Plasminogen Activator Inhibitor-1 Regulates Myoendothelial Junction Formation

Katherine R. Heberlein, Adam C. Straub, Angela K. Best, Mark A. Greyson, Robin C. Looft-Wilson, Poonam R. Sharma, Akshaya Meher, Norbert Leitinger, Brant E. Isakson

Rationale: Plasminogen activator inhibitor-1 (PAI-1) is a biomarker for several vascular disease states; however, its target of action within the vessel wall is undefined.

Objective: Determine the ability of PAI-1 to regulate myoendothelial junction (MEJ) formation.

Methods and Results: MEJs are found throughout the vasculature linking endothelial cells (ECs) and vascular smooth muscle cells. Using a vascular cell coculture we isolated MEJ fractions and performed two-dimensional differential gel electrophoresis. Mass spectrometry identified PAI-1 as being enriched within MEJ fractions, which we confirmed in vivo. In the vascular cell coculture, recombinant PAI-1 added to the EC monolayer significantly increased MEJs. Conversely, addition of a PAI-1 monoclonal antibody to the EC monolayer reduced the number of MEJs. This was also observed in vivo where mice fed a high fat diet had increased PAI-1 and MEJs and the number of MEJs in coronary arterioles of PAI-1−/− mice was significantly reduced when compared to C57Bl/6 mice. The presence of MEJs in PAI-1−/− coronary arterioles was restored when their hearts were transplanted into and exposed to the circulation of C57Bl/6 mice. Application of biofibrin-conjugated PAI-1 to the EC monolayer in vitro confirmed the ability of luminal PAI-1 to translocate to the MEJ. Functionally, phenylephrine-induced heterocellular calcium communication in the vascular cell coculture was temporally enhanced when recombinant PAI-1 was present, and prolonged when PAI-1 was absent.

Conclusion: Our data implicate circulating PAI-1 as a key regulator of MEJ formation and a potential target for pharmacological intervention in diseases with vascular abnormalities (eg, diabetes mellitus). (Circ Res. 2010;106:1092-1102.)

Key Words: myoendothelial junction ■ plasminogen activator inhibitor-1 ■ endothelial cell ■ smooth muscle cell

In diseases that exhibit vascular abnormalities, increased circulating plasminogen activator inhibitor-1 (PAI-1) is considered a major biomarker; however its function in these diseases remains unclear. In the vasculature, fibrinolysis and the plasminogen activator (PA) system are regulated by PAI-1 through inhibition of urokinase PA (uPA) and tissue PA (tPA), maintaining an important balance between matrix degradation and cellular adhesion. Inhibition of PAs disrupts the activation of plasminogen into plasmin, negatively regulating localized matrix degradation and maintaining a stable scaffold for cells to adhere to. Decreases in PAI-1 results in excessive proteolytic activity and increased matrix degradation, creating an unstable extracellular matrix (ECM) scaffold which disrupts cellular attachment and thereby the invasion by endothelial cell (EC) extensions into the ECM. Conversely, large increases in PAI-1 can inhibit overall matrix degradation, preventing the growth of EC extensions into the ECM. Therefore, the enzymatic balance of proteolytic activity and its regulation by PAI-1 is important in the formation of EC cell extensions.

In the resistance vasculature, EC extensions that penetrate the ECM-rich internal elastic lamina (IEL) form myoendothelial junctions (MEJs), which are locations within a vessel where ECs establish heterocellular contact or apposition with vascular smooth muscle cells (VSMCs). The MEJ is unique to the resistance vasculature and hypothesized to be a highly organized cell-signaling microdomain that facilitates heterocellular communication between ECs and VSMCs (for review see). Several additional studies correlate changes in MEJ regulation with multiple vascular pathologies such as diabetes mellitus, where changes in the vasoreactivity of diseased vessels are associated with potential changes in the number of MEJs. Despite the suggested importance of MEJs in the maintenance of vasomotor tone, there are...
currently no documented mechanisms regarding the regulation of MEJ formation and their potential role in vascular pathologies.

To test the hypothesis that PAI-1 can regulate MEJ formation we isolated in vitro MEJs and determined the enriched expression of PAI-1 at the MEJ and confirmed its presence at the MEJ in vivo. Modulation of PAI-1 activity at the EC luminal surface was reflected by changes in both in vitro and in vivo MEJ formation, where increases in PAI-1 augmented the number of MEJs and decreased PAI-1 activity had the opposite effect. Heterocellular communication between the two cell types, presumably occurring at the MEJ was also affected in response to changes in PAI-1 activity. We therefore suggest that circulating PAI-1 regulates MEJ formation and can alter heterocellular signaling mediated through MEJs in the resistance vasculature.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mice

Wild-type mice, strain C57Bl/6 and PAI-1<sup>−/−</sup> mice, strain B6.129S2-Serpine1<sup>tm1Mlg</sup>/J were males 8 to 10 weeks of age and used according to the University of Virginia Animal Care and Use Committee guidelines. Mice used for high fat comparison were C57Bl/6 mice fed a caloric-rich diet (5.45 kcal/g, 0.2% cholesterol, 35.5% fat; Bio-Serv).

Vascular Cell Coculture

Vascular cell cocultures were assembled as described.<sup>22</sup> Cells were derived from human umbilical vein (Cell Applications, Inc, San Diego) and grown in M199 (Gibco) supplemented with 10% FBS (Gibco), 1% glutamine (Gibco), 1% penicillin/streptomycin (Gibco), EC media also contained endothelial cell growth supplement (5 μg/mL, BD Biosciences); Additional cell lines were derived from human coronary artery (Lonza Walkersville Inc). Endothelial cells were grown in EBM-2 MV (Lonza) supplemented with Lonza bullet kits (Lonza), VSMCs were grown in SmBM (Lonza) supplemented with Lonza bullet kits (Lonza). Seeding densities of 7.5x10<sup>4</sup> VSMCs and 3.6x10<sup>4</sup> ECs were used.

Recombinant PAI-1 (rPAI-1) (0.1 μg/mL; Technoclone) and PAI-1 monoclonal antibody (mAb) (10 μg/mL; Technoclone) were added every 24 hours to ECs 48 hours before isolation. Biotin-conjugated rPAI-1 (Cell Sciences) was added to ECs 30 minutes before isolation.

