Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Drug Targeting of Plasminogen Activator Inhibitor-1 Inhibits Metabolic Dysfunction and Atherosclerosis in a Murine Model of Metabolic Syndrome

Hekmat B. Khoukaz,* Yan Ji,* Drew J. Braet, Manisha Vadali, Ahmed A. Abdelhamid, Cory D. Emal, Daniel A. Lawrence, William P. Fay

OBJECTIVE: Enhanced expression of PAI-1 (plasminogen activator inhibitor-1) has been implicated in atherosclerosis formation in humans with obesity and metabolic syndrome. However, little is known about the effects of pharmacological targeting of PAI-1 on atherogenesis. This study examined the effects of pharmacological PAI-1 inhibition on atherosclerosis formation in a murine model of obesity and metabolic syndrome.

APPROACH AND RESULTS: LDL receptor-deficient (*ldlr*$^{-/-}$) mice were fed a Western diet high in cholesterol, fat, and sucrose to induce obesity, metabolic dysfunction, and atherosclerosis. Western diet triggered significant upregulation of PAI-1 expression compared with normal diet controls. Addition of a pharmacological PAI-1 inhibitor (either PAI-039 or MDI-2268) to Western diet significantly inhibited obesity and atherosclerosis formation for up to 24 weeks without attenuating food consumption. Pharmacological PAI-1 inhibition significantly decreased macrophage accumulation and cell senescence in atherosclerotic plaques. Recombinant PAI-1 stimulated smooth muscle cell senescence, whereas a PAI-1 mutant defective in LRP1 (LDL receptor-related protein 1) binding did not. The prosenescent effect of PAI-1 was blocked by PAI-039 and R2629, a specific anti-LRP1 antibody. PAI-039 significantly decreased visceral adipose tissue inflammation, hyperglycemia, and hepatic triglyceride content without altering plasma lipid profiles.

CONCLUSIONS: Pharmacological targeting of PAI-1 inhibits atherosclerosis in mice with obesity and metabolic syndrome, while inhibiting macrophage accumulation and cell senescence in atherosclerotic plaques, as well as obesity-associated metabolic dysfunction. PAI-1 induces senescence of smooth muscle cells in an LRP1-dependent manner. These results help to define the role of PAI-1 in atherosclerosis formation and suggest a new plasma-lipid-independent strategy for inhibiting atherogenesis.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ cellular senescence ■ fibrinolysis ■ metabolic syndrome ■ muscle, smooth ■ obesity ■ plasminogen activator inhibitor-1

The fibrinolytic system plays an important role in the pathogenesis of atherosclerosis by several mechanisms, including regulation of intravascular fibrin deposition, extracellular matrix turnover, and cell migration.[1–3] The central reaction of the fibrinolytic system is the conversion of plasminogen to plasmin by tPA (tissue-type plasminogen activator) and uPA (urinary-type plasminogen activator). Plasmin, a serine protease, degrades fibrin and several other proteins in plasma, the extracellular matrix, and on the plasma membrane of vascular cells.

Correspondence to: William P. Fay, MD, Division of Cardiovascular Medicine, University of Missouri, 5 Hospital Drive, CE344–DC095.00, Columbia, MO 65212. Email fayw@missouri.edu

*These authors contributed equally to this article.

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Nonstandard Abbreviations and Acronyms

AT	adipose tissue
FGF	fibroblast growth factor
HbA1c	hemoglobin A1c
LDL	low-density lipoprotein
Ldlr$^{-/-}$	low-density lipoprotein-receptor-deficient
LRP1	low-density lipoprotein receptor-related protein 1
ND	normal diet
PAI-1	plasminogen activator inhibitor-1
SA-βgal	senescence-associated β-galactosidase
SMC	smooth muscle cell
TNF-α	tissue necrosis factor-α
tPA	tissue-type plasminogen activator
uPA	urinary-type plasminogen activator
WD	Western diet
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Highlights

- PAI-1 (plasminogen activator inhibitor-1) inhibitors significantly decrease atherosclerosis formation in a murine model of obesity and metabolic syndrome.
- Pharmacological targeting of PAI-1 inhibits macrophage accumulation and cell senescence in atherosclerotic plaques.
- PAI-1 stimulates vascular smooth muscle cell senescence by binding to LRP1 (low-density lipoprotein receptor-related protein 1).
- Pharmacological PAI-1 inhibition exerts durable antiobesity and prometabolic effects in LDL-receptor-deficient (*ldlr*$^{-/-}$) mice fed Western diet for up to 24 weeks.
- Overall, these results suggest that pharmacological targeting of PAI-1 may be an effective strategy to inhibit obesity-associated atherosclerosis and metabolic dysfunction.

PAI-1 (plasminogen activator inhibitor-1), the primary inhibitor of tPA and uPA, is a key regulator of fibrinolysis and cell migration.[4] PAI-1 over-expression is associated with atherosclerosis in humans,[5,6] particularly in individuals with the metabolic syndrome,[7] which is characterized by visceral obesity, insulin resistance, dyslipidemia, hypertension, and increased PAI-1 expression.[8,9]

Several pharmacological inhibitors of PAI-1 have been developed and studied.[10–12] These compounds have been proposed as novel treatments for multiple human diseases, including thrombosis, kidney disease, obesity, inflammatory bowel disease, and pathological components of aging.[13–23] However, no published studies have examined the effects of pharmacological PAI-1 inhibition on atherosclerosis. It is important to study this issue for several reasons. First, there are likely to be significant differences in the biological consequences of genetic versus pharmacological suppression of PAI-1.[24] For example, PAI-1 plays a functional role in the intracellular and extracellular compartments,[25] both of which would be affected by PAI-1 gene deletion. In contrast, pharmacological inhibitors might preferentially target the extracellular pool of PAI-1. Therefore, previous studies involving PAI-1 knockout mice[26–28] cannot be presumed to predict the effects of PAI-1 inhibitors on atherosclerosis. Second, pharmacological inhibitors of PAI-1 have begun to be studied in human clinical trials.[29] If any of these drugs become approved for human therapy, it will be essential to understand their effects on atherosclerosis, which is present in many individuals. Third, several lines of evidence suggest that drug-targeting of PAI-1 has the potential to inhibit atherosclerosis,[13,16] and thus could represent a new treatment strategy to prevent disease progression and complications, including myocardial infarction and stroke.

In this study, we examined the effects of PAI-039 and MDI-2268, highly specific, small molecule inhibitors of PAI-1,[11,15] on atherosclerosis formation in a murine model of the metabolic syndrome. Specifically, we studied LDL (low-density lipoprotein)-receptor-deficient (*ldlr*$^{-/-}$) mice fed a Western diet (WD) high in cholesterol, fat, and sucrose, which has been shown to generate the key features of human metabolic syndrome.[30] We also examined the effects of pharmacological PAI-1 inhibition on (1) atherosclerotic plaque and visceral adipose tissue (AT) inflammation, which play key roles in atherosclerosis progression and the metabolic dysfunction associated with obesity[8,31,32] and (2) vascular cell senescence, which is atherogenic.[33,34] Our results show that pharmacological targeting of PAI-1 inhibits atherosclerosis, AT inflammation and its downstream metabolic effects, as well as cell senescence and inflammation in atherosclerotic plaques. These results help to define the role of PAI-1 in atherogenesis and constitute the first report that drug-targeting of the fibrinolytic system inhibits atherosclerosis formation.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and in the Data Supplement.

