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BRIEF COMMUNICATION

Sildenafil (Viagra) Aggravates the Development of Experimental Abdominal Aortic Aneurysm

Chongyang Zhang, MS; Amy Mohan, A.A.S.; Hangchuan Shi, MD, MS; Chen Yan, PhD

BACKGROUND: cGMP-hydrolyzing phosphodiesterase type 5 (PDE5) regulates vascular smooth muscle cell (SMC) contraction by antagonizing cGMP-dependent protein kinase I (PKGI)–dependent SMC relaxation. SMC contractile dysfunction is implicated in the pathogenesis of aortic aneurysm. PDE5 inhibitors have been used for treating erectile dysfunction, such as drug Viagra (sildenafil). However, a few clinical cases have reported the association of Viagra usage with aortic dissection, and reduced PDE5A expression was found in human aortic aneurysm tissues. Therefore, we aimed to investigate the effect of sildenafil on experimental abdominal aortic aneurysm (AAA), the most common form of aortic aneurysm in elderly men.

METHODS AND RESULTS: AAA was induced in C57BL/6J male mice by periaortic elastase in combination with blocking elastin/collagen formation via 3-aminopropionitrile fumarate salt for 35 days. PDE5A protein levels detected by immunostaining were significantly reduced in mouse AAA. Sildenafil application in drinking water significantly aggravated aortic wall dilation and elastin degradation with pre-existing moderate AAA. The phosphorylation level of myosin light chain 2 at Ser19, a biochemical marker of SMC contraction, was significantly reduced by sildenafil in AAA. Proximity ligation assay further revealed that the interaction between cGMP and PKGI was significantly increased by sildenafil in AAA, suggesting an elevation of PKGI activation in AAA.

CONCLUSIONS: Sildenafil treatment aggravated the degradation of elastin fibers and progression of experimental AAA by dysregulating cGMP and contractile signaling in SMCs. Our findings may raise the caution of clinical usage of Viagra in aneurysmal patients.

Key Words: abdominal aortic aneurysm ■ elastase ■ elastin degradation ■ sildenafil ■ Viagra

In the healthy aorta wall, the media layer is composed of contractile smooth muscle cells (SMCs) and elastin fibers, which maintain viscoelastic property of the aorta. Aortic aneurysm (AA) is the permanent dilatation of the aorta resulting from progressive wall degeneration by loss of medial SMC and elastic matrix degradation,[1] which eventually leads to aortic rupture. Available evidence has suggested that SMC contractile dysfunction is associated with aberrant cellular properties implicated in aneurysm development.[1] For example, genetic mutations of SMC-specific contractile genes predispose humans to familial AA,[2,3] and synthetic SMCs acquire capabilities to produce extracellular matrix proteinases and proinflammatory mediators, which contribute to vascular wall degeneration.[1]

Smooth muscle contraction is primarily dependent on the phosphorylation status of myosin light-chain (MLC), which is regulated by the activities of myosin light-chain kinase (MLCK) and myosin light-chain phosphatase (MLCP).[4] MLCK activation leads to smooth muscle contraction, which is dependent on Ca^{2+} and calmodulin. In contrast, MLCP activation causes smooth muscle relaxation. cGMP serves

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as a critical mediator for SMC relaxation by lowering the intracellular Ca^{2+} concentration via multiple different mechanisms, such as suppressing Ca^{2+} influx and mobilization, promoting Ca^{2+} sequestration and extrusion.[4] cGMP also increases MLCP activity through activating cGMP-dependent protein kinase I (cGKI, also known as PKGI) that phosphorylates myosin-binding subunit of MLCP.[4] Thus, cGMP/PKGI antagonizes SMC contraction by facilitating MLC dephosphorylation at serine 19.[4]

cGMP-hydrolyzing phosphodiesterase type 5 (PDE5) is a cGMP-specific PDE and is highly expressed in vascular SMCs.[5] PDE5 functions as an important regulator in SMC contraction by hydrolyzing cGMP and antagonizing PKGI-mediated vascular relaxation.[5] Sildenafil is one of the PDE5 inhibitors and is well-known for treating erectile dysfunction (Viagra)[5] and pulmonary hypertension (Revatio).[4] Sildenafil promotes SMC relaxation through amplifying the effects of the endogenous cGMP/PKGI-dependent relaxation mechanisms.[6] Recent studies have indicated the potential association of cGMP-PKG signaling with AA. For example, a gain-of-function mutation in the gene encoding PKGI causes thoracic AA in humans.[7] The use of Viagra or other PDE5 inhibitors (such as tadalafil) have been occasionally reported to be associated with aortic dissection in men.[8–10] PDE5A expression levels were reduced in human AA tissues.[11] In the current study, we investigated the effect of sildenafil on the development of experimental mouse abdominal AA (AAA), the most common form of AA that mainly occurs in elderly men. Our findings indicate that sildenafil treatment aggravated aortic degeneration and the progression of AAA likely by dysregulating SMC contractile function.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. A complete description of Methods is provided in Data S1.

