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Hyperglycemia limits experimental aortic aneurysm progression

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Abstract

Objective—Diabetes mellitus (DM) is associated with reduced progression of abdominal aortic aneurysm (AAA) disease. Mechanisms responsible for this negative association remain unknown. We created AAAs in hyperglycemic mice to examine the influence of serum glucose concentration on experimental aneurysm progression.

Methods—Aortic aneurysms were induced in hyperglycemic (DM) and normoglycemic models by using intra-aortic porcine pancreatic elastase (PPE) infusion in C57BL/6 mice or by systemic infusion of angiotensin II (ANG) in apolipoprotein E-deficient ($ApoE^{-/-}$) mice, respectively. In an additional DM cohort, insulin therapy was initiated after aneurysm induction. Aneurysmal aortic enlargement progression was monitored with serial transabdominal ultrasound measurements. At sacrifice, AAA cellularity and proteolytic activity were evaluated by immunohistochemistry and substrate zymography, respectively. Influences of serum glucose levels on macrophage migration were examined in separate models of thioglycollate-induced murine peritonitis.

Results—At 14 days after PPE infusion, AAA enlargement in hyperglycemic mice (serum glucose ≥ 300 mg/dL) was less than that in euglycemic mice (PPE-DM: 54% ± 19% vs PPE: 84% ± 24%, $P < .0001$). PPE-DM mice also demonstrated reduced aortic mural macrophage infiltration (145 ± 87 vs 253 ±119 cells/cross-sectional area, $P = .0325$), elastolysis (% residual elastin: 20% ± 7% vs 12% ± 6%, $P = .0209$), and neovascularization (12 ± 8 vs 20 ± 6 vessels/high powered field, $P = .0229$) compared with PPE mice. Hyperglycemia limited AAA enlargement after ANG infusion in $ApoE^{-/-}$ mice (ANG-DM: 38% ± 12% vs ANG: 61% ± 37% at day 28). Peritoneal macrophage production was reduced in response to thioglycollate stimulation in hyperglycemic mice, with limited augmentation noted in response to vascular endothelial growth factor administration. Insulin therapy reduced serum glucose levels and was associated with AAA enlargement rates intermediate between

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euglycemic and hyperglycemic mice (PPE: 1.21 ± 0.14 mm vs PPE-DM: 1.00 ± 0.04 mm vs PPE-DM + insulin: 1.14 ± 0.05 mm).

Conclusions—Hyperglycemia reduces progression of experimental AAA disease; lowering of serum glucose levels with insulin treatment diminishes this protective effect. Identifying mechanisms of hyperglycemic aneurysm inhibition may accelerate development of novel clinical therapies for AAA disease.

Clinical Relevance—This report provides mechanistic insight into prior population-based clinical studies identifying a negative association between diabetes mellitus and abdominal aortic aneurysm (AAA). The inhibitory effects of hyperglycemia on aneurysm development are examined independent of other AAA risk factors. Further investigations into these or related mechanisms may accelerate the development of effective medical strategies to suppress progression of AAA disease.

Diabetes mellitus (DM) is an important contributor to the pathophysiology of many cardiovascular disorders, including abdominal aortic aneurysm (AAA) disease. Unlike other common demographic and environmental cardiovascular risk factors (eg, advanced age, male gender, and cigarette smoking), however, diabetes appears to reduce the risk for and progression of AAA disease.[1-7] Many features of DM may influence the pathophysiology of AAA disease; to date, however, the mechanism(s) responsible for the negative association have yet to be investigated in an in vivo experimental system. We superimposed hyperglycemia on experimental aortic aneurysm induction to identify potential mechanisms responsible for diabetic suppression of AAA disease.

Methods

Murine modeling

All proposed modeling experiments were reviewed and approved in advance by the Administrative Panel on Laboratory Animal Care Committee at Stanford University. Animal care and experimental procedures were conducted in compliance with Stanford Laboratory Animal Care Guidelines (http://labanimals.stanford.edu/). Male mice (aged 10 to 12 weeks), either C57BL/6, or apolipoprotein E-deficient ($ApoE^{-/-}$) on a C57BL/6 background, were used for all experiments (Jackson Laboratories, Bar Harbor, Me). Adequate inhaled isoflurane anesthesia was maintained for all invasive procedures. After survival procedures, mice were recovered in individual cages with unrestricted access to chow and water. All mice were maintained on normal chow diets.

Induction of DM

Hyperglycemia was induced by intraperitoneal (IP) injection of streptozotocin (STZ: 50 mg/kg; Sigma Aldrich, St. Louis, Mo) dissolved in citrate buffer for 5 consecutive days as specified by the Animal Models of Diabetic Complications Consortium protocol (http://www.amdcc.org). STZ induces necrosis and inflammation of the pancreatic islet beta cells; multiple injections of low-dose STZ produce a delayed but progressive increase in serum glucose levels in mice, resulting in insulin-dependent DM.[8] Control mice received a citrate buffer injection without STZ. Mice were monitored for at least 3 weeks after STZ injection, and blood glucose levels and body weights were measured weekly. Hyperglycemia was defined by casual blood glucose levels of ≥300 mg/dL before AAA creation and at sacrifice.

