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PLASMIN INHIBITION INCREASES MMP-9 ACTIVITY AND DECREASES VEIN WALL STIFFNESS DURING VENOUS THROMBOSIS RESOLUTION

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Abstract

Introduction—Deep venous thrombosis (DVT) resolution involves the plasmin and the matrix metalloproteinase (MMP) system. This study tested the hypothesis that pharmacological inhibition of the plasmin system would impair DVT resolution and worsen vein wall damage.

Methods—A rat model of stasis DVT by inferior vena cava (IVC) ligation was performed with intravenous control saline or aprotinin (AP; 2.8 mg/kg at operation), and harvest of thrombosed IVC at 7 days. After laser Doppler imaging, DVT were separated, weighed, and vein wall stiffness was assessed by tensiometry. Thrombus and vein wall tissue analysis included total collagen by colorimetric assay, cytokines, chemokines, and d-dimer by ELISA, urokinase-plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) by immuno-blotting, MMP-2 and -9 by zymography, and neutrophil (PMN) and monocyte (ED-1) leukocytes by immunohistochemistry.

Results—DVT weights were 2 fold greater in the AP treated rats ($P<.05$), but no significant differences in thrombus perfusion, collagen, or d-dimer levels were found. Vein wall stiffness was reduced 50% ($P<.05$), suggesting less biomechanical injury. The total vein wall MMP-9 was increased ($P<.05$) 5 fold in the AP group compared with controls, while MMP-2 was elevated but had not reached significance. No difference was found in vein wall TNFα, TGFβ, vein wall or thrombus monocytes, PMN, or uPA/PAI-1 ratio between groups.

Discussion—AP inhibition of the plasmin system was associated with larger thrombi but less vein wall injury, but no difference in other measures of resolution, possibly because of increased vein wall MMP-9 activity. These data suggest an important redundant mechanism for DVT resolution.

Keywords

inflammation; thrombosis; venous; plasmin; matrix metalloproteinase; fibrin

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INTRODUCTION

Post thrombotic syndrome (PTS) is a common clinical problem after deep venous thrombosis (DVT), of which the incidence has not decreased over the last 25 years, with treatment costs in the hundreds of millions of dollars.[1] The end result of DVT resolution is valvular dysfunction and stiff vein walls that clinically manifest as venous hypertension with pain, swelling, and sometimes recalcitrant ulceration.[2, 3] Anticoagulants, such as low molecular weight heparin (LMWH), are effective when used for prophylaxis and treatment of DVT, but no evidence exists that these agents decrease vein wall scarring[2, 4] Clinically, larger and increased time to thrombus resolution may increase the risk of PTS.[5]

The formation of plasmin from plasminogen by the action of tissue-type (tPA) and urokinase-type (uPA) plasminogen activators is thought to be the main pathway by which fibrin deposition is regulated in the vascular tree and by which pericellular fibrinolysis, required for cell migration in tissues and thrombi, is activated.[6-9] Aprotinin (AP; Trasylol® West Haven, CT), is a naturally occurring polypeptide that reversibly inhibits serine proteases including the plasminogen activators such as urokinase-type plasminogen activation (uPA). Serine proteases mediate a variety of reactions in the body, including activation of the coagulation cascade, the fibrinolytic system, and inflammatory responses. At low plasma concentrations, AP attenuates fibrinolysis via plasmin inhibition.[10, 11]

Matrix metalloproteinases (MMP) also play an important role in the degradation of extracellular matrix and basement membrane components to allow the migration of vascular smooth muscle cells, as well as contribute to acute and chronic fibrotic diseases such as interstitial lung disease, interstitial nephritis, and vascular disease.[12-15] MMP's are secreted in a latent proform, and physiological activation of proMMP's may involve plasmin.[16, 17] Plasmin directly activates proMMP-1, -3, -9, -10, and -13,[14, 16, 18] although other stimuli may cause MMP activation.[19] Furthermore, several active MMP's can activate other proMMP's, thus representing positive feedback mechanisms.[20-24] MMP-2 and -9 are both collagenolytic and elastolytic and are involved with vessel remodeling,[25,26] although, unlike other MMP's, these are not known to be directly fibrinolytic. [27] However, recent work has suggested reciprocal activation *in vivo* of the MMP and plasmin systems in a mouse model of DVT resolution; namely, that uPA upregulation may compensate partially for MMP inhibition.[28]

This study tested the hypotheses that early inhibition of plasmin via AP would result in larger thrombi, greater vein wall damage, and a reciprocal increase in MMP activity in a rat model of DVT.

