Jackson Immunoresearch Laboratories, Baltimore, PA) was applied to whole plaques or cell dispersions slices. The first primary antibody was anti-ubiquitin(conjugates) (1:100, Sigma) and the second primary antibodies were either anti-α-SMA (1:500, Dako) or anti-macrophage CD-68 (1:500, Dako). Observation of fluorescence was performed using a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss, Inc., Oberkochen, Germany).

**TUNEL Staining**

Apoptosis was evaluated in-situ by the TUNEL procedure, using a commercially available kit (Apoptag® Peroxidase In Situ Apoptosis Detection Kit, Chemicon) and following manufacturer’s instructions. Briefly, after deparaffinizing and rehydrating, Proteinase K (20 µg/mL) was applied to
tissue sections for 10 minutes at room temperature. Following rinsing, permeabilization of the tissue was obtained by incubation with 0.05% triton X-100 in 0.05% sodium citrate for 5 s. Endogenous peroxidase was blocked with 2% H2O2 for 15 minutes. Subsequently, slides were incubated with equilibration buffer (5 min at RT), terminal deoxynucleotidyl transferase (TdT) enzyme (60 min at 37°C), stop/wash buffer (10 min at RT), protein block (Dako, 7 min at RT). Antidigoxin-peroxidase was then applied (30 min at RT) and followed by DAB substrate. A second labeling of macrophage CD68 or smooth muscle cell α-SMA was also performed as described above. Slides were finally counterstained, dehydrated and mounted. Rat regressive mammary gland tissue was used as a positive control for apoptosis (Intergen Company, Purchase, New York). Omission of TdT enzyme from the labeling procedure served as a negative control.

**Sirius Red Staining**

The interstitial collagen content of carotid plaques was evaluated by Sirius red. Specimen sections were deparaffinized, rehydrated and incubated with 0.1% Sirius red in saturated picric acid for 60 minutes. After incubation in 1% acetic acid for 30 minutes and rinsing, slides were counterstained in hematoxylin, differentiated in acid alcohol solution, rehydrated and mounted. Slides were visualized under both bright-field and polarized light microscope, and pictures taken with identical exposure settings for all sections. The content of collagen type I and II, identified by birefringence under polarized light\(^5\), was evaluated as percent of the plaque area.

**Statistics**

For clinical data, Western blotting and proteasome activity variables were compared by use of the Student t-test or the \(\chi^2\) test. Comparison among multiple groups was performed by ANOVA followed by Tukey-Kramer post-hoc analysis. Correlation was calculated with Pearson product moment. Data are expressed as percentage or mean±SE for continuous variables and by percentage for qualitative variables. Statistical significance was assumed for P<0.05.