Isolation of MEJ Fractions

Following 6 days in culture, VSMC monolayers were scraped into lysis buffer and repeated for EC monolayers. The MEJ fractions were collected by removing the denuded Transwell membranes into lysis buffer, and vortexing. All fractions were sonicated and spun at 2500 rpm for 5 minutes and the supernatant collected. Measurements represent the average number of beads per micron squared ± SE.

Protein fractions were run on 10% SDS-PAGE Gels, transferred to nitrocellulose and imaged on a Li-Cor Odyssey Imager.<sup>23</sup>

Antibodies and Protein

Secondary antibodies: phalloidin conjugated to Alexa 488 or Alexa 594, donkey anti-rabbit or donkey anti-mouse Alexa 488 or Alexa 594, all from Invitrogen. Goat anti-rabbit or anti-mouse IRDye 680 or 800CW was used for immunobLOTS (Li-cor Biosciences). Primary antibodies: SMα-actin (monoclonal, Sigma); VE-cadherin, tPA and rPA (all polyclonal, Santa Cruz Biotechnologies); GAPDH (monoclonal, Zymed), PAI-1 polyclonal (Abcam), PAI-1 monoclonal (immunoblot analysis, BD Biosciences), Cx37 and Cx40 (polyclonal, ADI), Cx43 (polyclonal, Sigma) Cx45 (polyclonal, kind gift of Steinberg, Washington University), anti-rabbit 10 nm gold beads were from The Jackson Laboratory.

2D-DIGE Analysis

Individual protein fractions were labeled with Cy2, Cy3 or Cy5 and run per manufacturer specifications (Amersham Biosciences). IPG strips were transferred into gradient SDS-Gel (9% to 12% SDS). Image scans were made using Typhoon TRIO (Amersham Biosciences), analyzed by Image QuantTL software (GE-Healthcare). The ratio change of protein differential expression was obtained by in-gel DeCyder software analysis.

Mass Spectrometry

Proteins of interest were digested in-gel and MALDI-TOF MS and TOF/TOF tandem MS/MS were performed. Peptide mass and associated fragmentation spectra were searched in the National Center for Biotechnology Information nonredundant (NCBInr) database. Candidates with protein score confidence interval percent (C.I.%) or Ion C.I.% greater than 95 were considered significant.

Immunostaining

Immunohistochemistry on the vascular cell coculture (VCCC) was performed as described.<sup>22</sup>

Quantification of MEJs Using the VCCC

The number of F-actin filled pores per micrometer was quantified using Metamorph (Universal Imaging Corps, version 7.5.6.0), Phalloidin staining visualized F-actin within pores of the Transwell.

Immunolabeling on TEM Sections

Visualization of proteins by TEM was performed as described.<sup>25</sup> Quantification of electron-dense gold-beads was performed by immunolabeling for PAI-1 with minimum 5 TEM images per coronary arteriole, 10 μm apart. The areas of each component (EC, MEJ, and VSMC) were quantified using Metamorph software calibrated to measuring area (μm<sup>2</sup>). The EC and VSMC monolayers were traced from apical to basal lateral membrane. For MEJs, cellular extensions penetrating the IEL were defined by a line across the basal lateral membrane from which the MEJ originated, dissecting the junction from the monolayer, tracing the extension through the IEL to the base of the adjacent basal lateral membrane. The area inside these lines defined the area of the MEJ. The number of gold beads in each area (EC, MEJ, or VSMC) was counted. Measurements represent the average number of beads per micron squared ± SE.
Quantification of PAI-1 on Actin Bridges In Vivo

Quantification of PAI-1 on actin bridges formed between ECs and VSMCs in vivo was performed as previously described.25

Ultrastructure Electron Microscopy

Coronary arterioles were fixed in 4% paraformaldehyde and 2% gluteraldehyde at 4°C and ultrastructural TEM images were obtained as described.3 We quantified the total number of MEJs within a vessel using a minimum of 5 TEM images per coronary arteriole. To quantify the radial length of a vessel, a single line was traced along the EC basal lateral membrane with this distance measured using calibrated Metamorph software. Cellular extensions that penetrated the traced EC basal lateral membrane, IEL and came within 250 nm of membranous contact between ECs and VSMCs was counted as one MEJ. Numbers represent the average number of MEJs per 10 μm radial length ± SE. A minimum radial diameter of 150 mm per mouse and 10 μm between each TEM section were used.

Heart Transplants

Heart transplants were performed as described (see Online Figure I).26 Five days postsurgery, donor hearts were harvested for TEM.

Recombinant PAI-1 Tail Vein Injections

Fifty microliters (μL) of rPAI-1 (1000 ng/μg) or saline was injected into PAI-1−/− mice via lateral tail vein every 12 hours for five days. Tissue was harvested for TEM.

Calcium Imaging

Fura-2 acetoxymethyl ester was loaded onto EC and VSMC monolayers as described for Fluo-4 acetoxymethyl ester (see Online Figure II).27 The VSMCs were stimulated with 10 μmol/L phenylephrine (PE) and EC intracellular calcium concentrations [Ca2+]i were recorded as described.27

Statistics

Significance for all experiments was at P<0.05 and determined by one-way ANOVA (Bonferroni post hoc test); error bars are ±SE using Origin Pro 6.0 software.

Results

Isolation and Characterization of MEJ Proteins

For initial experiments demonstrating isolation of EC, VSMC, and MEJ protein fractions we used phalloidin to mark cellular components of the VCCC. In Figure 1A and 1D, an intact VCCC with cell monolayers and actin extensions within the pores of the Transwell (eg, in vitro MEJs22) is clearly observed. The formation of junctions is also confirmed by expression of vascular specific connexins in isolated VSMC, EC, and MEJ fractions (as previously described22 and Online Figure III). After the EC and VSMC monolayers were removed by scraping, the actin extensions within the Transwell pores remained (Figure 1B and 1E). When the scraped membranes were vortexed with lysis buffer, in vitro MEJs were no longer visible via phalloidin staining (Figure 1C and 1F). The three fractions were analyzed via silver stain (Figure 1G) and GelCode Blue (Figure 1H), demonstrating an abundance of proteins in each fraction. Immunoblots demonstrated labeling of MEJ and EC fractions for VE-Cadherin (Figure 1I) and SMα-actin (J), and GAPDH (K). Bar in A is 20 μm and representative for A through C; bar in D is 10 μm and representative for D through F. Arrows in A and B indicate pores of the Transwell insert.
son between each fraction of the same spot. Using Qualitative DeCyder analysis, all spots with increased protein expression in the MEJ fraction were identified. Of these, three spots (arrows 1 to 3) of similar molecular weight and pH had greater than 2.5 fold increase in protein expression in the MEJ fraction as compared to VSMC and EC fractions (Figure 2A). Using DeCyder software, spots 1 to 3 are represented quantitatively as protein expression peaks, where each spot is identified by a magenta tracer (Figure 2B). Mass spectrometry identified each of these spots as PAI-1, with minimal 99.9% confidence in protein identification (Figure 2C). It is likely, although not confirmed that each spot represents a separate glycosylation isoform of PAI-1. Results were confirmed using coronary artery EC, VSMC, and MEJ fractions (Online Figure IV).

