A Novel mRNA Binding Protein Complex Promotes Localized Plasminogen Activator Inhibitor-1 Accumulation at the Myoendothelial Junction

Katherine R. Heberlein, Jenny Han, Adam C. Straub, Angela K. Best, Christoph Kaun, Johann Wojta, Brant E. Isakson

Objective—Plasminogen activator inhibitor-1 (PAI-1) has previously been shown to be key to the formation of myoendothelial junctions (MEJs) in normal and pathological states (eg, obesity). We therefore sought to identify the mechanism whereby PAI-1 could be selectively accumulated at the MEJ.

Methods and Results—We identified PAI-1 protein enrichment at the MEJ in obese mice and in response to tumor necrosis factor (TNF-α) with a vascular cell coculture. However, PAI-1 mRNA was also found at the MEJ and transfection with a PAI-1–GFP with TNF-α did not demonstrate trafficking of the protein to the MEJ. We therefore hypothesized the PAI-1 mRNA was being locally translated and identified serpine binding protein-1, which stabilizes PAI-1 mRNA, as being enriched in obese mice and after treatment with TNF-α, whereas Staufen, which degrades PAI-1 mRNA, was absent in obese mice and after TNF-α application. We identified nicotinamide phosphoribosyl transferase as a serpine binding protein-1 binding partner with a functional α-like microtubule binding domain. Application of peptides against the microtubule binding domain significantly decreased the number of MEJs and the amount of PAI-1 at the MEJ.

Conclusion—We conclude that PAI-1 can be locally translated at the MEJ as a result of a unique mRNA binding protein complex. (Arterioscler Thromb Vasc Biol. 2012;32:1271-1279.)

Key Words: plasminogen activators ■ NAMPT ■ SERBP1 ■ myoendothelial junctions ■ Staufen

The myoendothelial junction (MEJ) is a heterocellular junction located predominantly within the resistance vasculature and comprises vascular smooth muscle cells (VSMCs) and endothelial cell (EC) extensions that extend from each cell type into the internal elastic lamina and form a close apposition with the opposing cell membrane. The MEJ is presumed to be a highly organized signaling microdomain, acting as a conduit for heterocellular signaling within the vasculature. Indeed, deregulation of the MEJ is associated with several disease states, but the mechanism for how these changes are occurring has not been fully described. Most recently, changes in the formation of MEJs were shown to be regulated by plasminogen activator inhibitor-1 (PAI-1) at the MEJ. The expression of PAI-1 directly affected MEJ formation both in vitro and in vivo, with potential effects on signaling between the 2 cell types. However, despite its importance in MEJ formation, the mechanism for PAI-1 localization to the MEJ remains undefined.

Although a common means of achieving subcellular organization is through protein trafficking, recent focus has highlighted a role for localization of messenger RNA (mRNA), which suggests that the ability to localize a single transcript that acts as a template for translating several copies of the same protein may be more energy efficient for the cell. Additionally, localized protein translation can facilitate the rapid accumulation of a protein in response to various stimuli. For mRNA localization to occur, the transcript of interest is typically bound by an mRNA-binding protein (RBP), forming an mRNA-RBP complex, which is trafficked to the targeted area of a cell. Importantly, the mRNA-RBP complex must be stabilized or anchored to the cytoskeleton within the targeted area of the cell to achieve asymmetrical distribution of the protein.

To test the hypothesis that PAI-1 localization to the MEJ can be achieved through localization of PAI-1 mRNA, we looked at the expression of PAI-1 as well as 2 RBPs, the PAI-1 mRNA stabilizing RBP, serpine binding protein-1 (SERBP1) and Staufen, an RBP associated with PAI-1 mRNA degradation, and in response to inflammatory stimulation. We also looked at a
novel role for the protein nicotinamide phosphoribosyl transferase (NAMPT; or PBEF/visfatin) as an anchoring component for the PAI-1 mRNA-RBP complex at the MEJ. In response to disease conditions, we show the first evidence for a PAI-1 mRNA localization mechanism to the MEJ. The increases in PAI-1 correlate with simultaneous increases in SERBP1 and NAMPT, which we suggest act together as a novel PAI-1 mRNA-RBP complex at the MEJ. Disrupting the ability of the complex to anchor at the MEJ inhibits PAI-1 localization to the MEJ and ultimately prevents the effects of tumor necrosis factor (TNF-α) on MEJ formation. Because the MEJ is hypothesized to play an integral role in the regulation of normal vascular function, understanding how its formation is regulated, especially during vascular disease states such as those associated with the metabolic syndrome, may provide valuable therapeutic targets in the future.

