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Plasminogen activation: a mediator of vascular smooth muscle cell apoptosis**in atherosclerotic plaques**

Short title: Plasmin induces VSMC apoptosis in atherosclerosis

Patrick Rossignol^{*}, Aernout Luttun[□], Jose Luis Martin-Ventura[§], Florea Lupu^{**}, Peter Carmeliet[□],
Désiré Collen^{*□}, Eduardo Anglès-Cano[§], and Henri Roger Lijnen^{*}

^{*}Center for Molecular and Vascular Biology, University of Leuven, Campus Gasthuisberg, O&N, Herestraat 49, B-3000 Leuven, Belgium [□]Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology (VIB), Campus Gasthuisberg, O&N, Herestraat 49, B-3000 Leuven, Belgium [§]INSERM U698, CHU Bichat-Claude Bernard, 46 rue Henri Huchard, 75877-Cedex Paris 18, France ^{**} Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA.

Corresponding author : H. R. Lijnen

Center for Molecular and Vascular Biology, K.U.Leuven, Campus Gasthuisberg, O&N, Herestraat 49, B-3000 Leuven, Belgium

Tel : +32 16 34 57 71

Fax : +32 16 34 59 90

Email : roger.lijnen@med.kuleuven.ac.be

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SUMMARY

Background: Apoptosis of vascular cells is considered to be a major determinant of atherosclerotic plaque vulnerability and potential rupture. Plasmin can be generated in atherosclerotic plaques and recent in vitro data suggest that plasminogen activation may trigger vascular smooth muscle cell (VSMC) apoptosis.

Objective : To determine whether plasminogen activation may induce aortic VSMC apoptosis ex vivo and in vivo.

Methods and results: Mice with single or combined deficiencies of ApoE and PAI-1 were used. Ex vivo incubation of plasminogen (1.3 μ M) with isolated aortic tunica media from PAI-1-deficient mice induced plasminogen activation and VSMC apoptosis, which was inhibited by α_2 -antiplasmin. In vivo, levels of plasmin, active caspase 3 and VSMC apoptotic index were significantly higher in atherosclerotic aortas from mice with combined ApoE^{-/-} and PAI-1^{-/-} deficiencies than in those from littermates with single ApoE deficiency. A parallel decrease in VSMC density was also observed.

Conclusions: These data strongly suggest that, in vivo, plasminogen activation may contribute to VSMC apoptosis in atherosclerotic plaques.

Keywords : apoptosis ; atherosclerosis ; genetically altered mice ; plasminogen; vascular smooth muscle cell.

Apoptosis is considered to be a major determinant of atherosclerotic plaque vulnerability and potential rupture¹⁻³. Little is known, however, about the mechanisms that trigger apoptosis of vascular cells in atherosclerotic plaques. In human atherosclerotic specimens, apoptotic vascular smooth muscle cells (VSMC) were observed in areas with loss of pericellular adhesion³, indicating that pericellular proteolysis could be involved⁴. Proteases capable of degrading pericellular matrix components are indeed secreted by resident vascular cells and infiltrating inflammatory cells.⁵ Components of the fibrinolytic system, including plasmin(ogen), plasminogen activators (either tissue-type, t-PA, predominantly expressed by VSMC⁶⁻⁸, or urokinase-type, u-PA, predominantly expressed by infiltrating macrophages), and plasminogen activator inhibitor-1 (PAI-1),⁹⁻¹¹ are present in human atherosclerotic lesions. The activity of plasmin promotes cell migration, regulates growth factor activity (e.g. Transforming Growth Factor- β , TGF- β)¹² and induces extracellular matrix proteolysis, either directly, via degradation of adhesive glycoproteins, such as fibronectin¹³ or laminin¹⁴ or indirectly, via activation of matrix metalloproteinases¹⁵. Actually, it was recently demonstrated, using primary cultures of rat and human arterial VSMCs, that t-PA constitutively secreted by these cells can generate plasmin on the cell surface and induces thereby the proteolysis of extracellular matrix proteins, cell retraction and finally cell detachment leading to VSMC apoptosis⁸.

