

The Protein Synthesis Inhibitor Anisomycin Induces Macrophage Apoptosis in Rabbit Atherosclerotic Plaques through p38 Mitogen-Activated Protein Kinase

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ABSTRACT

Because macrophages play a major role in atherosclerotic plaque destabilization, selective removal of macrophages represents a promising approach to stabilize plaques. We showed recently that the protein synthesis inhibitor cycloheximide, in contrast to puromycin, selectively depleted macrophages in rabbit atherosclerotic plaques without affecting smooth muscle cells (SMCs). The mechanism of action of these two translation inhibitors is dissimilar and could account for the differential effects on SMC viability. It is not known whether selective depletion of macrophages is confined to cycloheximide or whether it can also be achieved with translation inhibitors that have a similar mechanism of action. Therefore, in the present study, we investigated the effect of anisomycin, a translation inhibitor with a mechanism of action similar to cycloheximide, on macrophage and SMC viability. *In vitro*, anisomycin induced apoptosis of macrophages in a concentration-dependent manner, whereas SMCs were only affected at higher concentrations. *In vivo*, anisomycin selectively decreased the macrophage content of rabbit atherosclerotic plaques through

apoptosis. The p38 mitogen-activated protein kinase (MAPK) inhibitor SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole] prevented anisomycin-induced macrophage death, without affecting SMC viability. SB202190 decreased anisomycin-induced p38 MAPK phosphorylation, did not alter c-Jun NH₂-terminal kinase (JNK) phosphorylation, and increased extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. The latter effect was abolished by the mitogen-activated protein kinase kinase 1/2 inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl)butadiene ethanolate], although the prevention of anisomycin-induced macrophage death by SB202190 remained unchanged. The JNK phosphorylation inhibitor SP600125 did not affect anisomycin-induced macrophage or SMC death. In conclusion, anisomycin selectively decreased the macrophage content in rabbit atherosclerotic plaques, indicating that this effect is not confined to cycloheximide. p38 MAPK, but not ERK1/2 or JNK, plays a major role in anisomycin-induced macrophage death.

Atherosclerosis is a chronic inflammatory disease of the large- and medium-sized arteries and is characterized by the formation of plaques in the intima. Advanced atherosclerotic plaques have a large necrotic core that is surrounded by numerous macrophage-derived foam cells and is separated from the lumen by a protective fibrous cap consisting of smooth muscle cells (SMCs) and extracellular matrix (Lusis,

2000; Libby and Theroux, 2005). Because macrophages are a prominent feature in all stages of atherosclerosis and because their numbers are highly increased in shoulder regions of plaques that tend to rupture (van der Wal et al., 1994), macrophages are believed to play a role in plaque destabilization (Takahashi et al., 2002). They produce matrix metalloproteinases (Galis and Khatri, 2002; Johnson, 2007) and induce SMC death (Boyle et al., 2001), resulting in decreased synthesis of interstitial collagen and further thinning of the extracellular matrix. In line with these findings, selective removal of macrophages from atherosclerotic plaques via macrophage-specific initiation of cell death represents a promising strategy to stabilize rupture-prone plaques. We

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ABBREVIATIONS: SMC, smooth muscle cell; MAPK, mitogen-activated protein kinase; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; MEK, mitogen-activated protein kinase kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl)butadiene ethanolate; JNK, c-Jun NH₂-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; H/E, hematoxylin/eosin; BAL, bronchoalveolar; AN, anisomycin; ANOVA, analysis of variance.

showed recently that the protein synthesis inhibitor cycloheximide selectively decreased the macrophage content of rabbit atherosclerotic plaques (Croons et al., 2007). We hypothesized that macrophages are more dependent on protein synthesis for survival than SMCs because arterial macrophage-derived foam cells consume 3 times more oxygen than SMCs (Björnheden and Bondjers, 1987). Moreover, inhibition of translation in SMCs induces a modulation toward a differentiated, quiescent, contractile phenotype (Martin et al., 2004), which makes them even more resistant to inhibition of protein synthesis. However, in contrast to cycloheximide, the protein synthesis inhibitor puromycin induced cell death of both macrophages and SMCs (Croons et al., 2008). The major dissimilarity between these two protein synthesis inhibitors is their mechanism of action (Azzam and Algranati, 1973; Stöcklein and Piepersberg, 1980), which is in part responsible for their differential effect on SMC viability (Croons et al., 2008). Cycloheximide inhibits peptide bond formation, and the nascent peptide remains attached to the polyribosome (Grollman, 1967; Stöcklein and Piepersberg, 1980). In contrast, puromycin is an aminoacyl-tRNA structure analog that causes the release of unfinished polypeptide chains (Azzam and Algranati, 1973). The presence of unfinished polypeptide chains activates endoplasmic reticulum-stress associated cell death pathways (Croons et al., 2008), which are in part responsible for puromycin-induced SMC death. However, it is not known whether selective depletion of macrophages is confined to cycloheximide or also can be achieved with protein translation inhibitors that have a similar mechanism of action such as anisomycin. Therefore, in the present study, we investigated the effect of anisomycin on macrophage and SMC viability and the role of mitogen-activated protein kinases (MAPKs) herein.

Materials and Methods

Cell Culture. The murine macrophage cell line J774A.1 (American Type Culture Collection, Manassas, VA) was grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 20 U/ml polymyxin B sulfate in a humidified 5% carbon dioxide incubator at 37°C. Rabbit alveolar macrophages were obtained from New Zealand White rabbits fed a normal chow by bronchoalveolar lavage. In brief, rabbits were sacrificed with an overdose of sodium pentobarbital (CEVA Santé Animale, Libourne, France). The lungs were carefully removed and lavaged four times with 50 ml of phosphate-buffered saline (Invitrogen). Cells were washed twice with phosphate-buffered saline and resuspended in serum-containing RPMI 1640 medium. SMCs were isolated from rabbit aorta by collagenase type 2 (Worthington Biochemicals, Freehold, NJ) and elastase (Sigma-Aldrich, St. Louis, MO) digestion (60–90 min, 37°C) at 300 and 5 U/ml final concentration, respectively, and cultured in serum-containing Ham's F-10 medium (Invitrogen). After overnight incubation at 37°C, nonadherent cells were washed away, and medium was replaced. Cells were treated with anisomycin (Sigma-Aldrich) at different concentrations (0–40 µM) for 24 h. For rescue experiments, the p38 MAPK phosphorylation inhibitor SB202190 (Sigma-Aldrich), the mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor U0126 (Sigma-Aldrich), and the c-Jun NH₂-terminal kinase (JNK)/stress-activated protein kinase (SAPK) phosphorylation inhibitor SP600125 (Axxora Life Sciences, Inc., San Diego, CA) were used.