**PAI-1 at the MEJ**

To verify expression of PAI-1 at the MEJ in vitro, we immunoblotted isolated VSMCs, EC and MEJ fractions and showed enrichment of PAI-1 in MEJ fractions (Figure 3A). Using confocal microscopy to image transverse sections of the VCCC, we also confirmed the expression of PAI-1 in the pores of the VCCC membrane, where PAI-1 colocalized with F-actin regardless of it’s location in the Transwell pores (Figure 3B). Associated substrates for PAI-1, active and inactive uPA, but not tPA, were increased in MEJ fractions (Online Figure V), supporting our identification of PAI-1 at the MEJ in vitro. Quantified immunohistochemistry of whole mount tissue preps from fixed C57Bl/6 and PAI-1/H/H11002 mesenteric, cremasteric and coronary microvascular beds labeled for PAI-1 confirm the presence of PAI-1 on actin bridges in vivo (Figure 3C). Quantified immunolabeling for PAI-1 on TEM sections of C57Bl/6 coronary arterioles showed expression of PAI-1 at the MEJ in vivo (Figure 3D, as well as mesenteric and cremasteric arterioles, Online Figure VI).

The presence of PAI-1 is crucial for invasion of EC extensions into the ECM. To determine whether PAI-1 is similarly necessary for the formation of MEJs, we depleted PAI-1 activity in each monolayer using a PAI-1 specific mAb or increased PAI-1 activity using rPAI-1. Depletion of
activity caused a significant reduction in MEJs, but only when applied to EC or EC/VSMC monolayers (Figure 4A and Online Figure VII). Likewise, addition of rPAI-1 caused a significant increase in MEJs only in the EC and EC/VSMC-treated monolayers (Figure 4B and Online Figure VII). The identification of PAI-1 was corroborated in coronary artery cells with immunoblots for PAI-1 at the MEJ as well as changes in MEJ formation in response to changes in PAI-1 (Online Figure VII).

To verify the ability of PAI-1 to regulate MEJ formation in vivo, we performed TEM ultrastructure analysis on coronary arterioles isolated from C57Bl/6 and PAI-1−/− mice. Vessels from C57Bl/6 mice had significantly more MEJs (Figure 5A and 5I) than those from PAI-1−/− mice (Figure 5B and 5I). This was also demonstrated in mesenteric and cremasteric arterioles (Online Figure VIII). Conversely, coronary arterioles isolated from C57Bl/6 mice fed a high fat diet had increased PAI-1 expression (Online Figure IX) and signific-

Figure 3. Localization of PAI-1 at the MEJ. Immunoblots of VSMC, EC, and MEJ fractions isolated from the VCCC blotted for PAI-1 and GAPDH as a loading control. Normalized quantification of protein expression for PAI-1 in each fraction is given in the adjacent histogram, n=4 (A). Immunocytochemistry of a single focal plane of a transverse section of the VCCC labeled for PAI-1 (green) and actin (red; phalloidin) demonstrate colocalized expression of both proteins regardless of the location in the Transwell pores (B). In vivo, the expression of PAI-1 on actin bridges that form between ECs and VSMCs (ie, MEJs) in mesentery, cremaster and coronary microvascular beds is quantified using confocal microscopy in both C57Bl/6 and PAI-1−/− mice, n=3 mice per experimental paradigm, 5 images per mouse (C). A representative TEM image of a MEJ from a mouse coronary arteriole labeled for PAI-1 with 10 nm gold particles is shown and quantified as number of beads per micrometer squared in D, n=3 mice per experimental paradigm, 5 images per mouse. Enlargement of white box in B is shown on right. In C, arrow indicates PAI-1 labeling. In D, “L” indicates lumen. Enlargement of red box insert in D is shown on right. Bar in B is 5 μm; bar in D is 0.5 μm. *P<0.05.

Figure 4. Effects of PAI-1 on MEJ formation in vitro. Metamorph analysis of changes in the number of MEJs per micrometer following inhibition of PAI-1 activity by application of 10 μg/mL PAI-1 specific mAb to the EC, EC and VSMC, or VSMC monolayers is shown in A. Analysis of changes in the number of MEJs per micrometer following increases in PAI-1 activity by application of 0.1 μg/mL rPAI-1 to the EC, EC and VSMC, or VSMC monolayers is shown in B. *P<0.05. For each condition (A and B), n=7 Transwells per condition, 10 images per Transwell.
cantly more MEJs (Figure 5C and 5I) as compared to C57Bl/6 mice fed a standard chow diet. To test whether circulating PAI-1 could enhance MEJ formation in vivo, we performed heterotypic heart transplants. Transplantation of a C57Bl/6 heart into a C57Bl/6 mouse (Figure 5D) or a PAI-1−/− heart into a PAI-1−/− mouse (Figure 5E) produced no change in MEJ formation (Figure 5A, 5B, 5I, respectively). However, PAI-1−/− hearts transplanted into a C57Bl/6 mouse, increased MEJ formation similar to that seen in C57Bl/6 mice (Figure 5F and 5I). This data further suggests that circulating PAI-1 interacts with ECs to influence formation of MEJs. To verify that increases in MEJ formation were attributable to increases in PAI-1, we injected saline or rPAI-1 into PAI-1−/− mice via tail vein, to increase circulating PAI-1 (Online Figure X). Saline injected PAI-1−/− mice showed no change in MEJ formation (Figure 5G) when compared to noninjected PAI-1−/− (Figure 5A, 5B, and 5I). Increases in circulating PAI-1 (injected rPAI-1) produced similar increases in MEJ formation (Figure 5H) as compared to those seen following the heart transplants and C57Bl/6 mice (Figure 5A, 5E, and 5I) suggesting changes in MEJ formation are likely attributable to PAI-1.