Mouse Model of AAA

Ten-week-old wild-type C57BL/6J male mice were treated with 0.2% 3-aminopropionitrile fumarate salt (BAPN) (w/v) in drinking water.[12] Periaortic application of porcine pancreas elastase or heat-deactivated elastase (sham) were blinded performed on infrarenal abdominal aorta as previously described with slight modifications.[12] Mice were treated with vehicle or sildenafil (100-mg tablets; Viagra, Pfizer) prepared in drinking water to a concentration of 400 mg/L (0.6 mmol/L) as previously described,[13] resulting in the ingestion of ≈60 to 100 mg/kg per day, starting from the seventh day post-surgery until harvest.[13] Procedures followed were in accordance with protocols approved by the University Committee on Animal Resources at the University of Rochester.

Immunofluorescence Staining and Proximity Ligation Assay

Aortic sections were stained with anti-PDE5 polyclonal, anti-total myosin light chain 2 (MLC) polyclonal, anti-phospho-myosin light chain 2 at Ser19 (pMLC) polyclonal, anti-PKGIα/β monoclonal, anti-calponin polyclonal, anti-MYH11 polyclonal, anti-α-SMA monoclonal, anti-LMOD1 polyclonal, anti-F4/80 monoclonal, anti-Mac2 monoclonal, or anti-MMP9 polyclonal primary antibody for immunofluorescence. Proximity ligation assay was performed on aortic sections incubated with rabbit anti-cGMP polyclonal primary antibody and mouse anti-PKGIα/β monoclonal primary antibody using a Duolink In Situ Red Starter Kit.

Statistical Analysis

Assumptions of normality and equal variance were tested using R (version 3.6.2, https://www.R-project.org/) by Shapiro-Wilk test and Brown-Forsythe test. All tests were 2-sided, with a significance level for 2-sided tests set at 5%. Statistical analyses and plotting were conducted using GraphPad 8 software. All data are presented as mean±SEM.

RESULTS

PDE5A Expression is Reduced in SMCs of Mouse AAA Tissues

A previous study reported a reduction of PDE5A expression in human thoracic AA.[11] Here, we aimed to examine PDE5A expression in mouse AAA tissues. Mouse AAA was induced in C57BL/6J male mice by periaortic elastase application in combination with BAPN treatment to block elastin/collagen cross-links.[12] Because of the limitation of AAA tissue quantity, we used immunofluorescent staining to detect PDE5A protein. We found that PDE5A immunofluorescent staining intensities were significantly reduced in SMCs of AAA medial lesion areas compared with sham (0.62±0.03 versus 1.00±0.03; $P<0.0001$) (Figure 1A and 1B, Figure S1), consistent with the finding in human AA tissues. Immunostaining of AAA sections with SMC markers, including calponin, myosin heavy chain 11, leiomodin 1, and α-smooth muscle actin, revealed a dominant localization of SMC in the media lesion area (area inward of external elastic lamella) (Figure S2). These results suggest that loss of PDE5 function may be implicated in the pathogenesis of AA.

