METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

A Noncanonical Role for Plasminogen Activator Inhibitor Type 1 in Obesity-Induced Diabetes

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Obesity is a major risk factor for type 2 diabetes because of chronic hepatic inflammation and resultant insulin resistance. Hepatocyte growth factor (HGF) is responsible for resetting hepatic homeostasis after injury following activation by urokinase-type plasminogen activator (u-PA; encoded by the *PLAU* gene). Plasminogen activator inhibitor type-1 (PAI-1; encoded by the *SERPINE1* gene), a u-PA inhibitor and antifibrinolytic agent, is often elevated in obesity and is linked to cardiovascular events. We hypothesized that, in addition to its role in preventing fibrinolysis, elevated PAI-1 inhibits HGF's activation by u-PA and the resultant anti-inflammatory and hepatoprotective properties. Wild-type and PAI-1 knockout (KO) mice on a high-fat diet both became significantly heavier than lean controls; however, the obese KO mice demonstrated improved glucose metabolism compared with wild-type mice. Obese KO mice also exhibited an increase in conversion of latent single-chain HGF to active two-chain HGF, coinciding with an increase in the phosphorylation of the HGF receptor (HGFR or MET, encoded by the *MET* gene), as well as dampened inflammation. These results strongly suggest that, in addition to its other functions, PAI-mediated inhibition of HGF activation prohibits the resolution of inflammation in the context of obesity-induced type 2 diabetes. (*Am J Pathol* 2019, 189: 1413–1422; <https://doi.org/10.1016/j.ajpath.2019.04.004>)

Type 2 diabetes (T2D) is characterized by an increase of hepatic glucose, impaired insulin secretion, and obesity. It is thought that in the development of T2D, overnutrition because of increased caloric intake leads to a dysfunction in metabolic homeostasis from the overwhelming presence of adipose tissue.¹ This dysregulation is characterized by elevated proinflammatory cytokine production that comes from macrophages in the liver and adipose tissue, ultimately leading to insulin resistance.^{1–6}

Cytokines secreted by the liver reflect the presence and intensity of inflammation and are part of how the liver attempts to maintain systemic homeostasis during stress, infection, and/or injury.^{7,8} On the other hand, proper homeostasis of the liver itself is regulated by a protein known as hepatocyte growth factor (HGF), a mitogen for liver repair and maintenance that is transcribed in response to the proinflammatory cytokine IL-6.⁹ Among its other physiological functions, HGF plays a key role in resetting the

hepatic homeostatic threshold after injury.^{10,11} In liver, this is achieved after cleavage of latent, single-chain HGF (scHGF) to its active two-chain form (tcHGF) by the urokinase-type plasminogen activator (u-PA).^{12,13} u-PA's activity is regulated by plasminogen activator inhibitor type-1 (PAI-1), an acute phase protein and antifibrinolytic agent, which is consistently up-regulated in obesity and linked to elevated cardiovascular anomalies in obese patients.^{14–16} Another critically important outcome of hepatic PAI-1 is

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the inhibition of u-PA that subsequently prevents activation of HGF.^{12,17,18} As HGF must be active to signal through its receptor, MET,^{12,19,20} and as evidence suggests that active tcHGF can have a palliative role in diabetes,^{21,22} possibly because of its role in regulating the insulin receptor,²³ it is reasonable to hypothesize that in addition to its role in preventing fibrinolysis, an obesity-induced increase in PAI-1 protein could lead to the accumulation of nonactive scHGF, thereby exacerbating the disease process.

Although active tcHGF can induce IL-6 production in hepatocytes,²⁴ it suppresses immune cell IL-6 production in a glycogen synthase kinase-3 β —dependent manner.²⁵ Therefore, in the earliest developmental stages that can lead to nutrition-induced T2D, tcHGF is a likely candidate for inducing IL-6 production in hepatocytes while simultaneously suppressing the early, macrophage-mediated production of cytokines. On the other hand, over time, as prolonged, obesity-induced stress generates PAI-1 production that coincides with the development of T2D, the expectation would be that PAI-1—mediated inactivation of HGF would no longer be able to induce hepatocellular IL-6 or suppress its expression from immune cells.