To examine de novo protein synthesis, cells were treated with anisomycin (35 µM) for 1 h and pulse-labeled for 30 min at 37°C with

5 µCi 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, 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. Evaluation of cell viability was based on the incorporation of the supravital dye neutral red by viable cells (Löwik et al., 1993).

To examine 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, then supplemented with 5-µl volumes of DNase-free RNase A (2 mg/ml) 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 Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 7.4). After electrophoresis in 2% agarose E-gel (Invitrogen), DNA laddering was visualized under UV light.

Western Blot Analysis. Cells were lysed in an appropriate volume of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Cell lysates were heat 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 containing 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 Femo Maximum Sensitivity Substrate (Pierce Chemical, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). The following rabbit primary antibodies were used: anti-caspase-3, anti-extracellular signal-regulated kinases (ERKs) 1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-JNK/SAPK (clone 56G8), anti-phospho-JNK/SAPK (Thr183/Tyr185), anti-p38 MAPK, and anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology Inc., Danvers, MA). Peroxidase-conjugated secondary antibodies were purchased from Dako Denmark A/S (Glostrup, Denmark).

In Vitro Treatment of Atheroma-Like Lesions. Male New Zealand White rabbits (3.3–3.9 kg, *n* = 8) were fed a diet supplemented with cholesterol (1.5%) for 14 days. After anesthesia with sodium pentobarbital (30 mg/kg i.v.), a nonocclusive, biologically inert, flexible silicone collar was placed around both carotid arteries and closed with silicone glue to induce atheroma-like lesions (i.e., intimal thickenings consisting of SMCs and macrophages) (Booth et al., 1989; Kockx et al., 1992; De Meyer et al., 1997). After another 14 days, while continuing the cholesterol diet, the animals were sacrificed by an overdose of sodium pentobarbital. Carotid arteries were prepared free from surrounding tissues and released from the collars. Two rings were cut from each collared segment and were incubated in serum-containing Ham's F-10 medium for 3 days in the presence or absence of 4 µM anisomycin. The medium was refreshed daily. After treatment, the carotid artery rings were formalin fixed for 24 h.

In Vivo Treatment of Atheroma-Like Lesions. Atheroma-like lesions were induced in the carotid artery of male New Zealand White rabbits (3.3–3.9 kg, *n* = 13) by positioning a silicone collar and feeding a cholesterol-rich diet (see above). Fourteen days after collar placement, an osmotic minipump (type 2ML1; Alzet, Cupertino, CA) was connected to each collar (Croons et al., 2007). The pumps delivered 10 µl of solution (saline, 4 or 20 µM anisomycin) per hour locally to the carotid artery for 3 days. Thereafter, the rabbits were heparinized (150 U/kg) (Leo Pharmaceuticals, Ballerup, Denmark) and sacrificed by an overdose of sodium pentobarbital. Two rings were cut from each collar-wrapped segment; one was formalin-fixed for 24 h, and another was snap-frozen in liquid nitrogen.

Histological Examination. Formalin-fixed carotid artery rings were paraffin-embedded and stained with hematoxylin/eosin (H/E) or Verhoeff's elastin. Immunohistochemical detection was carried out using an indirect antibody conjugate technique (Kockx et al., 1992; De Meyer et al., 2000). The following mouse primary antibodies were used: anti- α -SMC actin (clone 1A4; Sigma-Aldrich) and anti-rabbit macrophage (clone RAM11; DakoCytomation Denmark A/S) on paraffin sections and anti-CD31 (JC/70A; DakoCytomation Denmark A/S) on frozen sections. Rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (DakoCytomation Denmark A/S) was used to detect α -SMC actin. The avidin-biotin complex staining kit (Vectorstain ABC-PO kit; Vector Laboratories, Burlingame, CA) was applied to visualize RAM11 and CD31. Fluorescent double staining for caspase activation and SMCs was performed on frozen sections with a caspase detection kit (Calbiochem, San Diego, CA) and mouse anti- α -SMC actin antibody (clone 1A4; Sigma-Aldrich). The latter antibody was visualized by Alexa fluor 546-labeled anti-mouse secondary antibody (Invitrogen).

The images were analyzed using a color image analysis system (Image-Pro Plus 4.1; MediaCybernetics, Inc., Bethesda, MD). Fluorescence images were taken with a confocal laser scanning microscope (LSM510; Carl Zeiss Inc., Thornwood, NY) and further analyzed using LSM510 imaging software (Carl Zeiss Inc.). RAM11-positive areas and α -SMC actin-positive areas were determined in six random regions of interest ($600 \times 450 \mu\text{m}$ each). Fragmented nuclei were counted in four random regions of interest ($160 \times 120 \mu\text{m}$ each) on H/E-stained sections and expressed as the number of intact nuclei per 10^{-2}mm^2 . The area of the intima and the media was measured via planimetry on sections stained for elastin.

Statistical Analysis. All statistical analyses were carried out with SPSS 14.0 software (SPSS Inc., Chicago, IL). Differences were considered significant at $p < 0.05$. The statistical tests that were used are mentioned in the figure legends and table.

Results

Macrophages Were More Sensitive to Anisomycin-Induced Apoptosis than Rabbit Aortic Smooth Muscle Cells. J774A.1 macrophages, rabbit bronchoalveolar (BAL) macrophages, and SMCs isolated from rabbit aorta were treated in vitro with the protein synthesis inhibitor, anisomycin. Cell death was initiated in both cell types in a concentration-dependent manner (Fig. 1A). There was a marked difference in sensitivity between macrophages and SMCs, the latter being more resistant to anisomycin treatment (Fig. 1A). Both J774A.1 macrophages and SMCs treated with $35 \mu\text{M}$ anisomycin showed signs of apoptosis, such as caspase-3 cleavage and DNA laddering (Fig. 1B). At this concentration, anisomycin blocked de novo protein synthesis by $96 \pm 1\%$ and $93 \pm 1\%$ in J774A.1 macrophages and SMCs, respectively.

Anisomycin Decreased the Macrophage, but Not the SMC Content, of Rabbit Carotid Artery Rings in Vitro. Collared carotid artery segments from hypercholesterolemic rabbits were exposed to $4 \mu\text{M}$ anisomycin for 3 days in vitro. Thereafter, RAM11 and α -SMC actin immunostains were performed to quantify the amount of macrophages and SMCs, respectively. In the atheroma-like lesions, anisomycin decreased the macrophage content but did not significantly affect the amount of SMCs (Fig. 1C). In addition, the SMC content of the media was not affected by anisomycin. Higher concentrations of anisomycin did not further decrease the macrophage content in the intima (data not shown).