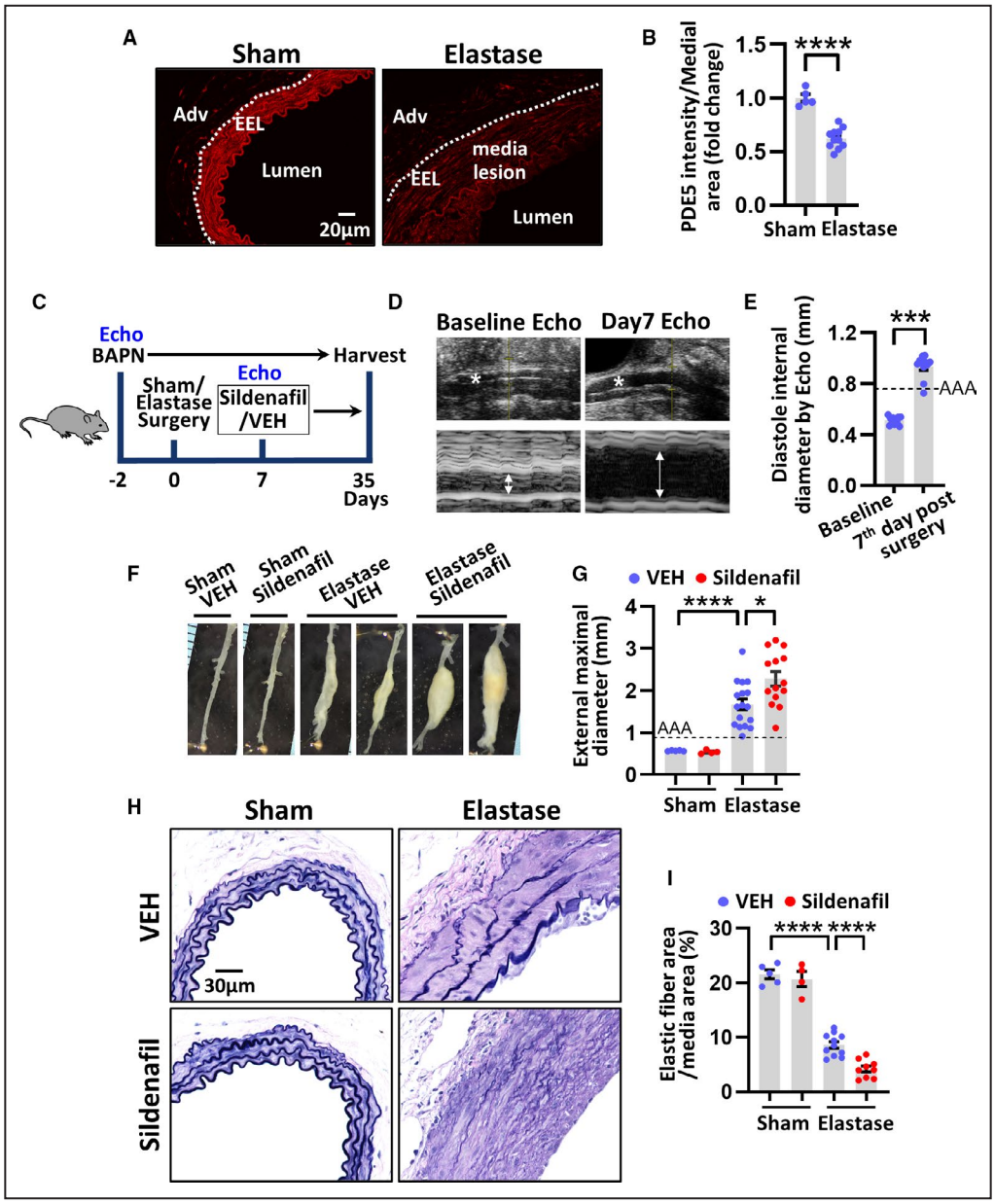

Figure 1. cGMP-hydrolyzing phosphodiesterase type 5 (PDE5A) expression is reduced in media smooth muscle cells (SMCs) of mouse abdominal aortic aneurysm (AAA) tissues, and sildenafil aggravates development of AAA.

AAA was induced by periaortic elastase application in combination with 3-aminopropionitrile fumarate salt (BAPN) treatment in C57BL/6J mice. **A** and **B**, PDE5 immunostaining of sham abdominal aorta (left panel) or AAA (right panel) cross-section (**A**) and staining intensity of PDE5 in media lesion area of AAA, normalized to the averaged amount of staining intensity of sham at media (**B**). External elastic lamina (EEL) is indicated with the dashed line. Adv indicates adventitia. Sham (n=5), AAA (n=11). Data were analyzed by unpaired Student's *t*-test. **C**, Experiment design to test the effect of sildenafil treatment on the development of pre-existing AAA. **D** and **E**, M-mode (**D**) and aorta diastole internal diameter (**E**) at baseline or on the seventh day post-surgery assessed by Echo. The * indicates abdominal aorta. Dash line indicates 50% dilation compared with normal abdominal aortic diameter at baseline. n=11 mice. Data were analyzed by nonparametric paired Wilcoxon test. **F**, Representative infrarenal abdominal aortas. **G**, Quantification of the maximal abdominal aortic width. Aneurysm formation was defined as the increase in the external width of the infrarenal aorta by ≥50% compared with that in the sham/vehicle group. Sham/vehicle (n=5), sham/sildenafil (n=4), elastase/vehicle (n=17), elastase/sildenafil (n=14). Data were analyzed by parametric Welch ANOVA with Dunnett's T3 post-hoc test. **H**, Viagra deteriorated elastin degradation by Van Gieson staining in AAA. **I**, Elastic fiber content in media area. Sham/vehicle (n=5), sham/sildenafil (n=4), elastase/vehicle (n=11), elastase/sildenafil (n=9). Data were analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. All data are expressed as mean±SEM. Each dot represents one animal. **P*<0.05, ****P*<0.001, *****P*<0.0001. VEH indicates vehicle.