Induction of AAA

Experimental AAAs[9] were created in hyperglycemic and euglycemic mice by using one-time intra-aortic porcine pancreatic elastase (PPE) infusion in C57BL/6 (14-day model) or by continuous subcutaneous angiotensin II (ANG) infusion in $ApoE^{-/-}$ cohorts (28-day model), as previously described by our laboratory.[10-12] In hyperglycemic mice, aneurysm induction

began 3 weeks after completion of STZ injections and confirmation of hyperglycemia as defined above.

Two distinct but complementary murine AAA modeling systems were used to ensure, to the greatest extent possible, that experimental results were not model-specific.[13] Briefly, in the PPE model, elastase (E-1250, Lot 102K7685; Sigma Aldrich) was infused directly into the aorta (0.03 mL of 15 U/mL of PPE over 5 minutes), producing either hyperglycemic (PPE-DM, n = 22) or normoglycemic AAA (PPE, n = 21) mice. In the ANG model, AAA was created by subcutaneous infusion of angiotensin II (Lot 37K5102; Sigma Aldrich) at 1000 ng/kg/min continuously over 28 days by a Alzet mini-osmotic pump (DURECT Corp, Cupertino, Calif) in $ApoE^{-/-}$ mice, producing either hyperglycemic (ANG-DM, n = 6) or normoglycemic AAA (ANG, n = 8) mice. In summary, four experimental AAA groups were created, distinguished by the method of aneurysm induction (PPE or ANG) and the presence or absence of diabetes (STZ injection or vehicle alone).

Sacrifice was performed by intentional sodium pentothal overdose. The two distinct experimental AAAs used (PPE and ANG) in this study have different model-specific end points; therefore, sacrifice was performed respectively at that time.[9] PPE-DM and PPE mice were euthanized at post-PPE infusion day (POD) 14; select aortae were harvested for histologic analysis (n = 10 and 9, respectively) and substrate zymography (n = 12 in both groups). ANG-DM and ANG mice were euthanized at post-pump placement day (POD) 28 for histologic analysis only (n = 6 and n = 8, respectively). Before the study group-specific end point, mice losing weight rapidly or appearing lethargic or immobile were euthanized immediately, regardless of experimental progress or group assignment.

Exogenous insulin administration

To evaluate the consequences of reducing serum glucose levels, insulin therapy was instituted in a separate cohort of PPE-DM mice. Subcutaneous insulin pellets (LinShin, Toronto, Ontario, Canada) were implanted in each mouse before AAA induction according to manufacturer's protocol, releasing 0.1 U insulin/d (PPE-DM + Insulin, n = 6), to examine the influence of reduced glucose levels on aneurysm progression. After implantation, blood glucose levels were monitored weekly and compared with those in PPE (n = 6) and PPE-DM (n = 6) mice. A significant response to insulin was confirmed when serial blood glucose levels consistently measured below 200 mg/dL. All mice of this cohort were sacrificed at POD 14 for histologic analysis.

Serial in vivo aortic diameter measurement

Aortic diameter was measured in all mice by transabdominal ultrasound imaging at 40 MHz (Vevo770; Visualsonics, Toronto, Ontario, Canada), by examiners blinded to study group assignment at baseline and after AAA induction, on PODs 7 and 14 (all models) and PODs 21 and 28 for ANG only. Percent change in AAA growth rate was calculated as [100 × (current aortic diameter – baseline aortic diameter/baseline aortic diameter]. The sensitivity, accuracy, and reproducibility of similar murine AAA high-frequency ultrasound measurement protocols has been demonstrated previously.[12,14,15]

Thioglycollate-induced peritonitis

Intra-aortic infusion of thioglycollate induces mural inflammation and aneurysmal degradation when administrated with plasmin[16]; mechanisms responsible for thioglycollate-induced macrophage stimulation are likely similar to those initiated by porcine pancreatic elastase.[17] We used IP thioglycollate injection (1 mL of 4% solution, Becton Dickinson, Sparks, Md)[17] to induce chemical peritonitis in hyperglycemic and normoglycemic C57BL/6 mice to evaluate the effect of hyperglycemia and vascular endothelial growth factor (VEGF) on macrophage

migration. Since VEGF signaling plays an integral role in aneurysm pathogenesis,[12] and VEGF may promote macrophage chemotaxis and activation[18,19] in addition to stimulating neovessel production, exogenous VEGF was administered to determine its influence on macrophage production in the setting of hyperglycemia. Selected mice from each group (thioglycollate with or without hyperglycemia) were simultaneously treated with recombinant mouse VEGF (VEGF164; R&D Systems, Minneapolis, Minn) every other day to evaluate hyperglycemic influences on macrophage chemotaxis in response to VEGF stimulation.

Four days after thioglycollate injection, peritoneal lavage was performed with phosphate-buffered saline (PBS), and 2.5 mL of lavage solution was collected and centrifuged to remove erythrocytes. After resuspension in 1 mL of PBS, cells were counted using a Fuchs-Rosenthal Chamber (Hausser Scientific, Horsham, Pa).

Histopathology and immunohistochemistry

At sacrifice, aortae were harvested for histologic analysis after left ventricular injection of 4% paraformaldehyde (PFA) solution in PBS. Further fixation was achieved by overnight immersion in 4% PFA at 4°C. After paraffin embedding, blocks were sectioned at 4 μm for Elastica-Masson (EM) and immunohistochemical (IHC) staining. Primary antibodies for IHC staining were either rat antimouse MAC-2 for macrophages (Cedarlane Laboratories, Burlington, Ontario, Canada) or rabbit antimouse CD31 for endothelial cells (Lab Vision, Fremont, Calif).