Anisomycin Selectively Decreased the Macrophage Content in Rabbit Atheroma-Like Lesions in Vivo. Anisomycin was locally administered in vivo by connecting an

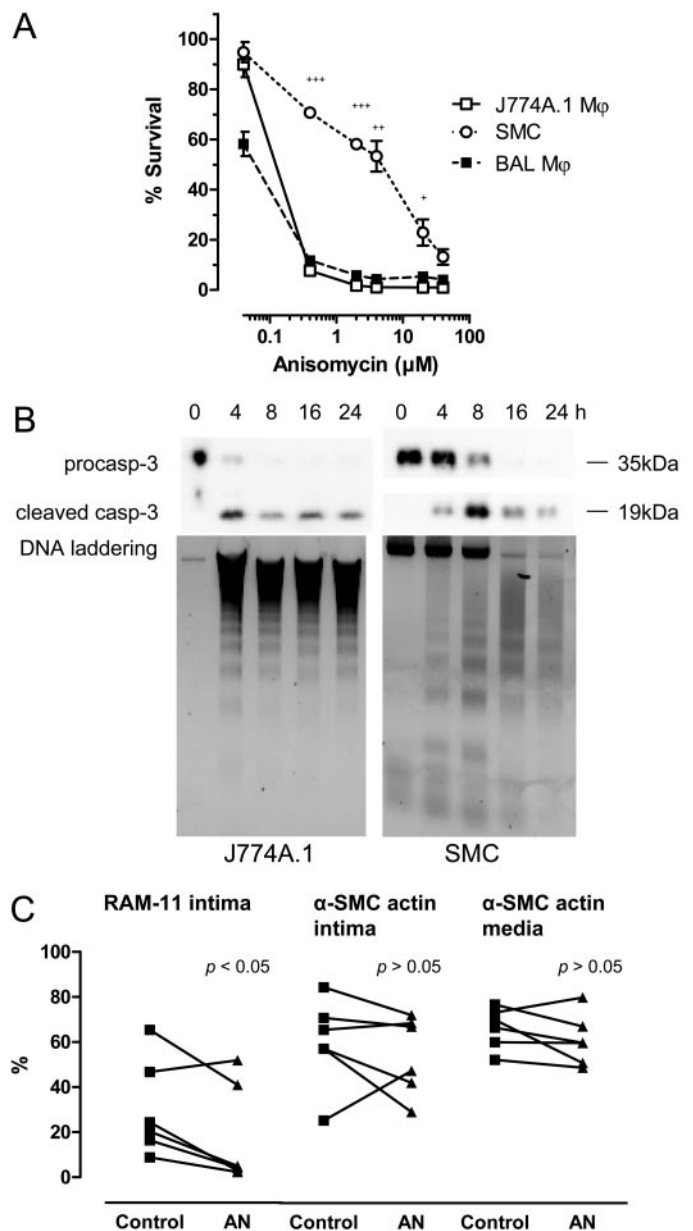


Fig. 1. Effect of anisomycin on macrophages and smooth muscle cells in culture or in rabbit atherosclerotic carotid artery rings. **A**, J774A.1 macrophages (M ϕ), rabbit BAL macrophages, and rabbit aortic smooth muscle cells (SMCs) were treated for 24 h with anisomycin (0.04–40 μM). Data are presented as mean \pm S.E.M. of three independent experiments all performed in duplicate. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$ J774A.1 versus SMCs (independent samples t test). **B**, J774A.1 macrophages (left) or rabbit aortic SMCs (right) were treated with $35 \mu\text{M}$ anisomycin for 0 to 24 h. Cleavage of procaspase-3 (top) and DNA laddering (bottom) were analyzed using Western blotting and agarose gel electrophoresis, respectively. **C**, atherosclerotic rabbit carotid artery rings were incubated in vitro with $4 \mu\text{M}$ anisomycin for 3 days. Macrophages and SMCs were quantified, and data are presented as paired values ($n = 6$ rabbits). *, $p < 0.05$ versus control (Wilcoxon matched pairs signed-rank test).

osmotic minipump to a collar around the rabbit carotid artery. The area of the atheroma-like lesion or the media was not affected by anisomycin treatment (Table 1). Anisomycin (20 μM in the osmotic minipump) significantly decreased the macrophage content (Fig. 2A) and increased the amount of nuclear fragments (Fig. 3A) in the intima, without affecting the α -SMC-positive area either in the intima or in the media (Fig. 2B). In the media, the number of nuclear fragments tended to increase,

TABLE 1

Effect of in vivo treatment of atherosclerotic rabbit carotid arteries with 4 or 20 μM anisomycin in the osmotic minipump for 3 days on intimal and medial area

Data are shown as mean \pm S.E.M.; $n = 5-11$; $p > 0.05$ (Kruskal-Wallis test).

	Control	Anisomycin	
		4 μM	20 μM
Intimal area	0.25 \pm 0.04	0.26 \pm 0.03	0.20 \pm 0.04
Medial area	0.60 \pm 0.04	0.58 \pm 0.02	0.56 \pm 0.03

although this effect was statistically not significant ($p = 0.14$, Fig. 3B). Fluorescein isothiocyanate-labeled Val-Ala-DL-Asp(O-methyl)-fluoromethylketone labeling for caspase activation was present in the intima and rarely in the media but did not colocalize with α -SMC actin staining (Fig. 3C). The number of intact nuclei in the media consistently did not change significantly after anisomycin treatment (25 ± 1 versus 24 ± 1 nuclei/ 0.01 mm^2 media in control versus anisomycin, respectively). The staining for the endothelial cell marker CD31 was similar in anisomycin-treated plaques compared with control plaques ($94 \pm 5\%$ versus control).

Anisomycin Altered Phosphorylation Patterns of Mitogen-Activated Protein Kinases. Western blot analysis of MAPK signaling in macrophages (J774A.1 or BAL) and SMCs did not reveal major differences between the cell types (Fig. 4). Similar to J774A.1 macrophages, p38 MAPK and JNK/SAPK were hyperphosphorylated in primary rabbit

macrophages. Although JNK phosphorylation was much more difficult to pick up compared with p38 MAPK phosphorylation in primary rabbit macrophages. ERK1/2 was rapidly dephosphorylated in J774A.1 and BAL macrophages. In SMCs, p38 MAPK, JNK/SAPK, and ERK1/2 showed a transient phosphorylation status (increased phosphorylation followed by dephosphorylation).