Sildenafil Aggravates Development of AAA and Elastin Degradation

Next, we examined the effect of sildenafil on the progression and expansion of pre-existing AAAs in mice. Mice were provided with BAPN in drinking water daily starting from 2 days before surgery until the end of the study (Figure 1C). Mice were subjected to surgery with periaortic elastase application or sham on the infrarenal abdominal aorta. Abdominal aorta size was monitored by ultrasound imaging before surgery as baseline and on the seventh day following surgery. The diastole internal diameter of the aorta by ultrasound imaging exceeded 50% dilation compared with baseline, indicating AAA occurrence (0.94±0.03 mm versus 0.51±0.01 mm; $P<0.001$) (Figure 1D and 1E). Mice were then randomly grouped and treated with sildenafil or vehicle daily until harvest on day 35 post-surgery (Figure 1C). Compared with sham/vehicle controls, elastase induced remarkable dilatation in maximal aortic width, which was an increase of ≈193% (1.67±0.13 mm versus 0.57±0.004 mm; $P<0.0001$) (Figure 1F and 1G). Sildenafil aggravated AAA dilation (2.28±0.17 mm; $P<0.05$), with a 36.54% additional increase in aortic width compared with the vehicle AAA group. The images of AAA from all animals are shown in Figure S3.

Elastin degradation, as a key factor, contributes to aneurysmal wall degeneration and dilation.[1] Elastase-induced AAA exhibited evident degradation of elastic fibers via Van Gieson elastic staining, while sham groups showed no appreciable damage (8.62%±0.62% versus 21.58%±0.82%; $P<0.0001$) (Figure 1H and 1I). Sildenafil-treated AAA manifested more severe destruction of elastin depicted by breaks and fiber thinning compared with the elastase/vehicle group (4.22%±0.56%; $P<0.0001$).

Effect of Sildenafil on MLC Phosphorylation in SMCs of AAA

Increased evidence has suggested that SMC contractile dysfunction is associated with aortic dissection and aneurysm.[1–3] SMC contraction is trigged by Ca^{2+}-dependent activation of MLCK and subsequent phosphorylation of MLC at serine 19. MLC phosphorylation (pMLC) has been frequently used as a biochemical indicator of vascular SMC contraction. Thus, we examined the effect of sildenafil on SMC contractile function by immunostaining for pMLC and total MLC in aortic tissues. We found that MLC staining was not significantly different in medial lesion areas of AAA between the elastase/vehicle and the elastase/sildenafil groups (25.51±3.12 versus 26.76±3.04; Figure 2A and 2C, Figure S4A). In adjacent sections, pMLC staining signals were significantly decreased in medial lesion areas of the AAA group compared with the sham group (26.43±1.86 versus 42.47±1.72; $P<0.0001$) (Figure 2B and 2D, Figure S4B). Interestingly, pMLC signals were further reduced in the sildenafil-treated AAA group (20.15±0.95; $P<0.01$). Figure 2E shows the ratio of pMLC to MLC per medial lesion area for each animal. These results suggest that sildenafil may reduce aortic SMC contractility by suppressing MLC phosphorylation.

Effect of Sildenafil on PKGI Activation in SMCs of AAA

We hypothesize that sildenafil reduces MLC phosphorylation through PKGI-mediated dephosphorylation of MLC. cGMP binds to allosteric sites in the PKGI regulatory domain and activates PKGI.[4] To determine the effect of sildenafil on PKGI activation, cGMP and PKGI binding was detected by proximity ligation assay (PLA) (Figure 3C through 3E, Figure S5A). Compared with sham groups, elastase AAA showed increased PLA signals in media lesion areas (1.66±0.16 versus 1.00±0.06; $P<0.05$). The PLA signal was further increased in the sildenafil-treated AAA group (2.96±0.27; $P<0.0001$), indicating increased cGMP and PKGI binding upon sildenafil treatment. Immunofluorescence staining showed that PKGI expression in AAA was not significantly changed by sildenafil (0.81±0.06 versus 0.79±0.03; Figure 3A and 3B, Figure S5B). These results suggest that PKG activation is upregulated by sildenafil treatment in aortic SMCs of AAA.

