Plasminogen Activator Inhibitor-1 Regulates Myoendothelial Junction Formation

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Plasminogen Activator Inhibitor-1 Regulates Myoendothelial Junction Formation

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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 biofin-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
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−/− mice, strain B6.129S2-Serpine1tm1Mlg/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. 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 7.5×104 VSMCs 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. All steps were performed at 4°C.

Immunoblots

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

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 tPA (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 (polycrinal, 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 (NCBI) 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.

Quantification of MEJs Using the VCCC

The number of F-actin filled pores per micrometer was quantified using Metamorph software calibrated to ion C.I.%. 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 \( \frac{1}{100} \) nm of membranous contact between ECs and VSMCs was counted as one MEJ. Numbers represent the average number of MEJs per 10\( \mu \)m radial length \( \pm \)SE. A minimum radial diameter of 150\( \mu \)m per mouse and 10\( \mu \)m 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 (\( \mu L \)) of rPAI-1 (1000 ng/\( \mu g \)) 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 acetoxyethyl ester was loaded onto EC and VSMC monolayers as described for Fluo-4 acetoxyethyl ester (see Online Figure II).27 The VSMCs were stimulated with 10\( \mu \)mol/L phenylephrine (PE) and EC intracellular calcium concentrations \([\text{Ca}^{2+}]_i\) 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 \( \pm \)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 MEJs) 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 described 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 using 2D-DIGE proteomic analysis. Representative images compare EC and VSMC fractions (Figure 2A, top), MEJ and VSMC fractions (Figure 2A, middle) and MEJ and EC fractions (Figure 2A, bottom) from the same gel. Gel images for each fraction were obtained and overlaid, allowing direct quantitative compari-
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 its 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−/− 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 significant

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 increased MEJ fractions, nor in the controls (Figure 6A through 6D). The sum of the data suggests that increased PAI-1 could result in relocation of PAI-1 to the MEJ, we applied biotin-conjugated rPAI-1 to the VSMC or increased MEJ formation similar to that seen in C57Bl/6 mice (Figure 6F and 6I). 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.

Effects of PAI-1 on Ca2+ Signaling at the MEJ

To test the functional effect of changes in the number of MEJs 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.11,12,28,29 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, 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, increases in 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.13

Plasminogen activator inhibitor-1 is a biomarker for several vascular disease states, including diabetes 1,3,5,34–36 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 1992, 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 elsewhere).52 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, elsewhere). 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.

Figure 7. Heterocellular Ca2+ communication in vascular cell cocultures with increases and decreases in PAI-1 activity. Schematic representing the setup for measuring EC [Ca2+]i following PE stimulation of the VSMC (A). The maximum values of EC [Ca2+]i following PE stimulation is quantified, and conditions include application of PAI-1 (+PAI-1); application of rPAI-1 plus glycyrrhetinic acid (+rPAI-1+GA); control; control+GA, decreases in PAI-1 using a mAb against PAI-1 (-PAI-1); decreases in PAI-1 plus glycyrrhetinic acid (-PAI-1+GA); and no MEJs (B). In C, the temporal change in EC [Ca2+]i after stimulation of VSMC with PE following application of rPAI-1 (+rPAI-1), control; decreases in (-PAI-1); and no MEJs are shown. The time required to reach maximum EC [Ca2+]i fluorescent intensity after VSMC stimulation with PE is shown for each condition: application of rPAI-1 (+rPAI); control; and decreases in PAI-1 (-PAI-1) (D). For all images, VCCCs were treated for the final 48 hours of culture, *P<0.05; n=3 Transwells per experimental condition.
Acknowledgments

We are grateful to Brian Duling, Scott Johnstone, Jeremy Ross, and Jenny Han for critical reading and discussion of the manuscript; Jessica Connelly for the kind gift of human microvascular coronary artery cells; the University of Virginia Histology Core for sectioning of VCCCs; Jan Redick and Stacey Guillot at the Advanced Microscopy Core for services relating to electron microscopy; and Hong Pei in the Robert M. Berne Cardiovascular Research Center Animal Surgery Core Facility. We also thank Thomas Geer (Olympus America) for valuable help in microscope design.

Sources of Funding

This work was supported by NIH grants HL088554 (to B.E.I.) and HL084422 (to N.L.) and an American Heart Association Scientist Development Grant (to B.E.I.).

Disclosures

None.

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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 vasoactivity 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.