Differential Effect of the Protein Synthesis Inhibitors Puromycin and Cycloheximide on Vascular Smooth Muscle Cell Viability

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ABSTRACT

Recent evidence indicates that the protein synthesis inhibitor cycloheximide triggers selective macrophage death in rabbit atheroma-like lesions without affecting smooth muscle cells (SMCs) or the endothelium, thereby favoring a stable plaque phenotype. In this study, we report that puromycin, a protein synthesis inhibitor with a different mode of action but with similar ability to inhibit de novo protein synthesis, did not reveal plaque-stabilizing effects. The macrophage and the SMC content readily decreased in puromycin-treated atheroma-like lesions in rabbit carotid arteries. Moreover, puromycin induced apoptosis in macrophages and SMCs in vitro. Puromycin-treated SMCs showed signs of endoplasmic reticulum (ER) stress, as demonstrated by CCAAT/enhancer-binding protein homologous protein (CHOP) protein expression, splicing of X-box-binding protein 1 mRNA, and phosphorylation of eukaryotic translation initiation factor 2α. The ER stress inducer thapsigargin up-regulated CHOP protein expression in SMCs without affecting their viability, indicating that ER stress not necessarily results in cell death. Puromycin, but not thapsigargin, activated the ER stress-related caspase-12. Treatment of SMCs with a combination of cycloheximide and puromycin inhibited ER stress and partially improved SMC viability. In addition, puromycin, but not cycloheximide or thapsigargin, induced intracellular accumulation of polyubiquitinylated proteins in SMCs, whereas the proteasome function was not affected. Taken together, puromycin, in contrast to cycloheximide, induces SMC apoptosis, thereby favoring an unstable plaque phenotype. SMC death upon puromycin treatment could only be partially prevented by cycloheximide, which completely blocked ER stress. However, other or additional mechanisms, such as increased polyubiquitination of proteins, might be involved in puromycin-induced SMC death.

Atherosclerotic plaque destabilization and rupture are one of the main causal events of acute coronary syndromes and sudden death (Lutgens et al., 2003; Shah, 2003; Libby and Theroux, 2005). Because plaques tend to rupture at sites of inflammation (Lafont, 2003; Koenig and Khuseyinova, 2007), macrophages are major players in this process. Indeed, lesions macrophages induce apoptosis of smooth muscle cells (SMCs) via Fas/Fas-L interactions, production of proinflammatory oxygen species, and the release of proinflammatory cytokines (Boyle et al., 2001). Moreover, macrophages secrete matrix metalloproteinases, which results in breakdown of interstitial collagen fibers and thinning of the protective fibrous cap

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ABBREVIATIONS: SMC, smooth muscle cell; ER, endoplasmic reticulum; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; 4-PBA, sodium 4-phenylbutyrate; zATADfmk, z-Ala-Thr-Asp-fluoromethyl ketone; eGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; XBP, X-box-binding protein; elf2α, eukaryotic translation initiation factor 2α; MDC, mouse ornithine decarboxylase; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; UPR, unfolded protein response; ANOVA, analysis of variance; PM, puromycin; CHX, cycloheximide.
coding for components of the translation machinery is specifically inhibited, ribosome biosynthesis is blocked, and autophagic cell death is initiated (Raught et al., 2001). More recently, we demonstrated that the protein synthesis inhibitor cycloheximide was able to selectively clear macrophages from atheroma-like lesions by means of apoptotic cell death recently, we demonstrated that the protein synthesis inhibitor puromycin, which has a different mechanism of action compared with cycloheximide.

Materials and Methods

In Vitro Treatment of Atheroma-Like Lesions. Male New Zealand White rabbits (2.7–3.5 kg; from atheroma-like lesions by means of apoptotic cell death recently, we demonstrated that the protein synthesis inhibitor cycloheximide was able to selectively clear macrophages from atheroma-like lesions by means of apoptotic cell death without affecting the viability and functionality of SMCs and the endothelium (Croons et al., 2007). In this way, cycloheximide promotes plaque stability. In the present study, we addressed the question whether protein synthesis inhibitors in general can selectively clear macrophages in atherosclerotic plaques. To investigate this hypothesis, we used the protein synthesis inhibitor puromycin, which has a different mechanism of action compared with cycloheximide.