DISCUSSION

In the present study, we demonstrated experimentally that chronic sildenafil treatment aggravated the progression of pre-existing aortic elastin degradation and AAA dilatation induced by periaortic elastase in mice. Our finding is consistent with clinical case reports of aortic dissections in a few patients after abuse of PDE5 inhibitors sildenafil or tadalafil.[8–10] Our finding of PDE5A downregulation in mouse AAA is also in line with the report of PDE5A reduction in human aortic tissue obtained from Marfan, tricuspid, and bicuspid thoracic aneurysm samples,[11] suggesting that PDE5A downregulation may contribute to the pathogenesis of aortic dissection and/or aneurysm. Although the causes of AA in the thoracic and abdominal aorta are different, they share many common pathological features such as elastic fiber degeneration and SMC dysfunction and loss.[1] Given that PDE5 inhibitor–induced aortic dissections occurred more often in the thoracic parts of human aortas, the role of PDE5 inhibitors in the experimental model of thoracic AA also deserves to be investigated in the future. PDE5 inhibitors have been clinically used in patients with erectile dysfunction or pulmonary hypertension, and these patients

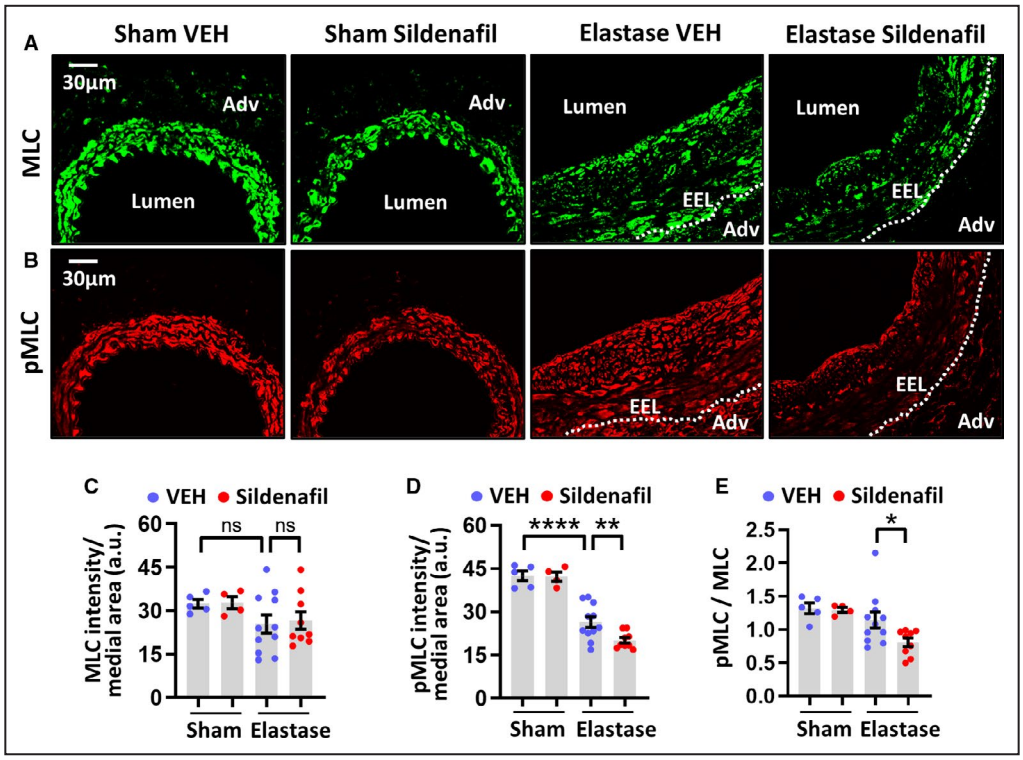

Figure 2. Effect of sildenafil on myosin light chain 2 (MLC) phosphorylation in media smooth muscle cells (SMCs) of abdominal aortic aneurysm (AAA).
A, Immunostaining of total MLC2 in cross-sections of abdominal aorta. **B**, Immunostaining of MLC2 phosphorylated at Ser19 (pMLC) in adjacent sections. **C**, Staining intensity of MLC in sham aorta media or media lesion area of AAA. Data were analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. **D**, Staining intensity of pMLC in sham aorta media or media lesion area of AAA. Data were analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. **E**, Ratio of pMLC staining intensity per media lesion area to that of MLC for each animal. The elastase/vehicle group was compared with the elastase/sildenafil group by nonparametric Mann–Whitney test. **C** through **E**, Sham/vehicle (n=5), sham/sildenafil (n=4), elastase/vehicle (n=11), elastase/sildenafil (n=9). All data are expressed mean±SEM. Each dot represents one animal. **P*<0.05, ***P*<0.01, *****P*<0.0001. Adv indicates adventitia; a.u., arbitrary unit; EEL, external elastic lamina; ns, not significant; and VEH, vehicle.

often have cardiovascular diseases. Thus, caution may be taken when using PDE5 inhibitors in patients with, in particular, a predisposition to aortic diseases or cardiovascular risk factors.

