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Hyperglycemia Modulates Plasminogen Activator Inhibitor-1 Expression and Aortic Diameter in Experimental Aortic Aneurysm Disease

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Abstract

Background—Extracellular matrix degradation is a sentinel pathologic feature of abdominal aortic aneurysm (AAA) disease. Diabetes mellitus, a negative risk factor for AAA, may impair aneurysm progression through its influence on the fibrinolytic system. We hypothesize that hyperglycemia limits AAA progression through effects on endogenous plasminogen activator inhibitor-1 (PAI-1) levels and subsequent reductions in plasmin generation.

Methods—Experimental AAAs were induced in diabetic and control mice via the intra-aortic elastase infusion method. Serial transabdominal high-frequency ultrasound examinations were performed to monitor aortic diameter following elastase infusion. Circulating PAI-1 and plasmin α 2-antiplasmin (PAP) complex concentrations were determined by ELISA and local expression of PAI-1 levels was examined by RT-PCR and immunohistochemistry.

Results—Hyperglycemia was associated with reduced AAA diameter, increased plasma PAI-1 concentration and reduced plasmin generation. Aneurysmal aortic PAI-1 gene expression increased in parallel with plasma concentration, with peak expression occurring early after aneurysm initiation.

Conclusions—Hyperglycemia increases PAI-1 expression and attenuates AAA diameter in experimental AAA disease. These results emphasize the role of the fibrinolytic pathway in AAA pathophysiology, and suggest a candidate mechanism for hyperglycemic inhibition of AAA disease.

INTRODUCTION

Extracellular matrix (ECM) dissolution is a characteristic feature of abdominal aortic aneurysm (AAA) disease. Matrix metalloproteinases (MMPs) degrade ECM proteins including collagen and elastin, an activity that is essential in aneurysm progression.¹ Secreted as inactive zymogens, MMPs are processed into active enzymes by other MMPs or by serine proteases

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such as plasmin, a trypsin-like proteolytic enzyme also capable of directly degrading components of the vascular wall.² Plasmin is the key enzyme in the fibrinolytic pathway and results from the activation of plasminogen by plasminogen activators (urokinase-type: uPA; and tissue-type: tPA), a reaction regulated by the primary endogenous inhibitor, plasminogen activator inhibitor-1 (PAI-1).²

Prior studies have suggested a clinical association between PAI-1 and AAA risk. The 5G allele variant within the PAI-1 promoter region is associated with reduced PAI-1 expression, increased plasmin production and increased MMP activation.³ Patients with familial AAAs are twice as likely to carry this single nucleotide polymorphism (SNP) as unaffected individuals⁴, and carriers may also experience more rapid aneurysm expansion.⁵ PAI-1 levels are elevated in diabetes,⁶ a condition known to reduce the prevalence and progression of AAA disease.^{7–10} Diabetes-related reductions in plasmin generation and subsequent ECM proteolysis could plausibly explain the protective effect conferred by diabetes against AAA disease (Fig 1).

To explore the potential mechanisms inhibiting AAA progression in the setting of diabetes, we investigated the influence of hyperglycemia on PAI-1 generation in experimental aortic aneurysms.

MATERIALS AND METHODS

Experimental models

All animal work was reviewed and approved by the Administrative Panel on Laboratory Animal Care committee at Stanford University. Inhaled isofluorane anesthesia was used for all operative procedures. Hyperglycemia was induced in male C57/BL6 mice (20 to 30g, 10 weeks of age, Jackson Laboratory, Bar Harbor, ME) via consecutive intraperitoneal injection of streptozotocin (STZ: 50 mg/kg, Sigma Aldrich, St. Louis, MO) dissolved in citrate buffer for five days as specified by the Animal Models of Diabetic Complications Consortium protocol (<http://www.amdcc.org>). STZ induces a necrotic and inflammatory response of the pancreatic islet beta cells; multiple injections of low-dose STZ produce a delayed but progressive increase in blood glucose in mice.¹¹ Control mice received a citrate buffer injection without STZ. Serum glucose levels and body weight were monitored for three or more weeks following STZ injection.