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Internucleosomal DNA Fragmentation. Cells were lysed in 0.5 ml of hypotonic lysis buffer supplemented with 200 µg of proteinase K. Lysates were incubated for 1 h at 50°C, and then they were supplemented with 5-µl volumes of 2 mg/ml DNase-free RNase A and incubated for an additional hour at 37°C. The samples were precipitated overnight with 1/10 volume of 3 M sodium acetate and 1 volume of isopropanol. DNA pellets were air-dried and dissolved in water. After electrophoresis in 2% agarose gel (Invitrogen), DNA laddering was visualized under UV light.

Inhibition of de Novo Protein Synthesis. Rabbit aortic SMCs and J774A.1 macrophages were treated for 1 to 4 h with 35 µM cycloheximide or puromycin, and then they were pulse-labeled for 30 min at 37°C with 5 µCi of Pro-mix L-[35S] in vitro cell labeling mix (GE Healthcare, Chalfont St. Giles, UK) in cysteine/methionine free Dulbecco’s modified Eagle’s medium. After homogenization of cells in hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100), labeled proteins were precipitated with 10% trichloroacetic acid, resuspended in 0.2 N NaOH, and measured by liquid scintillation counting. Alternatively, in vitro translation of enhanced green fluorescent protein (eGFP) was evaluated in a cell-free system. Purified pGEM4Z-GFP-A64 (a gift from Dr. Van Tendeloo, University Hospital Antwerp, Antwerp, Belgium) containing the eGFP coding sequence downstream of a T7 promoter was Spel-linearized, purified with a QIAquick PCR purification kit (QIAGEN, Venlo, The Netherlands) and used as DNA template for an in vitro transcription reaction using the T7 mMessage mMachine transcription kit (Ambion, Austin, TX). Transcription reactions were carried out at 37°C for 2 h. Unincorporated nucleotides were removed by size exclusion chromatography on RNase-free NucAway spin columns (Ambion). Subsequently, 0.5 µg of purified eGFP mRNA was in vitro translated in the presence or absence of cycloheximide or puromycin using L-[35S]methionine (GE Healthcare) and the Rabbit Reticulocyte Lysate System (Promega, Madison, WI) for 90 min at 30°C. Small aliquots of translated product were loaded on 4 to 12% NuPage SDS gels (Invitrogen), and they were analyzed for eGFP via Western blotting.

Western Blot Analysis. Cells were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad, Hercules, CA). Cell lysates were then heated denatured for 3 min and loaded on 4 to 12% NuPage SDS gels (Invitrogen). After gel electrophoresis, proteins were transferred to an Immobilon-P Transfer membrane (Millipore Corporation, Billerica, MA) according to standard procedures. Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk (Bio-Rad) for 1 h. After blocking, membranes were probed overnight at 4°C with primary antibodies in antibody dilution buffer (Tris-buffered saline containing 0.05% Tween 20 and 1% nonfat dry milk), followed by 1-h incubation with secondary antibody at room temperature. Antibody detection was accomplished with SuperSignal West Pico or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Chemical, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). The following mouse primary antibodies were used: monoclonal anti-caspase-3 (clone 19) (BD Biosciences Transduction Laboratories, Lexington, KY), anti-C/EBP homologous protein (CHOP) (clone B-3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-cleavage (clone 6C1; Sigma-Aldrich), and anti-phospho aktin (clone AC-15; Sigma-Aldrich). Rabbit antibodies used in this study include anti-cleaved caspase-3, anti-caspase-12, anti-ubiquitin, anti-caspase-12, anti-caspase-12, anti-ubiquitin, and anti-caspase-12.
factor 2α (eIF2α), anti-phospho-eIF2α (Ser 51) (Cell Signaling Technology Inc., Danvers, MA), and anti-eGFP (Clontech, Mountain View, CA). Peroxidase-conjugated secondary antibodies were purchased from Dako Denmark A/S.