After primary incubation, sections were incubated with a biotinylated secondary antibody (Biocare Medical, Concord, Calif) and avidin-biotin complex (Vectastain ABC kits; Vector Laboratories, Burlingame, Calif) according to the manufacturers' protocols. Color development was performed using the DAB color development system (Dako Corporation, Carpinteria, Calif). Tissue sections were counterstained with hematoxylin. Negative control experiments were performed by replacing the primary antibody with nonspecific immunoglobulin G.

Medial elastin density was defined by the ratio of elastin/total medial-area.[20,21] For mural neovascularization, cross-sections were divided into four quadrants (original magnification ×400), and CD31-positive stained neovessels, rather than individual cells, in each quadrant were counted. Mean numbers of neovessels per high-powered field (HPF) with standard deviations are reported. To quantify macrophage number, MAC-2-positive stained individual cells were counted (original magnification ×200) per cross-sectional area (CSA).

Gelatin zymography

Snap-frozen AAA samples were pooled in each cohort for gelatin zymography. After extraction from harvested aortae, total protein concentration was determined using the BCA protein assay kit (Pierce-Thermo Scientific, Rockford, Ill). A uniform amount of protein was applied to the 10% gelatin zymogram gel (Bio-Rad, Hercules, Calif), and electrophoresis was performed according to the manufacturer's protocol. Densitometric analysis of lytic bands indicative of MMP activity was performed with National Institutes of Health Image-J software.

Statistical analysis

Data are represented as mean ± standard deviation (SD). Determination of significance between two groups of continuous variables was performed using the *t*-test; adjustments for multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by the Scheffe post hoc test. Significance was determined at the level of $P < .05$. All calculations were performed using StatView J 5.0 software (SAS Institute Inc, Cary, NC).

Results

STZ injection increases serum glucose levels, reduces body weight

Murine blood glucose levels and body weights were monitored weekly after STZ injection. Serum glucose concentration increased significantly within 2 weeks of the initial injection (370 ± 67 mg/dL STZ-treated vs 152 ± 29 mg/dL vehicle, $P < .0001$) and persisted until sacrifice (459 ±53 vs 148 ± 22 mg/dL, $P < .0001$). Body weight was lower in hyperglycemic compared with glycemic mice (23.6 ± 2.4 vs 26.7 ± 2.4 g, $P < .0001$) immediately before AAA induction.

Hyperglycemia reduces experimental AAA diameter

Although mean aortic diameters increased in both PPE-DM and PPE groups after elastase infusion, absolute growth was significantly less in PPE-DM mice (POD 7: 40% ± 15% vs 54% ± 15%, $P = .0091$; POD 14: 54% ± 19% vs 84% ± 24%, $P < .0001$). By POD 7, PPE-DM aneurysms were significantly smaller than PPE ($P = .0016$); this difference became more pronounced by POD 14 ($P < .0001$, Fig 1, A). AAA growth and diameter were also significantly reduced by hyperglycemia in the angiotensin II/ApoE $^{-/-}$ model (growth through POD 21: ANG-DM 29% ± 12% vs ANG 58% ± 30%, $P = .0474$; growth through POD 28: 38% ± 12% vs 61% ± 37%, $P = .1705$; and diameter at POD 14, $P = .0474$, and POD 21, $P = .0472$; Fig 1, B).

Hyperglycemia attenuates AAA mural neovascularity and macrophage infiltration

Mural neovascularity was significantly reduced in PPE-DM compared with PPE mice (12 ± 8 vs 20 ± 6 vessels/HPF, $P = .0229$, Figs 2, A, B, C). In both the PPE-DM and PPE groups, mural neovessel density was positively correlated with aneurysm diameter (Fig 2, D). MAC-2 immunostaining demonstrated reduced medial macrophage infiltration in PPE-DM mice (145 ± 87 vs 253 ± 119 cells/cross-sectional area, $P = .0325$; Fig 3, A, B, C). Medial macrophage density was also positively correlated with aortic diameter and neovascularity in both groups (Fig 3, D, E).

Hyperglycemia attenuates AAA MMP-9 activity, preserves medial elastin

Pro-MMP-9 activity was significantly reduced in PPE-DM compared with PPE mice (relative density: 0.63 ± 0.31 vs 1.00 ± 0.13, $P = .0259$, Figs 4, A and B). No significant differences in pro- or active MMP-2 activity were noted (Fig 4, C and D). EM staining revealed relative aortic medial elastin preservation in PPE-DM mice (Fig 4, E and F), quantified by an increased elastin/total medial-area ratio vs that present in PPE mice (20% ± 7% vs 12% ± 6%, $P = .0209$; Fig 4, G). Similar evidence of elastin preservation was present in ANG-DM vs ANG mice (data not shown).