Induction of AAAs was performed three weeks after STZ or buffer injection. Hyperglycemia, defined as casual blood glucose levels of ≥ 300 mg/dl, was confirmed prior to AAA creation in diabetic mice. Retroperitoneal exposure was achieved via laparotomy, and porcine pancreatic elastase (PPE, Sigma Aldrich, St. Louis, MO) was infused directly into the infrarenal aorta (0.03 ml of 15 U/ml of PPE over 5 min) as previously described.¹² These procedures created 2 experimental AAA groups; hyperglycemic (DM-AAA, n=16) and euglycemic mice (AAA, n=16).

Maximum aortic diameter was measured on all mice before elastase infusion and prior to sacrifice via transabdominal 40 MHz B-mode ultrasonography imaging (Visualsonics, Ontario, Canada). Images were obtained in the transverse scan plane on the Vevo 770 imaging system using the RMV 704 microvisualization scan head (spatial resolution: 40 microns). The sensitivity, accuracy, and reproducibility of similar murine AAA measurement protocols has been demonstrated previously.^{13,14} Mice were euthanized at baseline before PPE infusion and at 3, 7, and 14 days after PPE infusion. Prior to euthanization, 700 μ l of blood was collected in citrate via direct cardiac puncture from the anesthetized animal. Following sacrifice, aortae were either fixed with 4% paraformaldehyde-buffered solution at 100 mmHg for immunohistochemical analysis or snap-frozen in liquid nitrogen for RNA preparation.

Histological and Immunohistochemical Staining

Following pressure perfusion fixation, aortic tissue was immersed overnight in 4% PFA solution overnight at 4°C, embedded in paraffin and sectioned at 4µm for hematoxylin and eosin (H&E), Elastica-Masson (EM) and immunohistochemical (IHC) staining. For IHC staining, endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol and non-specific binding sites blocked with 10% normal serum (Dako Inc., Carpinteria, CA). Sections were incubated for one hour at room temperature with avidin-conjugated rabbit anti-mouse PAI-1 polyclonal antibody (Abcam, Cambridge, MA), rat anti-mouse MAC-2 monoclonal antibody (Cedarlane, Burlington, NC) or rabbit anti-mouse MMP-9 polyclonal antibody (Abcam) followed by the appropriate biotinylated secondary antibody (Biocare Medical, Concord, CA) and color development performed using the DAB color development system (Dako Inc.). Negative control experiments were performed by replacing the primary antibody with non-specific IgG antibody.

ELISA

Citrated blood samples (9 volumes blood: 1 volume citrate) collected immediately prior to sacrifice were centrifuged at 5000rcf for 10 minutes at 4°C to isolate the plasma. Total PAI-1 and plasmin- α 2-antiplasmin (PAP) complex protein in the plasma samples were measured using respective ELISA kits according to manufacturer's protocol (PAI-1: Molecular Innovations, Novi, MI and PAP Complex: Technoclone GmbH, Vienna, Austria).

RNA isolation and preparation, quantitative real-time PCR

Snap-frozen aortic samples were homogenized with QIAzol Lysis Reagent (Invitrogen, Carlsbad, CA) followed by RNA isolation per manufacturer's protocol. RNA was quantitated by Nanodrop, and RNA quality and integrity were verified using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA). For quantitation of gene transcription, total RNA was reverse transcribed to cDNA using Iscript (BioRad, Hercules, CA). Real-time PCR was performed on the ABI PRISM 7900HT Sequence Detection System with TaqMan on Demand Gene Expression Probe for PAI-1 (PAI-1 Mm00435858_m1, Serpine1, Applied Biosystems, Foster City, CA). Expression levels were normalized to corresponding 18S internal controls.

Statistical Analysis

All fold changes for RT-PCR were calculated by the method of $\Delta\Delta C_t$. Data from the RT-PCR assay and the ELISA between the treated and control groups were compared using the 2-tailed paired Student t test; statistical analysis for multiple comparisons was performed using the one-way analysis of variance (ANOVA) for continuous variables followed by the Scheffe post hoc test. Results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as $P < .05$.