**XBP1 mRNA Splicing.** Total RNA was isolated from cultured cells using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA). Alternative splicing of X-box-binding protein (XBP) 1 mRNA was examined by RT-PCR using XBP1-specific primers (forward primer, 5'-GATCCTGAGAGTGTCGAGGTG-3' and reverse primer, 5'-GATGTCAGCTAAGAGGACTGTCG-3') and the Superscript One-Step RT-PCR kit (Invitrogen). Thermostable parameters were as follows: reverse transcription at 50°C for 30 min, denaturation at 94°C for 2 min, and 40 cycles consisting of incubation at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. PCR products were analyzed on 4% E-gels (Invitrogen).

**Real-Time Quantitative RT-PCR.** cDNA was prepared from cultured cells using the FastLane Cell cDNA kit (QIAGEN). TaqMan gene expression assays for CHOP (assay ID, Mm00492907_m1; Applied Biosystems, Foster City, CA) were then performed in duplicate on an ABI Prism 7300 sequence detector system (Applied Biosystems) in 25-μl reaction volumes containing 1X Universal PCR Master Mix (Applied Biosystems). The parameters for PCR amplification were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression of mRNA species was calculated using the comparative threshold cycle method. All data were controlled for quantity of cDNA input by performing measurements on the endogenous reference gene β-actin (assay ID, Mm00607939_s1; Applied Biosystems).

**Microarray Analysis.** Total RNA was prepared from cultured cells using the Absolutely RNA Miniprep kit (Stratagene). All RNA samples were treated with RNase-free DNAse I. RNA quality was verified on an Agilent 2100 bioanalyzer using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). Samples were then analyzed by the Microarray Facility of the Flanders Interuniversity Institute for Biotechnology (VIB, Leuven, Belgium) using the Whole Mouse Genome Oligo Microarray kit (Agilent Technologies), representing more than 41,000 mouse genes and transcripts. To define differential gene induction, we used a 5-fold threshold value.

**Transient Transfection.** The proteasome sensor vector p26ProSensor-1 (Clontech) containing ZsGreen, fused to the mouse ornithine decarboxylase (MODC) degradation domain and under control of the immediate early cytomegalovirus promoter, was propagated in Escherichia coli TOP10 and purified using the Plasmid Midi kit (QIAGEN). Rabbit aortic SMCs (2.5 × 10⁶ cells) were transiently transfected with 5 μg of purified plasmid DNA via Nucleofector technology (program U-25) using the Human aSMC Nucleofector kit (Amaza GmbH, Koeln, Germany).

**Statistical Analysis.** All data are presented as mean ± S.E.M. Statistical analyses were carried out with SPSS 14.0 software (SPSS Inc., Chicago, IL). Differences were considered significant at p < 0.05.

**Results**

**Puromycin Decreased Macrophage and Smooth Muscle Cell Content in Rabbit Atheroma-Like Lesions by Induction of Apoptotic Cell Death.** To examine the effects of puromycin on atherosclerotic plaques, collared carotid artery rings from hypercholesterolemic rabbits were treated in vitro with puromycin for 3 or 7 days. Thereafter, RAM11 and α-SMC actin immunostains were performed to quantify the amount of macrophages and SMCs, respectively. The RAM11-positive area in the intima was significantly reduced both after 3 and 7 days of treatment (Fig. 1, A and C). The α-SMC actin area was decreased after 7 days of incubation, both in the intima and in the media (Fig. 1, B and C). Compared with control, puromycin induced a progressive decrease in the amount of cells with intact nuclei (Table 1), together with an increase of positive TUNEL labeling (Fig. 1D) from 3 ± 3 to 24 ± 9 positive cells/mm² intima (p ≤ 0.05) and from 7 ± 7 to 56 ± 35 positive cells/mm² media (p < 0.05, Wilcoxon sign rank test; n = 9). Puromycin did not affect the intimal area, but the medial area was reduced after 3 and 7 days of treatment (Table 1).