Reagents

PAI-039, an orally available, specific PAI-1 inhibitor,[15,35] was from Pfizer, Cambridge, MA. MDI-2268, an orally available, specific PAI-1 inhibitor, has been described previously.[11] Recombinant human PAI-1 (Catalog No. PAI-A) was from Molecular Innovations, Novi, MI. PAI-1-I91L (a recombinant

human PAI-1 mutant with increased stability, normal antiprotease activity, and normal LRP1 [LDL receptor-related protein 1]-binding affinity) and PAI-1-I91L, K80/207A (a recombinant human PAI-1 mutant with increased stability, normal antiprotease activity, normal vitronectin binding affinity, and a >20-fold reduction in LRP1-binding affinity) were generated and purified as described.[36] R2629, a rabbit anti-LRP1 antibody that does not cross-react with other LDL receptor family members,[37] was a gift from Dudley Strickland, PhD, University of Maryland.

Mice

Ldlr$^{-/-}$ mice, which had been back-crossed >10 generations into the C57BL/6J genetic background, were from Jackson Laboratory, Bar Harbor, ME (stock number 002207). Mice were fed normal laboratory diet (ND) containing 62% of total calories from carbohydrates, 25% from proteins, <0.02% total cholesterol, and 3% sucrose by weight (No. 5053, LabDiet, Richmond, IN) from the time of weaning until initiation of WD, as specified for each experiment. WD contained 42% of total calories from fat, 0.2% total cholesterol, and 34% sucrose by weight (TD.88137, Envigo, South Easton, MA). WD pellets lacked or contained either PAI-039 (5 mg/g of diet)[38] or MDI-2268 (400 µg/g of diet). Animals consumed diet and water ad libitum. The University of Missouri Animal Care and Use Committee approved all experiments.

Plasma Assays

Plasma PAI-1 antigen was measured either by a Luminex multiplex assay[39] or a standard ELISA using antimurine PAI-1 capture antibody (Molecular Innovations clone H34G6; coating concentration 1 µg/mL) and biotinylated antimurine PAI-1 detection antibody (Molecular Innovations, ASMPAI-GF-BIO, 1 µg/mL). The Luminex PAI-1 antigen assay detects active, latent, and complexed forms of PAI-1, with the signal generated by tPA-PAI-1 complex being about 25% that of an equimolar amount of free, active PAI-1. Plasma PAI-1 activity was measured using a Luminex multiplex assay.[39] Plasma total cholesterol, HDL (high-density lipoprotein) cholesterol, LDL cholesterol, and triglycerides were measured by the University of Missouri Veterinary Diagnostic Lab. Blood samples for lipid analyses were collected after mice had fasted 4 hours. HbA1c (hemoglobin A1c) was measured with a Siemens DCA Vantage Analyzer, per manufacturer's instructions.

Gene Expression

Expression of PAI-1 and TNF-α (tissue necrosis factor-α) genes were assessed by quantitative real-time reverse transcriptase-PCR, as described.[40] PCR primers were: PAI-1: 5′-GCTGCAGATGACCACAGCGGG-3′ and 5′-CCGCAGTACTGATCTCATTC-3′; TNF-α: ThermoFisher Scientific (Catalog No. 4331182); and 18S ribosomal RNA: 5′-CCTGGATACCGCAGCTAGGA-3′ and 5′-GCG GCGCAATACGAATGCCCC-3′.

Quantitative Assessment of Atherosclerotic Plaques

Atherosclerotic plaque formation was quantified in the aortic sinuses of Valsalva, ascending thoracic aorta, aortic arch, descending thoracic aorta, abdominal aorta, and carotid arteries, which were perfused with saline followed by 4% paraformaldehyde at physiological pressure upon euthanasia. To quantify atherosclerosis formation in the aortic root, three cross-sections (each 10 µm thick), mounted in OCT compound and harvested at sequential intervals of 100 µm, beginning at the level of the aortic valve leaflets, were prepared for each animal and stained with Oil Red O and hematoxylin. The length of aortic root assessed in our studies (≈300 microns) was shorter than that advised by the American Heart Association guidelines, which recommend assessing plaque formation throughout the entire aortic root, that is, a distance of ≈800 microns.[41] Plaque size in aortic root microscopic images was analyzed using ImagePro software (Media Cybernetics, Rockville, MD). Atherosclerosis in the aortic arch, descending thoracic aorta, and abdominal aorta was assessed by fastidiously removing perivascular AT from the excised aorta, longitudinally incising it, staining it with Oil Red O, and performing en face quantification of total plaque area with computerized software, as described.[42] This approach is consistent with American Heart Association guidelines. Atherosclerosis in the common carotid arteries (from their origin from the aortic arch to just distal to the bifurcation into internal and external carotid artery) was measured by isolating these segments by dissection, measuring their surface area by computer-assisted planimetry of images viewed through a dissecting microscope, staining them with Oil Red O, rinsing them, incubating them in chloroform/methanol to extract Oil Red O, measuring the A520 of extracts in a spectrophotometer, and normalizing results to total surface area of the segment (ie, A520/mm^2).[43]

Histochemical Analyses of Vascular Segments and AT

Macrophage invasion into the fibrous cap of atherosclerotic plaques was assessed at the level of the aortic sinuses. Cross-sections were immunostained with anti-Mac-3 antibody (BD Pharmingen, Catalog No. 553322, concentration 2 µg/mL). The entire fibrous cap of the largest atherosclerotic plaque within each cross-section was selected as a region of interest. The false-color segmentation technique was used to quantify macrophage content in the fibrous cap, as described.[44] Smooth muscle cell (SMC)-α actin immunostaining was performed with anti-α-actin antibody (Santa Cruz biotechnology, Catalog No. sc-32251, concentration 1 µg/mL).[44] Picrosirius red staining for collagen was performed by incubating tissue cross-sections with Weigert hematoxylin (Sigma Aldrich, Catalog No. HT1079-1SET), rinsing, and incubating with Direct red 80 (0.1%, Sigma Aldrich, Catalog No. 365548-5G) diluted in picric acid solution (1.2%, Fisher Scientific, Catalog No. 5860-32).[45] Paraformaldehyde-fixed cross-sections of epididymal fat pad were prepared and immunostained with anti-Mac-3 antibody, as described for atherosclerotic segments of aorta. Crown-like structures (representing macrophage clusters encircling an adipocyte) per high-power field were counted. Immunostaining of epididymal fat pads for LRP1 (Abcam, Catalog No. AB92544, concentration 4.6 µg/mL) and fibrin(ogen; Abcam, Catalog No. AB34269, dilution 1:200) was performed, and positive staining was quantified using the false color segmentation technique. For all immunohistochemical staining procedures, control experiments involving substitution of an isotype control primary antibody were performed to confirm assay specificity (Figure I

in the Data Supplement). Mean adipocyte cross-sectional area was determined by counting the number of adipocytes in 4 random fields of defined area within a histological cross-section of AT and dividing the total area by the number of adipocytes.