We also observed an increase in cGMP binding to PKGI in AAA with sildenafil, a critical step for PKG activation. This result suggests an enhanced PKGI activation in mouse AAA by sildenafil, which is consistent with the finding that a gain-of-function mutation in human PKGI contributes to thoracic AA and aortic dissection.[7] Activation of cGMP-PKG signaling is well-known to suppress SMC contraction by decreasing MLC phosphorylation.[4] We indeed observed less pMLC in AAA tissues with sildenafil treatment, suggesting a reduction of SMC contractile function. Therefore, it is possible that chronic sildenafil treatment causes contractile dysfunction, which leads to the decrease of aortic wall elasticity (viscoelasticity), increase of aortic wall stress, and deterioration of AAA development in the abdominal aortic loci with pre-existing damage (Figure 3F). Increasing evidence has suggested that SMC contractile dysfunction contributes to AA and aortic dissections. For example, human genetics studies have revealed that genetic mutations of SMC contractile genes, such as *ACTA2* (encoding SMC-specific alpha-actin, α-SMA)[2] and *MYH11* (encoding SMC-specific myosin heavy chain),[3] result in familiar thoracic AA or dissections. A recent study has also reported a decrease of maximum contraction in SMCs ≈30% from patients with sporadic AAA compared with normal SMCs.[14] Thus, maintaining the normal aortic SMC contractile function is believed to be important for reducing wall stress in response to the pulsatile blood flow and high pressure in aortas.

Previous studies that investigated the contribution of angiotensin II (Ang II)–induced hypertension on AAA have indicated that AAA induction by Ang II is independent of the blood pressure–elevating effects of Ang II.

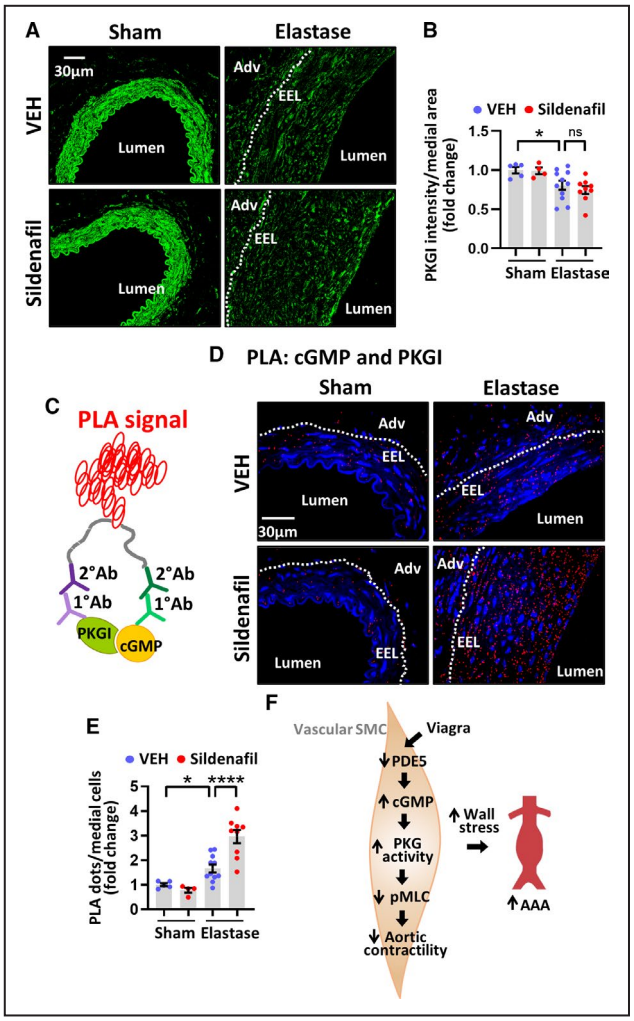

Figure 3. Effect of sildenafil on cGMP-dependent protein kinase I (PKGI) activation in media smooth muscle cells (SMCs) of abdominal aortic aneurysm (AAA).
A, Immunostaining of PKGI in cross-sections of abdominal aorta. **B**, Staining intensity of PKGI in the media lesion area of AAA, normalized to the averaged amount of staining intensity of sham at media. Data were analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. **C**, Proximity ligation assay (PLA) principal paradigm. **D**, PLA detected cGMP and PKGI interaction in cross-sections of abdominal aorta. **E**, Number of PLA dots were normalized to total cell number in the media lesion area. Data were analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. **B** and **E**, Sham/vehicle (n=5), sham/sildenafil (n=4), elastase/vehicle (n=11), elastase/sildenafil (n=9). **F**, Proposed model: chronic sildenafil treatment causes contractile dysfunction of SMCs via activation of cGMP-PKG signaling, which leads to the decrease of aortic contractility, increase of aortic wall stress, and subsequent deterioration of AAA development with pre-existing damage. All data are expressed as mean±SEM. Each dot represents one animal. *P<0.05, ****P<0.0001. Adv indicates adventitia; EEL, external elastic lamina; ns, not significant; PDE5, cGMP-hydrolyzing phosphodiesterase type 5; pMLC, phosphomyosin light chain 2 at Ser19; and VEH, vehicle.