To determine whether exposing the EC luminal surface to increased PAI-1 could result in relocation of PAI-1 to the MEJ, we applied biotin-conjugated rPAI-1 to the VSMC or EC monolayers of the VCCC thirty minutes before isolation. Following application of biotin-conjugated rPAI-1 to the EC monolayer resulted in a more rapid increase in EC Ca2+ signaling as a result changes in PAI-1 activity in vitro, we measured heterocellular Ca2+ communication from the VSMCs to the ECs by stimulating VSMCs with PE (Figure 7A). Following stimulation of the VSMCs with PE, there were no changes in the maximum EC Ca2+ response regardless of EC treatment with rPAI-1(+rPAI-1) or mAb to PAI-1(PAI-1). The addition of gap junction blocker glycyrrhetinic acid (+GA) or inhibiting MEJ formation through collagen coating of the Transwell (Online Figure XI) significantly decreased the EC Ca2+ response (Figure 7B). However, the addition of rPAI-1 to the EC monolayer resulted in a more rapid increase in EC Ca2+ as compared to EC monolayers with depleted PAI-1 (-PAI-1; Figure 7C and 7D). None of the conditions altered the expression of vascular connexins at the MEJ (Online Figure XII). These data suggest that PAI-1 can enhance heterocellular communication, which is likely a result of increased MEJ formation.

**Discussion**

In the present study we provide evidence for the biomarker PAI-1 in the regulation of MEJ formation and function. Plasminogen activator inhibitor-1 is the major regulator of the PA system. Components of the PA system, uPA and tPA activate plasminogen to plasmin, which degrades ECM. Specifically, inhibition of uPA by PAI-1 decreases matrix degradation, maintaining a localized area of structured matrix scaffold to facilitate cellular invasion of EC extensions into the ECM.11,28,29 Because of its role in the formation of cellular extensions and regulation of matrix degradation, disruption of the balance between PA components, namely changes in PAI-1 activity, can result in decreased cellular invasion. In the resistance vasculature, cellular invasion by the ECs or VSMCs is required for functional MEJs to form. The disruption of MEJ function has been indirectly implicated in vascular diseases such as diabetes mellitus19–21; however, no studies regarding regulation of MEJ formation have been reported.

To identify regulatory proteins enriched at the MEJ, we developed a method for isolating in vitro MEJ fractions using the VCCC as an in vitro model of the MEJ.22–27,30 Isolation of specific EC, VSMC, and MEJ protein fractions was confirmed using immunoblot analysis for cell-type specific markers, which not only demonstrated successful isolation of each monolayer, but isolation of the EC and VSMC components of the MEJ as well. This is the first time that MEJs have been directly isolated, either in vivo or in vitro and we believe this method will enhance the capacity to investigate the function of MEJs. Although a method to isolate protein from MEJs in vivo would be ideal, thus far any method to isolate and characterize MEJs outside of immunohistochemistry, usually using TEM (eg, elsewhere16), has proven elusive.

Using simultaneous 2D-DIGE analysis, we identified three spots representing proteins with increased expression in the MEJ (in vitro) as compared to ECs and VSMCs and using mass spectrometry these spots were determined to be PAI-1. Expression of PAI-1 at the MEJ was confirmed both in vitro using confocal microscopy and in vivo using TEM. Because all three spots from the 2D-DIGE were found at the same molecular weight but demonstrated different isoelectric focusing, it is possible that each spot represents one of the glycosylated isoforms of PAI-1.31 It has been suggested that the glycosylation state of PAI-1 may be useful in determining the protein’s origin12 and further investigation regarding the glycosylated isoforms of PAI-1 could provide insight for the origin of PAI-1 expressed at the MEJ in vivo.

Because of the ability of PAI-1 to regulate matrix degradation and cellular adhesion to the ECM, it is considered an integral component of EC invasion into the ECM.11 We therefore tested the hypothesis that MEJ formation mimics EC invasion of the ECM as regulated by PAI-1. Using the VCCC, PAI-1 activity (by addition of rPAI-1) promoted an increase in the number of actin extensions (ie, in vitro MEJs) that formed. Conversely depletion of endogenous PAI-1 activity (using a mAb specific for PAI-1) decreased the number of actin extensions, suggesting PAI-1 plays a critical role in the formation of MEJs. Correlating with these data, TEM analysis of isolated PAI-1−/− arterioles demonstrated significantly less MEJs as compared to C57Bl/6 mice. Ultrastructure analysis also revealed a thicker IEL in the knockout vessels as compared to wild type. These data are in agreement with recent evidence that shows PAI-1−/− cells exhibit increased collagen production, which correlates with an
Figure 5. Effects of PAI-1 on MEJ formation in vivo. Ultrastructural TEM images of coronary arterioles at low magnification (top) and higher magnification (bottom) from C57Bl/6 (A), PAI-1−/− hearts (B), and C57Bl/6 fed a high-fat Western diet (C). TEM was used to visualize MEJs in coronary arterioles for each of the following experimental paradigms: C57Bl/6 hearts transplanted into recipient C57Bl/6 mice (D), PAI-1−/− hearts transplanted into recipient PAI-1−/− mice (E), and PAI-1−/− hearts transplanted into recipient C57Bl/6 mice (F). TEM analysis of coronary arterioles isolated from saline injected PAI-1−/− mice (G) and rPAI-1 injected PAI-1−/− mice (E) are also shown. The number of MEJs per 10 μm radially is quantified for images A through H in I. Bar in each low magnification image (top image) is 2 μm, bar in A (higher magnification, top) is 2 μm and representative for all higher magnification images. In all images, * denotes vessel lumen and for each image, the lumen is located above the EC monolayer. EC and VSMC monolayers are separated by IEL for all images; arrows indicate MEJ in each magnified image. In I, *P<0.05 when compared to C57 animals. For images A through F and H, n=3 mice per experimental paradigm, 5 images per mouse. In G, n=2 mice, 5 images per mouse.
increase in TGF-β activity through sustained activation by integrins.35