Cell and Vascular Senescence Analyses

Human coronary artery SMCs (Cascade Biologics, Portland, OR, Catalog No. C0175C, lot number 1689414, from a 32 year-old female) were cultured in supplemented Medium 231 (Cascade Biologics) and passaged 6 to 8× to render them more susceptible to senescence, which was defined by positive expression of SA-βGal (senescence-associated β-galactosidase). To study the effects of PAI-1 on SMC senescence, cells were grown to ≈50% confluence, after which recombinant human PAI-1 was added to media and cells were cultured an additional 24 hours. In some experiments, PAI-039 or vehicle control was added to media along with PAI-1. Cells were fixed and scored for expression of SA-βGal using the Senescence Detection Kit (Catalog No. AB65351, Abcam, Cambridge, MA). Total cellular senescence in paraformaldehyde-fixed murine aortic arches was assessed by incubating them with fluorescein di-β-d-galactopyranoside, as described.[46] The fluorescence of the solution was measured and normalized to tissue weight. To specifically assess cell senescence in atherosclerotic plaques, paraformaldehyde-fixed, OCT mounted cross-sections of the aortic root were incubated 17 hours in citrate-phosphate buffer (37 mmol/L citric acid, 126 mmol/L sodium phosphate, pH 6.0) containing 150 mmol/L NaCl, 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, Invitrogen Catalog No. B1690), 2 mmol/L $MgCl_2$, 5 mmol/L potassium ferrocyanide, and 5 mmol/L potassium ferricyanide. Specimens were imaged using a Zeiss Axiovert 200M microscope and Leica DFC290 camera. Overlapping fields of view at 63× magnification were merged into a single image of the entire vascular cross-section using MetaMorph v.7.8.12 software. The region of plaque with the most intense X-gal staining (determined with examiner blinded to treatment group) was selected for comparison to aortic segments from other mice.

Hepatic Analysis

Triglyceride content of hepatic tissue was measured using the Cayman Chemical Triglyceride Colorimetric Assay Kit (Catalog No. 10010303).

Statistical Analyses

Results are shown as mean±SEM. Student *t* test and 1-way ANOVA were used to compare experimental groups, as appropriate, after first demonstrating that the data sets passed normality and equal variance tests. If either test failed, the Mann-Whitney *U* test was used for 2-group comparisons. When 1-way ANOVA was performed, the Holm-Sidak method was used for post hoc testing. The effect of drug treatment on mouse body weight was analyzed with a piecewise linear model with an autoregressive error structure to accommodate the serial correlation due to repeated measurements on the same animals. Ninety-five percent confidence regions were calculated for each growth trajectory. The effect of drug treatment on diet consumption was analyzed by 2-way ANOVA with an interaction effect between diet group and time. The false discovery rate method was used to correct significance levels for multiple testing.[47]

RESULTS

To determine if WD upregulates PAI-1 expression in *ldlr*$^{-/-}$ mice, males who had been fed ND since weaning were divided into 2 matched groups, one fed WD (n=6, mean age 12.6±0.3 weeks) the other continued on ND (n=6, mean age 12.6±0.3 weeks; $P>0.95$ versus WD group). After 4 weeks, plasma PAI-1 antigen was significantly increased in the WD group compared with ND controls (Figure IIA in the Data Supplement). Consistent with these results, PAI-1 gene expression, assessed by quantitative real-time reverse transcriptase-PCR analysis of RNA isolated from epididymal fat pads, was significantly higher in WD-fed *ldlr*$^{-/-}$ mice than in ND controls (Figure IIB in the Data Supplement). These results support the use of WD-fed *ldlr*$^{-/-}$ mice to model the metabolic syndrome, which is characterized by increased PAI-1 expression and a predisposition to atherosclerosis.[7]

Pharmacological Targeting of PAI-1 Inhibits Obesity and Atherosclerosis

To determine the effects of pharmacological PAI-1 inhibition on atherosclerosis, we fed male adult *ldlr*$^{-/-}$ mice WD containing or lacking PAI-039 for 12 to 24 weeks. Mice receiving PAI-039 (n=12) were 27±3 weeks old when commencing WD versus 27±3 weeks old for control mice receiving WD lacking PAI-039 (n=12; $P>0.9$). PAI-039 treatment significantly inhibited plasma PAI-1 activity but had no significant effect on plasma PAI-1 antigen (Figure IIC in the Data Supplement). Body weight and diet consumption were measured weekly. Mice not treated with PAI-039 continued to gain weight throughout the 24 weeks of WD feeding. In contrast, PAI-039 treatment significantly attenuated weight gain, resulting in near plateau of body weight beyond 12 weeks of treatment (Figure 1A) but did not decrease food consumption compared with control (Figure 1B). At 12 weeks of WD, 7 mice from each group were euthanized for atherosclerosis studies. Atherosclerosis formation in the aortic root, aortic arch, and thoracic aorta was significantly less in mice treated with PAI-039 compared with controls (Figure 1C and 1D). At 24 weeks of WD consumption, the remaining 5 mice in each group were euthanized. Atherosclerosis formation was still significantly less in the aortic arch and thoracic aorta of PAI-039-treated animals than in controls (Figure 1D). Aortic root atherosclerosis was not assessed at the 24 week time point because a technical error in slide preparation resulted in loss of all samples. We quantified atherosclerosis in the carotid arteries of mice after 24 weeks of WD. As with the aorta, carotid atherosclerosis was significantly less in PAI-039-treated mice than in controls (Figure 1E). Thus, pharmacological inhibition of PAI-1 with PAI-039 was associated with a significant and sustained decrease in obesity and atherosclerosis formation.

To confirm the specificity of PAI-1 drug targeting, we also studied the effects of a recently described PAI-1