Insulin therapy increases AAA diameter

Serum glucose levels declined from 465 ± 26 mg/dL preinsulin to 120 ± 69 mg/dL 2 weeks after insulin treatment ($P < .0001$). Despite lower serum glucose levels, body weight did not change significantly between institution of insulin therapy and sacrifice. AAA diameter in all three groups (PPE, n = 6; PPE-DM, n = 6; PPE-DM + insulin, n = 6) was inversely related to serum glucose levels in a dose-response fashion (Fig 5), with larger aneurysm enlargement in PPE-DM + insulin compared with PPE-DM mice (POD 14: 1.14 ± 0.05 vs 1.00 ± 0.04 mm, respectively; $P = .0002$). PPE-DM mice demonstrated reduced aortic mural neovascularization, macrophage infiltration, and elastolysis compared with the other two groups (Table).

VEGF does not augment macrophage production in hyperglycemic mice

Significantly fewer macrophages were present in dialysate obtained from hyperglycemic mice (n = 9) after IP thioglycollate (1.4 ± 0.3 vs 2.2 ± 0.3 × 10^7 cells/mL in euglycemic mice; n =

9; P< .0001; Fig 6). VEGF164 administration augmented recovery of thiogly-collate-induced peritoneal macrophages in euglycemic mice (*P* = .0171) but did not alter dialysate macrophage density in DM mice (Fig 6).

Discussion

Hyperglycemia impairs aneurysm progression in two complementary, but mechanistically distinct, murine models of AAA disease. To our knowledge, these are the first experiments to identify candidate mechanisms of hyperglycemic AAA suppression in vivo. Reduced AAA diameter in hyperglycemic mice is accompanied by reduced aortic mural neovascularization, macrophage infiltration, and medial elastolysis. Insulin-mediated reductions in serum glucose levels partially negate the protective effects of hyperglycemia on aneurysm progression.

Many metabolic consequences of diabetes increase risk for adverse cardiovascular events.[22,23] In the particular case of AAA disease, however, several of these consequences may paradoxically serve to limit proteolysis and aneurysm expansion. Aortic mural neovascularity plays a prominent role in aneurysm pathogenesis,[24,25] and hyperglycemia reduces neoangiogenesis in wound healing[26,27] and aortic tissue.[28] In murine models of wound healing, hyperglycemia inhibits neovessel formation by interfering with hypoxia-inducible factor 1 (HIF-1) and HIF-related activation of the nuclear hypoxia response element (HRE), down-regulating activation of VEGF expression and the angiogenic response.[26] Although circulating VEGF levels are frequently increased in diabetic individuals and mice,[29] and diabetes promotes neovascularization in proliferative retinopathy,[30] VEGF levels in select target organs may be depressed by diabetes secondary to impaired HIF signaling.[27]

VEGF-induced macrophage recruitment is a critical feature of the angiogenic response, and macrophages themselves produce VEGF as well as MMP-9.[18,31] As was apparent from the results of our chemical peritonitis experiments, and consistent with prior observation,[32,33] hyperglycemia impairs monocyte/macrophage production in response to exogenous VEGF administration. These results, combined with evidence that angiogenesis inhibitors attenuate experimental AAA progression,[12] suggest that aortic neovessel formation and macrophage infiltration are interdependent and essential stimuli for aneurysm progression, and that both appear to be significantly inhibited in the setting of hyperglycemia.

MMP activity is essential to the progression of AAA disease.[34-36] In this study, reduced aortic mural macrophage density was associated with diminished MMP-9 activity and relative elastin preservation. In addition to limiting macrophage infiltration, hyperglycemia may inhibit MMP production by activated monocytes. Glycation may modify monocyte/extracellular matrix interactions by collagen crosslinking; exposure to glutaraldehyde and high glucose concentrations produces similar reductions in MMP and interleukin-6 expression from activated monocytes in collage lattices.[37] Because MMP activity is essential for endothelial cell migration and neovascularization, as well as mural macrophage infiltration,[17] these candidate mechanisms may act synergistically to reduce aortic aneurysm inflammation and disease progression in diabetes.

Although effective in increasing serum glucose levels, streptozotocin-induced hyperglycemia is more analogous to type 1 (insulin-deficient) than type 2 (insulin-resistant) diabetes. Consistent with their age and risk factor status, most AAA patients have insulin-resistant diabetes as a feature of the metabolic syndrome, which may, in its aggregate effects, promote cardiovascular inflammation and AAA progression. Streptozotocin exposure also induces significant weight loss and thus may reduce systemic inflammatory responses through immune suppression (anergy). To control for these limitations, future experiments are planned to use alternative methods and models, including aneurysm induction in the db/db leptin receptor-

deficient mouse[38] to characterize the influence of insulin resistance on AAA disease progression. Homozygous db/db mice with genetic mutation of the leptin receptor develop hyper-glycemia associated with obesity and hyperinsulinemia, more closely approximating type 2 diabetes.

Beyond influences on mural neovascularization and macrophage infiltration and activation, additional mechanisms of diabetic AAA suppression almost certainly exist. On the basis of current evidence, alternative or complimentary mechanisms include hyperglycemic influences on the fibrinolytic system,[39] receptors of advanced glycation end-products,[40] and progenitor cell function.[26] Examination of patterns of gene expression throughout the time course of experimental AAA progression, particularly by application of transcriptome-wide message and protein profiling techniques, will provide additional insights into alternative mechanisms of aneurysm suppression in the setting of hyperglycemia.