The fibrinolytic system plays a complex role in atherosclerosis, as assessed in experimental models using mice with targeted inactivation of its main components.¹⁶ Thus, lack of PAI-1 in transgenic mice with combined apolipoprotein E and PAI-1 deficiencies (*ApoE^{-/-}:PAI-1^{-/-}*) resulted in elevated plasmin levels, accompanied with extracellular matrix desorganization, increased accumulation of macrophages and a reduced density of myofibroblasts in advanced atherosclerotic lesions¹⁷. On the basis of the deleterious effect of plasmin on VSMC survival,⁸ we

sought to investigate if plasminogen activation by VSMCs *ex vivo* on the one hand, and the increased plasmin generation within *ApoE^{-/-}:PAI-1^{-/-}* aortic plaques *in vivo* on the other hand could lead to VSMC apoptosis. We demonstrate that aortic VSMC apoptosis can be induced *ex vivo* by plasminogen activation. *In vivo*, VSMC apoptosis and plasmin activity were colocalized within atherosclerotic plaques. This mechanism may contribute to plaque destabilization and rupture.

METHODS

Reagents and animals

Ten weeks old mice with targeted inactivation of the gene encoding PAI-1 (*PAI-1*^{-/-}) and wild-type (*WT*) mice of the same genetic background (75% C57Bl/6 and 25% 129SV) and of either sex were obtained as described elsewhere¹⁸.

Human or murine plasminogen, plasmin, and α 2-antiplasmin were obtained as described^{19,20}.

Primary monoclonal antibodies used are described below. Secondary antibodies used were goat anti-mouse-Alexa fluor® 568 (Molecular Probes, Eugene, Oregon, USA) and biotinylated rabbit anti-mouse Ig (Dako, Glostrup, Denmark).

Animal models

For ex vivo experiments, *WT* and *PAI-1*^{-/-} mice were anesthetized and exsanguinated by perfusion at physiological pressure via cardiac puncture with a 0.9% NaCl solution. The aorta was dissected, the adventitia was extruded as described²¹, and the aorta was cut first longitudinally (to achieve de-endothelialization), and then transversely in 6 pieces of equal size. These sections were incubated in a humidified CO₂ incubator at 37°C for 4 hours in Dulbecco's modified Eagle's Medium (without phenol red) containing 2 mM glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin, with and without 1.3 μ M plasminogen, and in the absence or the presence of 1 μ M α 2-antiplasmin. The aorta fragments were transferred to Jung tissue freezing medium™ (Leica Instruments, Nussloch, Germany) and snapfrozen in precooled 2-methyl butane.

For in vivo experiments, *ApoE*^{+/+}:*PAI-1*^{-/-} mice were intercrossed with *ApoE*^{-/-}:*PAI-1*^{+/+} mice to generate breeding pairs with heterozygous deficiency of ApoE and PAI-1 (*ApoE*^{+/-}:*PAI-1*^{+/-}), which sired *ApoE*^{-/-}:*PAI-1*^{-/-} mice and *ApoE*^{-/-}:*PAI-1*^{+/+} littermate offspring with a mixed genetic

background of 87.5% C57Bl/6 and 12.5% 129/SvJ, as described¹⁷. Mice were kept on a regular chow diet for 5 weeks, and then fed a cholesterol /cholate rich diet for 25 weeks, as described¹⁷. Aorta embedding procedures were previously described¹⁷.

For all surgical procedures, mice were anesthetized by intraperitoneal injection of Nembutal (60 mg/kg ; Abbott Laboratories, North Chicago, IL, USA). All procedures were approved by the University Ethical Committee (P03112) and were performed in accordance with the guidelines of the International Society on Thrombosis and Haemostasis²².

Protein assays

For immunoblotting of plasmin(ogen) and active caspase 3, equal protein amounts from whole protein extracts (from in vivo experiments) were electrophoresed on a 15% acrylamide gel, under reducing conditions. The nonspecific sites of the membranes were blocked with 10% non-fat dry milk in Tris Buffered Saline (TBS) containing 1% Tween 20 (TBST). The membranes were incubated with a rabbit anti-human/mouse active caspase 3 antibody (R&D systems) or a rabbit anti-murine plasmin(ogen)²³ antibody overnight at 4°C in TBST containing 1% non-fat dry milk. Then, the membranes were washed and incubated with an anti-rabbit peroxidase-conjugated secondary antibody in TBST containing 1% non-fat dry milk. The membranes were washed with TBST, followed by detection with enhanced chemiluminescence (ECL kit, Amersham).

Fibrinolytic activity was monitored by fibrin overlay (containing traces of plasminogen, with and without aprotinin) of non-fixed 8- μ m arterial cryostat sections at 37°C for 24 hours²⁴.

Histological and immunohistochemical studies

Tissue sections (8 μ m thick) were stained with hematoxylin-eosin under standard conditions.