**Puromycin Induced Apoptosis of Macrophages and Smooth Muscle Cells in Culture.** Mouse J774A.1 macrophages, vascular SMCs isolated from rabbit aorta, and C2C12 myoblasts were treated in vitro with the protein synthesis inhibitor puromycin. Cell death was initiated in all cell types in a concentration- and time-dependent manner (Fig. 2, A and B). Macrophage and SMC death induced by puromycin was characterized by cleavage of procaspase-3 and internucleosomal DNA fragmentation, typical of apoptosis (Fig. 2C).

**Puromycin and Cycloheximide Inhibited de Novo Protein Synthesis to a Similar Extent.** Because SMCs are highly resistant to cycloheximide (Croons et al., 2007), but not to puromycin treatment, we tested the ability of both compounds to function as de novo protein synthesis inhibitors. Macrophages and rabbit SMCs were treated with equimolar amounts (35 μM) of puromycin or cycloheximide, and de novo protein synthesis was assessed by measuring the incorporation of L-[³⁵S]methionine and cysteine. De novo protein synthesis was equally inhibited in both cell types (Fig. 3A). Similar results were obtained in a cell-free system using in vitro-transcribed eGFP mRNA (Fig. 3B).

**Puromycin, but Not Cycloheximide, Induced Endoplasmic Reticulum Stress.** Real-time RT-PCR showed that both puromycin and cycloheximide up-regulated transcription of the ER stress marker C/EBP homologous protein (CHOP) in J774A.1 macrophages and C2C12 myoblasts (Fig. 4A). However, CHOP protein accumulated only in cells treated with puromycin (Fig. 4B). Moreover, puromycin, but not cycloheximide, induced splicing of XBP1 mRNA in both macrophages and SMCs (Fig. 4C), and phosphorylation of eIF2α in SMCs (Fig. 4D). In J774A.1 macrophages already high basal levels of phosphorylated eIF2α were present and did not change after puromycin treatment (Fig. 4D).

**Puromycin, but Not Thapsigargin, Led to Caspase-12 Activation and SMC Death.** The puromycin-induced CHOP protein expression was accompanied by a decrease in SMC viability (Fig. 5A). The combination of puromycin and cycloheximide (35 μM each) did no longer trigger CHOP expression, and it partially restored SMC viability compared with puromycin treatment (Fig. 5A). ER stress inducer thapsigargin (2 nM) resulted in CHOP expression without affecting SMC viability (Fig. 5A), demonstrating that CHOP expression is not necessarily lethal for SMCs. Treatment of SMCs with both thapsigargin and cycloheximide (2 nM and 35 μM, respectively) failed to up-regulate CHOP expression, and it did not induce SMC death (Fig. 5A). It is noteworthy that puromycin, but not thapsigargin, cleaved and activated caspase-12 in C2C12 myoblasts in a time-dependent manner (Fig. 5B).
Although the viability of SMCs treated with the combination of 4-PBA and puromycin was statistically improved compared with puromycin-treated cells, still 93% of the cells died (Fig. 6A). Moreover, 4-PBA could not prevent phosphorylation of eIF2α and CHOP protein expression (Fig. 6B), indicating that ER stress was still present. The caspase-12 inhibitor zATADfmk did not affect SMC viability or CHOP protein expression upon puromycin treatment (data not shown).

Puromycin-Induced SMC Death Was Not Associated with Differential Gene Transcription. To identify other potentially important pathways linked to puromycin-induced
Induced Accumulation of Polyubiquitinated Proteins in SMCs. Although both puromycin and cycloheximide induced CHOP gene transcription compared with the untreated control, differentially expressed genes between puromycin- and cycloheximide-treated cells could not be identified.

Puromycin, but Not Cycloheximide or Thapsigargin, Induced Accumulation of Polyubiquitinated Proteins in SMCs. Because puromycin stimulates formation of polyubiquitinated defective ribosomal products (Lelouard et al., 2004), we examined polyubiquitination in puromycin- versus cycloheximide- or thapsigargin-treated SMCs via Western blotting. Polyubiquitinated proteins accumulated in puromycin-treated cells, but they decreased after cycloheximide treatment, and they were unaffected by thapsigargin treatment (Fig. 7, A and B). To examine whether puromycin inhibits the proteasome, SMCs were transfected with a plasmid encoding ZsGreen fused to the MODC degradation domain. After transfection, MODC accumulated in cells treated with the proteasome inhibitor MG132 (10 μM), which was used as a positive control, but not in untreated controls, or puromycin- or cycloheximide (35 μM)-treated cells, indicating that puromycin did not affect proteasome function (Table 2).