Conclusions

Hyperglycemia in experimental aneurysm models is associated with attenuated mural neovascularization, macrophage infiltration, and medial elastolysis, all essential features of human AAA disease. Understanding of inhibitory mechanisms associated with hyperglycemia may translate into effective new therapeutic strategies to reduce the morbidity and mortality associated with AAA disease.

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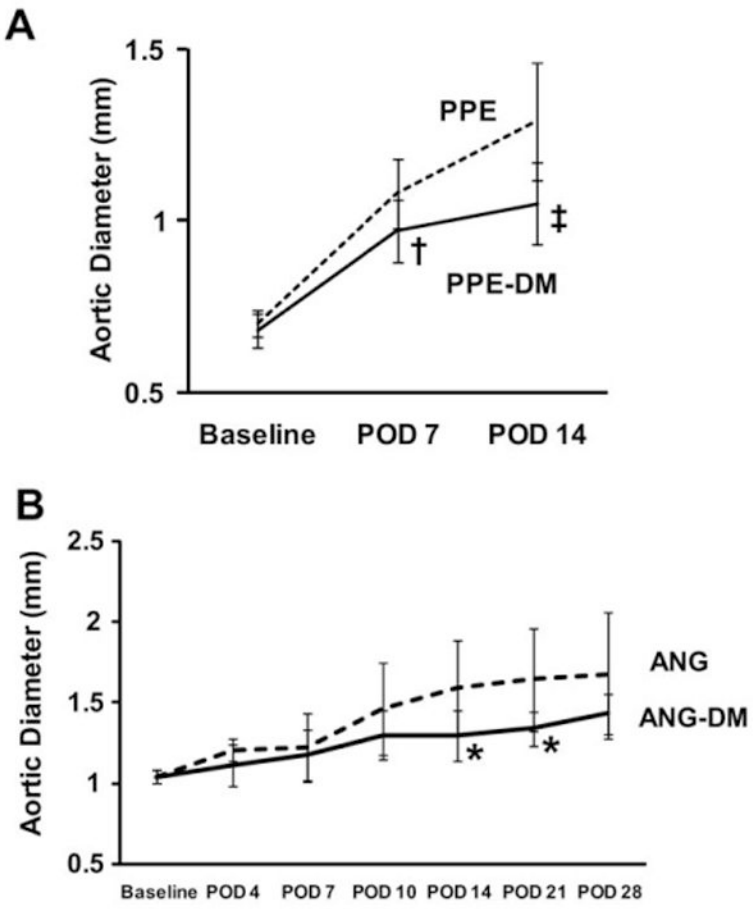

Fig 1.

Aortic diameters in (A) PPE vs PPE-DM mice (†*P* = .0016, POD 7; ‡ *P* < .0001, POD 14) and (B) ANG vs ANG-DM mice (**P*= .0474, POD 14; **P*= .0472, POD 21). The *range bars* show the standard deviation. *ANG*, angiotensin II; *DM*, diabetes mellitus; *POD*, postoperative day; *PPE*, porcine pancreatic elastase.