SB202190, but Not SP600125, Selectively Prevented Anisomycin-Induced Macrophage Death. J774A.1 macrophages or SMCs were preincubated with the p38 MAPK phosphorylation inhibitor SB202190 or the JNK/SAPK phosphorylation inhibitor SP600125 for 1 h, followed by 24 h of treatment with anisomycin (0.4 or 4 μM). SB202190 (10 μM) partially prevented anisomycin-induced macrophage cell death (Fig. 5A). In contrast to macrophages, SB202190 was unable to affect anisomycin-induced SMC death (Fig. 5A). SP600125 (10 or 50 μM , respectively) had no effect on aniso-

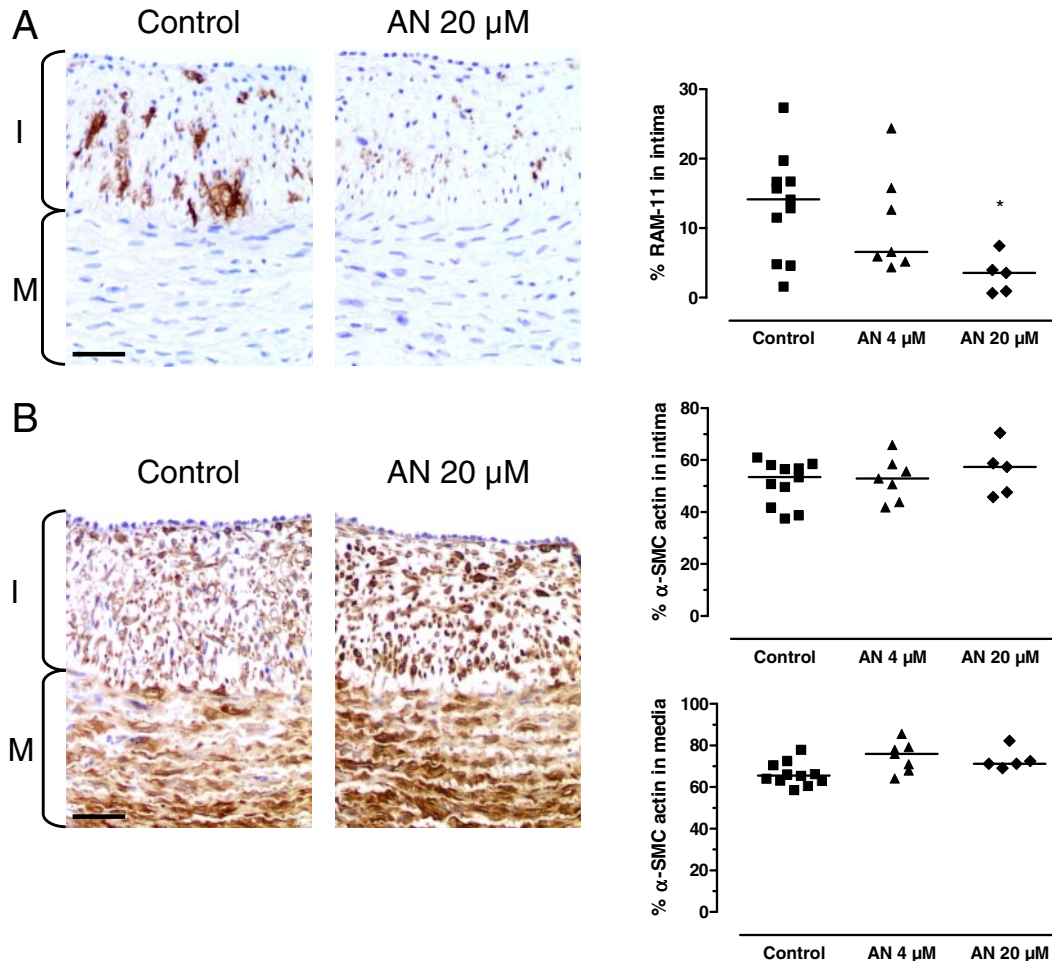


Fig. 2. In vivo effect of anisomycin locally administered for 3 days via an osmotic minipump that was connected to a collar around atherosclerotic rabbit carotid arteries. A, immunoreactivity (left) and quantification (right) of RAM11 (brown = macrophages). B, immunoreactivity (left) and quantification (right) for α -SMC actin (brown = SMCs), treated for 3 days with saline (control) or 4 or 20 μM anisomycin (AN) in the osmotic minipump. I, intima; M, media; bar, 50 μm . Individual values are shown with the median. *, $p < 0.05$ versus control (Kruskal-Wallis test followed by Dunn's multiple comparison test).