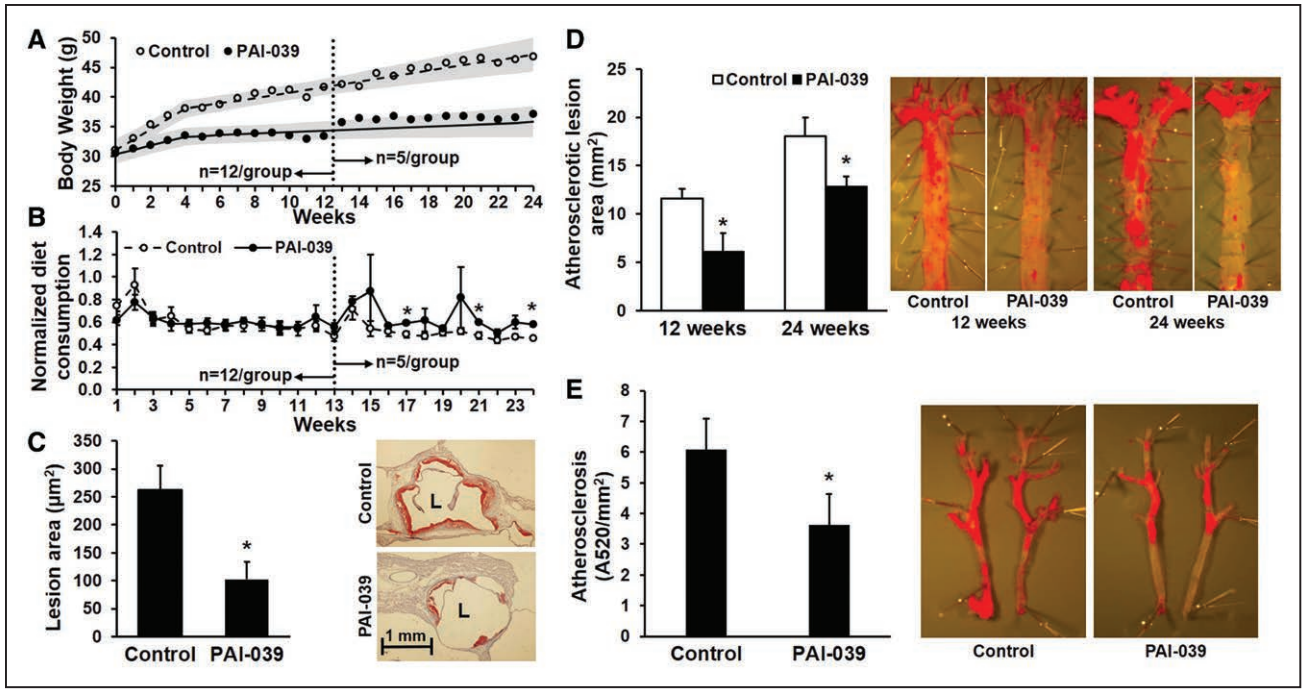

Figure 1. PAI-039 inhibits obesity and atherosclerosis without decreasing diet consumption.
Ldlr$^{-/-}$ mice (n=12/group) were fed Western diet (WD) containing (●) or lacking (○) PAI-039. Mean body weight and diet consumption were measured weekly. After 12 wk, 7 mice from each group were euthanized for atherosclerosis quantification. The remaining mice (n=5/group) were continued on WD and euthanized at week 24 for atherosclerosis quantification. **A**, Mean body weights. Shaded areas represent 95% CIs. Differences between groups were statistically significant ($P<0.05$) for all data points beyond week 2. **B**, Mean diet consumption/mouse/wk, normalized to body weight, that is, (diet consumed [g]/mouse/wk)/(body weight [g]); *$P<0.05$ vs control. **C**, Aortic root atherosclerosis, assessed after 12 wk of WD; n=7/group; *$P<0.05$. Representative Oil Red O-stained images are shown. L, lumen. **D**, Aortic arch and thoracic aorta atherosclerosis after 12 wk (n=7/group) and 24 wk (n=5/group) of WD; *$P<0.05$ vs control. Representative Oil Red O-stained images are shown. **E**, Carotid artery atherosclerosis after 24 wk of WD; *$P<0.05$ vs control. Representative images are shown.

inhibitor on weight gain and atherosclerosis formation. For this experiment, *ldlr*$^{-/-}$ mice were fed WD containing or lacking MDI-2268 for 12 weeks. MDI-2268 is structurally unrelated and significantly more potent than PAI-039 in vivo and was dosed >10-fold lower than for PAI-039 (400 μg/g of diet versus 5 mg/g, respectively). Mice receiving MDI-2268 (n=7; 3 females, 4 males) were 19±2 weeks old when commencing WD versus 19±2 weeks old for mice receiving WD alone (n=6; 3 females, 3 males; $P>0.7$). Control mice not receiving MDI-2268 continued to gain weight throughout the experiment. In contrast, MDI-2268-treated mice failed to gain weight, despite diet consumption similar to that of controls (Figure 2A and 2B). MDI-2268-treated mice appeared healthy throughout the experiment. At 12 weeks of WD, atherosclerosis formation in the aortic arch, thoracic and

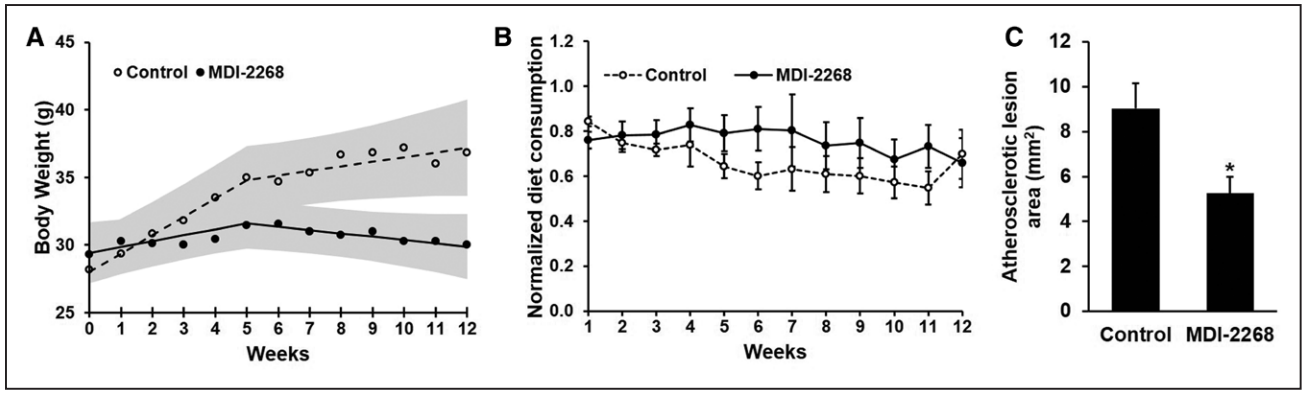

Figure 2. MDI-2268 inhibits obesity and atherosclerosis, without affecting diet consumption.
Ldlr$^{-/-}$ mice (n=6–7/group) were fed WD containing (●) or lacking (○) MDI-2268 for 12 wk. Mean body weight and diet consumption were measured weekly. **A**, Mean body weights. Shaded areas represent 95% CIs. Differences between groups were statistically significant ($P<0.05$) for all data points beyond week 8. **B**, Mean diet consumption, normalized to body weight, that is, (diet consumed [g]/mouse/wk)/(body weight [g]); no statistically significant difference was achieved at any time point between groups. **C**, Aortic arch, thoracic, and abdominal aorta atherosclerosis, assessed after 12 wk of WD; *$P<0.05$ vs control.

abdominal aorta was significantly less in MDI-2268-treated mice compared with controls (Figure 2C).

Pharmacological Targeting of PAI-1 Inhibits Macrophage Invasion Into Atherosclerotic Plaque Without Altering Plaque SMC or Collagen Content

We performed histochemical studies of atherosclerotic plaques to gain insights into the mechanisms by which pharmacological PAI-1 inhibition altered atherogenesis. Macrophage accumulation in the fibrous cap was significantly decreased in mice treated with either PAI-039 or MDI-2268, as compared with controls (Figure 3A and 3B). However, atherosclerotic plaque SMC and collagen content were not significantly altered by pharmacological inhibition of PAI-1 with MDI-2268 (Figure 3C and 3D).