Herein, we characterized the PAI-1 knockout (KO) mouse to definitively show the role PAI-1 plays in HGF activation and subsequent inflammatory-mediated induction of diabetes. Wild-type (WT) and KO mice were either fed normal chow or a high-fat diet (HFD) for a period of 20 weeks, and parameters related to the development of T2D were assessed. The results demonstrate that in WT mice, overnutrition leads to weight gain, glucose intolerance, and insulin resistance, as well as elevated PAI-1 levels. The increase in PAI-1 corresponds with a decrease in the conversion of scHGF to tcHGF and subsequent reductions in MET phosphorylation; these events coincide with classic, unresolved chronic inflammation. When PAI-1 KO mice are fed the HFD, with the exception of weight gain, the phenotype is abrogated, including the deleterious effects of obesity-mediated inflammation. As active HGF is known to have a palliative role in T2D, these positive changes are attributed to unabated HGF activation when PAI-1 is absent.

Materials and Methods

Mice

For most experiments, 6- to 8-week-old male C57BL/6J (WT) and B6.129S2-Serpine1<tm1Mlg>/J (PAI-1 KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were fed either an HFD, consisting of 60% kcal fat and 20% kcal each protein and carbohydrate (D12492; Research Diets, New Brunswick, NJ), or standard chow. The animals were fed the diets for 20 weeks, and body weight was recorded weekly until termination of the experiment. For some experiments, animals were fed an accelerated fast food diet for 20 weeks that maintains a higher than normal fat content but that also includes a higher

than normal carbohydrate component.²⁶ Animals with exon 16 of MET specifically deleted from their hepatocytes were generated by crossing MET^{fl/fl} mice (a gift from Snorri Thorgeirsson, currently available at The Jackson Laboratories as 016974, Mettm1Sst) with animals expressing Cre recombinase under an α -fetoprotein enhancer-driven albumin promoter.²⁷ All animals were housed under specific pathogen-free conditions in the animal facility at the University of Pittsburgh (Pittsburgh, PA). This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the NIH.²⁸ The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (assurance number A3187-01).

I.P. Glucose Tolerance Test

A 1 g/kg dose of glucose was administered intraperitoneally to fasted mice (12 hours) at 20 weeks after normal or HFD feeding. Blood glucose readings were taken at 0 (before glucose injection), 30, 60, 90, and 120 minutes after glucose injection and measured using a Bayer Breeze2 glucometer (Bayer AG, Leverkusen, Germany).

HOMA-IR Data

The homeostatic model assessment—estimated insulin resistance (HOMA-IR) index was calculated as a measure of insulin sensitivity, as follows:

$$\text{HOMA-IR} = [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (U/mL)}] \div 405.$$
²⁹

Glucose-Stimulated Insulin Secretion

A 2 g/kg dose of glucose was injected intraperitoneally after overnight fasting (12 hours). Blood glucose was measured immediately before injection and 15 minutes after injection using a Bayer Breeze2 glucometer.

Serum PAI-1 Measurement

PAI-1 levels were measured in the serum of WT and PAI-1 KO animals fed an HFD or standard chow at 2.5, 7.5, 11, and 20 weeks of feeding by a Milliplex Mouse Serum Adipokine Panel (Millipore, Billerica, MA).

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR was performed, as previously described.³⁰ The primer sequences for the RNAs encoding murine ribosomal protein L15 and PAI-1 are as follows: *Rpl15*, 5'-GGCGACCTGGAAGTCCAACT-3' (forward) and 5'-CCATCAGCACACAGCCTTC-3' (reverse); and *Serpine1*, 5'-GATCAGTACTGCGGATGCCA-3' (forward) and 5'-CCCCTTGCCAGTAAGTCAC-3' (reverse).