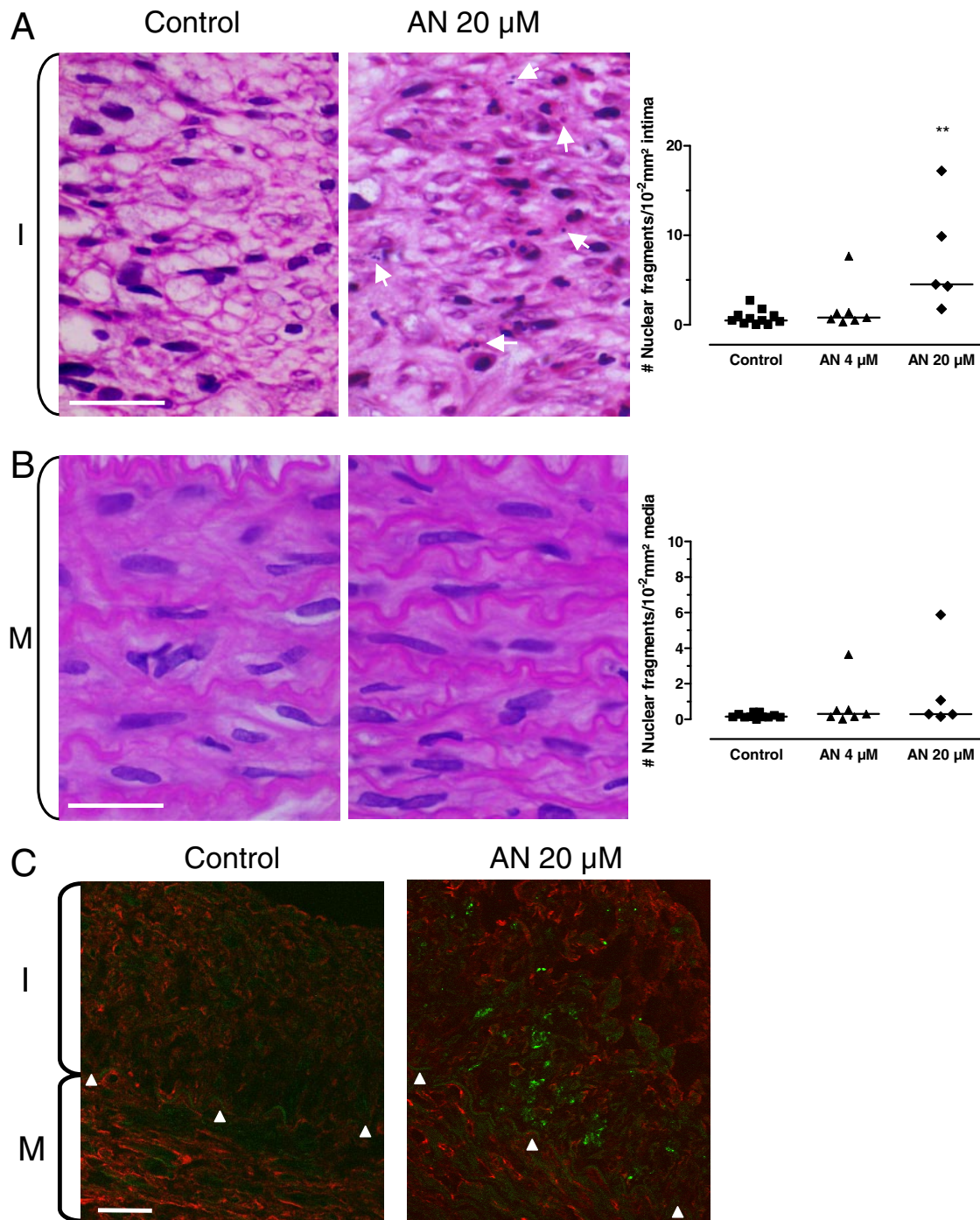


Fig. 3. Determination and characterization of cell death induced by AN (4 or 20 μM) locally administered for 3 days via an osmotic minipump that was connected to a collar around atherosclerotic rabbit carotid arteries. Nuclear fragments were counted in the intima (A) and in the media (B) on H/E stainings and expressed per 10^{-2}mm^2 . Individual values are shown with the median. *, $p < 0.05$ versus control (Kruskal-Wallis test followed by Dunn's multiple comparison test). C, apoptotic cells were visualized using fluorescein isothiocyanate-labeled Val-Ala-DL-Asp(O-methyl)-fluoromethylketone labeling (green), whereas smooth muscle cells were detected by immunohistochemical staining for α -SMC actin (red). Bar, 25 μm ; I, intima; M, media; arrowheads, fragmented nuclei; triangles, internal elastic lamina.

mycin-induced macrophage or SMC death (Fig. 5B). Western blot analysis showed that expression levels of phosphorylated p38 MAPK, JNK/SAPK, and ERK1/2 after 1 h of treatment with anisomycin (0.4 μM) were different between macrophages and SMCs ($p < 0.05$, independent samples t test) (Fig. 6). SB202190 (10 μM) decreased anisomycin-induced phosphorylation of p38 MAPK in macrophages and in SMCs (Fig. 6). SB202190 also modestly increased ERK1/2 phosphoryla-

tion but had no effect on JNK phosphorylation in both cell types. SP600125 (10 or 50 μM , respectively) decreased anisomycin-induced phosphorylation of JNK/SAPK in macrophages and in SMCs and had no effect on the phosphorylation patterns of p38 MAPK or ERK1/2 (Fig. 7). In macrophages, SP600125 (50 μM) further decreased anisomycin-induced JNK/SAPK phosphorylation (data not shown) but caused $80 \pm 6\%$ cell death after 24 h of treatment.

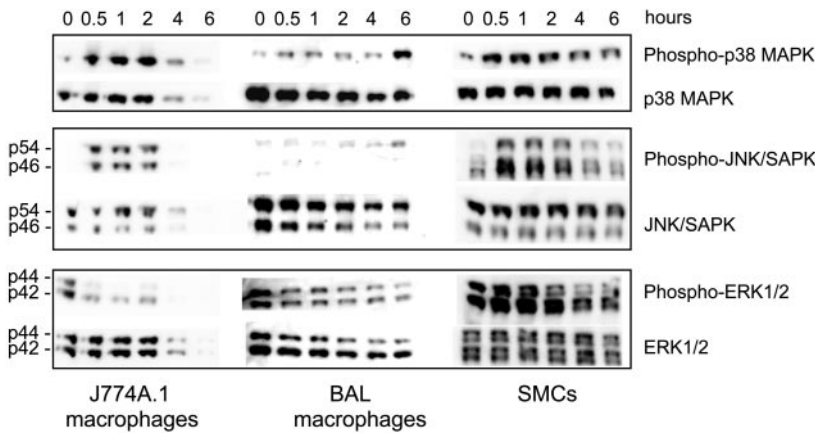


Fig. 4. Western blot analysis of phosphorylation patterns of MAPK in J774A.1 macrophages, rabbit bronchoalveolar macrophages, and rabbit aortic SMCs before and after anisomycin treatment. Cells were treated with 35 μ M anisomycin for 0 to 6 h.

The Protective Effects of SB202190 on Anisomycin-Induced J774A.1 Macrophage Death Were Due to Inhibition of p38 MAPK Phosphorylation but Not to Increased ERK Phosphorylation. J774A.1 macrophages were pretreated for 1 h with different concentrations of the MEK1/2 inhibitor U0126, alone or in combination with SB202190, followed by 24 h of treatment with anisomycin (0.4 μ M). U0126 is commonly used to inhibit ERK1/2 phosphorylation/activation (English and Cobb, 2002) but induced only a very small amount of cell death ($14 \pm 3\%$) at the highest concentration used (10 μ M). Moreover, the protective

effects of SB202190 on anisomycin-induced macrophage death were not altered by U0126 (Fig. 8A). However, Western blot analysis clearly showed that U0126 concentration-dependently abolished the increase in ERK phosphorylation induced by SB202190 to levels even lower than those obtained by anisomycin alone (Fig. 8B).

Discussion

We showed previously that the protein synthesis inhibitor cycloheximide selectively decreases the macrophage content