RESULTS

AAA diameter is reduced in hyperglycemic mice

Immediately prior to elastase infusion, three weeks after initiation of STZ or buffer injections, blood glucose levels in the DM-AAA mice averaged 421 ± 14 mg/dL vs. 147 ± 6 mg/dL in AAA mice, $P < .05$. There was no difference in mean aortic diameter between groups at baseline; DM-AAA mice had significantly decreased aortic diameters at POD 7 (n=4) and POD 14 (n=4) compared to AAA mice (also n=4 at each time point; POD7: DM-AAA 0.92 ± 0.02 mm vs. AAA 1.08 ± 0.05 mm, $P < .05$; POD14: DM-AAA 1.05 ± 0.03 mm vs. AAA 1.25 ± 0.05 mm, $P < .05$; Fig 2).

PAI-1 expression is increased in hyperglycemic mice

PAI-1 expression was analyzed both systemically and within the aortic wall. Plasma PAI-1 levels were significantly increased in DM-AAA compared to AAA mice at baseline and after elastase induction (Fig 3). Expression increased at POD3 in both cohorts compared to baseline levels (POD3 vs. Baseline: DMAAA 1.4 fold increase, AAA 1.9 fold increase, $P < .05$) following elastase infusion. Subsequent plasma levels returned to near baseline (Fig 3).

Aortic PAI-1 gene expression was also increased in DM-AAA mice prior to elastase infusion, and peak expression increased further in both cohorts early in the time course of aneurysm progression (POD3 vs. Baseline: DM-AAA 4 fold increase, AAA 5 fold increase, Fig 4). Morphometric comparison of medial elastin content and structural integrity was performed at POD 14 (Fig 5A–D, $n=4$ DM-AAA and $n=4$ AAA) demonstrating preserved elastin in the DM-AAA mice. Aortic mural PAI-1 expression was most prominent within the media and intima with greater staining intensity of PAI-1 visualized in the DM-AAA mice compared to the AAA mice (Fig 5E–H).

Reduced circulating plasmin in hyperglycemic mice

To examine the downstream effects of increased PAI-1 on plasmin generation, we measured the levels of PAP complexes in the plasma samples of both DM-AAA and AAA mice. Plasmin is rapidly bound in the circulation by α_2 -antiplasmin; the resulting complex (PAP) serves as an indirect measure of plasmin generation. PAP complexes were significantly reduced in DM-AAA mice compared to AAA mice at all time points after elastase induction of AAA; this effect was observed by POD 3 (DM-AAA $194 \pm 18\text{ng/mL}$ vs. AAA $366 \pm 14\text{ng/mL}$, $P < .05$) and persisted until POD14 (DM-AAA $463 \pm 8\text{ng/mL}$ vs. AAA $618 \pm 10\text{ng/mL}$, $P < .05$; Fig 6).

Hyperglycemia reduced aortic macrophage and MMP-9 expression

Inflammatory markers including macrophage (MAC-2 staining) and MMP (MMP-9 staining) expression were compared by immunohistochemistry in DM-AAA ($n=4$) and AAA mice ($n=4$). DM-AAA mice had decreased MAC-2 and MMP-9 stained cells per cross sectional area (CSA) compared to AAA mice at POD 14 (MAC-2: DM-AAA $218 \pm 13\text{cells/CSA}$ vs. AAA $495 \pm 14\text{cells/CSA}$, $P < .05$; MMP-9: DM-AAA $50 \pm 4\text{cells/CSA}$ vs. AAA $166 \pm 6\text{cells/CSA}$, $P < .05$; Fig 7).

DISCUSSION

Epidemiologic risk factors for AAA disease include advanced age, male gender, cigarette smoking, family history, hypertension, obesity, hypercholesterolemia and concomitant atherosclerotic disease.^{7, 8} Although diabetes is a recognized risk factor for most acquired cardiovascular pathologies, recent observations consistently identify a negative association with AAA disease in both men and women.^{7–10} The purpose of this study was to examine the influence of serum glucose levels on the fibrinolytic system and subsequent progression of experimental AAA disease.