**Methods**

**Mice**

Wild-type mice, strain C57Bl/6, were males 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).

**Ultrastructure Electron Microscopy**

Coronary arteries were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde, and ultrastructural transmission electron microscopy (TEM) images were obtained as described. The total number of MEJs within a vessel using a minimum of 5 TEM images per coronary arteriole was quantified as previously described. Briefly, the radial length of a vessel was measured using calibrated Metamorph software. Numbers represent the average number of MEJs per 10-μm radial length ± SEM. A minimum radial diameter of 150 μm per mouse and 10 μm between each TEM section were used.

**Vascular Cell Coculture**

Vascular cell cocultures (VCCC) were assembled as described. Cells were derived from human coronary artery (Lanza, Walkersville, MD). Endothelial cells were grown in MCD8131 (Gibco), supplemented with EC Lonza bullet kits (Lonza); VSMC were grown in DMEM/F12 (Gibco) supplemented with VSMC Lonza bullet kits (Lonza). Seeding densities of 7.5×10^4 VSMC and 3.6×10^4 EC were used. Recombinant human tumor necrosis factor-α (10 ng/mL; R&D Systems) was added 18 hours before isolation. Peptides with a sequence directed against the microtubule binding domain of NAMPT (MKQKMWSIENIAFGSGGG) using Roche Universal ProbeLibrary Assay Design Center, PAI-1 was performed as previously described. Primer design was done using Roche Universal ProbeLibrary Assay Design Center, PAI-1 was performed as previously described.

**Isolation of MEJ Fractions**

In vitro VCCC fractions were collected as previously described. Briefly, VSMC and EC monolayers were scraped into lysis buffer. The MEJ fractions were collected by vortexing the denuded membrane in lysis buffer. Fractions for coimmunoprecipitations were disrupted using a dounner and spun at 500 g for 5 minutes and the supernatant collected. Fractions for coimmunoprecipitations were disrupted using a dounner and spun at 500 g for 5 minutes and the supernatant collected. All steps were performed at 4°C. The RNA extraction and cDNA preparation from isolated VCCC fractions was performed according to the RNeasy 96 protocol (Qiagen). The real-time PCR was performed as previously described. Primer design was done using Roche Universal ProbeLibrary Assay Design Center, PAI-1 UPL probe No. 15: Amplicon Size (bp) 102-OSM (forward primer: 5'-tccagcagctgtaacctg-3', reverse primer: 5'-gctggacagctgcatct-3'); GAPDH UPL probe No. 60: Amplicon Size (bp) 66-OSM (forward primer: 5'-aggcaagctgtaaccac-3', reverse primer: 5'-gccaatcaagcatactc-3'). Data were analyzed using LightCycler Software Version 3.5 (Roche, Basel, Switzerland). PAI-1 antigen in each cell fraction was measured using ELISA for total and active PAI-1, as previously described (Technoclone, Vienna, Austria). All measurements were performed in triplicate.

**Antibodies and Protein**

Secondary antibodies were phallloidin 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 immunoblotts (Li-cor biosciences). Primary antibodies were PAI-1 polyclonal, SERBP1 monoclonal, Staufen monoclonal and PBEF monoclonal (all from Abcam), and GAPDH (monoclonal, Zymed). Anti-rabbit and anti-mouse 10-nm gold beads and anti-mouse 15-nm gold beads were from Electron Microscopy Services.