Figure 1 Changes in plasminogen activator inhibitor type-1 (PAI-1) levels with developing obesity. **A:** PAI-1 was analyzed by Western blot analysis in livers from C57BL6/J wild-type (WT) animals fed either standard chow (lean) or a diet of 60% kcal of fat and 20% kcal each protein and carbohydrate [high-fat diet (HFD)] at 12 weeks after the start of feeding. Blots were then stripped and reprobed with actin as a loading control. Each lane is from a different animal. **B:** Serum PAI-1 levels were measured in WT animals after 2.5, 7.5, 11, and 20 weeks of feeding the animals either a lean diet or an HFD. Unpaired *t* tests were performed. **C:** Hepatic PAI-1 gene expression was measured by quantitative real-time RT-PCR after 20 weeks in WT and PAI-1 knockout (KO) mice fed either a lean diet or an HFD. One-way analysis of variance with a Tukey posttest was performed. **D:** Weight gain was monitored weekly in WT and PAI-1 KO mice fed either a lean diet or an HFD for 20 weeks. Two-way analysis of variance with a Bonferroni posttest was performed. Data are expressed as means $\pm$ SEM (**B–D**). *n* = 5 mice per group (**D**). **P* < 0.05, ***P* < 0.01.

Tissue Lysate Preparation and Western Blot Analysis

Protein lysates from snap-frozen tissues were prepared and probed, as previously described,²⁴ using a detergent-free lysis buffer (10 mmol/L Tris-HCl, pH 7.5) with standard inhibitors. Amiloride and aprotinin were also added to the lysis buffer as they specifically inhibit u-PA and plasminogen activities, respectively. Once homogenized, the samples were centrifuged at $40,000 \times g$ for 30 minutes at 4°C. The supernatants were saved, and the pellets were resuspended by sonication in 1% SDS-Tris buffer with inhibitors. Supernatants were enriched for cytosolic proteins; pellets represented a nuclear-, matrix-, and membrane-enriched fraction. The bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL) was used to determine protein concentrations. Protein lysates were separated onto 10% SDS-PAGE gels. After transfer, membranes were stained with Ponceau S. Western blot analyses were performed with antibodies to PAI-1 (sc-8979; Santa Cruz Biotechnology, Dallas, TX), HGF (AF2207; R&D Systems, Minneapolis, MN), phosphorylated MET (Y1349; number 3121; Cell Signaling Technology, Danvers, MA), and total MET (number 3127; Cell Signaling Technology) in 5% fish gelatin in Tris-buffered saline with 0.1% Tween. Secondary antibodies, from Jackson ImmunoResearch (West Grove, PA), were used in 1% fish gelatin in Tris-buffered saline

with 0.1% Tween. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). The blots were visualized using BioBlot BXR (Laboratory Product Services, Rochester, NY). Numbers obtained from ImageJ version 1.50i (NIH, Bethesda, MD; <https://imagej.nih.gov>) analyses were analyzed using Prism software version 7.0d (GraphPad, San Diego, CA).

Enzyme-Linked Immunosorbent Assay

Hepatic IL-6 and tumor necrosis factor- α protein concentrations were measured by enzyme-linked immunosorbent assay (Abcam, Cambridge, MA), as previously described.²⁹ Briefly, cytokine concentration was determined from liver lysates, and data are displayed as picograms of cytokine per milligrams of liver tissue.