Prior evidence has indirectly linked activation of the fibrinolytic system with clinical aneurysm progression; plasma concentrations of tissue-type plasminogen activator, plasmin α_2 -antiplasmin complexes, and D-dimers (plasmin-mediated fibrin degradation products) are increased in AAA patients and correlate with aneurysm diameter and volume of mural thrombus.¹⁵ tPA levels also correlate with risk for AAA expansion and rupture.¹⁶ Our data suggests that plasmin activation or fibrinolytic tone is directly associated with increased experimental aneurysm progression, consistent with prior experimental observations relating

variable plasminogen, urokinase or plasminogen activator inhibitor-1 (PAI-1) expression with AAA modulation.^{17, 18}

These experiments also confirm and extend prior observations regarding the inhibitory influence of hyperglycemia on experimental AAA progression. In these constructs, hyperglycemia increased PAI-1 plasma levels and mRNA for aortic tissue expression within aneurysm tissue. *In vitro*, PAI-1 is produced and secreted in culture medium by platelets and endothelial and smooth muscle cells, and high glucose and insulin levels both induce PAI-1 gene expression.¹⁹ Recent clinical investigations including the Insulin Resistance Atherosclerosis Study²⁰ have correlated plasma PAI-1 levels with risk for diabetes development in patients with the metabolic syndrome. The mechanism by which serum glucose influences PAI-1 gene expression is uncertain; *in vitro* findings suggest that glucose activates the Rho/Rho-kinase pathway in endothelial cells, mesangial cells, and vascular smooth muscle cells (SMCs). High glucose induces intracellular oxidant stress in various cell types, and Rho-kinase activation under these hypoxic conditions regulates several transcription factors that bind to and activate the PAI-1 promoter.²¹

The increased PAI-1 expression following elastase infusion in both hyperglycemic and euglycemic mice likely represents a generalized response to the ensuing mural inflammation. Increased Interleukin-1 β (IL-1 β) expression is present early in inflammation and decreases as monocyte infiltration stabilizes.²² IL-1 β suppresses production of tPA and uPA in AAA SMCs²² and HepG2 cells express PAI-1 in response to IL-1 stimulation.³ Although expression was increased in all mice following elastase infusion, in our experiments PAI-1 levels were consistently higher in hyperglycemic mice at all time points sampled.

Not surprisingly, increased PAI-1 levels were associated with reduced plasmin activation. Plasmin is a common activator of several proteolytic systems and PAP levels have correlated to aneurysmal expansion in humans.²³ In addition to activating zymogen forms of MMP-1, -2, -3, -9, -10, and -13 either directly or indirectly, plasmin may also independently degrade other ECM proteins including fibrin, laminin and fibronectin.² Previous work in our laboratory has demonstrated a significant reduction in proMMP-9 proteolytic activity in hyperglycemic compared to euglycemic mice with concurrent preservation of aortic medial elastin. Interestingly, provision of insulin to hyperglycemic mice after AAA induction normalizes blood glucose levels with subsequent aneurysm expansion and elastolysis increased to near that observed in euglycemic mice (data not shown). In euglycemic AAA mice we have also demonstrated that prior treatment with tranexamic acid, a direct plasmin inhibitor, inhibited progression of experimental aneurysm disease.¹⁴ In combination with these previous observations, the current data suggest that hyperglycemia limits experimental aneurysm progression at least in part via PAI-1 mediated inhibition of plasmin production, decreasing MMP activity, elastolysis and mural degeneration.

Most patients with aneurysm disease have coexisting type-2 diabetes; however, we employed an insulin-deficient model hyperglycemic model for these experiments to isolate and examine the influence of serum glucose alone on PAI-1 stimulation and avoid incorporating other potentially confounding features of insulin resistance and the metabolic syndrome. Prior mechanistic studies examining glucose-dependent PAI-1 induction have similarly used pure D-glucose in culture,²² to allow for a direct cause-effect analysis. Streptozotocin induces apoptosis and eventual necrosis of the pancreatic beta cells, thereby causing a persistent hyperglycemia rather than a transient model of increasing blood glucose levels. The elastase model is generally accepted to be a 14-day model of AAA disease;¹² experimental AAAs induced with STZ carried out to longer durations of 28 days also exhibit blood glucose levels > 400mg/dL at sacrifice with attenuated aortic diameter compared to control mice (results of absolute difference between groups remaining similar to day 14 values). Future experiments