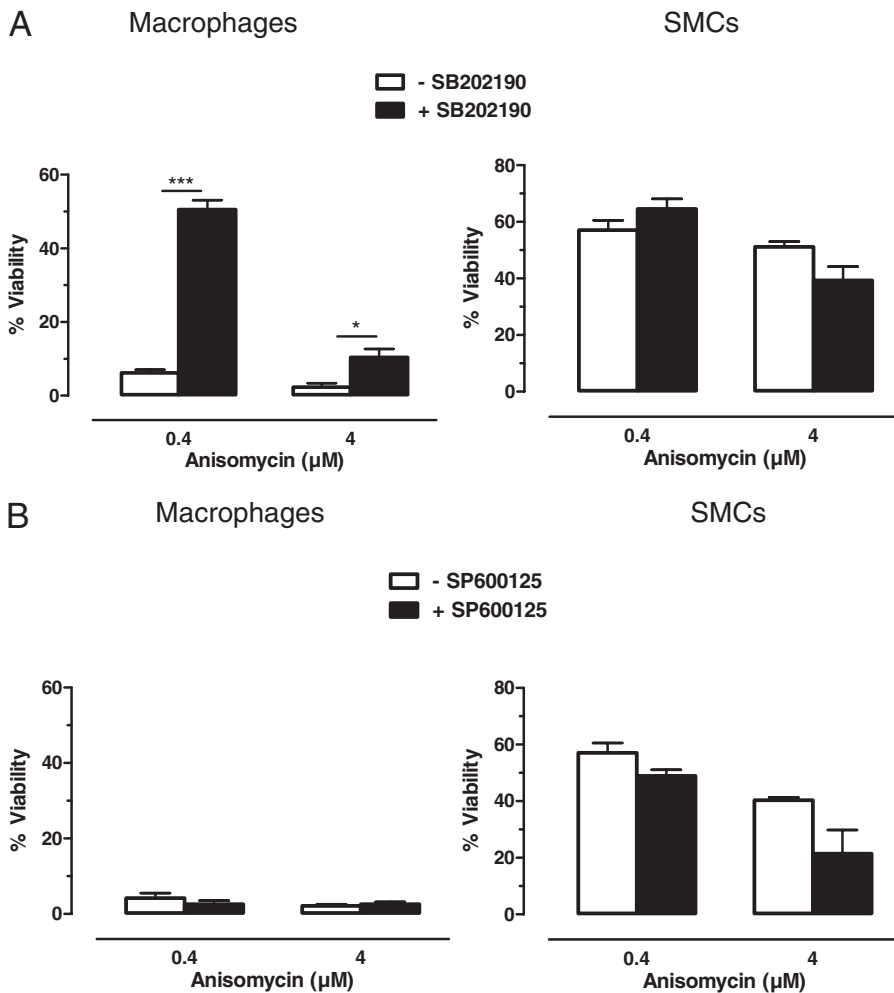


Fig. 5. Effect of the p38 MAPK phosphorylation inhibitor SB202190 and the JNK/SAPK phosphorylation inhibitor SP600125 on anisomycin-treated macrophages and SMCs. A, J774A.1 macrophages (left) or rabbit aortic SMCs (right) were pretreated for 1 h with 10 μ M SB202190, followed by 24 h of treatment with anisomycin (0.4 or 4 μ M). B, J774A.1 macrophages (left) or rabbit aortic SMCs (right) were pretreated for 1 h with 10 or 50 μ M SP600125, respectively, followed by 24 h of treatment with anisomycin (0.4 or 4 μ M). Cell viability was examined by neutral red assays. Data are presented as mean \pm S.E.M. of at least three independent experiments all performed in duplicate. *, $p < 0.05$; ***, $p < 0.001$ versus without SB202190 [analysis of variance (ANOVA), followed by Dunnett test].

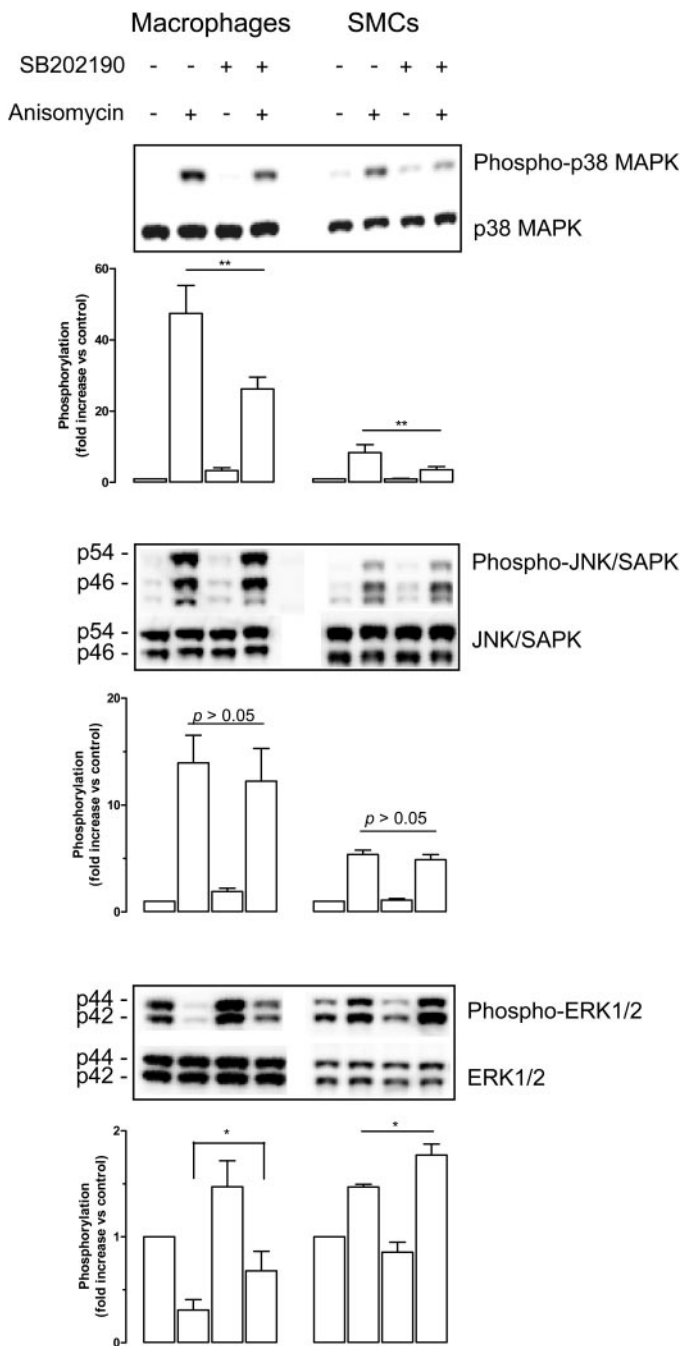


Fig. 6. Western blot analysis of the effects of SB202190 on phosphorylation of MAPK. J774A.1 macrophages (left) or rabbit aortic SMCs (right) were pretreated for 1 h with 10 μ M SB202190 followed by 1 h of treatment with anisomycin (0.4 μ M). The effect of SB202190 on phosphorylation patterns of MAPK was evaluated by Western blot analysis, and the optical density of the phosphorylated MAPK protein bands was normalized to the total MAPK protein bands. Data represent the mean \pm S.E.M. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ versus without SB202190 [ANOVA with treatment (+/-SB202190) and cell type (macrophages, SMCs) as between-subject factors].

of rabbit atherosclerotic plaques (Croons et al., 2007). In agreement with these findings, we show here that anisomycin also selectively decreases the macrophage content of rabbit atherosclerotic plaques through macrophage apoptosis. Therefore, selective depletion of macrophages is not confined to cycloheximide but also can be achieved with protein translation inhibitors that have a similar mechanism of action.