For example, apolipoprotein E knockout mice infused with chronic Ang II increased mean arterial pressure and formed AAA, whereas administration of hydralazine lowered systolic blood pressure but did not prevent AAA formation.[15] Therefore, the effect of PDE5 inhibition on AAA is not likely dependent on its effect on blood pressure regulation. It is worth further examination of the effect of Viagra on Ang II–induced AAA in the future.

Multiple cell types participate in AAA development at different stages. Immunostaining of macrophage markers in sham or AAA (Figure S6) showed that F4/80- or Mac2-positive cells are significantly increased in AAA tissues, while no significant difference was detected with sildenafil treatment. This suggests that the impact of macrophage infiltration by sildenafil, if any, is not likely a major factor in this AAA model, particularly at the late stage. We do not exclude the possibility of Viagra to affect macrophage function in other AAA models. It also remains unclear whether PDE5 inhibition affects endothelial function in AAA, which warrants exploration in the future with endothelial-specific PDE5A knockout or transgenic mice.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data S1. Supplemental Materials and Methods
Figure S1–S6

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Supplemental Material

Data S1.

Supplemental Materials and Methods

Mouse model of AAA

All mice were bred and housed in animal facility of University of Rochester Medical Center under a 12:12 hour light-dark cycle. Standard chow and water ad libitum were available before experiment. During the experiment, 10-week-old wild type C57BL/6J male mice were treated with 0.2% 3-aminopropionitrile fumarate salt (BAPN) (Sigma, A3134) (w/v) in drinking water. BAPN is an irreversible inhibitor of lysyl oxidase which is critical in maintaining homeostasis of the elastic lamina.[12] BAPN treatment started from 2 days before surgery until the end of study.[12] For surgery, mice were anesthetized with inhaled isoflurane. Periaortic application of porcine pancreas elastase (Sigma, E1250) were blinded performed as previously described with slight modifications.[12] Briefly, the connective tissue surrounding infrarenal abdominal aorta was cleaned off from approximately 2 mm below the left renal artery to the bifurcation. A 6mm x 9mm piece of whatman paper were placed on the exposed aortic adventitia for 5 minutes and 40µl of elastase (7.6 mg protein/mL, 4 units/mg protein) were applied directly to the whatman paper. After that, the exposed area was washed gently with saline twice and the abdomen was closed routinely in layers. For sham groups, heat deactivated elastase (100°C for 30 minutes), instead of active elastase, was applied topically to the aorta. The rest of the surgical procedure was identical to the active elastase groups. Mice that received sham surgery or elastase surgery were assigned before the surgery. Extended Release Buprenorphine (0.5mg/kg subcutaneous injection) were used as analgesic prior to surgery and mice were monitored for 3 days post-surgery for recovery. Mice occasionally died shortly within the 3-day monitoring due to the surgery were excluded from following experiments. Only male mice were used because male gender is a major risk factor for AAA. Abdominal aorta size were assessed by ultrasound imaging before surgery as baseline, and assessed on the 7th day post-surgery of active elastase application. Mice were then randomly assigned into four groups on the same day right after ultrasound imaging: sham/vehicle (n=5), sham/sildenafil (n=4), elastase/vehicle (n=17), elastase/sildenafil (n=14). Treatment of drinking water with vehicle or sildenafil to mice started from the 7th day post-surgery till harvest. Animals in each group were caged together and treated in the same way until harvest, and drinking water containing vehicle or sildenafil was prepared daily from the stock solution, therefore investigators were not blinded to group allocation in terms of drug treatment. Total 3 separate experiments were included. Sildenafil was prepared in drinking water as described previously.[13] Briefly, sildenafil citrate (100mg tablets; Viagra; Pfizer) was dissolved in drinking water (pure water acidified with citric acid to pH 5.2) to a concentration of 400 mg/L (0.6 mM), sterile filtered, and given ad libitum to mice, resulting in the ingestion of approximately 60-100 mg/kg/day (based on an average water consumption of 3–5 ml/day for an adult mouse).[13]Sildenafil water solution was stored at 4°C for a maximum of 3 weeks.[13] The IC_{50} for sildenafil inhibition of PDE5A is 4 nM.[5] Using this approach, the average concentration of circulating sildenafil was 70 nM over a 24 hour period.[13] Vehicle is pure water acidified with citric acid to pH 5.2 without sildenafil. All mice were euthanized at 35 days post-surgery. All animals were used in accordance with the guidelines of the National Institutes of Health and American Heart Association for the care and use of laboratory animals.