may utilize the db/db leptin receptor-deficient mouse to incorporate features of insulin resistance into this model. We attribute decreased proteolytic activity in DM-AAA to be in part due to decreased plasmin; however, further studies using the PAI-1 knock out mouse will directly correlate PAI-1 expression and proteolytic inhibition. In the particular case of abdominal aortic expansion, other consequences of hyperglycemia may potentially limit disease progression; high glucose levels have been reported to reduce aortic neovessel formation²⁴ and impair macrophage localization and glycosylate matrix proteins, reducing their propensity to enzymatic cleavage.²⁵ *In vitro* studies have demonstrated exposure of activated monocytes to glycated type I collagen lattices induces a marked reduction in MMP and interleukin-6 secretion.²⁵ Consistent with these findings, our results demonstrated reduced local macrophage density and MMP-9 expression in hyperglycemic mice. Reductions of inflammatory cellularity and cytokine production certainly represent a potential synergistic mechanism in the spectrum of possible AAA inhibitory actions associated with diabetes.

In conclusion, the current study highlights the potential importance of the fibrinolytic system in aneurysm pathogenesis and suggests a mechanism by which elevated glucose levels may inhibit aneurysm enlargement and clinical progression. Further investigations are warranted to determine whether systemic or local pharmacologic manipulation of the fibrinolytic system may prove effective in limiting progression of early AAA disease.

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Fig 1.

Potential mechanism accounting for diabetes-related aneurysm inhibition beginning with increased PAI-1 levels observed in diabetic patients. Reductions in plasmin generation are hypothesized to decrease aortic wall degradation and aneurysm progression.

Fig 2.

Average aortic diameter of AAA (n=16 total; n=4 per time point) and DM-AAA (n=16 total; n=4 per time point) cohorts. Baseline (Base) indicates measurement prior to elastase infusion compared to subsequent post-operative days. (AAA vs. DM-AAA: POD7, POD14 * $P < .05$; AAA: all vs. Base [†] $P < .05$; DM-AAA: all vs. Base [§] $P < .05$).

Fig 3. Circulating PAI-1 levels in AAA (n=16 total; n=4 per time point) and DM-AAA (n=16 total; n=4 per time point) plasma samples. (AAA vs. DM-AAA: all * $P < .05$; AAA: POD3 vs. Base † $P < .05$; DM-AAA: POD3 vs. Base § $P < .05$).

Fig 4.

Quantitation of PAI-1 mRNA isolated from aortic tissue of AAA and DM-AAA mice at baseline (n=8 total; n=4 per group), POD3 (n=8; n=4 per group) and POD7 (n=8; n=4 per group). Expression levels were normalized to corresponding 18S internal controls. Aortic samples were pooled together in groups of (n=2) for isolation of mRNA. (AAA: POD3 vs. Base [†] $P < .05$).

Fig 5.

Representative images of EM staining at POD14 (n=8 total; n=4 per group) in AAA (A) 100× (B) 400× and DM-AAA (C) 100× (D) 400×. Black box in low resolution (100×) images represent area of magnified high resolution (400×) images. PAI-1 immunohistochemistry shown below in AAA (E) 100× (F) 400× and DM-AAA (G) 100× and (H) 400×. Increased elastin preservation and intensity of PAI-1 staining is observed in DM-AAA images.

Fig 6. Circulating PAP complex levels in AAA (n=16 total; n=4 per time point) and DM-AAA (n=16 total; n=4 per time point) plasma samples. (AAA vs. DM-AAA: all * $P < .05$; AAA: POD7, POD14 vs. Base † $P < .05$; DM-AAA: all vs. Base § $P < .05$).

Fig 7. Representative immunohistochemistry staining POD14 (n=8 total; n=4 per group) of DM-AAA (A, B) and AAA (C, D) aortic sections. Comparison of MMP-9 stained areas (black arrows, 100×) is shown in (A, C) and Mac-2 stained areas (black arrows, 100×) is shown in (B, D). (E) Quantitation of stained cell counts per cross sectional area of MMP-9 and Mac-2 in AAA and DM-AAA sections. (AAA vs. DM-AAA: * $P < .05$)