**Immunoblots**

Protein fractions were run on 10% SDS-PAGE gels, transferred to nitrocellulose, and imaged on a Li-Cor Odyssey Imager. In most cases, because GAPDH is highly ubiquitous in both the membrane and cytosolic fraction of a cell, GAPDH was used to normalize to protein expression as it is consistent between each protein fraction, as we have previously demonstrated, and loads identically to β-tubulin, a cytoskeletal loading protein (Figure I in the online-only Data Supplement).

**Coimmunoprecipitation**

Coimmunoprecipitations were performed as previously described. Briefly, SERBP1 or NAMPT antibody was conjugated to mouse IgG/IgM Dynabeads (Invitrogen) and incubated with equal amounts of EC, VSMC, or MEJ fractions isolated from the VCCC, as determined by a Bradford protein assay. The fractions were run on an SDS-PAGE gel, transferred to nitrocellulose, and imaged on a Li-Cor Odyssey Imager.

**Immunostaining**

Immunohistochemistry on the VCCC was performed as described. For all micrographs, VCCC are arranged with the EC monolayer above the VSMC monolayer.

**Quantification of MEJs Using the VCCC**

Quantification of MEJ fractions was performed as described.

**Immunolabeling on TEM Sections**

Visualization and quantification of proteins by TEM immunolabeling was performed as described.

**Generation of GFP-Tagged PAI-1 Construct**

The full-length PAI-1 sequence (Origene) was cloned into a pEGFP-C1 expression vector (kindly provided by Dr Doug Bayliss, University of Virginia) and validated by sequencing.

**Transfections**

For transfections, ECs were transferred to a cuvette containing 100 μL of Amaxa Nucleofector solution (Lonza) with 5 μg of the GFP-tagged PAI-1 construct. The cuvette was placed in the Nucleofector cuvette holder and subjected to electroporation (EC transfection program, S-005). After electroporation, the ECs were plated on VCCCs as per normal protocol (see above).

**Microtubule Binding Assay**

Microtubules were assembled per manufacturer specifications (Cytoskeleton, Inc). The microtubules were incubated with purified recombinant visfatin (rNAMPT), BSA, or a cocktail mix of microtubule associated proteins. The reactions were layered onto a cushion buffer and centrifuged at 100,000g at room temperature for 40 minutes.
The uppermost layer of supernatant and pellet was resuspended in 10 μL of 53 or 50 μL of 1× laemmli buffer, respectively. The samples were run on 10% SDS-PAGE gels, and proteins were detected by immunoblot analysis or Coomassie stain.4

**Mass Spectrometry**

Protein identification was performed as previously described.4

**Statistics**

Significance for all experiments was at $P<0.05$ and determined by 1-way ANOVA (Bonferroni post hoc test), unless otherwise denoted; error bars are ±SEM, using Origin Pro 6.0 software.