Plasminogen activator inhibitor-1 is a biomarker for several vascular disease states, including diabetes1,3,5,34–36 which is also associated with the dysregulation of MEJs.18 In C57Bl/6 mice fed a high fat diet there is a significant increase in body weight, blood glucose and PAI-1 levels, effectively mimicking diabetic conditions (Online Figure IX). Isolated coronary arterioles from these mice have significantly more MEJs and decreased IEL thickness as compared to the C57Bl/6, so we determined if increases in circulating PAI-1 were capable of regulating MEJ formation in vivo using heterotypic heart transplants. When hearts from PAI-1−/− mice were transplanted into a C57Bl/6 mouse and exposed to circulating PAI-1, MEJ formation within PAI-1−/− coronary arterioles was significantly increased five days post surgery. We confirmed that changes in circulating PAI-1 affected MEJ formation by injecting rPAI-1 into PAI-1−/− mice, demonstrating an increase in MEJ formation in isolated coronary arterioles similar to that seen in PAI-1−/− vessel transplanted into a C57Bl/6 and C57Bl/6 controls. Evidence shows that increases in available PAI-1 coincides with increased cellular invasion37 and our data supports a similar mechanism, whereby increases in MEJ formation occur in response to circulating PAI-1.

Application of rPAI-1 in vitro and heterotypic heart transplants suggest for the first time that increases in PAI-1 at the EC luminal membrane can effect MEJ formation. The application of biotin-conjugated rPAI-1 demonstrated the translocation of biotin-conjugated rPAI-1 from the luminal surface of ECs to the MEJ and further supports the hypothesized movement of PAI-1. It is well documented that VSMCs produce PAI-118,39 and it is likely that some of the endogenous PAI-1 expressed at the MEJ is VSMC-derived, however, the application of biotin-conjugated rPAI-1 to the VSMC monolayer showed no increase in PAI-1 at the MEJ, which coincides with experiments that show with application of mAb or rPAI-1 to the VSMC monolayer there is no change in the number of MEJs in vitro. Therefore, our data indicates that in conditions where circulating PAI-1 is increased, changes in MEJ formation are mediated through the movement of circulating PAI-1 from the luminal surface of ECs to the IEL. Although the internalization of PAI-1 for degradation occurs via a low density lipoprotein receptor mediated mechanism was first described in 199240,41, there is currently no defined mechanism for the uptake and translocation of circulating PAI-1 to areas of the IEL and subsequent sites of potential MEJ formation.

As a unique signaling microdomain, the MEJ is hypothesized to play a key role in the regulation of heterocellular Ca2+ signaling (reviewed elsewhere42). Modulation of PAI-1 activity in vitro using rPAI-1 or mAb to PAI-1 showed no variation in the maximal EC Ca2+ responses following PE stimulation of VSMCs. This indicated that the ECs could still respond normally to second messengers that are produced in the VSMCs and move through gap junctions at the MEJ (eg, elsewhere40,42). However, there was a significant difference in the rate of Ca2+ response between VCCCs treated with rPAI-1 and those with depleted PAI-1, correlating with an increase or decrease (respectively) in the number of MEJs (Figure 7). We interpret these data to mean that the number of MEJs, specifically gap junctions at the MEJ, dictates the time necessary for ECs to respond to second messengers from the VSMCs.

In concurrence with our Ca2+ data and the observed effects of PAI-1 on MEJ formation, we would hypothesize that mice deficient in PAI-1 have impaired vasoreactivity to agonist stimulation caused by a reduced number of MEJs. Indeed, it was recently reported that mesenteric artery rings from
PAI-1−/− mice have diminished sensitivity to acetylcholine (R. Korthuis, University of Missouri, Columbia, unpublished data, 2009). It is also well documented that in multiple diabetic models with upregulated PAI-1, there is increased sensitivity to vasoconstrictors (eg, PE).43–46 Our data supports this hypothesis, providing a potential mechanism for how PAI-1 may affect vasoreactivity through changes in MEJ formation. However, it is important to note that despite increasing evidence supporting a role for MEJs in pathological conditions, it is still unknown if these changes are compensatory or promoting the disease state.

In conclusion, after isolating proteins expressed at the MEJ and performing 2D-DIGE analysis, we have identified PAI-1, an important mediator of ECM degradation and major biomarker for several vascular diseases, as being enriched at the MEJ. Our data suggests localization of PAI-1 to the MEJ allows it to act as a key regulator of MEJ formation and function. The increase or decrease in MEJs in response to changes in PAI-1, including conditions that mimic diabetes, correlate with altered temporal Ca2+ responses in ECs following VSMC stimulation. Although this article does not present a mechanism for how PAI-1 regulates MEJ formation, it is the first evidence of a protein inducing the formation of MEJs, as is extensively demonstrated using innovative techniques both in vitro and in vivo. Although it remains to be seen if the changes in MEJ formation during vascular disease are beneficial, the accumulation of this work suggests that manipulation of PAI-1 at the MEJ may be an attractive pharmaceutical target to treat vascular associated diseases where heterocellular communication is aberrant.
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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Plasminogen activator inhibitor-1 (PAI-1) is a major biomarker for a variety of vascular pathologies and regulates the invasion of cellular extensions into the extracellular matrix.
- Myoendothelial junctions (MEJs) link endothelial and vascular smooth muscle cells and promote heterocellular communication within the resistance vasculature.
- Myoendothelial junctions are hypothesized to be associated with several vascular disease states.

**What New Information Does This Article Contribute?**

- PAI-1 expression is enriched at the MEJ in vitro and in vivo.
- The formation of MEJs is regulated by PAI-1 in vitro and in vivo and effects heterocellular Ca$^{2+}$ signaling at the MEJ in vitro.
- In mice fed a diabetogenic diet, increases in circulating PAI-1 resulted in increased MEJ formation.