Histology

Liver tissue harvested from WT and PAI-1 KO mice after 20 weeks of feeding was fixed in 10% formalin and embedded in paraffin. Sections were stained with an antibody specific for IL-6 (Abcam) for immunohistochemistry. Antigen retrieval was performed using a citrate buffer, pH 6.0, for IL-6 staining and microwaving. Staining was

Figure 2 Effects on glucose metabolism as a result of plasminogen activator inhibitor type-1 deficiency. **A–F:** After overnight fasting, i.p. glucose tolerance tests were given to mice in all cohorts at 20 weeks of feeding. Blood glucose levels were recorded after a 1 g/kg dose of glucose intraperitoneally over 120 minutes. Groups from **A** were separated to show clarity of statistical differences in **B** through **F**. Wild-type (WT) mice fed standard chow, open circles; WT mice fed a high-fat diet (HFD), closed circles; knockout (KO) mice fed lean chow, open triangles; and KO mice fed an HFD chow, closed triangles. Two-way analysis of variance with a Bonferroni posttest was performed. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group (**A–F**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

visualized using a chromogenic substrate. Scoring for hepatocellular and immune cell IL-6 was performed using random images ($n = 3$ per sample at $\times 400$ magnification) taken by one investigator (G.M.C.) and analyzed by a second investigator (K.K.) who was blinded to what the various samples were. Hepatocellular IL-6 was graded on an arbitrary scale of 1 to 5, with 5 representing the highest overall staining level. For immune cells, the total number of positive cells in each image were counted.

Statistical Analysis

Prior work from our laboratory has demonstrated that group sizes of five or greater are sufficient for generating statistically meaningful results. All experiments were

performed in at least triplicate. The differences between mean values were assessed by t -test or analysis of variance, with $P \leq 0.05$ considered significant. Significance was calculated using Prism software version 7. Data are displayed as means $\pm$ SEM. Densitometry and immunohistochemistry quantification was calculated using the ImageJ software.

Results

High-Fat Diet Induces PAI-1 and Obesity

PAI-1 is known to be elevated in obesity and T2D and is associated with cardiovascular complications.^{31–33} To test the hypothesis that PAI-1 negatively regulates HGF in

Figure 3 Effects of plasminogen activator inhibitor type-1 deficiency on insulin resistance. The homeostatic model assessment—estimated insulin resistance (HOMA-IR) index was calculated and glucose-stimulated insulin secretion (GSIS) was measured in mice from all cohorts at 20 weeks of feeding. **A:** HOMA-IR was calculated using fasting blood glucose and serum insulin levels ($[\text{fasting blood glucose (mg/dL)} \times \text{fasting insulin } (\mu\text{U/mL})]/405$). **B** and **C:** In the fasted state, GSIS was measured at 0 and 15 minutes after a 2 g/kg dose of glucose intraperitoneally in lean diet (**B**) or high-fat diet (HFD) fed (**C**) mice. Unpaired t tests were performed. Data are expressed as means $\pm$ SEM. $n = 5$ mice per group (**A–C**). * $P < 0.05$. KO, knockout; WT, wild type.

Figure 4 Plasminogen activator inhibitor type-1 (PAI-1) regulates conversion of latent hepatocyte growth factor (HGF) to its active two-chain form (tcHGF). Liver tissues were harvested from wild-type (WT) and PAI-1 knockout (KO) mice after 20 weeks of feeding. **A:** Western blot analysis for HGF was performed on cytoplasmic-enriched protein fractions from WT and PAI-1 KO mice after a high-fat diet (HFD) or standard chow feeding. **B:** After ensuring an overall evenness of protein loading using Ponceau S staining, the blots in **A** were quantified by densitometry. **C:** Western blot analysis for HGF was performed on membrane-enriched fractions from mice on an alternative, fast food diet (FFD, left panel) and results were quantified by densitometry (right panel). **B** and **C:** As the blots were probed with a polyclonal antibody that also detects the light chain (not depicted), the relative fraction of single-chain HGF (scHGF) converted to tcHGF was calculated using the formula: $100 \times [3/2 \text{ heavy chain of tcHGF (hcHGF)} / (3/2 \text{ hcHGF} + \text{scHGF})]$. The assumption was that the hcHGF represents two-thirds of the tcHGF. Unpaired *t* tests were performed. Each lane represents a separate animal. Data are expressed as means $\pm$ SEM (**B** and **C**). *n* = 5 mice per group (**B**). **P* < 0.05, ***P* < 0.01.