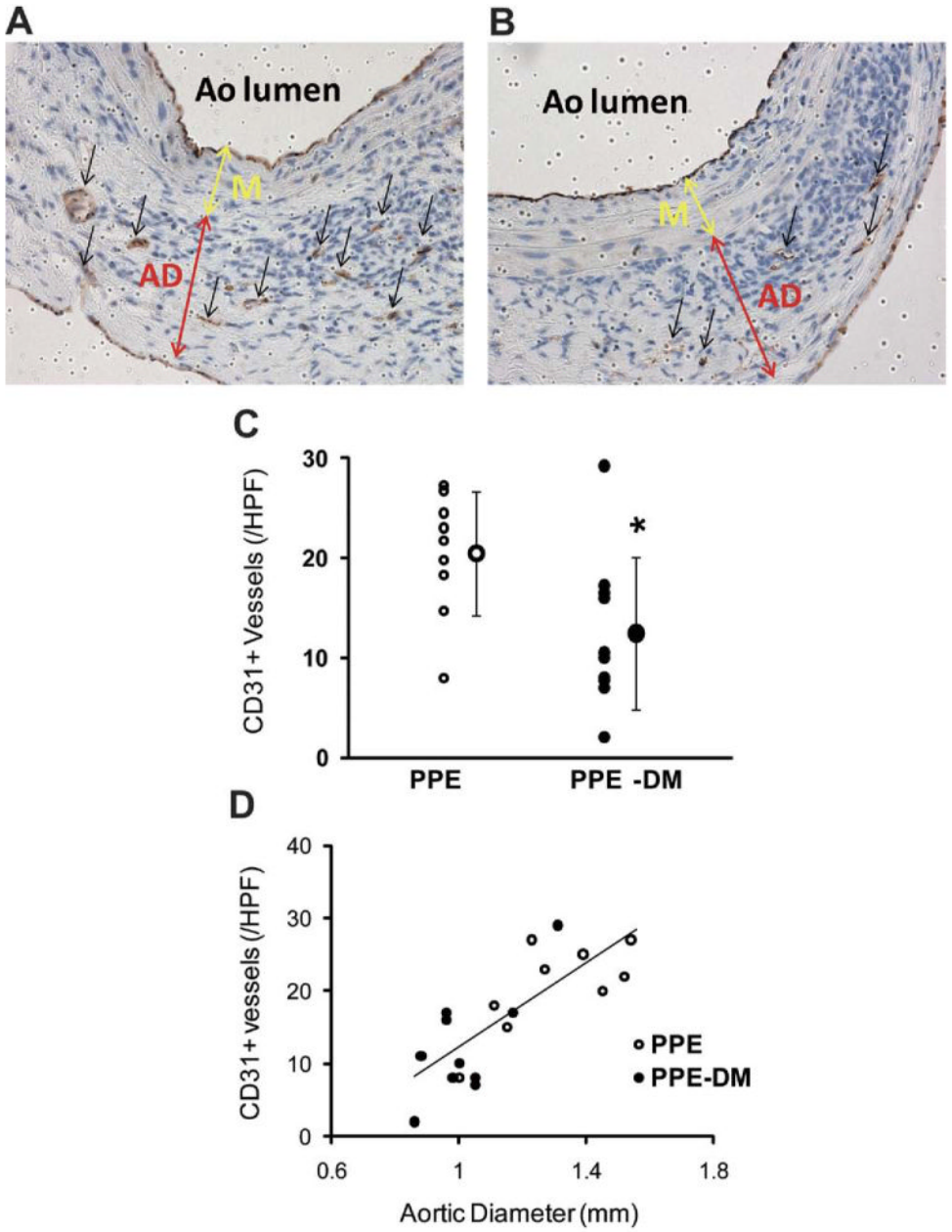

Fig 2.

Representative images of CD31 staining in **(A)** PPE and **(B)** PPE-DM (original magnification ×400). *AD*, adventitia; *Ao*, aorta; *M*, media. **C**, Number of medial and adventitial CD31+ vessels (A and B, *black arrows*) per high power field (*HPF*). The *range bars* show the standard deviation. **P* < .0229 vs PPE; *small circles* represent individual data points, *large circle* represents mean of cohort. **D**, Correlation between neovessel density and aortic diameter, $\gamma = 29.68X - 17.86$; correlation coefficient, $R = 0.794$; $P < .0001$; PPE (n = 9), PPE-DM (n = 10). *DM*, Diabetes mellitus; *PPE*, porcine pancreatic elastase.

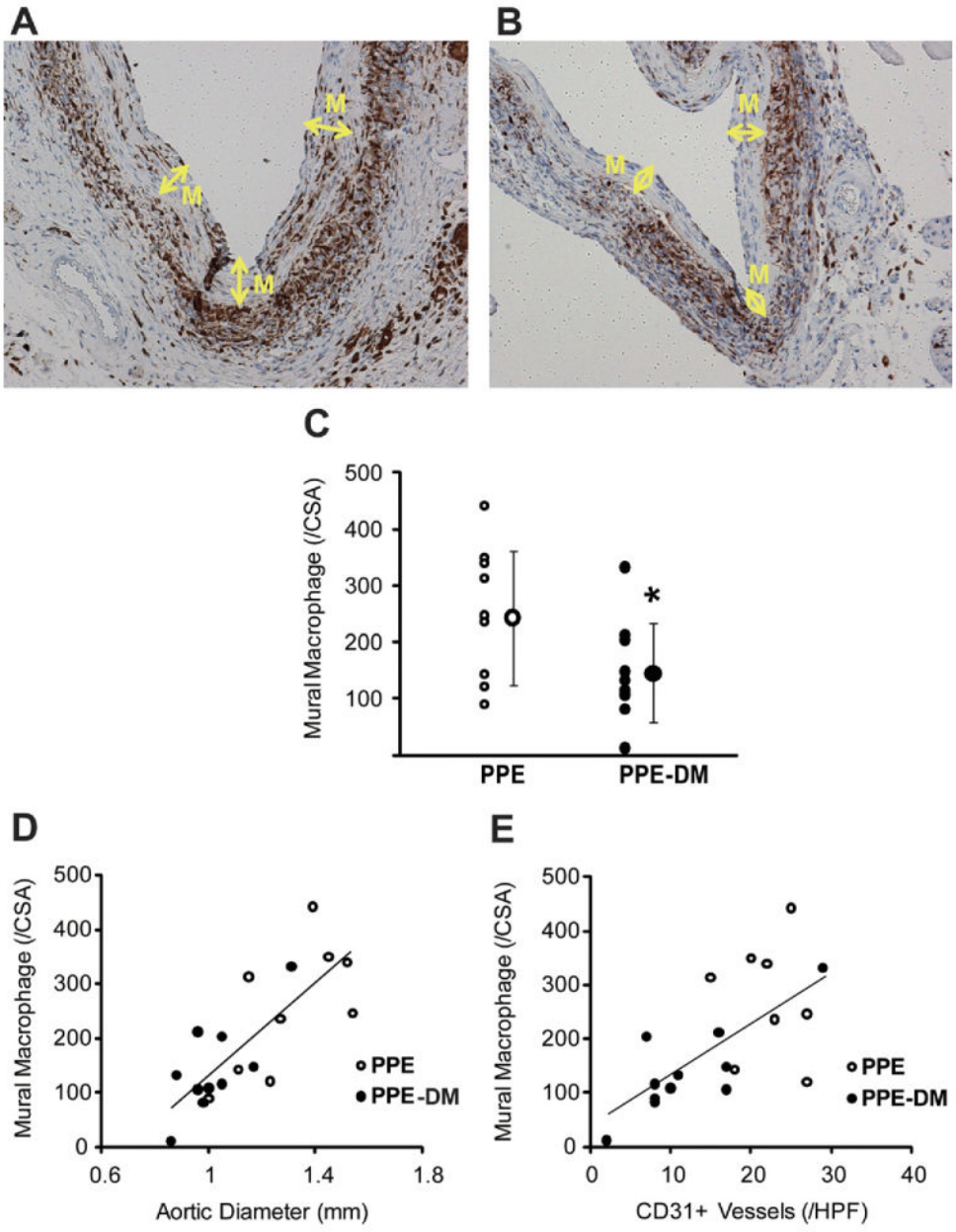

Fig 3.