METHODS

Animal model

Male Sprague-Dawley rats (250-450 g) were used for all studies and all protocols were approved by the University of Michigan Animal Care Protocol. For all surgical procedures, the rats underwent general anesthesia with isoflurane/O_2 with full physiological monitoring. Stasis thrombosis was induced by IVC ligation as described.[29, 30] Briefly, a laparotomy with ligation of the IVC below the renal veins and all visible side branches was performed. Rats were injected with Trasylol® (2.8 mg/kg AP; Bayer Pharmaceuticals Corporation, West Haven, CT) or control saline via tail vein at time of operation. At 7 days, the thrombosed IVC was carefully dissected and removed for histological analysis while tissue studies were done after thrombus-vein wall separation.

Laser Doppler

The Lisca laser Doppler (Lisca, Inc., Lingköping, Sweden) was used to assess *in vivo* micro-vascular IVC blood flow, based on the aforementioned techniques.[29-31] Flow through the exposed IVC region of interest was assessed before ligation and at harvest. Depth was constant for all rodents to ensure consistent estimation of the mid-coronal IVC section. These scans were saved and accompanying image software was used to estimate the mean flow by using a standardized area of analysis. Flow intensities were reported as % baseline flow.

Tensiometric vein wall analysis

Each harvested IVC was divided longitudinally and force extension curves were generated for each segment using an Instron Tensiometer (model 5542, Instron Corporation, Canton, MA) with data analysis as described.[30, 32]

Collagen Assay

Thrombus collagen (Types I-V) content was estimated using a commercially available kit according to manufacturer's instructions (Biocolor LTD, Belfast, North Ireland). Total collagen content was corrected to mg thrombus weight, with an estimated sensitivity of 0.5 mcg/mL.[30, 33]

D-dimer ELISA

Thrombus d-dimer content was estimated using a commercially available kit for human d-dimer with cross reactivity to rat according manufacturer's instructions (Diagnostica Stago, Germany).[34] D-dimer content was corrected to mg thrombus weight.

Chemokine/cytokine ELISA

Tissue homogenate of the IVC's for rat TNFα, βFGF, TGFβ, and RANTES were performed with species specific primary antibodies quantified using a double ligand technique as has been described for similar chemokines.[30, 35, 36] Quantification of peptide mediators was normalized to total protein in the sample.

SDS-PAGE Gelatin Zymography

As described[28, 30] homogenized IVC tissue and thrombus were subjected to substrate zymography for MMP-2 and -9 using pre-cast 10% SDS-polyacrylamide gels containing 1 mg/mL of gelatin (unless otherwise stated, all zymography supplies were from Novex, San Diego, CA). Densitometry analysis was performed using a FOTO/Analyst CCD CAMERA (Fotodyne, Hartland, WI) and GEL-Pro Analyzer software version 3.1 (Media Cybernetics, Silver Springs, MD). Pro and active MMP-2 and -9 activity optical densities were summed and normalized to IVC or thrombus protein.

Western immunoblotting for uPA and PAI-1

Protein was isolated from IVC tissue and thrombus using TRIzol Reagent (Life Technologies, Carlsbad, CA) and dissolved in 1% SDS. Rabbit anti-rat uPA (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-rat PAI-1 (1:2500; BD Biosciences, Franklin Lakes, NJ) monoclonal antibodies were used. Blotting was performed and immunoreactive bands visualized as described.[30, 33]

Immunohistochemical staining

Immunohistochemical staining was performed on the paraffin embedded tissue sections (10 μm) as described.[28, 37] Anti-ED-1 (1:100; Serotec, Oxford, UK) and anti-PMN (1:500, Accurate Chemical, Westbury, CT) antibodies were used. In a blinded fashion, cellular positive

staining quantification was determined by direct counting of cells in 5 hpf (1000X) radially around the IVC wall or thrombus and totaled.