obesity-induced diabetes, the model was first verified by examining male WT mice fed an HFD for up to 20 weeks. Mice fed standard chow were also monitored for the duration of the study and considered lean controls. Although there are other models, the HFD-fed mouse model is a well-accepted model for studying obesity-induced insulin resistance.³⁴ On immunoblotting, bands of approximately 55 kDa, corresponding to the reported size of full-length PAI-1, as well as secondary, lower-molecular-weight bands were elevated in the livers of animals after 12 weeks of feeding (Figure 1A). Furthermore, serum PAI-1 levels, as measured by a multiplex assay in WT mice, gradually became elevated and reached significance at 20 weeks of HFD feeding when compared with lean mice (Figure 1B). Although RNA levels did not reach significance, HFD-fed WT mice also exhibited a trend toward elevated gene expression for mRNAs encoding PAI-1 protein in the liver compared with lean mice, after 20 weeks of feeding (Figure 1C). RNAs from PAI-1 KO mice on standard and HFD served as negative controls.

Next, the effect of HFD was assessed on weight gain in the WT and KO mice. Figure 1D shows the body weights of all cohorts of WT and KO over 20 weeks of HFD or

standard chow feeding. The overall body weight of HFD-fed WT animals significantly increased compared with lean controls, beginning at 9 weeks after initiation of the diet. For the KO animals, although there was also a difference observed after 9 weeks on the diet, consistent differences in weight between lean and HFD were only noted after 14 weeks of feeding. There were no significant differences observed in body weight between the HFD or lean groups of either WT or KO mice throughout the study, although there was a trend toward a slightly lower weight gain for the KO mice on the HFD versus their WT counterparts.

PAI-1 Alters Glucose Metabolism and Insulin Sensitivity

To determine the effect of PAI-1 on glucose metabolism after HFD feeding, an i.p. glucose tolerance test was performed after 20 weeks of feeding (Figure 2). In both WT and KO animals, HFD negatively altered glucose metabolism when compared with their lean counterparts, starting at 30 minutes after glucose challenge (Figure 2, A–C). Interestingly, significant differences were observed when comparing lean WT with KO animals from 30 to 90 minutes

Figure 5 Plasminogen activator inhibitor type-1 regulates hepatocyte growth factor signaling through the hepatocellular MET receptor. **A:** Western blot analyses were performed on membrane-enriched liver lysates for phosphorylated MET (p-MET) and total MET protein on animals fed normal chow or a high-fat diet (HFD). The controls (Cs) for the Western diet using normal chow (lean) liver lysates were from an animal fed an HFD; and for the HFD blots, the C was from an animal fed normal chow. **B:** Densitometric analyses of the blots shown in **A** was performed. Analyses of individual blots are shown along with the calculated ratio of p-MET/MET (far right). **C:** Mice with a conditional deletion of the MET receptor from hepatocytes [conditional knockout (cKO)] and wild-type (WT) mice on a lean diet were subjected to a glucose tolerance test. Unpaired *t* tests were performed. Data are expressed as means $\pm$ SEM (**B** and **C**). *n* = 4 (**C**, cKO mice); *n* = 3 (**C**, WT mice on a lean diet). **P* < 0.05.

after glucose challenge (Figure 2D), indicating that the absence of PAI-1 positively affects basal glucose metabolism. When comparing the WT with KO HFD-fed mice, the only significant difference was observed at 30 minutes after glucose challenge (Figure 2E). However, the most striking finding was that no significant differences in glucose tolerance were observed when comparing WT lean animals with KO HFD-fed animals (Figure 2F). This suggests that PAI-1 deficiency is able to combat obesity-induced glucose intolerance after 20 weeks of HFD feeding.