The following primary antibodies were used: for immunodetection of plasmin- α 2-antiplasmin complexes, 7 AP (a mouse monoclonal antibody that recognizes neoantigen epitopes in the

complexes but neither free plasmin nor α 2-antiplasmin²⁵), for plasmin(ogen) a rabbit anti-murine plasminogen/plasmin²³, for active caspase 3 a rabbit anti-human/mouse active caspase 3 (R&D systems) and for VSMC detection a mouse anti-human smooth muscle actin (DAKO). Biotinylated secondary antibodies were applied in combination with the Vectastain system (ABC kit, Vector Laboratories Inc, Burlingame, CA, USA), using the appropriate negative controls.

VSMC were detected in paraffin embedded sections from in vivo experiments using a primary mouse monoclonal antibody against human α -actin and a secondary goat anti-mouse antibody labelled with Alexa fluor® 568. A TUNEL reaction, using the appropriate negative controls (yielding FITC staining of apoptotic nuclei, with negligible background), and a nuclear counterstaining with DAPI were then performed. Cryosections from ex vivo experiments were also submitted to the TUNEL and DAPI reactions. Apoptotic VSMCs were defined as doubly (FITC and DAPI: ex vivo) or triply labelled cells (DAPI, FITC, and Alexa fluor 568: in vivo), and an apoptotic index was calculated using the formula: (number of TUNEL-positive VSMC/total VSMC)x100.

Images of the same microscopic (Zeiss Axioplan 2) fields were taken with each filter set (DAPI, FITC and Alexa 568), using a Zeiss AxioCam HRc digital camera with Zeiss Axiovision 3.0.6.38 SP4 Imaging Software, and were merged by using Adobe Photoshop software. Quantifications (areas and cell numbers) were performed by computer-assisted image analysis with the Zeiss KS300 Version 3.0 SP6 software. Briefly, the positive and negative cells within a defined area were counted automatically using a fixed threshold contrast. The operating system and the video-adaptor were Microsoft Windows 2000 SP1, and Matrox Meteor_II PCI frame grabber, respectively. Morphometric analyses were performed blinded for the genotype; for ex vivo experiments 18 areas in 6 sections (each 160 μ m apart) randomly selected through the aortic

segment were analyzed, and for in vivo experiments 18 areas randomly chosen in 3 sections (each 160 μm apart) were analyzed, the first one taken at the point where the cardiac valves were first visible.

Transmission electron microscopy was performed as described ²⁶.

Statistical analysis

The statistics were performed with the Statview 5.0 software. Results are expressed as mean \pm SEM or median (range). Comparisons were made by one-way analysis of variance with Scheffe's F test, or Wilcoxon signed ranks, or Mann-Whitney U-test, as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

Aortic VSMC apoptosis parallels plasminogen activation ex vivo

In order to isolate the effect of plasminogen activation on VSMC apoptosis from other cellular components of the vascular wall, we performed ex vivo experiments on isolated aortic tunica media from *WT* and *PAI-1^{-/-}* mice. The tunica media were incubated with plasminogen at 1.3 μM for 4 h, with or without 1 μM α_2 -antiplasmin. Plasmin generation developed faster in *PAI-1^{-/-}* than in *WT* aortas, as assessed by in situ fibrin zymography (Fig. 1A). VSMC apoptotic levels, as assessed by TUNEL, was not significantly different between both genotypes in the absence of plasminogen (n=9, $P=0.1$). Following plasminogen activation, *PAI-1^{-/-}* aortas displayed a five-fold increase in VSMC apoptosis (n= 9, $P= 0.03$) that was inhibited by α_2 -antiplasmin, thus suggesting a plasmin-dependent effect (Fig. 1B-C). In contrast, apoptotic levels in *WT* aortas remained unchanged following the incubation with plasminogen (n=9, $P>0.99$; Fig. 1 B-C).

Increased plasmin generation and VSMC apoptosis in atherosclerotic plaques from *ApoE^{-/-}:PAI-1^{-/-}* mice.