PAI-039 Attenuates Visceral AT Inflammation, Hyperglycemia, and Hepatic Triglyceride Content

Visceral fat accumulation, assessed by weighing epididymal fat pads harvested 12 and 24 weeks after initiation of WD, was significantly less in PAI-039-treated mice than in controls (Figure 4A). Formation of macrophage crown-like structures in epididymal white AT was significantly less in PAI-039-treated mice than in controls (Figure 4B and 4C). Consistent with these results, quantitative reverse transcriptase-PCR analysis revealed a significant decrease in TNF-α gene expression in epididymal white AT of PAI-039-treated mice compared with controls (Figure 4D). There was no significant effect of PAI-039 on mean adipocyte size (Figure 4E). Consistent with the inhibitory effects of pharmacological targeting of PAI-1 on obesity and AT inflammation, there was a significant reduction in blood glucose, assessed by measurement of HbA1c after 24 weeks of WD, in PAI-039-treated mice (6.5±0.8%) versus controls (8.1±0.5%; n=5/group; $P<0.05$), indicating that PAI-039 reduces WD-induced hyperglycemia. PAI-039 significantly decreased liver triglyceride content, assessed after 12 weeks of WD, but had no significant effect on liver weight, assessed after 24 weeks of WD (Figure IIIA and IIIB in the Data Supplement). PAI-039 had no significant effect on plasma total cholesterol, LDL cholesterol, HDL cholesterol, or triglycerides concentrations, assessed after 12 and 24 weeks of WD (Figure IIIC through IIIF in

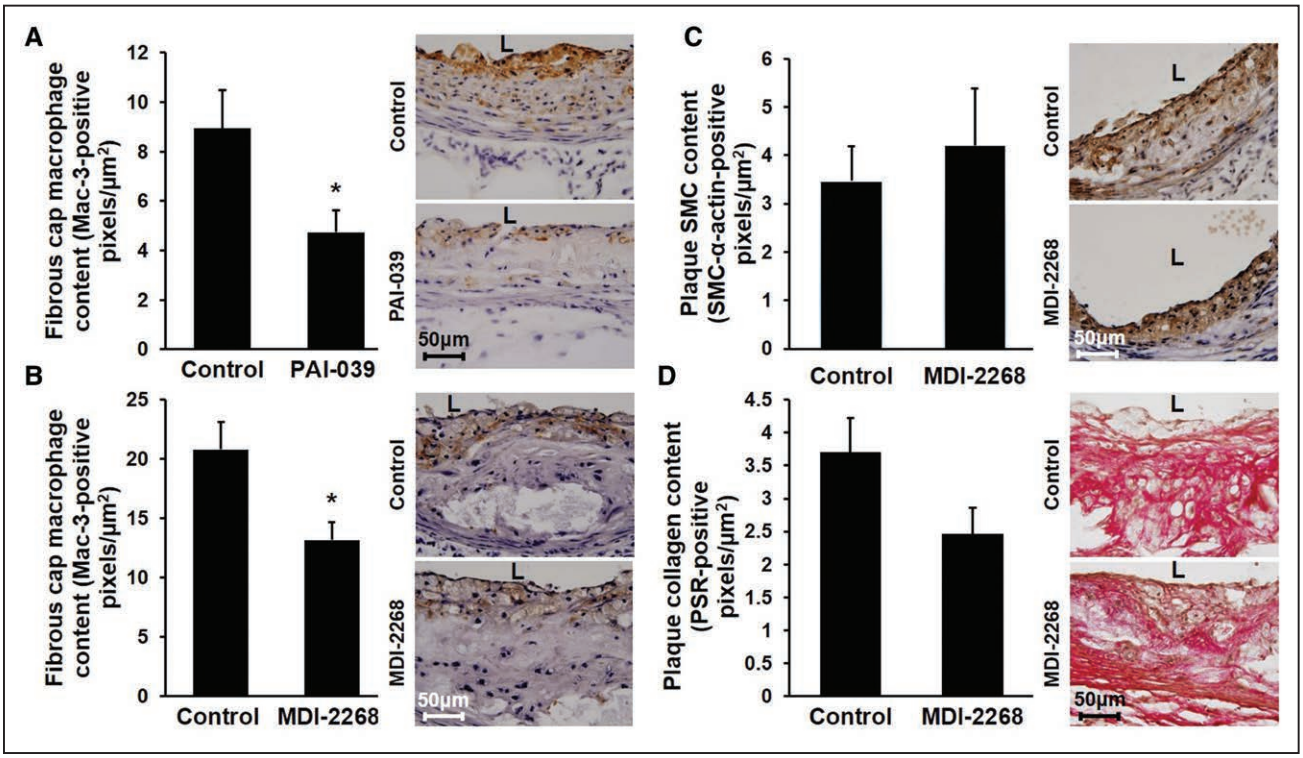

Figure 3. Pharmacological targeting of PAI-1 (plasminogen activator inhibitor-1) inhibits macrophage invasion into atherosclerotic plaques without altering their smooth muscle cell (SMC) or collagen content.
Ldlr$^{-/-}$ mice were fed Western diet containing or lacking PAI-1 inhibitor for 12 wk, after which aortic root atherosclerotic plaque composition was assessed. **A**, PAI-039 decreases fibrous cap macrophage content. Quantified data (n=5/group; *$P<0.05$) and representative images demonstrating macrophage invasion (brown color) are shown. **B**, MDI-2268 decreases fibrous cap macrophage content. Quantified data (n=6–7/group; *$P<0.05$) and representative images are shown. **C**, MDI-2268 does not significantly affect intimal SMC content, assessed by SMC-α actin immunostaining (brown color). Quantified data (n=4–6/group, difference between groups did not achieve statistical significance; $P>0.6$) and representative images are shown. **D**, MDI-2268 does not significantly affect plaque collagen content, assessed by picrosirius red (PSR) staining. Quantified data (n=6–7/group, difference between groups did not achieve statistical significance; $P>0.08$) and representative images are shown. L indicates lumen.