Concomitant with enhanced glucose tolerance, improved insulin resistance was observed in HFD-fed PAI-1 KO mice. HOMA-IR was used to estimate insulin sensitivity in all cohorts of mice. A higher HOMA-IR score equates to more severe insulin resistance. As expected, the lean WT and KO mice had a low HOMA-IR score that became elevated in WT mice after HFD feeding (Figure 3A). However, when KO mice were fed the HFD, their HOMA-IR score was significantly decreased, compared with WT HFD animals. This correlates with results from an *in vivo* glucose-stimulated insulin secretion test. When fasted lean WT and PAI-1 KO mice were challenged with glucose, they both exhibited significant increases in glucose-stimulated insulin secretion 15 minutes after glucose challenge, indicating that WT and KO mice on a normal diet are able to mount a normal insulin response (Figure 3B). In contrast, HFD feeding in WT mice thwarted insulin sensitivity, during which the mice remained insulin insensitive 15 minutes after glucose challenge (Figure 3C). However, in accordance with the HOMA-IR results, HFD-

fed PAI-1 KO mice remained insulin sensitive, with a significant increase in insulin secretion 15 minutes after glucose challenge.

PAI-1 Regulates Activation of HGF and Subsequent Signaling through MET

HGF signaling through MET is known to ameliorate the effects of experimental stressors on pancreatic islets in animal models of diabetes^{21,22} and has been shown to alter insulin resistance,²³ possibly in a macrophage-specific manner.^{35,36} In liver, u-PA is known to process latent, scHGF to active, tcHGF^{12,13} in a high-affinity, stoichiometric manner.³⁷ As PAI-1 is a known inhibitor of u-PA,³⁸ we hypothesized that excess hepatic PAI-1 might disrupt HGF activation in liver, leading to impaired signaling through MET. Western blot analyses of cytoplasm-enriched liver lysates from WT and KO mice on the lean diet show no significant difference in the conversion of scHGF to tcHGF. However, when mice were fed the HFD for 20 weeks and PAI-1 became elevated, there was a significant increase in the amount of tcHGF versus scHGF in the PAI-1 KO mice compared with WT mice, indicating increased conversion had taken place (Figure 4).

Although u-PA and PAI-1 are known to regulate HGF activation in liver, there are also several other known activators and inhibitors of HGF that can regulate activation, such as tissue plasminogen activator,¹³ HGF activator³⁹ and its inhibitors, HAI-1⁴⁰ and HAI-2,⁴¹ matriptase, and hepsin.⁴² To determine whether the correlation between excess

Figure 6 Absence of plasminogen activator inhibitor type-1 prevents high-fat diet (HFD)–mediated induction of cytokines. Enzyme-linked immunosorbent assays were performed on hepatic lysates for tumor necrosis factor (TNF)– α (A) and IL-6 (B), and results were analyzed by one-way analysis of variance with a Tukey posttest. Data are expressed as means $\pm$ SEM. * P < 0.05, ** P < 0.01. KO, knockout; WT, wild type.

PAI-1 and impaired HGF cleavage observed in the WT mice fed an HFD (Figure 1) was unique to this model or a more general phenomenon, an alternative HFD known as the Western or fast food diet, which has a lower fat content but a higher carbohydrate load, was used. First, Western blot analyses were performed to confirm that PAI-1 was similarly elevated in animals on the fast food diet (data not shown). Next, Western blot analyses were performed for HGF, this time using membrane-enriched samples to specifically assess the activation status of HGF on the cell surface. Although efficient HGF processing occurs in membranes from WT animals fed a lean diet, processing in animals fed the fast food diet is impaired (Figure 4C).