To investigate the involvement of the plasminogen activation system in apoptosis of vascular cells in vivo and its relevance for atherosclerosis, we analyzed atherosclerotic and non-atherosclerotic aorta fragments from *ApoE^{-/-}:PAI-1^{+/+}* and *ApoE^{-/-}:PAI-1^{-/-}* mice. In both genotypes, plasmin activity was increased in extracts from aortic areas with plaques, as compared to areas without plaques, being six-fold more abundant (n=4, $P<0.05$) in plaques from *ApoE^{-/-}:PAI-1^{-/-}* mice as compared to plaques from *ApoE^{-/-}:PAI-1^{+/+}* mice (Fig. 2A, upper panel). Activated caspase-3 (Fig. 2A, lower panel) was barely detectable in areas without plaques, in

either genotype (n=4, $P=0.96$) but was concomitantly increased six-fold in plaques from *ApoE*^{-/-}:*PAI-1*^{-/-} mice (plaque vs non plaque, n=4, $P=0.003$) but not in *ApoE*^{-/-}:*PAI-1*^{+/+} mice (n=4, $P=0.24$). Similarly, caspase-3 activity levels were higher in plaques from *ApoE*^{-/-}:*PAI-1*^{-/-} as compared to plaques from *ApoE*^{-/-}:*PAI-1*^{+/+} mice (n=4, $P=0.04$). In short, active caspase-3 levels paralleled plasmin levels in atherosclerotic plaques. Tissue section analysis confirmed that plasminogen activation as assessed by plasmin- α_2 -antiplasmin complexes (data not shown), plasmin detection (Fig. 2B) and apoptosis (activated caspase 3 in Fig. 2C, TUNEL in Fig. 2D and electron microscopy analysis in Fig. 2E), were present within the aortic intima only in areas with plaques, in both genotypes. Apoptosis within atherosclerotic plaques was a patchy phenomenon, but apoptotic cells were mainly located in the fibrous cap and were predominantly foam-cells.

We used the TUNEL to quantitatively compare the VSMC index in plaques from both genotypes. Since about 25 % of the examined areas did not contain α -actin positive cells, to overcome a potential bias, we calculated the VSMC apoptotic index as a ratio (apoptotic VSMC/ total VSMC)x100, each parameter being the average of the 18 values collected from the 18 areas (from 3 sections, 100 μ m apart) evaluated per mouse. *ApoE*^{-/-}:*PAI-1*^{-/-} plaques had a significantly lower VSMC density (13 \pm 1% vs. 23 \pm 2%, $P<0.05$, n=5) but the total cellular density was not significantly different between both genotypes. Although the overall apoptotic cell density was not significantly increased in *ApoE*^{-/-}:*PAI-1*^{-/-} mice plaques, the VSMC apoptotic index was significantly higher within atherosclerotic plaques from *ApoE*^{-/-}:*PAI-1*^{-/-} (1.5% (0-9.5), n=8) as compared to *ApoE*^{-/-}:*PAI-1*^{+/+} mice (0% (0-0.5), n=7, $P<0.03$). Averaging in each mouse the indices calculated in each individual area (i.e omitting areas without α -actin positive cells, where the apoptotic index could not be calculated) yielded similar trends (not shown).

DISCUSSION

Cell death by apoptosis is considered to be a major determinant of atherosclerotic plaque vulnerability¹⁻³. VSMCs can stabilize atherosclerotic plaques by maintaining the tensile strength of the fibrous cap via the synthesis of collagen isoforms and protease inhibitors. VSMC apoptosis may therefore potentially contribute to the instability and rupture of plaques. Although apoptosis has been detected in human plaques, the mechanisms that trigger this apoptosis and its contribution to VSMC loss, remain unclear.

It has been reported that VSMCs in atherosclerotic lesions may undergo apoptosis in response to effectors secreted by infiltrating inflammatory cells. Macrophages, for instance, may contribute to VSMC apoptosis by direct cell-cell contact, Fas-L/Fas signaling, nitric oxide and TNF- α production^{27, 28}. However, VSMC autocrine destruction has not been demonstrated as yet, although a study suggested that self autodestruction may occur via Fas-mediated apoptosis by relocating Fas-L to the VSMC surface.²⁹ t-PA, an other glycoprotein constitutively expressed by VSMCs³⁰, can be located at the cell surface where it transforms plasminogen into plasmin, which triggers proteolysis-induced cell detachment and apoptosis.⁸ The hypothesis that this sequence of reactions may be participating in the apoptosis of vascular cells on atherosclerotic plaques in vivo, was evaluated in this study. The well characterized *ApoE*^{-/-} atherosclerosis prone mouse model mimics several features of human atherosclerosis^{31, 32}, including the lack of VSMC apoptosis in the normal media^{1,2,35}. Moreover, apoptosis is a heterogeneous and patchy phenomenon in this murine model, as also observed in humans^{2,36}. The combined deficiency of PAI-1 with *ApoE*^{-/-} allows a better assessment of the effects of plasminogen activation on atherosclerosis progression in vivo while discarding possible direct effects of PAI-1 on

apoptosis^{33 34}. Indeed, in both genotypes VSMC apoptosis was undetectable in aortic areas without atherosclerotic plaques.