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**Results**

Previous data have shown that in response to a high-fat diet, there is a significant increase in MEJ formation, as well as systemic expression of PAI-1, in vivo.4 To verify that these increases in MEJ formation correlated with an increase in PAI-1 specifically at the MEJ, we performed TEM analysis with immunogold labeling for PAI-1. Quantified immunolabeling for PAI-1 on isolated coronary arteries from C57Bl/6 mice on a normal or high-fat diet shows that there is a significant increase in PAI-1 protein expression at the MEJ in response to a high-fat diet (Figure 1A and Figure II in the online-only Data Supplement). To further elucidate the
mechanism for PAI-1 localization at the MEJ in response to a high-fat diet, we used an in vitro model of the MEJ, the VCCC, and treated the ECs with TNF-α to increase PAI-1 and mimic the low level chronic inflammatory conditions of a high-fat diet and metabolic syndrome. High glucose, an inflammatory-independent stimuli to increase PAI-1, did not show increases in PAI-1 confined to MEJs (Figure III in the online-only Data Supplement). We confirmed the ability of TNF-α to mimic a high-fat diet, showing there was a significant increase in MEJ formation in vitro (Figure 1B), and used PAI-1-specific ELISA for both total and active PAI-1 to show an increase in PAI-1 protein especially at the MEJ, as compared with EC or VSMC fractions (Figure 1C). To determine if the increase in PAI-1 at the MEJ was the result of protein trafficking, we transfected ECs with a GFP-tagged PAI-1 construct before culturing the cells on the VCCC. Movement of the GFP-tagged PAI-1 in response to TNF-α was monitored using quantitative immunoblot analysis, comparing endogenous and GFP-tagged PAI-1 expression at the MEJ (Figure 1D). Because we detected no increase in GFP-tagged PAI-1 at the MEJ after treatment with TNF-α, this suggested that the enrichment of PAI-1 at the MEJ in response to inflammatory conditions was not attributable to a protein trafficking mechanism. For this reason, we used quantitative r-PCR on isolated EC, VSMC, and MEJ fractions and showed there was a global and significant increase in PAI-1 mRNA for all 3 cell fractions, in response to TNF-α (Figure 1E). In conjunction with these data as well as previous work demonstrating rough endoplasmic reticulum at the MEJ, we verified the expression of the PAI-1 RBP, SERBP1, at the MEJ (Figure 1F). Because we detected no increase in SERBP1 expression in response to TNF-α, we transfected ECs with a SERBP1 construct before culturing the cells on the VCCC.4,22,27 Using a microtubule binding assay to determine the ability of NAMPT to bind to the cytoskeleton, although SERBP1 does not contain a cytoskeletal binding domain, proteomic analysis of the in vitro MEJs indicated that the protein nicotinamide phosphoribosyl transferase (NAMPT), which contains a conserved microtubule binding domain (MBD), is highly enriched at the MEJ (Figure 3A). Using quantified immunoblot analysis, we confirmed the expression of SERBP1 at the MEJ in vivo and showed that there was a significant increase in SERBP1 at the MEJ in response to TNF-α (Figure 2A). Additionally, we looked at expression of the RBP, Stauken, which has been associated with the degradation of PAI-1 mRNA. Using immunohistochemistry on transverse sections of the VCCC (Figure 2D) and quantified immunoblot analysis of isolated VCCC fractions (Figure 2E), we show that in response to TNF-α, there was a significant decrease in Stauken expression at the MEJ in vitro (Figure 2F). These data suggested that in response to inflammatory conditions that mimic the metabolic syndrome, there is an organization of RBPs that associate with the stabilization of PAI-1 mRNA at the MEJ in vitro and in vivo.

Localization of mRNA requires that the mRNA-RBP complex be anchored to the targeted area within a cell,
microtubules, we showed that purified recombinant NAMPT (rNAMPT) precipitated with microtubules when centrifuged together, indicating there was a strong interaction between NAMPT and microtubules (Figure 3D). Control experiments done in parallel support the interaction, showing that on its own, rNAMPT does not precipitate (Figure V in the online-only Data Supplement). To further confirm a role for NAMPT as an anchoring component of a PAI-1 RBP complex, we used immunohistochemistry on transverse sections of the VCCC colabeled for SERBP1 and NAMPT. In response to TNF-α, SERBP1 and NAMPT colocalized together at the MEJ (Figure 4A). We next used isolated VCCCs fractions and show both SERBP1 and NAMPT coimmunoprecipitated together in the MEJ fractions, and this interaction was enhanced in response to TNF-α (Figure 4B). Dual immunostaining for SERBP1 and NAMPT on TEM sections from isolated mouse coronary arteries further supported a role for NAMPT as part of the PAI-1 RBP complex, again showing a colocalization of the 2 proteins at the MEJ in response to a high-fat diet (Figure 4C). Last, quantification of the distance between NAMPT and SERBP1 at the MEJs of coronary arteries showed a significant decrease in distance under high-fat conditions (Figure 4D).