**Discussion**

Because plaque macrophages promote matrix degradation (Newby, 2005) and cell death of SMCs (Martinet and Kockx, 2001), selective removal of macrophages from plaques is con-
sidered a promising strategy to stabilize rupture-prone atherosclerotic plaques (De Meyer et al., 2003; Martinet and De Meyer, 2007). We recently demonstrated that the protein synthesis inhibitor cycloheximide was able to selectively induce apoptosis of macrophages in rabbit plaques without affecting the viability and functionality of SMCs or the endothelium (Croons et al., 2007). The major finding of the present study is that the protein synthesis inhibitor puromycin, in contrast to cycloheximide, did not reveal plaque-stabilizing effects. On the contrary, puromycin induced apoptotic cell death of both macrophages and SMCs in treated plaques.

Puromycin and cycloheximide are protein translation inhibitors; yet, their mechanism of action is not identical. Puromycin is a structural analog of aminoacyl-tRNA, and it leads to the release of unfinished polypeptide chains (Azzam and Algranati, 1973), thereby inhibiting protein elongation. Cycloheximide, in contrast, inhibits protein synthesis by binding exclusively on cytoplasmic (80S) ribosomes of eukaryotes (Stöcklein and Piepersberg, 1980). Despite this different mode of action, we demonstrated that the potency of cycloheximide and puromycin to inhibit de novo protein synthesis in macrophages or SMCs was similar, confirming a study in rat primary hepatocytes (Sidhu and Omiecinski, 1998).

Puromycin is commonly used to increase the production of truncated or misfolded proteins, also known as defective ribosomal products (Lelouard et al., 2004). If these misfolded proteins accumulate in the lumen of the ER, the unfolded protein response (UPR) is activated (Shen et al., 2004). UPR, in turn, uses an evolutionarily conserved signaling pathway during which the signal of unfolded proteins activates a set of ER-located stress sensors. Finally, this situation leads to attenuation of general protein synthesis via phosphorylation of eIF2α and changes in gene expression by processing the mRNA of the transcription factor XBP1 and up-regulation of another transcription factor, named CHOP (Rutkowski and Kaufman, 2004; Xu et al., 2005). In addition to these adaptive responses, the UPR initiates proapoptotic pathways (Rutkowski and Kaufman, 2004; Xu et al., 2005). Therefore, we examined whether changes in UPR response could explain puromycin-induced SMC death. Both cycloheximide and puromycin up-regulated CHOP mRNA in macrophages and SMCs, which is in agreement with previous reports showing enhanced transcriptional levels of CHOP in rat liver after intravenous injection of cycloheximide (Ito et al., 2006; Kumagai et al., 2006). However, only puromycin-treated cells showed accumulation of CHOP protein. Given the equal in-

**Fig. 3.** Inhibition of de novo protein synthesis by puromycin and cycloheximide. A, rabbit aortic SMCs and J774A.1 macrophages were treated with 35 μM puromycin (PM) or 35 μM cycloheximide (CHX) for 1 or 4 h, respectively, and then they were pulse labeled with L-[35S]methionine/cysteine for 30 min. Lysates of treated cells were measured by liquid scintillation counting. ***p < 0.001 versus control (ANOVA, followed by Bonferroni’s test for SMCs or Dunnett’s T3 test for macrophages). B, purified eGFP mRNA was in vitro translated using L-[35S]methionine and the Rabbit Reticulocyte Lysate System in the presence of various concentrations of puromycin or cycloheximide. Translated eGFP was analyzed by Western blotting.