Representative images of MAC-2 staining in **(A)** PPE and **(B)** PPE-DM (original magnification ×200). *M*, Media. **C**, Number of mural MAC-2+ cells per cross-section are shown. The *range bars* show the standard deviation. **P* = .0325 vs PPE. **D**, Correlation between macrophage density and aortic diameter, $\gamma = 418.04X - 284.35$; correlation coefficient, $R = 0.777$, $P < .0001$. **E**, Correlation between macrophage and neovessel density, $\gamma = 9.53X + 41.51$; correlation coefficient $R = .662$; $P < .01$. Data for D and E are from PPE (n = 9) and PPE-DM (n = 10) mice. *DM*, Diabetes mellitus; *PPE*, porcine pancreatic elastase.

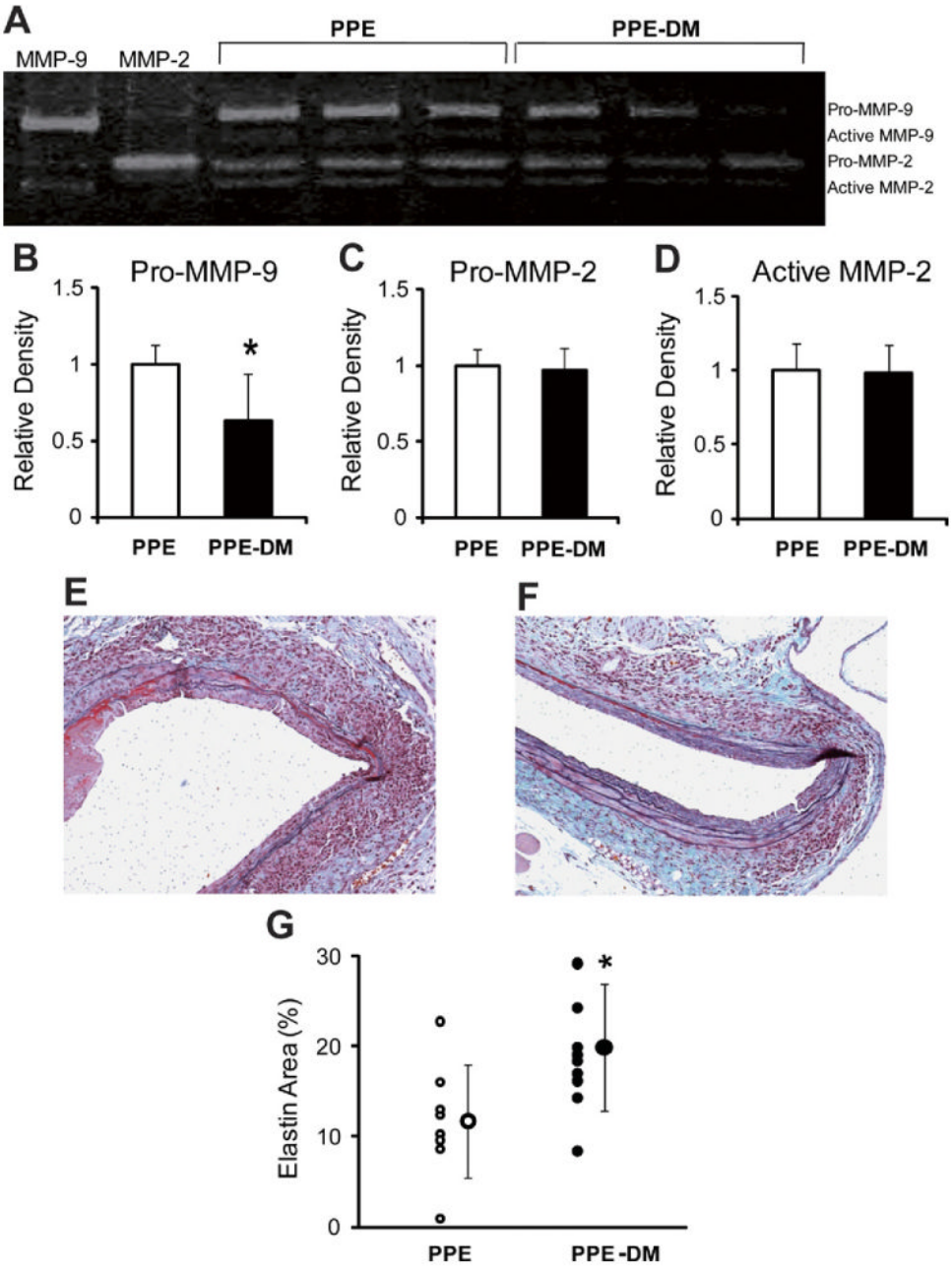

Fig 4.