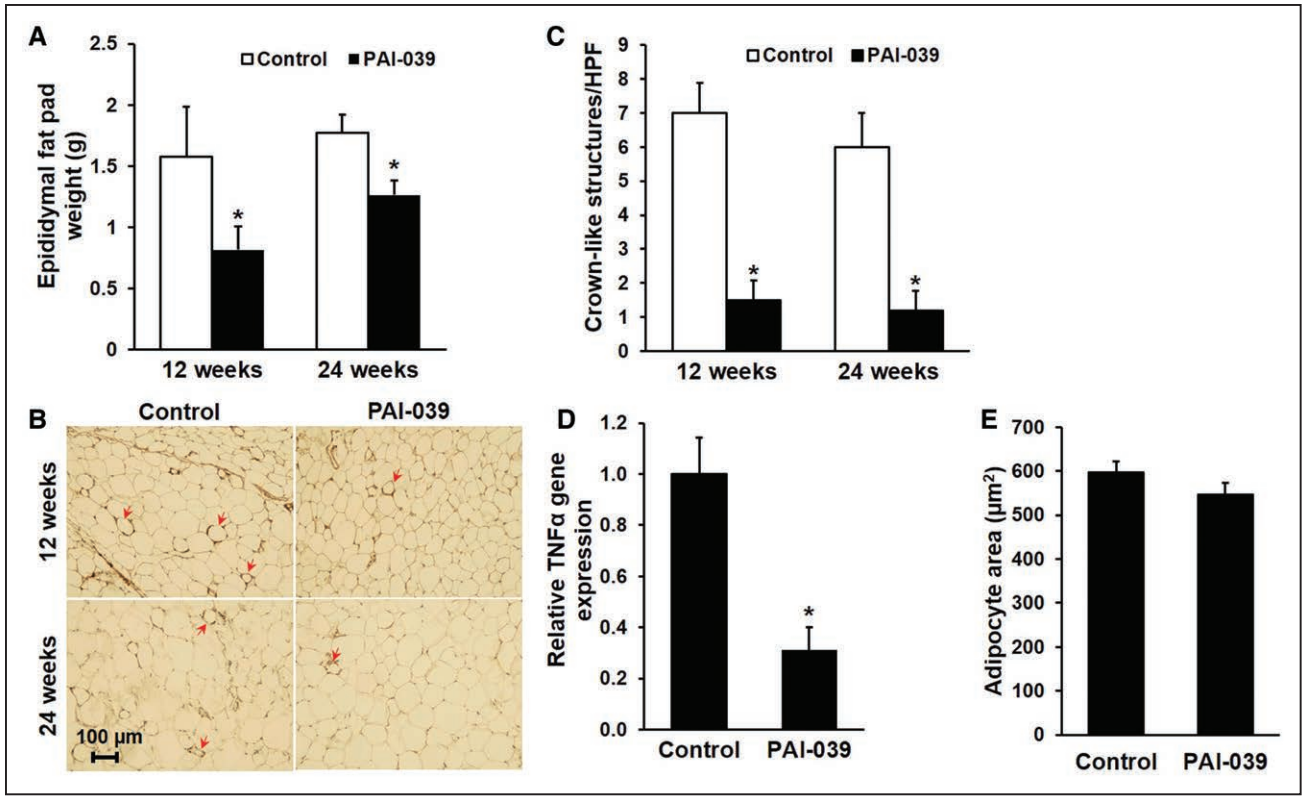

Figure 4. PAI-039 decreases visceral white adipose tissue mass and inflammation.
Epididymal white adipose tissue of *Idlr*$^{-/-}$ mice was harvested and analyzed after 12 and 24 wk of Western diet (WD) with or without PAI-039. **A**, Mean combined weights of left and right epididymal fat pads; n=5–7 mice/group; *P<0.05 vs control. **B**, Macrophage (Mac-3) immunostaining, demonstrating peri-adipocyte crown-like structures (arrows). **C**, Quantification of crown-like structure formation; n=4–5/group, *P<0.01; HPF indicates high-power field. **D**, TNF-α (tissue necrosis factor-α) gene expression, assessed by real-time reverse transcriptase-PCR analysis after 24 wk of WD; n=3–5 mice/group; *P<0.01 vs control. **E**, Mean adipocyte size (cross-sectional area), assessed after 12 wk of WD; n=4–5/group; P>0.3.

the Data Supplement). Recent studies suggest that fibrin deposition in AT plays a key role in AT inflammation and metabolic dysfunction.[48] However, we found no significant effect of pharmacological PAI-1 inhibition on epididymal white AT fibrin content (Figure IVA and IVB in the Data Supplement). LRP1, which binds PAI-1, has been implicated in the pathogenesis of obesity.[49] However, we found no significant effect of pharmacological PAI-1 inhibition on visceral white AT LRP1 content, assessed by quantitative immunohistochemistry (Figure IVC and IVD in the Data Supplement). As a whole, these results suggested that the antiatherosclerotic effects of pharmacological PAI-1 inhibition are mediated, at least in part, by attenuation of diet-induced visceral adiposity, AT inflammation, glucose intolerance, and steatohepatosis.

PAI-1 Stimulates SMC Senescence, Which Is Blocked by PAI-039 and Anti-LRP1 Antibody

Senescence of vascular cells, including SMCs, plays a significant role in atherogenesis.[33,34] To determine if PAI-1 induces SMC senescence, human coronary artery SMCs were incubated with or without recombinant PAI-1 for 24 hours, after which expression of SA-βGal, a specific marker of cell senescence, was measured. PAI-1 significantly increased SMC senescence, and this effect was inhibited by PAI-039 (Figure 5A). To elucidate potential mechanisms, SMCs were pretreated with or without R2629, a specific anti-LRP1 antibody, then incubated with or without recombinant PAI-1. R2629 significantly inhibited the prosenescent effect of PAI-1, while also exerting a minor prosenescent effect when incubated with SMCs in the absence of PAI-1 (Figure 5B). To further test the hypothesis that PAI-1 stimulates SMC senescence by binding LRP1, SMCs were incubated with vehicle control, a recombinant active, stable form of PAI-1 (I91L), or a recombinant PAI-1 mutant defective in LRP1 binding due to mutations at amino acids 80 and 207 (while also containing the activity-stabilizing I91L mutation).[36] Whereas PAI-1-I91L (1 µg/mL) significantly increased SMC senescence, PAI-1-I91L, K80/207A, the PAI-1 mutant defective in LRP1 binding, had no significant effect (Figure 5C).

PAI-039 Inhibits Vascular Senescence In Vivo

To determine the effects of pharmacological PAI-1 inhibition on vascular senescence in vivo, SA-βGal activity

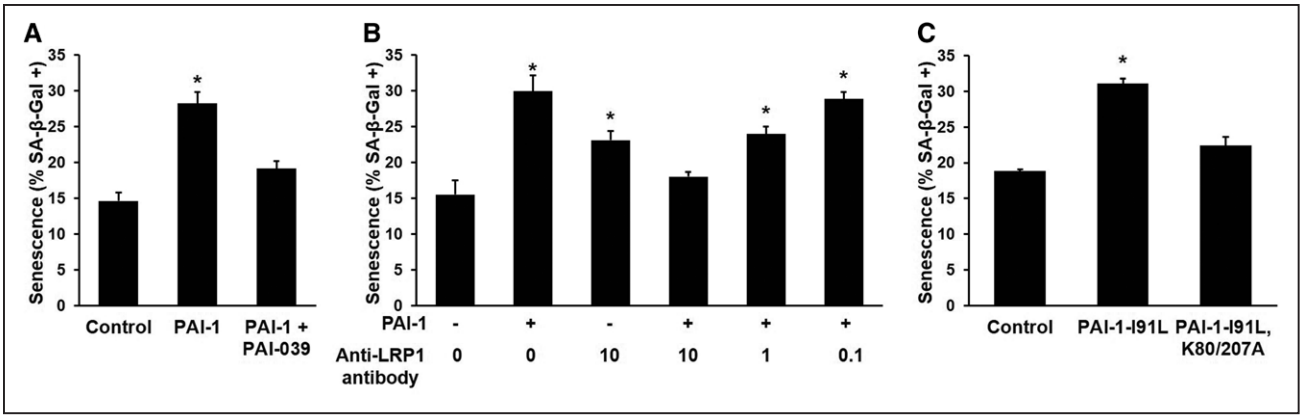

Figure 5. PAI-1 (plasminogen activator inhibitor-1) stimulates smooth muscle cell (SMC) senescence in an LRP1 (low-density lipoprotein receptor-related protein 1)-dependent manner.
A, SMCs were incubated for 24 h with recombinant PAI-1 (10 μg/mL), recombinant PAI-1 and PAI-039 (25 μmol/L), or vehicle control, after which SA-βGal (senescence-associated β-galactosidase) expression (% positive cells) was measured; n=5–7/group; **P*<0.05 vs other groups. **B**, SMCs (passage number 6–8) were incubated 12 h with or without anti-LRP1 antibody (at indicated concentrations [μg/mL]), after which PAI-1 (1 μg/mL, +) or vehicle control (–) was added. Cells were incubated an additional 24 h, after which SA-βGal expression was measured; n=5/group, **P*<0.05 vs control (untreated SMCs). **C**, SMCs (passage number 7) were incubated 24 h with PAI-1-I91L (recombinant PAI-1 mutant with increased stability, normal antiprotease activity, and normal LRP1-binding affinity, concentration 1 μg/mL), PAI-1-I91L,K80/207A (recombinant PAI-1 mutant with increased stability, normal antiprotease activity, and a >20-fold reduction in LRP1-binding affinity; concentration 1 μg/mL), or vehicle control, after which SA-βGal expression was measured; n=4/group; **P*<0.001 vs other groups.

was assessed in aortic arches of *ldlr*$^{-/-}$ mice after 12 weeks of WD. Vascular cell senescence was significantly decreased in PAI-039-treated animals compared with controls fed WD alone (Figure 6A). We used a histochemical assay to specifically assess cell senescence in atherosclerotic plaques. This analysis suggested that cell senescence was significantly reduced in atheroma from mice treated with PAI-039 compared with controls (Figure 6B). These in vivo data, together with the in vitro SMC data, suggest that PAI-1 promotes atherosclerosis by direct effects on vascular cells, including SMCs, by pathways that are inhibited by PAI-039 and require PAI-1 binding to LRP1.