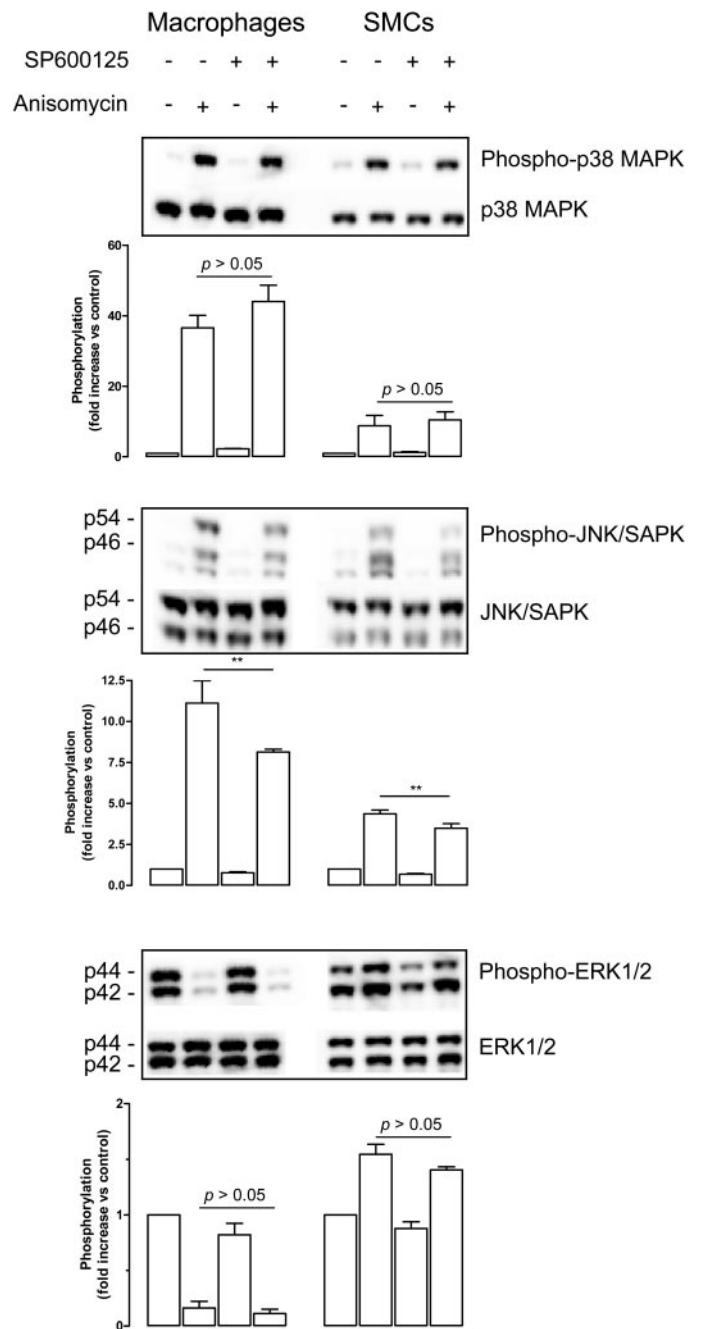


Fig. 7. Western blot analysis of the effects of SP600125 on phosphorylation of MAPK. J774A.1 macrophages (left) or rabbit aortic SMCs (right) were pretreated for 1 h with 10 or 50 μ M SP600125, respectively, followed by 1 h of treatment with anisomycin (0.4 μ M). The effect of SP600125 on phosphorylation patterns of MAPK was evaluated by Western blot analysis, and the optical density of the phosphorylated MAPK protein bands was normalized to the total MAPK protein bands. Data represent the mean \pm S.E.M. of three independent experiments. **, $p = 0.01$ versus without SP600125 [ANOVA with treatment (+/-SP600125) and cell type (macrophages, SMCs) as between-subject factors].

These findings are important in terms of atherosclerotic plaque stabilization because macrophages and their secretory products have a tremendous impact on plaque destabilization, accounting for most acute coronary syndromes (Halvorsen et al., 2008). Therefore, selective, clean, and safe removal of macrophages within the atherosclerotic plaque is thought to be beneficial for plaque stability. However, be-

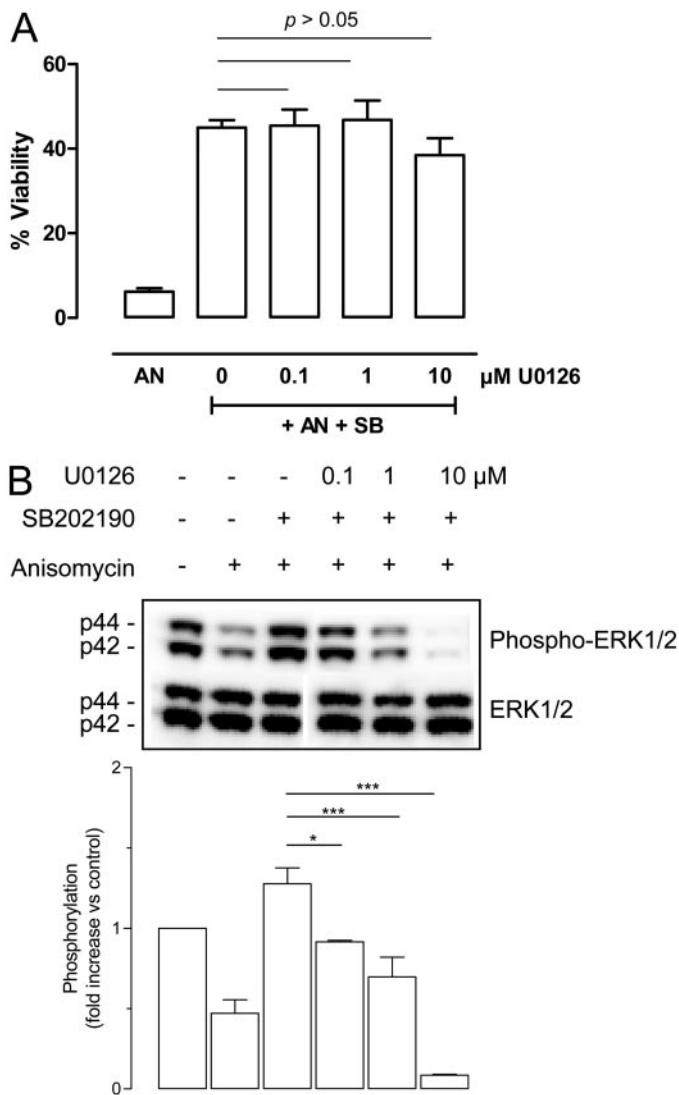


Fig. 8. Effect of the MEK1/2 inhibitor U0126 on the protective effect of SB202190 on anisomycin-induced macrophage death. **A**, J774A.1 macrophages were pretreated for 1 h with SB202190 (SB, 10 μ M) alone or in combination with U0126 (U, 0.1–10 μ M), followed by 24 h of treatment with AN (0.4 μ M). Cell viability was examined by neutral red assays. Data are presented as mean \pm S.E.M. of three independent experiments all performed in duplicate. $p > 0.05$ versus AN + SB (ANOVA, followed by Dunnett test). **B**, J774A.1 macrophages were pretreated for 1 h with SB202190 (10 μ M) alone or in combination with U0126 (0.1–10 μ M) followed by 1 h of treatment with anisomycin (0.4 μ M). The effect of U0126 on the SB202190-induced increase in ERK phosphorylation was evaluated by Western blot analysis. The optical density of the phosphorylated ERK1/2 protein bands was normalized to the total ERK protein bands. Data represent the mean \pm S.E.M. of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$ versus SB + AN (ANOVA, followed by Dunnett test).