Aprotinin assay

Blood was drawn from rats 1 hour after Trasylol® injection. AP plasma concentration was determined using a commercially available kit according to manufacturer's instructions for use with rat serum (Unitest Protenin assay, Technoclone Ltd, Dorling, UK).

Statistical analysis

All data are represented as mean ± SE. Two-tailed unpaired Student's T-test was used for comparison between groups (Sigma Plot, SPSS, Inc. Chicago, IL). $P \leq .05$ was assigned significance.

RESULTS

Aprotinin causes larger DVT but a similar cellular and cytokine composition

Thrombus weights are a simple and reliable measure of thrombus resolution.[28, 29,37] The AP assay [38] confirmed significant plasma AP concentrations at one hour after injection ($P < .001$). Thrombus size was increased nearly 2-fold in AP animals compared with controls ($P = .048$) (Figure 1a). Qualitatively, thrombi in the AP group were less solid and more gel-like than controls. There were no differences in thrombus fibrin, measured indirectly by d-dimer[34] (Figure 1b), or thrombus collagen (Figure 1c) content between groups. In addition, there was no difference in thrombus perfusion measured by laser Doppler between groups (56±7% to 51 ±8% arbitrary units of baseline non-thrombosed blood flow, n=6-7) and values were consistent with 7 day re-established flow.[30] Evaluation of thrombus concentrations of TNFα, TGFβ, βFGF and cellular counts of ED-1 and PMN showed similar values (data not shown).

Aprotinin reduces vein wall damage

Vein wall stiffness is the approximate inverse of vein wall compliance, and is a biochemical measure of vein wall injury during DVT resolution.[30, 32] Vein wall stiffness was ~2 fold less in the AP group compared with controls ($P = .05$) (Figure 2). Vein wall inflammation is associated with elevation of cytokines and chemokines.[30, 35] RANTES is a chemokine that plays an active role in recruiting leukocytes into inflammatory sites as well as being released by platelets. Vein wall RANTES concentration was increased 2-fold in AP treated rats compared with controls ($P = .035$) (Figure 3). Similarly, there was also no difference in vein wall concentrations of inflammatory cytokines TNFα, βFGF, or TGFβ between groups (data not shown). There was no difference between groups in the number of PMN or ED-1 positive cells in vein wall (data not shown).

Vein wall and thrombus matrix reorganization

Total uPA antigen in the thrombi was increased 3-fold in controls compared to the AP group ($P=.11$), suggesting a trend in increased total uPA activity in control thrombi (Figure 4a). There was no significant difference in uPA concentration in vein walls between groups (.011±.002 vs .010±.002 OD/mg protein; n=5-6; $P=.80$). To better evaluate whether uPA balance was altered, PAI-1 Ag was also evaluated by WB as uPA is inhibited by PAI-1. Thus, the uPA/PAI-1 ratio is indicative of the overall balance of uPA activity per molecule.[33] There were no differences between groups in uPA/PAI-1 ratios measured in thrombus (AP=.67±.19 vs. control= .94±.17; n=3-4; $P=.34$) or vein wall (AP=.52±.03 vs. control=.57±.20; n=3-4; $P=.81$), suggesting that there was no difference in overall uPA activity per molecule between groups.

Conversely, total MMP-9 activity of the AP group was increased 5-fold in the vein wall (P=.048) and increased 2-fold in the thrombus (P=.19) (Figure 4b). Similar increases were found analyzing solely pro and active forms of MMP-9 (data not shown). Total MMP-2 activity in the vein wall was increased 2-fold in the AP group (P=.10), but less so in the thrombus as compared with control (P=.18).