To confirm the importance of mRNA localization of PAI-1 at the MEJ, we designed a TAT-tagged peptide designed against the MBD of NAMPT and treated ECs of the VCCC to inhibit the ability of NAMPT to anchor the PAI-1 mRNA-RBP complex to microtubules at the MEJ. Quantified immunoblots for PAI-1 on isolated MEJ fractions show that in response to TNF-α or TNF-α plus a scrambled peptide, there is a significant increase in PAI-1 expression at the MEJ (Figure 5A). However, in the presence of the MBD peptide
and TNF-α, there was no change in PAI-1 expression as compared with nontreated samples (Figure 5A), confirming the importance of PAI-1 mRNA trafficking in the accumulation of PAI-1 protein at the MEJ. It has previously been shown that PAI-1 expression regulates MEJ formation. Therefore, using the same experimental conditions, we also show that by disrupting the microtubule binding capacity of NAMPT, we also inhibit the affects of TNF-α on MEJ formation as well (Figure 5B).

Discussion

In the present study, we provide evidence for a novel PAI-1 mRNA localization mechanism and highlight its importance in the regulation of MEJ formation in response to inflammatory conditions that are typical of the metabolic syndrome. The MEJ is reported to be a key component for the maintenance of normal vascular function, and it has been suggested that changes in the regulation of the MEJ may play an important role in the progression of vascular disease. Indeed, recent evidence now shows there is a significant increase in MEJ formation in mice...
fed a high-fat diet and that the changes in MEJ formation directly correlated with changes in PAI-expression in vivo and in vitro. Importantly, although the previous work from our laboratory showed a novel role for PAI-1, the regulation of MEJ formation, the mechanism by which PAI-1 accumulated at the MEJ in response to a high-fat diet remained undefined. We now show that the significant increases in systemic PAI-1 seen in a high-fat diet are also seen at the subcellular level, where there is a significant increase in PAI-1 protein at the MEJ in vivo, as compared with ECs or VSMCs (Figure 1). These data suggest that in response to inflammatory conditions, there is a mechanism promoting a preferential increase in PAI-1 protein at the MEJ.

The accumulation of protein within a targeted area of a cell is probably accomplished by one of two mechanisms. Protein localization is either achieved by trafficking the protein of interest or trafficking of the mRNA for localized translation of the protein of interest, an important mechanism for subcellular organization of neurons. To identify the mechanism of PAI-1 localization to the MEJ, we chose to recapitulate the inflammatory state as seen in a high-fat diet in vitro using the VCCC. Treating the endothelial cells with a low dose of TNF-α to mimic the inflammatory conditions in vivo resulted in a significant increase in MEJ formation (Figure 1). When assessing the effects of TNF-α on PAI-1 expression in vitro, there was a global increase in PAI-1 protein and mRNA for all 3 VCCC fractions. However, despite TNF-α inducing a significant increase in PAI-1 mRNA in all 3 cell fractions, the fold increase in PAI-1 protein was greatest in the MEJ fractions (Figure 1). As such, there appeared to be a mechanism promoting the asymmetrical accumulation of PAI-1 protein at the MEJ. Interestingly, although there are several mechanisms that can promote a global increase in PAI-1 protein, the mechanism of PAI-1 localization to the MEJ appears to be a specialized response to inflammatory stimulation, as increases in glucose do not result in increased PAI-1 at the MEJ (Figure III in the online- only Data Supplement).

To further elucidate the mechanism for increased PAI-1 expression at the MEJ, we designed a GFP-tagged PAI-1 construct to track the movement of PAI-1 in response to TNF-α. If PAI-1 localization was the result of protein trafficking, there would be a significant increase in GFP-tagged PAI-1 protein at the MEJ, in vitro. It is important to note that the GFP-tagged construct does not contain an NF-kB promoter, which is the general mediator for TNF-α induced increases in PAI-1. Therefore, any increases in GFP at the MEJ would be the result of protein trafficking and not increased GFP-tagged protein production. Interestingly, despite a significant increase in endogenous PAI-1 protein, there was no increase in GFP-tagged PAI-1 at the MEJ, after exposure to TNF-α (Figure 1D). These data suggest that protein trafficking was not playing an important role in mediating PAI-1 localization to the MEJ, but rather the increases in PAI-1 protein at the MEJ were the result of a novel mRNA localization mechanism, promoting the stabilization of PAI-1 mRNA at the MEJ. Although these results do not negate additional mechanisms for PAI-1 localization to the MEJ, our results suggest that in response to TNF-α, there is no movement of intracellular PAI-1 protein to the MEJ. Subcellular organization of signaling microdomains is an important facet of the mRNA localization mechanism, that translational machinery is also present within the targeted area of a cell. Recent data showed the classic TEM evidence for the presence of ribosomes and endoplasmic reticulum in the MEJ, thereby supporting the ability for localized protein production at the MEJ. From this, we hypothesized that in response to disease conditions; the cell establishes an environment that promotes the stabilization of PAI-1 mRNA within the MEJ, resulting in increased PAI-1 protein at the MEJ.