A representative image of **(A)** zymographic and densitometric analysis of **(B)** pro-MMP-9, **(C)** pro-MMP-2, and **(D)** active MMP-2. **P* = .0259 vs PPE. Representative images of Elastica-Masson-stained aortic cross-sections in **(E)** PPE **(F)** and PPE-DM (original magnification ×200), and **(G)** the ratio of stained-elastin-area to media-area. **P* = .0209 vs PPE. *DM*, Diabetes mellitus; *MMP*, matrix metalloproteinase; *PPE*, porcine pancreatic elastase. The *range bars* show the standard deviation.

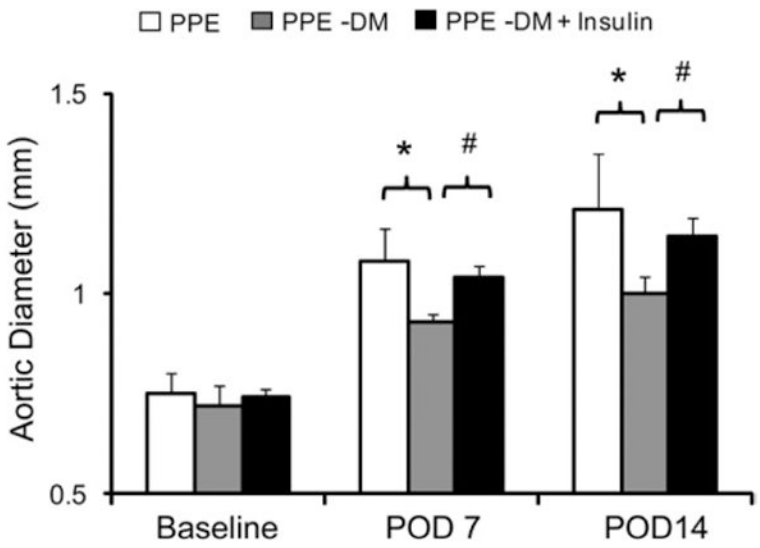

Fig 5.

Comparison of aortic diameter at baseline and after porcine pancreatic elastase *(PPE)* infusion in PPE (n = 6; *white bar)*, PPE-DM (n = 6; *grey bar*) and PPE-DM + insulin (n = 6; *black bar*) mice (POD 7 **P* = .0014, #*P*< .0001; POD 14 **P*= .0036, #*P* = .0002). The *range bars* show the standard deviation. *DM*, Diabetes mellitus; *POD*, postoperative day.

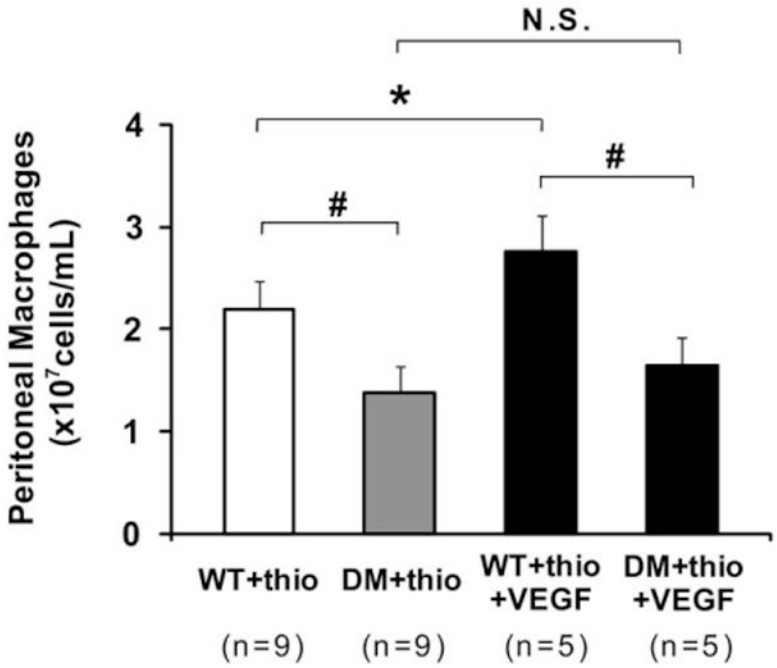

Fig 6.

Comparison of peritoneal macrophages in lavage solution from diabetes mellitus (*DM*) mice and control mice with thioglycollate-induced peritonitis. **P* = .0171, #*P* < .0001. The *range bars* show the standard deviation. *NS*, No significance; *thio*, thioglycollate; *VEGF*, vascular endothelial growth factor; *WT*, wild type.

Table

Cellular and elastin density as a function of diabetes status and insulin therapy in the porcine pancreatic elastase *(PPE)* abdominal aortic aneurysm model

Group	CD31 + neovessels (per HPF)	Mural macrophages (per CSA)	Elastin preservation ratio (%)
PPE	18.4 ± 2.9	268 ± 59	14.1 ± 4.7
PPE-DM	11.9 ± 1.7	172 ± 14	23.1 ± 1.6
PPE-DM + insulin	18.4 ± 3.9	263 ± 87	15.6 ± 8.6

CSA, Cross-sectional area; *DM*, diabetes mellitus; *HPF*, high-power field (original magnification ×400); *PPE*, porcine pancreatic elastase.