DISCUSSION

Plasmin is the primary fibrinolytic protease that directly degrades fibrin and the pericellular matrix, as well as activating other matrix-degrading enzymes, such as proMMP's.[39, 40] The purpose of this study was to use AP as a selective tool to inhibit plasmin activation, and determine its role on vein wall injury as well as MMP activation. We also used this strategy to determine if greater thrombus size was associated with increased vein wall damage. Four main conclusions can be drawn from this study; at 7 days after thrombosis, AP: 1) increases thrombus size, 2) increases total vein wall MMP-9 activity, 3) reduces vein wall stiffness, and 4) does not affect the cellular or inflammatory cytokine profile of the thrombus. This is counterintuitive in many respects, given the proximal position of plasmin mediated fibrinolysis, and is counter to what has been shown in the arterial system with plasmin dependent MMP-9 activation.[25] However, multiple pathways provide for plasmin activation and subsequent MMP-activation,[41] and prior work has suggested that the thrombus composition is more important than the size.[30, 32]

Thrombus weights are a simple and reliable measure of thrombus resolution.[37] Not surprisingly, AP increased thrombus size at 7 days, suggesting reduced thrombus resolution. This was expected because AP inhibits plasmin, which is largely responsible for fibrinolysis in DVT resolution.[6-9] Similarly, it has been shown that uPA, rather than tPA, is most important in thrombus resolution in the venous system.[9] Although thrombi were larger in the AP group, other measures of DVT resolution (thrombus perfusion, collagen, and d-dimer content)[28-30] did not show a significant difference between groups. Thus, although the thrombi were larger with AP, the resolution was similar. For example, recannalization with interim thrombus blood flow (LD imaging) and collagenolysis were likely similar. We speculate that the fibrin or platelet content may make up the size difference.

MMP-2 and -9 have collagenolytic and elastolytic activity, and are elevated in the vein wall during DVT resolution.[15, 28, 33] Previous studies in mice DVT model have suggested a reciprocal activation of the plasmin and MMP systems.[28] Consistently, studies have shown that thrombus resolution is impaired in veins of $uPA^{-/-}$ mice,[9] although MMP activity was not evaluated in that study. Pharmacological inhibition of the plasmin system was associated with increased vein wall MMP-9 activity, but not a significant increase of MMP-2. It is likely that the MMP-9 is derived from influxed monocytes, as few PMN are present.[30] Alternative mechanisms of MMP-9 activation accounted for this observed increase in MMP-9,[15, 41] or the single peri operative dose of AP may have only briefly impaired plasmin production. We found that an increased dose of AP (5 fold) showed a similar increase in thrombus size and similar decrease in vein wall stiffness (data not shown).

Although the thrombi were larger in the AP group, the vein wall damage was less, as reflected by the biomechanical measure of stiffness. Clinical and experimental studies suggest that the longer a DVT is in contact with the vein wall, the greater the resulting damage[42-44](Henke, unpublished). In this study, AP impaired DVT resolution and paradoxically reduced vein wall stiffness. As this is a full stasis model of DVT, persistent thrombus - vein wall contact occurs, and this mechanism of DVT genesis was the same between both the AP and control groups. This suggests that the resulting damage in the vein wall may be caused by direct activity of the plasmin - activated proteases rather than simply duration of thrombus - vein wall contact. This

study indirectly suggests that MMP's are not the main agents responsible for vein wall fibrotic injury, but are important for DVT resolution. Interestingly, this has been observed with PMN depletion in experimental stasis DVT whereby early loss of thrombus MMP-9 was associated with increased vein wall stiffness.[30] Similarly, over expression of uPA has been associated with cardiac injury in a mouse model.[45] Another possible explanation is that attenuation of the inflammatory response by AP caused reduced vein wall injury, or AP inhibited other matrix enzymes such as cathepsins or serum elastase.[46] The latter contribution may be most important, and is a subject of future study.

Cellular and inflammatory cytokine content was also similar between the groups, corrected to total protein in the sample. In contrast to uPA genetic deleted mice, we found no difference in thrombus monocytes. However, the leukocyte deficiency noted in uPA-/- mice in Singh's study was most striking at later time points. Although there was no difference in vein wall PMN or ED-1 cell counts between groups, impaired DVT resolution may explain the significant increase in chemokine RANTES as a compensatory response to increase leukocyte influx in the AP group. However, there was no difference in pro-inflammatory cytokine TNFα concentrations between groups. AP is also known to be able to attenuate inflammatory responses,[11] which may have influenced RANTES secretion. Alternatively, the increased RANTES may also reflect a greater platelet mass as RANTES is released from platelets after stimulation.