DISCUSSION

The metabolic syndrome, which is characterized by obesity, dyslipidemia, hypertension, glucose intolerance, and

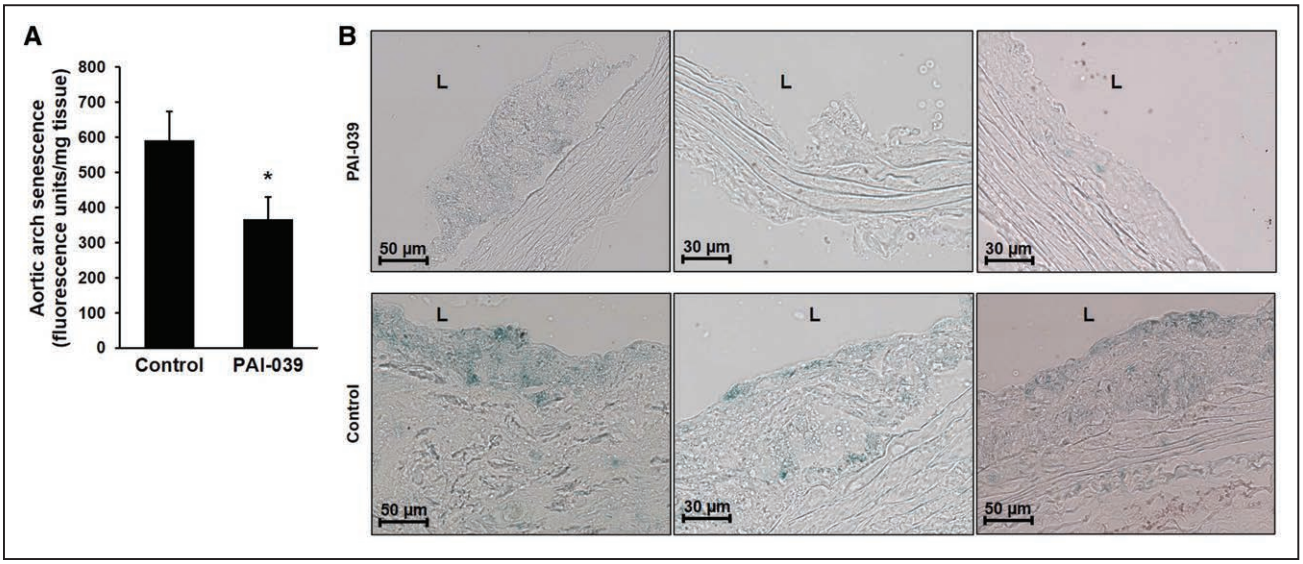

Figure 6. PAI-039 inhibits vascular senescence in vivo.
A, Total aortic cell senescence is decreased by PAI-039. *Ldlr*$^{-/-}$ mice were fed Western diet (WD) containing or lacking PAI-039 for 12 wk, after which aortas were perfusion-fixed with paraformaldehyde and excised. Periadventitial fat and other tissue were carefully excised. Cell senescence was determined by incubating aortic arches in solution containing fluorescein di-β-d-galactopyranoside for 6 h and measuring fluorescence of the solution, with results normalized to tissue weight; n=7 mice/group, **P*=0.05. **B**, *Ldlr*$^{-/-}$ mice were fed WD with or without PAI-039 for 12 wk. Paraformaldehyde-fixed aortas were excised and cross-sections from aortic roots were subjected to histochemical analysis to detect senescence-associated β-galactosidase activity (blue color). Representative images from 3 mice in each treatment group are shown. L indicates lumen.

atherosclerosis,[50,51] affects nearly 35% of all adults and 50% of those aged 60 years or older,[52] making it a dominant driver of the total societal burden of atherosclerosis. PAI-1, the primary inhibitor of tPA and uPA, is a key regulator of fibrinolysis and cell migration that has been strongly implicated in the pathogenesis of atherosclerosis, particularly in patients with the metabolic syndrome.[7,8] In this study, we demonstrated that PAI-039 and MDI-2268 inhibit atherosclerosis formation in mice with metabolic syndrome, which is the first demonstration that drug targeting of the fibrinolytic system inhibits atherogenesis. The similar effects of 2 unrelated PAI-1 inhibitors suggests a drug class effect on atherogenesis, rather than a potential off-target effect of a single compound. We also showed that pharmacological targeting of PAI-1 inhibits cell senescence and macrophage accumulation in atherosclerotic plaques, while also producing beneficial metabolic effects, including attenuation of obesity, AT inflammation, hyperglycemia, and steatohepatosis. These findings have important therapeutic implications and shed new light on the complex role of PAI-1 in regulating atherosclerosis and the metabolic dysfunction associated with obesity.

In murine models, the regulatory function of PAI-1 in atherosclerosis has been examined predominantly by using PAI-1-deficient mice. Eitzman et al[27] found that genetic deletion of PAI-1 inhibited carotid artery atherosclerosis, whereas Luttun et al[28] reported accelerated aortic atherosclerosis in PAI-1-deficient mice. Sjöland et al[26] found no significant effect of genetic PAI-1 deficiency on atherogenesis. However, results of gene knockout studies do not necessarily predict the effects of pharmacological PAI-1 inhibition on atherosclerosis and other PAI-1-dependent processes. We showed previously that genetic deletion of PAI-1 increases neointima formation in vein grafts,[44] while PAI-039 significantly decreases neointima formation in the same model.[24] This paradox is likely attributable to multiple factors, which we discussed previously.[24] The results of the current study suggest that drug targeting of PAI-1 may be a new strategy to inhibit atherosclerotic plaque growth, which is relevant to the many patients intolerant of statin therapy.[53] Pharmacological PAI-1 inhibition could also be employed in conjunction with statin therapy, not only to provide additional antiatherosclerotic effects, but also to inhibit thrombosis.