Myoendothelial junctions (MEJs) are predominantly located within the resistance vasculature and couple endothelial and vascular smooth muscle cells within the vessel wall. The MEJs are suggested to play a role in the regulation of vasoreactivity via heterocellular communication and have been implicated in several vascular diseases; however, how these structures are regulated is currently unknown. The formation of cellular extensions is governed by plasminogen activator inhibitor-1 (PAI-1), which is a major biomarker for microvascular diseases. We have identified PAI-1 at the MEJ and show a direct correlation between PAI-1 and the number of MEJs. We demonstrate that PAI-1 directly regulates MEJ formation and notably, we show there is an increase in MEJs in conditions that mimic type II diabetes. Microvascular dysfunction associated with type II diabetes can lead to hypertensive conditions and our data show that increases in the number of MEJs results in increased sensitivity to vasoconstrictors in vitro. In sum, these data are the first to demonstrate the ability of a single protein to regulate MEJ formation and provide a potential future pharmaceutical target for microvascular diseases associated with increased PAI-1.
SUPPLEMENTAL MATERIAL:

Plasminogen activator inhibitor-1 can regulate myoendothelial junction formation

Katherine Heberlein
Adam C. Straub
Angela K. Best
Mark A. Greyson
Robin C. Looft-Wilson
Poonam R. Sharma
Akshaya Meher
Norbert Leitinger
Brant E Isakson
SUPPLEMENTAL METHODS:

**Mice:** All wildtype C57Bl/6 mice or PAI-1<sup>−/−</sup> mice (Jackson) were males between 8-10 weeks of age and used according to the University of Virginia Animal Care and Use Committee guidelines. Mice used for high fat comparison were C57Bl/6 mice fed a caloric-rich diet (5.45 kcal/g, 0.2% cholesterol, 35.5% fat; Bio-Serv). Mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital.

**Vascular cell-co-culture:** A VCCC composed of EC and VSMC was assembled as originally described<sup>1</sup>. For these experiments, VSMC and EC were derived from human umbilical vein (both Cell Applications, Inc, San Diego). Endothelial cells were grown in M199 (Gibco) supplemented with 10% FBS (Gibco), 1% glutamine (Gibco), 1% penicillin/streptomycin (Gibco), and endothelial cell growth supplement (5 µg/mL, BD Biosciences); VSMC were grown in M199 supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine. Endothelial cells and VSMC were cultured on opposite sides of 24 mm diameter polyester Transwell inserts (0.4 µm pore width) at a seeding density of 7.5x10⁵ VSMC and 3.6x10⁵ EC for 6 days. All of these conditions induced MEJ formation similar to that seen in the resistance vasculature. So as to limit MEJ formation (e.g., as seen in aorta/carotid) using the VCCC, we coated Transwells with type I collagen. Additional cell lines were derived from human coronary artery (Lanza, Walkersville). Endothelial cells were grown in EBM-2 MV (Lanza) supplemented with Lonza bullet kits as per manufacturer’s instructions (Lanza), VSMC were grown in SmBM (Lanza) supplemented with Lonza bullet kits as per manufacturer’s instructions (Lanza). Seeding densities of 7.5x10⁴ VSMC and 3.6x10⁵ EC were used.

Recombinant PAI-1 (rPAI-1; 0.1 µg/mL) and PAI-1 mAbs (10 µg/mL) were added every 24 hours to the EC monolayer after the initial 24 hour EC incubation for a total of 48 hours. Biotin-conjugated rPAI-1 was added to the EC monolayer 30 minutes prior to isolation. For Ca²⁺ imaging experiments, cells were cultured on 12 mm diameter polyester Transwell inserts (0.4 µm pore width) at a seeding density of 1.9x10⁶ VSMC and 9.0x10⁵ EC, cells were treated with mAb to PAI-1 or rPAI-1 as described above.

**Isolation of MEJ fractions:** Following 6 days in culture, the VSMC monolayer was scraped with PBS into lysis buffer (PBS with 100 mmoles/L NaF, 0.5% NP-40, and 1% protease inhibitor cocktail (Sigma)) and repeated separately for the EC monolayer. The MEJ fractions were collected by removing the denuded Transwell membranes from the plastic insert, placing the membrane in lysis buffer, and vortexing for 5 minutes. All fractions were sonicated for 10 seconds, spun at 2500 rpm for 5 minutes, and the supernatant collected. All steps were performed at 4°C.

**Immunoblots.** Protein fractions in lysis buffer were mixed with 5X lamelli buffer and run on a 10% SDS-PAGE Gels, transferred to nitrocellulose and imaged on a Li-Cor Odyssey Imager<sup>25</sup>. Silver stains and GelCode Blue stains (both Thermo Scientific) were used as per manufacturer’s instructions.

**Antibodies and Protein:** Phalloidin conjugated to Alexa 488 or Alexa 594, donkey anti-rabbit or donkey anti-mouse Alexa 488 or Alexa 594 were all obtained from Invitrogen. Goat anti-rabbit or anti-mouse IRDye 680 or 800CW was used for immunoblots (Li-cor Biosciences). Primary antibodies used were: SMα-actin (monoclonal, Sigma); VE-cadherin (polyclonal, Santa Cruz Biotechnologies), GAPDH (monoclonal, Zymed), PAI-1 polyclonal (Abcam), PAI-1 monoclonal (used for inhibition of PAI-1 activity, Technoclone), PAI-1 monoclonal (used for immunoblot analysis, BD Biosciences), Cx37 and Cx43 (polyclonal, ADI), Cx43 (polyclonal, Sigma) Cx45 (polyclonal<sup>26</sup>), uPA and tPA (polyclonal, Santa Cruz Biotechnologies). Anti-rabbit 10 nm gold beads were obtained from Jackson Labs. Recombinant PAI-1 (rPAI-1) was obtained from Technoclone and biotin-conjugated rPAI-1 was obtained from Cell Sciences.

**2D-DIGE Analysis:** 2D-DIGE was performed by Applied Biomics (Hayward, California). In brief, pellets containing proteins from MEJ, VSMC and EC fractions were prepared and washed with washing buffer (10 mmoles/L Tris-HCl, 5 mmoles/L magnesium acetate, pH8.0). Cell pellets were resuspended in 200 µL 2-D cell lysis buffer (30 mmoles/L Tris-HCl, pH 8.8, containing 7 moles/L urea, 2 moles/L thiourea and 4% CHAPS). Cells were sonicated at 4°C, and incubated with shaking for 30 minutes at

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<sup>1</sup> For details on the VCCC assembly, see reference 1.

<sup>25</sup> For details on Odyssey imaging, see reference 25.

<sup>26</sup> For details on Cx45, see reference 26.
room temperature. Samples were spun for 30 min at 14,000 rpm and the supernatant collected. Protein samples were loaded at 3-8 mg/mL per sample.