**Fig. 4.** Evaluation of ER stress induction in macrophages and SMCs after treatment with puromycin or cycloheximide. J774A.1 macrophages, rabbit aortic SMCs or C2C12 myoblasts were treated with 35 μM puromycin or cycloheximide for the indicated times. Up-regulation of CHOP mRNA (A), expression of CHOP protein (B), splicing of XBP1 mRNA (C), and phosphorylation of eIF2α (D) was evaluated. Data represent mean ± S.E.M. of three independent experiments, each performed in duplicate. β-Actin was used as a loading control. *p < 0.05; ***p < 0.001 versus control (factorial ANOVA, followed by Dunnett’s T3 test).
Hibbition of protein synthesis upon exposure to puromycin or cycloheximide, differential expression of CHOP protein in puromycin-treated cells is surprising and hard to explain. It is possible that a fraction of CHOP mRNA escapes translational attenuation. Alternatively, puromycin, but not cycloheximide, induced splicing of XBP1 mRNA, one of the hallmarks of the UPR response. Furthermore, puromycin, but not cycloheximide, induced phosphorylation of eIF2α.

Although some of the UPR proteins and mechanisms involved in apoptosis induction are identified, little is known about how they are integrated and able to commit a cell to apoptosis (Xu et al., 2005). Overexpression of CHOP and
microinjection of CHOP protein have been reported to induce apoptosis via down-regulation of Bcl-2 protein, translocation of Bax protein from the cytosol to the mitochondria, and perturbation of the cellular redox state by depletion of cellular glutathione (McCullough et al., 2001). Nonetheless, several lines of evidence indicate that CHOP expression is not a priori responsible for puromycin-induced SMC death. First, inhibition of puromycin-induced CHOP expression by cycloheximide at the protein level only partially prevented puromycin-induced rabbit SMC death. This finding confirms previous results showing that CHOP−/− cells are still capable of undergoing ER stress−induced apoptosis, albeit with lower efficiencies (McCullough et al., 2001). Second, SMC viability was not affected by the well known ER−stress inducer thapsigargin that strongly stimulates CHOP expression, confirming previous observations (Martinet et al., 2007a). Third, only prolonged or severe ER stress seems to result in apoptotic cell death (Okada et al., 2004), and one of the pathways involved is caspase−12 activation (Lamkanfi et al., 2004; Momoi, 2004). In the present study, we showed that puromycin induced caspase−12 activation, whereas thapsigargin did not. Nonetheless, the caspase−12 inhibitor zATADfmk did not affect puromycin−induced SMC death. This finding is in agreement with the observation that cells lacking caspase−12 are still capable of undergoing ER stress−mediated cell death (Nakagawa et al., 2000). Furthermore, it has been reported that hypoxia−induced ER stress−mediated cell death could be prevented by the chemical chaperone 4−PBA, which was accompanied by an approximately 90% decrease in CHOP protein expression and an almost 30% restoration of basal pro− caspase−12 levels (Qi et al., 2004). However, in the present study, 4−PBA hardly affected SMC viability, eIF2α phosphorylation, and CHOP protein expression after puromycin treatment. Therefore, also mechanisms beyond ER stress have to be considered in puromycin−induced SMC death. Microarray analysis did not reveal differential gene expression in puromycin− and cycloheximide−treated SMCs, which excludes transcriptional activation of proapoptotic pathways. However, unlike cycloheximide or thapsigargin, puromycin triggered intracellular accumulation of polyubiquitinated proteins. Ubiquitination allows clearance of misfolded proteins through proteasomal degradation (Garcia−Mata et al., 2002). Because puromycin did not inhibit the proteasome, accumulation of polyubiquitinated proteins may reflect an overwhelming synthesis of misfolded proteins that cannot be degraded by the proteasome. These misfolded and/or truncated proteins potently activate apoptotic pathways, and they are prone to aggregation or other gain−of−function toxicities that may damage the cell (Hashimoto et al., 2003; Patterson et al., 2007).

In conclusion, puromycin induces apoptotic cell death of both macrophages and SMCs in rabbit atheroma. These findings are in contrast to the plaque−stabilizing effects that we showed previously with cycloheximide (Croens et al., 2007). However, SMC death upon puromycin treatment could only be partially prevented by cycloheximide, which completely blocked ER stress. Therefore, other or additional mechanisms, such as increased polyubiquitination of proteins, might be involved in puromycin−induced SMC death.

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