Next, it was ascertained whether the increase in active HGF observed in the KO animals on an HFD was able to affect signaling through MET and subsequent glucose sensitivity; Western blot and subsequent densitometric analyses for phosphorylated MET Y1349 and total MET were performed on membrane-enriched fractions from liver lysates (Figure 5, A and B). Although total MET levels were not significantly different, WT mice fed the HFD had a significant decrease in the phosphorylation status of MET (phosphorylated MET Y1349), relative to HFD-fed PAI-1 KO mice. This change was reflected as a decrease in the phosphorylated MET/MET ratio. To determine whether it was loss of HGF signaling on

Figure 7 Plasminogen activator inhibitor type-1 (PAI-1) deficiency contributes to differential expression of IL-6 by hepatocytes and macrophages. After 20 weeks of feeding, liver tissues were harvested and paraffin sections were stained with an IL-6–specific antibody. Slides were scored blindly (K.K.) for overall intensity of hepatocellular staining or, alternatively, the numbers of positive macrophages were counted per 40 $\times$ field. A: Representative images are shown from each group. Arrows point to macrophages. Insets depict magnifications of the areas with the denoted macrophage(s). B: Quantification results of blinded scoring for hepatocyte and macrophage staining. Data are expressed as means $\pm$ SEM (B). n = 4 WT mice, with the average counts from three random fields analyzed per mouse (B); n = 5 KO mice, with the average counts from three random fields analyzed per mouse (B). * P < 0.05. Original magnification: $\times$ 400 (A, main images); $\times$ 1200 (A, insets). HFD, high-fat diet; KO, knockout; WT, wild type.

hepatocytes that was responsible for impaired glucose metabolism, a glucose tolerance test was next performed on mice with a conditional hepatocellular deletion of the HGF receptor, MET, versus their WT counterparts. A discernible difference in glucose metabolism was observed, with a significant difference at 30 minutes after glucose challenge (Figure 5C).

PAI-1 Deficiency Results in Attenuated Macrophage-Mediated Inflammation

HGF signaling can induce IL-6 production in hepatocytes²⁴ or diminish IL-6 production in macrophages.²⁵ To determine whether the diet-induced alteration in HGF conversion and subsequent changes in signaling through MET affect cytokine production, two key hepatic proinflammatory cytokines, tumor necrosis factor- α and IL-6, were next examined. Significant increases were detected in hepatic protein production of both cytokines in WT animals on an HFD, relative to their PAI-1 KO counterparts, suggesting that the absence of PAI-1 results in an anti-inflammatory environment (Figure 6). Next, livers from animals on an HFD were stained for IL-6 to determine whether the location of the excess IL-6 in the WT animals could be identified. Although there was a slight trend upward in KO animals, levels of hepatocellular IL-6 were similar; however, there was a significant increase in positively stained immune cells in the WT animals, relative to the KOs (Figure 7). This was not caused by an increase in macrophage numbers as staining for F4/80 was similar in the two groups (data not shown).

Discussion

PAI-1, induced in response to chronic inflammation, has long been hypothesized to play a role in exacerbating vascular dysfunction in obesity and subsequent T2D because of its well-established ability in preventing plasmin formation^{14–16}; however, there is ample literature to suggest the role of PAI-1 is likely to be more complicated. For example, in the leptin-deficient ob/ob mouse model, disruption of PAI-1 results in reduced adiposity, improved metabolic profiles, and a reduction in the cytokine profile⁴³; however, it is the leptin, and not the PAI-1, that is directly related to thrombosis.^{44,45} Hence, the present study sought to determine whether PAI-1-mediated inhibition of HGF activation also plays a role in the development of obesity and T2D.

Because T2D is a polygenic and environmentally/lifestyle-induced disease, a variation of the classic, diet-induced model of T2D³⁴ was chosen to better ascertain the role of PAI-1 in developing T2D, preferring to use wild-type mice over the monogenic ob/ob diabetes model. Furthermore, a diet (20% kcal of each carbohydrate and protein and 60% kcal of fat) similar to that previously used by others