cause of the lack of suitable animal models for plaque instability (Ni et al., 2009), it remains to be identified whether selective depletion of macrophages through apoptosis contributes to plaque stability (Martinet et al., 2007; V. Croons, W. Martinet, and G.R.Y. De Meyer, submitted for publication).

Another unresolved question is why protein synthesis inhibitors like cycloheximide and anisomycin selectively induce apoptosis in macrophages, but not or to a lesser extent in SMCs. In both macrophages and SMCs, anisomycin equally inhibited protein synthesis, which excludes a different uptake of the drug or changes in protein expression as a possi-

ble explanation for selective macrophage death. However, divergent posttranslational modifications, such as phosphorylation and dephosphorylation, might be involved. It has been shown previously that MAPK play a major role in anisomycin-induced apoptosis (Stadheim and Kucera, 2002; Wang et al., 2005). Therefore, we investigated the effects of anisomycin on phosphorylation of MAPK and the potential relationship with anisomycin-induced macrophage or SMC apoptosis. There are three major branches of MAPK signaling, including p38, JNK/SAPK, and ERK (Seeger and Krebs, 1995; Widmann et al., 1999; Pearson et al., 2001; Raman et al., 2007; Junttila et al., 2008). p38 and JNK/SAPK are activated by stress stimuli (e.g., translation inhibitors, such as anisomycin) and have been associated with apoptosis (Ono and Han, 2000; Barr and Bogoyevitch, 2001; Junttila et al., 2008). However, depending on the stimuli and cellular conditions, these two pathways can also mediate cell proliferation and survival (Junttila et al., 2008). ERK is activated by growth factors and phorbol esters and contributes to cell proliferation, differentiation, and survival. Moreover, cross talk between these pathways influences the cellular outcome. During apoptosis, for example, the stress-activated JNK/SAPK and p38 pathways suppress the prosurvival activity of the ERK pathway, thereby lowering the threshold for JNK/p38-mediated apoptosis (Xia et al., 1995; Junttila et al., 2008). In this study, anisomycin modified phosphorylation of the three MAPK branches in both cell types. p38 MAPK and JNK/SAPK were hyperphosphorylated in macrophages, whereas ERK1/2 was rapidly dephosphorylated. In SMCs, p38 MAPK, JNK/SAPK, and ERK1/2 showed a transient phosphorylation status (increased phosphorylation followed by dephosphorylation). To further elucidate the involvement of MAPK phosphorylation in anisomycin-induced macrophage and SMC death, we subsequently used inhibitors of MAPK phosphorylation. The JNK/SAPK phosphorylation inhibitor SP600125 decreased anisomycin-induced JNK/SAPK phosphorylation to a similar extent in both cell types and did not have an effect on the other MAPK phosphorylation patterns. Pretreatment of macrophages or SMCs with SP600125 did not affect anisomycin-induced cell death. Therefore, JNK/SAPK phosphorylation is not involved in anisomycin-induced macrophage or SMC apoptosis. In contrast, the p38 MAPK phosphorylation inhibitor SB202190 prevented anisomycin-induced macrophage but not SMC death. Therefore, we investigated whether SB202190 affected anisomycin-induced alterations of MAPK phosphorylation in a different way in these two cell types. SB202190 decreased anisomycin-induced p38 MAPK phosphorylation in both macrophages and SMCs by approximately 50% and also modestly increased ERK phosphorylation equally in both cell types. Because the effect of SB202190 was similar in macrophages and SMCs, mechanisms other than modification of MAPK phosphorylation might account for anisomycin-induced decrease in SMC viability. Nevertheless, the levels of phospho-p38 MAPK were much lower in SMCs than in macrophages, which might explain the differential effect of SB202190 in the prevention of anisomycin-induced macrophage versus SMC death. Inhibition of the SB202190-induced increase of ERK1/2 phosphorylation by the MEK1/2 inhibitor U0126 had no effect on the prevention of anisomycin-induced macrophage death by SB202190. Therefore, p38 MAPK phosphorylation but not ERK1/2 dephosphorylation is involved in

anisomycin-induced macrophage apoptosis. An intriguing question is how p38 MAPK phosphorylation can orchestrate anisomycin-induced macrophage apoptosis. Upon activation, p38 MAPK phosphorylates several specific substrates, such as protein kinases and transcription factors, which can be involved in apoptosis (Widmann et al., 1999; Shi and Gaestel, 2002; Cuenda and Rousseau, 2007; Krishna and Narang, 2008). Various studies indicate that the role of p38 MAPK in apoptosis is cell type- and stimulus-dependent (Widmann et al., 1999; Shi and Gaestel, 2002). Therefore, it is very difficult to speculate about which downstream targets of p38 MAPK are involved in anisomycin-induced macrophage apoptosis. Nonetheless, we can exclude some of the possible substrates. Tumor suppressor protein p53 and CCAAT/enhancer-binding protein homologous protein, for example, can be activated by p38 MAPK and activate proapoptotic factors (Ono and Han, 2000; Shi and Gaestel, 2002; Jinlian et al., 2007). However, p53 is not detectable in J774A.1 macrophages and SMCs even after stimulation with protein synthesis inhibitors (Croons et al., 2007). In accordance, Higami et al. (2000) showed that p53 is not directly involved in cycloheximide-induced apoptosis. Similar to cycloheximide (Croons et al., 2008), CCAAT/enhancer-binding protein homologous protein was not detected at the protein level in macrophages or SMCs upon anisomycin treatment (data not shown). Other potential targets, such as MAPK-activated protein kinases and Cdc25s (Cuenda and Rousseau, 2007; Jinlian et al., 2007), may be involved in anisomycin-induced macrophage apoptosis through p38 MAPK.

In conclusion, our findings demonstrate that anisomycin decreases the macrophage content in rabbit atherosclerotic plaques, with limited effects on the viability of SMCs. p38 MAPK, but not JNK or ERK1/2, is involved in anisomycin-induced macrophage, but not SMC, death.

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