Corroborating previous in vitro studies⁸, our ex vivo experiments revealed that, in the presence of physiological circulating plasminogen concentrations, VSMC apoptosis was associated with plasmin generation in *PAI-1*^{-/-} aortic tunica media. In the absence of plasminogen, similar apoptotic rates were observed in *WT* and *PAI-1*^{-/-} VSMCs, suggesting that PAI-1 is not a pro-apoptotic factor in this model. Furthermore, we recently showed that fibroblasts in two or three dimensional culture systems became resistant to plasminogen activation-induced cell detachment and apoptosis when transfected either with PAI-1 or protease-nexin-1; these serpins inhibited plasminogen activation at the cell surface and subsequent pericellular proteolysis³⁵. These findings are in agreement with previous data indicating that addition of PAI-1 inhibited plasminogen activation-induced gel contraction and capillary regression, whereas anti-PAI-1 antibodies potentiated these processes³⁶.

Expression of the main components of the plasminogen/plasmin system has been documented in the atherosclerotic arterial wall of mice and humans^{9, 17}. To test whether this system may be involved in vivo in VSMC apoptosis within plaques, we have compared VSMC apoptosis in *ApoE*^{-/-}:*PAI-1*^{+/+} and *ApoE*^{-/-}:*PAI-1*^{-/-} mice. Our data show that PAI-1 deficiency is associated with strongly enhanced apoptosis within advanced atherosclerotic plaques in *ApoE* deficient mice, as assessed by active caspase 3 generation. Electron microscopy revealed that most apoptotic cells were foam cells, probably of macrophage and VSMC origin. Our TUNEL results suggest that among the plaque cells, VSMCs maybe particularly sensitive to plasmin-induced apoptosis, since the overall and non-VSMC apoptotic rates were not significantly different between both genotypes. In contrast, VSMCs were almost only detected within plaques from

ApoE^{-/-}:PAI-1^{-/-} mice. Moreover, we observed an associated decrease in VSMC cell density in *ApoE^{-/-}:PAI-1^{-/-}* mice, whereas the overall cell density did not significantly differ between both genotypes. A similar decrease in VSMC content and increase in caspase activity, was also observed in the inflammatory region of human carotid atherosclerotic plaques as reported recently³⁷.

The apoptosis observed in *ApoE^{-/-}:PAI-1^{-/-}* plaques appears to be dependent on plasminogen activation, as suggested by the six-fold higher plasmin activity detected in atherosclerotic extracts of *ApoE^{-/-}:PAI-1^{-/-}* as compared to *ApoE^{-/-}:PAI-1^{+/+}* mice, and by the colocalization of plasmin and active caspase 3 in situ. An extracellular pathway leading to plasmin induced cell detachment and apoptosis of VSMCs has recently been described.⁸ A similar plasmin(ogen)-dependent cell detachment mechanism has also been described for retinal ganglion cells³⁸, endothelial cells³⁶, fibroblasts³⁵, and cerebrovascular smooth muscle cells⁷, thus suggesting that autocrine production of plasminogen activators by VSMCs and other cell types may induce apoptotic autodestruction by cell surface-formed plasmin. As in vitro studies have revealed that plaque-infiltrating inflammatory cells may degrade pericellular matrix components⁴, it is possible that in our atherosclerotic plaque mouse model, paracrine expression of urokinase by macrophages may also contribute to vascular cell detachment and apoptosis. Of note, macrophages overexpressing urokinase show accelerated atherosclerosis in *ApoE^{-/-}* mice and had elevated lesion proteolytic activity that may cause plaque rupture⁴⁰. Other proteases (elastase, chymase, granzyme B, matrix metalloproteinases) secreted by macrophages, T-lymphocytes and mast cells, may also trigger apoptosis by breaking-down cell-extracellular matrix interactions⁴.

Plasmin may also activate growth factors such as latent TGF- β , which may induce VSMC apoptosis⁴¹. Interestingly, we found, in *ApoE^{-/-}:PAI-1^{-/-}* mice, more pronounced extracellular

matrix disorganisation, proMMP activation, and activation of latent TGF- β , as compared to *ApoE*^{-/-}:*PAI-1*^{+/+} mice¹⁷.