The stabilization of a transcript is achieved through binding of the mRNA by an RBP, and this mRNA-RBP complex anchors to the cytoskeleton within the targeted area of the cell, facilitating protein expression. Equally as important in targeted protein accumulation is mRNA degradation. Of interest, the PAI-1 transcript can be regulated by 2 separate RBPs and occurs when the transcript is bound by the PAI-1 RBP, SERBP1, whereas, conversely, a separate RBP, Staufen, has been shown to promote the degradation of PAI-1 mRNA when its expression is increased. Given the role for each of these proteins in the regulation of PAI-1 mRNA, we hypothesized that in response to TNF-α or a high-fat diet, there would be an increase in SERBP1 at the MEJ, in concurrence with a decrease in Staufen. This organization of proteins would create a subcellular microdomain that supports an increase in PAI-1 mRNA and protein at the MEJ. Indeed, both in vitro and in vivo, there appears to be a distinct distribution of the 2 RBPs in response to inflammatory stimulation, where SERBP1 is increased at the MEJ and Staufen is decreased (Figure 2). However, it is important to note that although SERBP1 appears to play an important role in the stabilization of PAI-1 mRNA at the MEJ, it does not have the ability to anchor the mRNA-RBP complex to the cytoskeleton at the MEJ. Therefore, for the localization to be maintained, the PAI-1 mRNA RBP complex requires an additional constituent to act as an anchoring protein.

Recent proteomic analysis of in vitro MEJs revealed a number of proteins whose expression levels were enriched at the MEJ under normal conditions. Interestingly, the protein NAMPT was not only identified as having increased expression at the MEJ but also contains a conserved microtubule binding domain, similar to the microtubule-associated protein, tau. Importantly, this provides NAMPT with the ability to anchor to microtubules, especially within the MEJ. In addition to being enriched at the MEJ, NAMPT expression is increased in several inflammatory disease states in which both PAI-1 and MEJ formation are also increased, such as obesity and diabetes. More specifically, it was recently shown that the protein levels of NAMPT and PAI-1 are positively correlated. Together with the identification of the conserved MBD, these data suggest there may be a potential role for NAMPT as a crucial anchoring component of the PAI-1 RBP complex in response to inflammatory conditions. Furthermore, the ability of NAMPT to act as an anchoring component for the PAI-1 RBP complex is dependent on its capacity to interact with microtubules and therefore we used a microtubule binding assay to demonstrate a strong and novel interaction between NAMPT and microtubules that is
Importantly, as NAMPT appears to be pleiotropic in function,\textsuperscript{16,36,38} it is unlikely that all NAMPT found at the MEJ is acting as an anchoring component for the PAI-1 RBP complex. Therefore, it becomes necessary to distinguish how NAMPT is regulated, especially in response to inflammatory conditions. Recent evidence now suggests that protein organization and function within the MEJ is facilitated by posttranslational modifications on the protein of interest.\textsuperscript{6} It is therefore possible that the regulation of NAMPT activity may also be achieved in part by posttranslational modifications, specifically within the MBD. Indeed, the protein tau contains 3 MBDs (that are conserved with NAMPT), and binding of tau to microtubules is regulated by phosphorylation of these domains.\textsuperscript{39,40} Specifically, the hyperphosphorylation of critical serine/threonines in the MBDs decreases the affinity of tau for microtubule binding.\textsuperscript{39–41}

Future investigation may show that the same is true for NAMPT and that differential phosphorylation of the NAMPT MBD might also play a role in determining the intracellular function of NAMPT, in response to different stimuli.