In the current study, we showed that PAI-1 promotes SMC senescence. This finding, which is consistent with previous studies demonstrating prosenescent effects of PAI-1 on other cell types,[13,14,54–58] is significant because it identifies a new trigger of SMC senescence, which itself has been identified as pro-atherosclerotic.[34] We also demonstrated that PAI-039 attenuates cell senescence in vivo in atherosclerotic plaques. Therefore, our data support the hypothesis that pharmacological targeting of PAI-1 inhibits atherosclerosis by attenuating vascular cell senescence.[33] Furthermore, we demonstrated that PAI-1's prosenescent effect is blocked by an antibody specific for LRP1, and that a PAI-1 mutant defective in LRP1 binding has no significant effect on SMC senescence. Heretofore, little has been known about the role of LRP1 in cell senescence.[59,60] Our results suggest that binding of PAI-1 to LRP1 initiates the process. LRP1 is a recognized PAI-1 receptor that triggers intracellular signaling pathways, including activation of JAK1/STAT1 (Janus kinase 1/signal transducer and activator of transcription 1).[24,61] Based on our findings and previously published data, we hypothesize that the antisenescent effect of PAI-039 is mediated by decreasing the pool of active, pericellular PAI-1, thereby inhibiting PAI-1 binding to uPA, which is expressed by SMCs.[62,63] This, in turn, will reduce the interaction of PAI-1 with LRP1, as PAI-1-uPA complex binds LRP1 ≈100-fold more avidly than either active or latent PAI-1.[36,64] Our studies also suggest another mechanism by which pharmacological inhibition of PAI-1 down-regulates atherogenesis, that is, by decreasing macrophage accumulation in developing plaques. Such an effect would be anticipated to attenuate formation of vulnerable plaques prone to rupture[65] and is consistent with a study demonstrating that pharmacological targeting of PAI-1 inhibits macrophage migration.[16] Together, our cell senescence and macrophage invasion data support the hypothesis that drug-targeting of PAI-1 exerts direct effects on the vascular wall that attenuate atherogenesis. This hypothesis is also supported by our previous demonstration of inhibition of neointima formation by pharmacological PAI-1 inhibition in mice without obesity or hyperlipidemia.[24] There are yet another potential mechanisms by which pharmacological PAI-1 inhibitors may act on the arterial wall to regulate atherosclerosis. Simone et al[20] demonstrated that PAI-039 fosters formation of the cleaved form of PAI-1 and increases SMC apoptosis by a pathway that appears to involve TNF-α weak inducer of apoptosis (TWEAK) and its receptor, FGF (fibroblast growth factor)-inducible 14 (FN14). This group also showed that PAI-039 and cleaved PAI-1 each inhibited intimal hyperplasia, a hallmark of atherogenesis, in the murine carotid artery ligation model.

In addition to direct vascular effects, pharmacological targeting of PAI-1 has the potential to attenuate atherogenesis indirectly by dampening systemic inflammatory pathways that stimulate atherosclerosis. The metabolic syndrome is characterized by fibrin formation and leukocyte accumulation in visceral AT, which leads to secretion of inflammatory cytokines that act systemically to promote obesity, insulin resistance, and endothelial cell activation.[48,66,67] LRP1 has also been implicated as a regulator of obesity.[49,68] We demonstrated that PAI-039 and MDI-2268 inhibit obesity. However, we found no significant effect of pharmacological PAI-1 inhibitors on visceral AT fibrin or LRP1 content, assessed by immunostaining. These results suggest that promotion of fibrinolysis or changes in LRP1 expression in AT did not play major roles in the suppression of obesity and hyperglycemia by PAI-1 inhibition in our model.

However, the methods employed in our study do not allow us to exclude the possibility that subtle changes in AT fibrin and LRP1 content, cleavage, or distribution contribute to the prometabolic and antiatherosclerotic effects of PAI-1 inhibition. Crandall et al[38] and Lijnen et al[19] demonstrated that PAI-039 inhibits short-term weight gain in mice with normal lipid metabolism. Our data significantly extend the prior work by showing that pharmacological inhibition of PAI-1 produces a durable antiobesity effect (ie, still present after 24 weeks of therapy, which is 6-fold longer than the previously published studies), including in obese mice with hyperglycemia and severe dyslipidemia. Unlike the studies of Crandall and Lijnen, we found no significant effect of PAI-039 on adipocyte size, raising the possibility that the attenuation of visceral obesity by pharmacological targeting of PAI-1 under the experimental conditions in our study might be linked to decreases in adipocyte number, possibly mediated by effects of PAI-1 targeting on preadipocyte survival or differentiation.[69] Further studies are warranted to address this issue. We showed in the current study that PAI-039 significantly inhibits macrophage accumulation and TNF-α expression in visceral white AT, while also inhibiting hyperglycemia and steatohepatosis, key features of the metabolic syndrome that are associated with atherogenesis.[70] These findings are consistent with a previous report demonstrating that TM5441, another PAI-1 inhibitor, significantly attenuates macrophage accumulation in white AT and insulin resistance in mice, assessed by glucose- and insulin tolerance testing.[71] Henkel et al[72] showed that pharmacological PAI-1 inhibition attenuates steatohepatosis. As a whole, our findings support the hypothesis that pharmacological inhibition of PAI-1 inhibits atherogenesis by significantly attenuating the AT inflammation and systemic metabolic dysfunction that are characteristic of the metabolic syndrome.

In conclusion, we have demonstrated that pharmacological targeting of PAI-1 significantly attenuates atherosclerosis in mice with obesity and features of the metabolic syndrome, a common clinical entity that is strongly associated with increased PAI-1 expression. Our results provide important insights into the role of PAI-1 in atherogenesis that complement and extend studies involving PAI-1 knockout mice. The finding that drug targeting of PAI-1 attenuates atherogenesis is highly significant from the clinical perspective. The fibrinolytic pathway is not directly involved in lipid metabolism, which is the target of currently used antiatherosclerotic drugs, such as statins and PCSK9 (proprotein convertase subtilisin/kexin type 9) inhibitors.[73,74] Thus, our results suggest a potential new direction in antiatherosclerotic therapy. They also suggest that the antiatherosclerotic effects of pharmacological PAI-1 inhibition involve multiple mechanisms, including attenuation of cell senescence and macrophage accumulation in atherosclerotic plaques, as well as reductions in visceral AT inflammation and glucose intolerance. We also have shown that PAI-1 promotes SMC senescence, a key step in atherogenesis, by an LRP1-dependent mechanism. Our data suggest that drug targeting of PAI-1 provides long-term attenuation of weight gain, even in the presence of severe metabolic dysfunction, a significant and clinically relevant advance from prior reports. More studies are indicated to define the effects of PAI-1 inhibitors on specific AT depots (ie, subcutaneous, perirenal, perivascular, and brown), including their size, degree of inflammation, and thermogenic properties. In future work, it also will be of interest to study the capacity of pharmacological PAI-1 inhibitors to reverse established obesity and atherosclerosis.

ARTICLE INFORMATION

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Affiliations

From the Department of Medicine (H.B.K, Y.J., D.J.B., M.V., A.A.A., W.P.F.) and Department of Medical Pharmacology & Physiology (W.P.F.), University of Missouri School of Medicine; Research Service, Harry S. Truman Memorial Veterans Hospital, Columbia, MO (W.P.F.); Department of Chemistry, Eastern Michigan University, Ypsilanti (C.D.E.); and Department of Internal Medicine, University of Michigan Medical School, Ann Arbor (D.A.L.).

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Disclosures

D.A. Lawrence and C.D. Emal have an equity interest in MDI Therapeutics, which holds an option to license MDI-2268 from the University of Michigan. The other authors report no conflicts.

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