The current data suggest a complex role for plasmin activators and the adjacent vein wall response. We are currently investigating the mediators involved with uPA -/- mice as well of some of the non-MMP mechanisms involved with DVT resolution. While native plasmin is essential for fibrinolysis, this may paradoxically worsen vein wall injury. The ultimate clinical goal is to provide rapid fibrinolysis and decrease the vein wall injury while conferring minimal bleeding risk.

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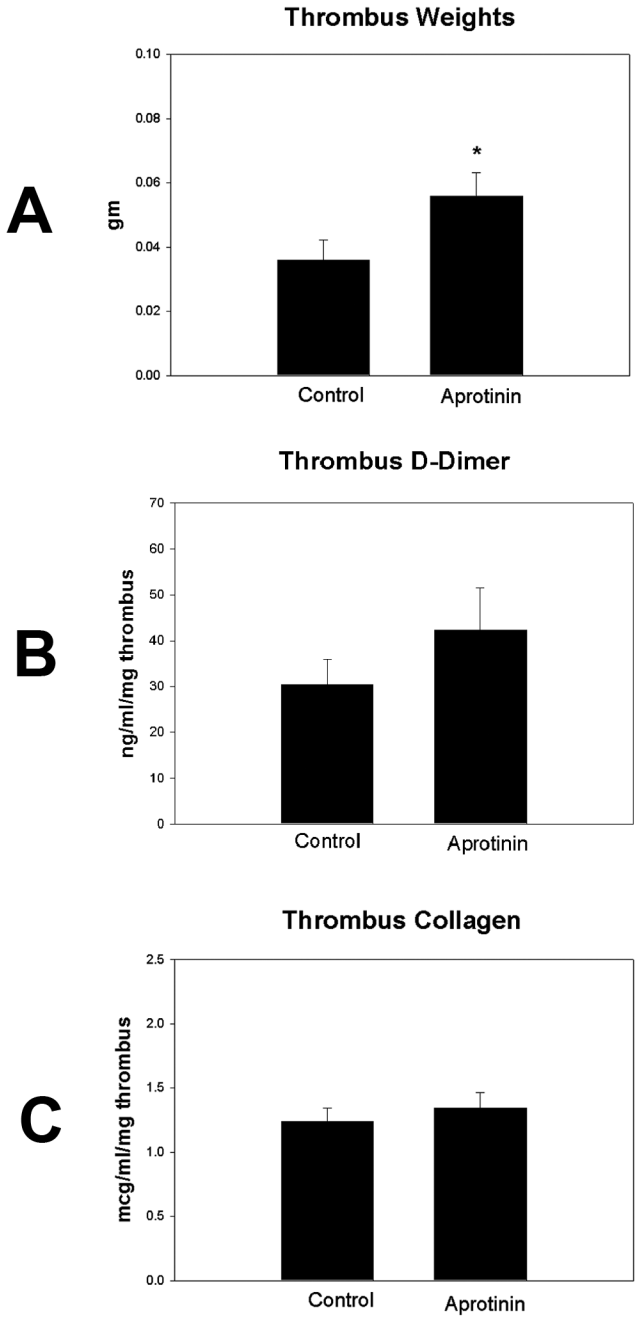

Figure 1.

a) Comparison of thrombus weights showed increased thrombus weight in the aprotinin group compared with control, suggesting that DVT resolution was impaired in the aprotinin group (*=P ≤ .05, n=12-14. b). Comparison of thrombus d-dimer concentrations by colorimetric assay showed no significant difference between groups, suggesting that fibrin concentration was not different between groups (n=3-4). c) Comparison of thrombus total collagen concentration by colorimetric assay showed no significant difference between groups (n=9-11).