Thirty µg of each cell lysate sample were labeled with 1.0 µl of a diluted CyDye, Cy2, Cy3 or Cy5 (1:5 diluted with DMF from 1 nmoles/µl stock), vortexed and incubated on ice for 30 minutes. One µl of 10 mmole/L lysine was added to each sample, vortexed and incubated on ice for 15 minutes. Samples were labeled with Cy2, Cy3 and Cy5 and mixed with 2X 2-D sample buffer (8 mols/L urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes), 100 µl destreak solution and rehydration buffer (7 mols/L urea, 2 mols/L thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes) for a total volume of 250 µl for the 13 cm IPG strip. Samples were mixed and spun before loading.

Samples were run using the protocol provided (Amersham BioSciences) at 20⁰C. Following isoelectric focusing, IPG strips were incubated in fresh equilibration buffer 1 (50 mmoles/L Tris-HCl, pH 8.8, containing 6 mols/L urea, 30% glycerol, 2% SDS and 10 mg/ml DTT) for 15 minutes with slow shaking. The strips were rinsed in fresh equilibration buffer 2 (50 mmoles/L Tris-HCl, pH 8.8, containing 6 mols/L urea, 30% glycerol, 2% SDS and 45 mg/ml DTT) for 10 minutes with slow shaking. The IPG strips were rinsed once in SDS-gel running buffer and transferred into gradient SDS-Gel (9-12% SDS-gel prepared using low fluorescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). The SDS-gels were run at 15⁰C.

Immediately following the SDS-PAGE, image scans were made using Typhoon TRIO (Amersham BioSciences). The scanned images were analyzed by Image QuantTL software (GE-Healthcare), and subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare). The ratio change of the protein differential expression was obtained from in-gel DeCyder software analysis.

Mass Spectrometry: Mass spectrometry was performed by Applied Biomics (Hayward, CA). Proteins of interest were digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C₁₈ (Millipore). Peptides were eluted from the Zip-tip with 0.5 µl of matrix solution (cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mmoles/L ammonium bicarbonate)) and spotted on the MALDI plate (model ABI 01-192-6-AB).

The MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each protein, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the National Center for Biotechnology Information non-redundant (NCBInr) database. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage allowed in the search parameters. Candidates with either protein score confidence interval percent (C.I.%) or Ion C.I.% greater than 95 were considered significant.

Immunostaining: Immunohistochemistry on the VCCC was performed as previously described on the VCCC¹. Quantification of MEJs using the VCCC: The number of F-actin filled pores per micrometer was quantified using Metamorph (Universal Imaging Corps, version 7.5.6.0). Phalloidin staining was used to visualize F-actin from EC and VSMC extensions (i.e., in vitro MEJs) within the pores of the Transwell, viewed transverse to the monolayers.

Immunolabeling on TEM Sections: Visualization of proteins on TEM sections was performed as described². Quantification for the number of electron-dense gold-beads was initially performed by immunolabeling for PAI-1 (as described above) with a minimum of 5 TEM images per coronary arteriole, 10 µm apart, from a minimum of three mice. The area of the EC monolayer, MEJ, or VSMC monolayer
was quantified using Metamorph software calibrated for measuring area ($\mu$m$^2$). EC and VSMC monolayers were traced from apical to basal lateral membrane. To quantify MEJ area, cellular extensions that penetrated the IEL were defined by a straight line was drawn across the basal lateral membrane from which the MEJ originated, dissecting the cellular extension from the monolayer above, tracing the entire length of the cellular extension through the IEL to the base of the adjacent monolayer’s basal lateral membrane and the area inside these lines was defined as the area of the MEJ. The number of gold beads in each set area (EC, MEJ or VSMC) was counted, being careful not to include electron-dense ribosomes. Measurements represent the average number of beads per micron squared ± SE.

**Quantification of PAI-1 on actin bridges in vivo:** Quantification of PAI-1 on actin bridges that form between EC and VSMC in vivo was performed as previously described$^2$.

**Ultrastructure Electron Microscopy:** Coronary arterioles (diameter 50-100 $\mu$m) obtained from each mouse were fixed in 4% paraformaldehyde and 2% gluteraldehyde at 4ºC and ultrastructural TEM images were obtained as described in$^3$. We quantified the total number of MEJs within a vessel using a minimum of 5 TEM images per coronary arteriole, using standard TEM protocol as described above. To quantify the radial length of a vessel, a single line was traced along the EC basal lateral membrane with this distance measured using calibrated Metamorph software. Cellular extensions that penetrated both the traced EC basal lateral membrane and IEL and came within <250 nm of membranous contact between EC and VSMC was counted as a single MEJ. The numbers given represent the average number of MEJs per 10 $\mu$m radial length ± SE. A minimum of three mice, with a minimum radial diameter of 150 mm total per mouse, and 10 $\mu$m longitudely between each TEM section, were used.

**Heart Transplants:** Heart transplants were performed as described$^4$. In brief, the donor heart was removed by excision of the ascending aorta below the brachiocephalic artery and pulmonary artery proximal to its bifurcation. The heart was transplanted into the recipient mouse through an abdominal midline incision and the donor ascending aorta was anastomosed to the recipient abdominal aorta. The donor pulmonary artery was anastomosed to the recipient inferior vena cava. Once adequate haemostasis was achieved, the donor heart was allowed to fill with blood and the wound closed. Mice were injected with 0.1 mL Buprenix + NaCl for a total of four times 48 hours post-surgery. Five days post-surgery, the donor hearts were harvested for TEM analysis.

**Recombinant PAI-1 Tail Vein Injections:** Fifty microliter ($\mu$L) of rPAI-1 (1000 ng/µg) was injected into PAI-1$^{+/+}$ via lateral tail vein every 12 hours for five days. Tissue was harvested for TEM analysis.

**Calcium Imaging:** Fura-2 AM (16.6 $\mu$moles/L in 0.1% DMSO mixed in MOPS-buffered saline) was loaded into EC and VSMC monolayers as previously described for Fluo-4 AM$^5$. A converted Olympus IX-71 with a Bioptek Transwell chamber and inflow and outflow pipettes was used to perfuse 37ºC MOPS-buffered saline over the EC and VSMC monolayers of the VCCC (see Supplementary Fig I). Ultra-rapid rapid image acquisition at 340 nm and 380 nm utilized a water-cooled Hamamatsu EM-CCD coupled to Slidebook imaging software running on a Dell Precision T5400 with dual 3 GHz processors and 8 GB of RAM. The VSMC were stimulated with 10 $\mu$moles/L phenylephrine (PE) and the EC intracellular calcium concentrations [Ca$^{2+}$], were recorded (as previously described$^6$).
SUPPLEMENTAL FIGURES

Supplementary Figure I: Gross anatomical of heterotypic heart transplant. “a” indicates anterior and “p” indicates posterior anatomical alignment, bold arrow indicates donor heart.