Previous work from our laboratory has shown that PAI-1 expression and activity is crucial for the regulation of MEJ formation.\textsuperscript{4} Therefore, if localization of PAI-1 mRNA is required for creating an asymmetrical distribution of PAI-1 at the MEJ, disrupting the PAI-1 mRNA localization mechanism should inhibit the effects of inflammation on MEJ formation. When the microtubule binding capacity of NAMPT was blocked, TNF-\(\alpha\) appeared to have no effect on PAI-1 expression at the MEJ (Figure 5). In sum, these data indicate there is an important role for the localization of PAI-1 mRNA in the mediation of MEJ formation during a pathological response (Figure 6). By disrupting the ability of the complex to anchor within the MEJ, the effects of inflammation on PAI-1 expression and MEJ formation can be reduced and thereby presents a future therapeutic target for vascular diseases that are also part of a larger metabolic syndrome.

Acknowledgments
We thank the University of Virginia Histology Core for sectioning and Jan Redick and Stacey Guillot at the University of Virginia Advanced Microscopy Core.

Sources of Funding
This work was supported by National Institutes of Health grant HL088554 (B.E.I.), an American Heart Association Scientist Development Grant (B.E.I.), an American Heart Association predoctoral fellowship (K.R.H.), and a National Research Science Award postdoctoral fellowship from the National Institutes of Health (A.C.S.).

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2012;32:1271-1279; originally published online March 1, 2012; doi: 10.1161/ATVBAHA.112.246371
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Supplemental Figure Legends**

**Supplemental Figure I:** Antibodies for both beta-tubulin (red; 680 nm) and GAPDH (green; 800 nm) were applied to a blot with 20 µg of protein from EC, MEJ, and VSMC lysates, showing a lack of difference between lysates for each commonly used loading controls.

**Supplemental Figure II:** **ImmunoTEM images of PAI-1 in response to high fat diet.** Representative TEM images of mouse coronaries from mice fed a normal (left) or high fat diet (right) are labeled for PAI-1 using 10 nm gold beads. In both images, EC is endothelial cell, VSMC is vascular smooth muscle cells and (*) denotes IEL. Scale bar is 0.5 µm and representative for both images.

**Supplemental Figure III:** **Effect of high glucose on PAI-1 expression on the vascular cell co-culture.** The VCCC was treated with 30 mM of glucose for 18 hours and each cell lysate collected and western blot was run with each sample. *=p<0.05.

**Supplementary Figure IV:** **Ribosomes on endoplasmic reticulum at the MEJ.** An electron microscopy image of an MEJ from the mouse coronary arteries demonstrating the presence of ribosomes on endoplasmic reticulum (rER) coming down into the MEJ (arrow). Scale bar is 0.5 µm.

**Supplemental Figure V:** **Microtubule binding assay for control conditions.** Coomassie blot for microtubule binding assay controls is shown in (A). Conditions include BSA and microtubule associated protein fraction (MAPF) with no microtubules present (-MT), BSA and MAPF with microtubules present (+MT) and microtubules only (-). In B, a control microtubule binding assay using rNAMPT at 30 and 15 micrograms, with no microtubules is shown using immunoblot analysis NAMPT. In A and B, arrow heads indicate protein fraction of interest. For both images, S is supernatant and P is pellet.
SUPP FIG III

VSMC  MEJ  EC
C    HG  C    HG  C    HG

![Image of protein expression levels with HG and C conditions for VSMC, MEJ, and EC cells.]

![Bar graph showing PAI-1/GAPDH expression levels for VSMC, MEJ, and EC cells under HG and C conditions.]

PAI-1/GAPDH

HG

VSMC  MEJ  EC
-    -    -
+    +    +

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**Fig. V**

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**Immunoblot**

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