(26% kcal of carbohydrate, 15% kcal of protein, and 59% kcal of fat^{34,46}), albeit lower in carbohydrates and higher in protein, was used. WT and KO mice, on an HFD or normal chow, were studied over a period of 20 weeks after commencement of diet. As obesity developed, there was a trend toward elevated PAI-1 in the sera of WT animals on the HFD as early as 7.5 weeks after the diet was initiated, with statistical significance reached at 20 weeks after initiation of the diet (Figure 1). Likely, this was because of excess PAI-1 produced by the liver in WT animals as an increase in hepatic PAI-1 was detected at 12 weeks after commencement of the diet using Western blot analyses. Surprisingly, although there was a trend toward a lesser weight gain in the KO versus WT mice on an HFD, unlike results with a similar study using the original diet⁴⁶ and in the ob/ob mouse model,⁴³ the absence of PAI-1 did not have an effect on the increase in weight, possibly because of our earlier implementation of the diet (6 versus 10 weeks^{43,46}; ie, it appears that in this model, the development of obesity occurs independently of PAI-1 production). However, similar to the other studies, PAI-1 was found to be an essential regulator of glucose metabolism; the absence of PAI-1 resulted in improved glucose tolerance and insulin resistance (Figures 2 and 3). Thus, this model was suitable for investigating the role of PAI-1 with regard to activation of HGF.

In liver, both u-PA and tissue-type plasminogen activator (t-PA) can facilitate activation of HGF by binding to and then cleaving the latent, single-chain form to the metabolically active two-chain HGF.¹³ As active HGF has been shown to be a positive effector in animal models of diabetes,^{21,22} and can regulate glucose metabolism,²³ and as the ability of PAI-1 to act as a physiological inhibitor of u-PA activity is well established,⁴⁷ we hypothesized that increased PAI-1 in response to obesity can prevent the activation of HGF and limit its hepatoprotective effects. In KO animals on an HFD, the conversion of latent HGF to its active form was accelerated, suggesting increased HGF turnover positively regulates glucose metabolism (Figure 4). These data were not unique in this sense since the absence of PAI-1, in the context of specific types of hepatic injuries, is also associated with an increase in active HGF.^{17,48,49} However, as impaired conversion of HGF in response to overnutrition occurs in the membrane (Figure 4C), it is likely that in the KO animals, the loss of PAI-1 leads to an increase in MET activation (Figure 5) and a subsequent improvement in the glucose/insulin axis (Figures 2 and 3). In support of this hypothesis, when i.p. glucose tolerance test studies were performed on animals with MET specifically deleted from their hepatocytes, at 30 minutes after injection there was a significant elevation in their glucose levels compared with their WT counterparts (Figure 5C), indicating HGF signaling on the hepatocyte is positively regulating glucose metabolism.

Excessive weight gain or morbid obesity often stimulates chronic inflammation, leading to elevations in circulating cytokines, such as IL-6, as well as acute phase proteins, such

as PAI-1; these proteins are all known to be produced in the liver by both hepatocytes and macrophages.^{4,5,50} These results showed that with an HFD, both IL-6 and tumor necrosis factor- α become elevated in livers from WT, but not KO, animals (Figure 6). This indicates that in addition to positively regulating glucose metabolism, the absence of PAI-1 also protected animals on an HFD from developing a chronic inflammatory response. The effects of cytokine suppression were exerted on macrophages (Figure 7). HGF can transiently induce IL-6 production in hepatocytes, yet suppress the excretion of IL-6 by macrophages^{24,25}; we posit that despite limited IL-6 production in hepatocytes, the HGF may act as a negative regulator of immune cell-mediated cytokine production in these animals. However, it is also possible that the presence of PAI-1 indirectly promotes cytokine production by inhibiting the anti-inflammatory effects of t-PA.⁵¹ Further studies will be needed to address this.

In conclusion, this study showed that, in WT animals fed an HFD, PAI-1 becomes elevated and prohibits activation of HGF. When PAI-1 is absent, there is an increase in the conversion of latent to active HGF and subsequent signaling through the MET receptor in liver. This increase in MET phosphorylation is associated with overall improved glucose metabolism and increased insulin sensitivity, as well as a decrease in the production of inflammatory cytokines by macrophages.

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