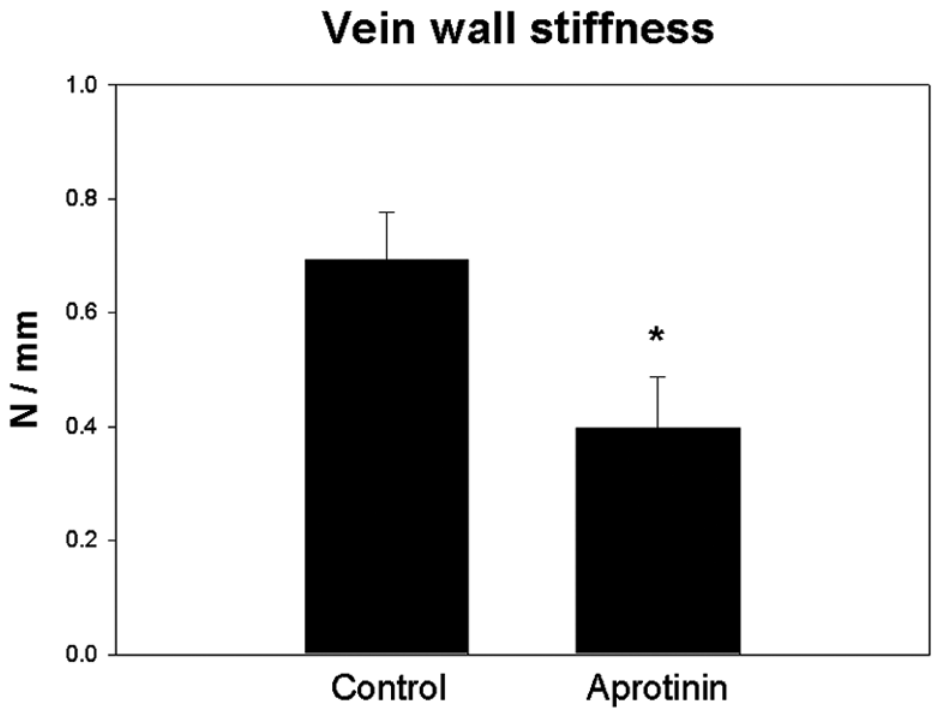

Figure 2.
Vein wall stiffness is an approximate converse of elasticity. Normal veins are elastic and compliant to accommodate blood volume changes. Comparison of vein wall stiffness by tensiometric analysis showed decreased stiffness in the aprotinin group compared to control, suggesting that vein wall damage was reduced in the aprotinin group (*= $P \leq .05$, n=4).

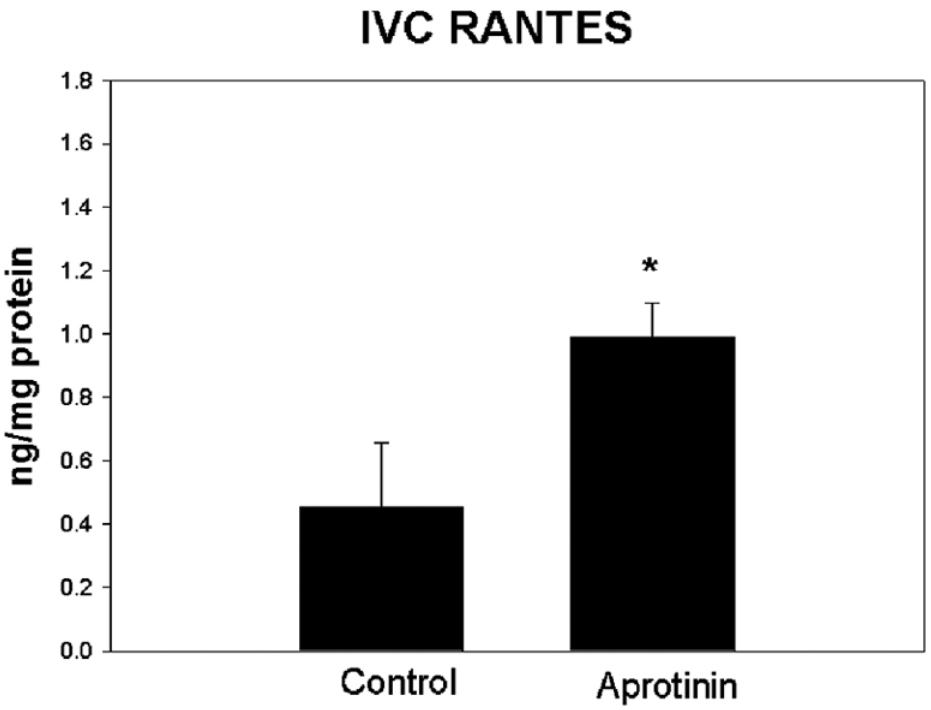

Figure 3.
Comparison of RANTES in vein wall showed increased concentration in the aprotenin group as compared with control (*= $P \leq .05$, n=6-7). Other pro-inflammatory mediators were not significantly altered by aprotinin treatment (TNFα, IL1β, TGFβ, bFGF) in the thrombus or vein wall.