Supplementary Figure II: Schematic of setup for measuring heterocellular calcium communication in the VCCC. Buffer flow rate of 1 mL/min was applied to the EC monolayer (top), 0.5 mL/min was applied to the VSMC monolayer (bottom) and 10 μmole/L of PE was applied to the VSMC monolayer. All components are maintained at 37°C.

Supplementary Figure III: Incidence of Connexins in VSMC, MEJ and EC fractions from the vascular cell co-culture. Immunoblots for Cx37, Cx40 and Cx43 in isolated VSMC, MEJ and EC fractions. In all images, black arrows represents 37 kDa.

Supplementary Figure IV: 2D-DIGE analysis of isolated MEJ protein fractions from coronary vascular cell co-culture. 2D-DIGE blots for isolated coronary VSMC, MEJ and EC fractions, comparing VSMC (red) to EC (green) (A, top), SMC (green) to MEJ (red) (A, middle) and EC (green) to MEJ (red) (A, bottom). Arrows labeled 1-3 represent three spots with increased protein expression in the MEJ fraction (greater than 2.5-fold). Using Quantitative DeCyder analysis, representative protein fluorescent intensity peaks for spots 1-3 in VSMC, MEJ and EC fractions are indicated by a magenta tracer (B). Mass spectrum of spots 1 (C, top), 2 (C, middle) and 3 (C, bottom) identify each spot as plasminogen activator inhibitor-1.

Supplementary Figure V: Incidence of tPA and uPA in VSMC, MEJ and EC fractions from vascular cell co-culture. Quantification of immunoblots for tPA (A), active uPA (B) and inactive uPA (C) in VSMC, MEJ and EC fractions isolated from the VCCC. In all images values are expressed as protein/GAPDH, * p<0.05.

Supplementary Figure VI: Localization of PAI-1 to cremasteric and mesenteric MEJs. Transmission electron microscopy images of mouse arterioles labeled for PAI-1 using 10 nm gold particles. Conditions include coronary arterioles treated with normal rabbit serum (A), cremaster arterioles (B) and mesenteric arterioles (C). Bar in A is 0.5µm; bar in B is 0.5µm and is representative for B and C. In all images VSMC and EC monolayers are labeled and IEL is indicated (*).

Supplementary Figure VII: Analysis of PAI-1 localization and effects on MEJ formation in coronary and umbilical vein vascular cell co-cultures. Quantification of immunoblots for PAI-1 in human microvascular coronary VSMC, MEJ and EC fractions (A), protein values are expressed as PAI-1/GAPDH. Representative transverse images of Transwells stained with phalloidin for control (top) and following inhibition of PAI-1 by application of 10 μg/mL PAI-1 mAb to the EC monolayer (- PAI-1, middle) and increased PAI-1 by application of 0.1 μg/mL rPAI-1 to the EC monolayer (+ PAI-1, bottom) are shown for umbilical vein VCCCs in B (quantified in Figure 4). The changes in MEJ numbers in experiments with umbilical vein cells were identical to experiments performed on recapitulated VCCC using human microvascular coronary EC and VSMC (C). Representative transverse images of Transwells plated with human microvascular coronary EC and VSMC and stained with phalloidin are shown in D. For all images, only the EC of the VCCC were treated for the final 48 hours of culture in 24 hr increments. Bars in B and C are 10µm and representative for all accompanying images.

Supplementary Figure VIII: Ultrastructure analysis of mesenteric and cremasteric vessels in C57Bl/6 and PAI-1-/- mice. Transmission electron microscopy images of mouse mesenteric arterioles isolated from C57Bl/6 mice (A) and PAI-1-/- mice (B). In (C) TEM images of mouse cremaster arterioles isolated from C57Bl/6 mice are shown and in (D), TEM images from
Supplementary Figure IX: Body weight, blood glucose levels and PAI-1 expression in high fat C57Bl/6 mice compared to normal C57Bl/6 mice. Body weight (grams) and blood glucose levels (mg/dL) measurements from normal C57Bl/6 (A, top, n=2) and C57Bl/6 + high fat (A, bottom). Quantification of total PAI-1 expression from C57Bl/6 mice and C57Bl/6 + high fat mice (B), image values are expressed as protein expression/GAPDH, *p<0.05

Supplementary Figure X: PAI-1 expression in saline and rPAI-1 injected PAI-1−/− mice. Quantification of total PAI-1 expression from PAI-1−/− mice injected with saline (PAI-1−/− + saline, n=2) and rPAI-1 (PAI-1−/− + rPAI-1). Image values are expressed as average protein expression/GAPDH, *p<0.05.

Supplementary Figure XI: In vitro MEJ formation using the vascular cell co-culture with collagen or fibronectin coated Transwells. Immunocytochemistry using phalloidin staining of F-actin (green) on transverse sections of fibronectin-coated VCCCs (A) or transverse sections of collagen-coated VCCCs (B). Arrows in A indicate actin bridges (i.e. in vitro MEJs); bar in A is 10 µm and representative for both images.

Supplementary Figure XII: Connexin expression at the MEJ in vitro, following treatments with rPAI-1 or mAb to PAI-1. Immunocytochemistry on transverse sections of the VCCC labeled for Cx37 (A), Cx40 (B), Cx43 (C) and Cx45 (D); conditions include application of 0.1 µg rPAI-1 (left), control (center) and application of 10 µg mAb to PAI-1 (right). Bar in A is 10 µm and is representative for all images.
Reference List


### Supp Fig IX

#### A

<table>
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<th>Weight (g)</th>
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<td>132.8 +/- 12.5</td>
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#### B

**PAI-1 protein expression**

![Image of PAI-1 protein expression](image)
Supp Fig X

![Bar graph showing PAI-1/GAPDH expression levels in PAI-1+/- + Saline and PAI-1+/- + rPAI-1 groups. The graph indicates a significant increase in PAI-1/GAPDH expression in the PAI-1+/- + rPAI-1 group compared to the PAI-1+/- + Saline group.](image)

The Western blot analysis shows:
- PAI-1: 50 KDa
- GAPDH: 37 KDa