Detection of cell apoptosis by TUNEL may have some potential limitations. Firstly, it is known that the TUNEL technique may overestimate the apoptotic rate, especially in highly proliferative cells⁴². However, the proliferation rate of VSMC is known to be low in advanced atherosclerotic plaques^{2, 43, 44}. Comparing the two *ApoE*^{-/-}:*PAI-1* genotypes, we observed no significant background, and our VSMC apoptotic rates were comparable to those observed in other studies using stringent criteria to define apoptosis⁴². Secondly, VSMCs within atherosclerotic plaques may lose α -actin immunoreactivity, thus compromising identification of VSMC-derived foam cells and determination of the apoptotic rate^{1, 42, 44}. However, active caspase 3 detection within atherosclerotic extracts corroborated our TUNEL data and, most importantly, it paralleled plasmin activity.

In summary, we have found that ex vivo plasmin generation may lead to apoptosis of murine VSMCs. In vivo, plasmin activity in atherosclerotic plaques was associated with VSMC apoptosis in *ApoE* deficient mice, suggesting that plasmin may induce VSMC apoptosis during atherogenesis. Bot et al.⁴⁵ reported that the serine protease inhibitor Serp-1 (which inhibits plasmin and plasminogen activators) impaired atherosclerotic lesion formation and stabilized plaques in *ApoE*^{-/-} mice. Therapeutic strategies aimed at preventing pericellular plasminogen activation might thus be beneficial to stabilize atherosclerotic plaques.

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FIGURE LEGENDS

Figure 1 : Plasminogen activation within the aortic wall leads to ex vivo VSMC apoptosis.

A. In situ fibrin zymography for 24 hours on de-endothelialized aortic tunica media from a *PAI-1*^{-/-} mouse in the absence (left panel) or in the presence of 10 k.I.U/ml aprotinin (right panel). The scale bar corresponds to 100 μ m.

B, C. De-endothelialized aortic tunica media from *WT* and *PAI-1*^{-/-} mice were incubated for 4 hours without (Pg 0) or with 1.3 μ M plasminogen (Pg+), without and with 1 μ M α_2 -antiplasmin (Pg+AP). n= 5 to 9 in each condition tested.

B. Representative *WT* (upper panels) and *PAI-1*^{-/-} (lower panels) mouse de-endothelialized aortas, stained with DAPI (blue, for DNA detection) after the TUNEL reaction (green, for DNA fragmentation). No increased apoptosis was detectable in *WT* aortas after 4 hours of plasminogen incubation, whereas *PAI-1*^{-/-} aortas displayed increased apoptotic levels (Pg+), inhibited in the presence of α_2 -antiplasmin (Pg + AP). The scale bar corresponds to 20 μ m.

C. VSMC apoptotic index (%) in aortas of *WT* and *PAI-1*^{-/-} mice.

Figure 2: Differential expression patterns of plasmin and active caspase 3 in aorta compartments of *ApoE*^{-/-}:*PAI-1*^{+/+} and *ApoE*^{-/-}:*PAI-1*^{-/-} mice.

A. Plasminogen (Pg), plasmin (Pn) (upper gel) and active caspase 3 (lower gel) were detected by Western blot under reducing conditions in the same extracts from aortic areas with (+) and without (-) atherosclerotic plaques of *ApoE*^{-/-}:*PAI-1*^{+/+} (*ApoE*^{-/-}) and *ApoE*^{-/-}:*PAI-1*^{-/-} mice.

B,C. Light microscopic analysis of an ascending aorta section in a representative *ApoE*^{-/-}:*PAI-1*^{-/-} mouse, after immunostaining for plasmin(ogen) (B) or active caspase

3 (C). Both were detected within atherosclerotic plaques but not in the tunica media nor in areas without atherosclerotic plaques.

D (TUNEL reaction) and D' (staining with DAPI) focused on a fibrous cap within an atherosclerotic plaque in a representative *ApoE*^{-/-}:*PAI-1*^{-/-} mouse. Magnification bar: 20 μm.

E. Transmission electron micrograph of the aortic intima of a representative *ApoE*^{-/-}:*PAI-1*^{-/-} mouse showing the presence of a VSMC with chromatin condensation and fragmentation (arrow), which represent distinctive ultrastructural features of apoptotic cells. A VSMC with normal nucleus is marked with asterisk. Magnification bar: 10 μm.