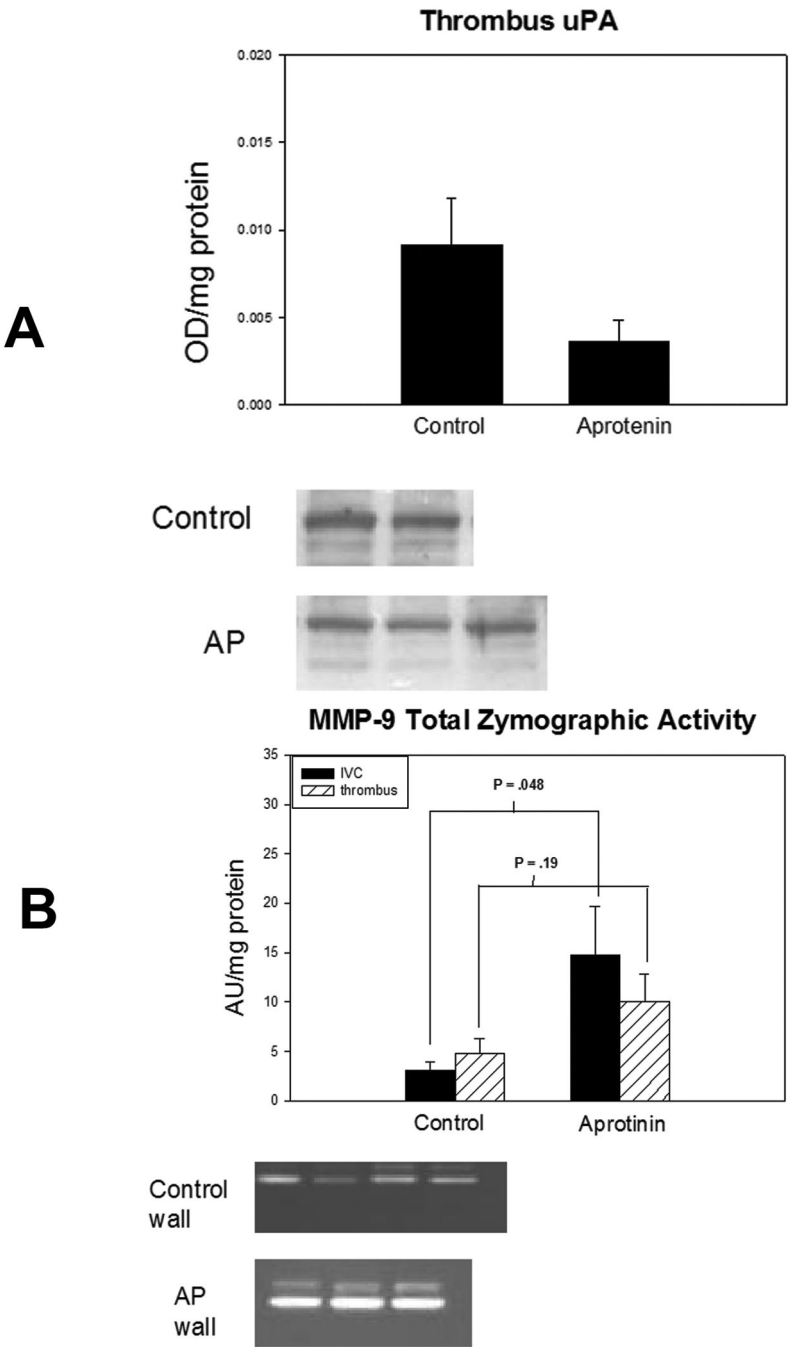

Figure 4.

a) Comparison of thrombus uPA between groups showed a trend of increased concentration in controls (n=6, P =.11). b) Total MMP-9 activity in thrombus and vein wall. The vein wall MMP-9 activity was significantly increased in the aprotinin group, and trended so in the thrombus, as compared with control (n=4-6).