Ultrasound imaging

A Vevo 2100 ultrasound imaging platform (FUJIFILM VisualSonics) was utilized to assess aneurysm progression in periaortic elastase induced AAA model as described previously.[12] Mice were anesthetized with isoflurane via nosecone, placed on a 37°C warming pad, in a supine position, and feet restrained. Body temperature, respiration rate and ECG were monitored. Heart rate was kept at a consistent range (500–550 beats per minute). Hair was removed from the abdomen and Aquasonic 100 ultrasound transmission gel was placed on abdomen to increase probe contact. The probe was applied on long axis to locate the aorta. Pulse wave (PW mode) doppler was used to confirm aortic flow, and M mode images were taken at the widest part of the aneurysm. Data for diastole internal diameter were shown for individual animals.

Morphometric analysis of maximal aortic width

At the end of the experiments, mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg), midazolam (5mg/kg) and heparin (1600 units/kg). Mice were euthanized via cervical dislocation when lost toe pinch response. Aortas were perfused with saline and fixed with 10% phosphate-buffered formalin (NBF) for 2 minutes. Whole aorta was dissected from the surrounding connective tissue and fixed with 10% NBF for 24 hours at 4°C. Subsequently aortas were further cleaned with a dissecting microscope. Pictures were taken with a digital camera with a ruler set aside. The adventitial circumferences at the maximal expanded portion of the aneurysm were quantified as the maximal abdominal aortic diameter. The maximum diameter of the abdominal aorta was analyzed using Image J software after adjusting the scale according to the ruler in aorta pictures. At least 3 measurements of the maximal expanded portion of the infrarenal aorta for each mouse were averaged before calculating the mean of each experimental group. Aortas were subsequently embedded in paraffin. Aortic cross sections (5 μm each) were collected serially from proximal to distal abdominal aorta in levels.

Van Gieson elastin staining

Paraffin sections were stained for elastic fibers using van Gieson Elastic Stain Kit (Thermo Scientific 87017) according to the manufacturer's instructions. The section from the level of largest diameter of AAA for each animal was selected for staining. Briefly, deparaffinized and hydrated sections were stained in elastic stain solution for 30 min and decolorize in differentiating solution. Following that, sections were rinsed in sodium thiosulfate solution shortly and stained in Van Gieson stain solution for 15min. Sections were viewed with a BX51 upright microscope (Olympus) using Olympus CellSens Standard acquisition software. The area of elastic fibers staining was quantified using ImageJ as described previously.[12] Eight visual fields (magnification 200) evenly distributed at media of every section were included to quantify the amount of elastin staining. All images were set to the same hue, saturation and brightness and measured for positive staining area. The elastin content is expressed as a percentage of elastic fiber area in media area.

Immunofluorescence staining

Sections were deparaffinized, followed by heat treatment with citrate buffer for antigen retrieval. Following that, sections were permeabilized by 0.2% Triton X-100/PBS for 10 minutes. Nonspecific binding sites were blocked with Dako serum-free blocking solution at room temperature for 1 hour. Sections were incubated with rabbit anti-PDE5 polyclonal primary antibody (1:500)(Cell Signaling Technology #2395), rabbit anti-phospho-myosin light chain 2 at Ser19 (pMLC) polyclonal primary antibody (1:300) (Cell Signaling Technology #3671), mouse anti-PKGIα/β monoclonal primary antibody (1:300) (Santa Cruz sc-271765), rabbit anti-calponin

polyclonal antibody (1:200)(Proteintech 13938-1-AP), rabbit anti-MYH11 polyclonal primary antibody (1:200)(Abcam ab53219), mouse anti-α-SMA monoclonal primary antibody (1:800)(Dako M0851), rabbit anti-LMOD1 polyclonal primary antibody (1:200) (Proteintech 15117-1-AP), rabbit anti-F4/80 monoclonal antibody (1:200)(Cell Signaling Technology #30325), rat anti-Mac2 monoclonal antibody (1:200)(Cedarlane CL8942AP), or rabbit anti-MMP9 polyclonal primary antibody (1:200)(Millipore AB19016) overnight at 4°C. Subsequently, the sections were incubated with Alexa Fluror-594 or 488 conjugated anti-rabbit, anti-mouse, or anti-rat secondary antibody for 1 hour at room temperature. Sections were subsequently incubated with DAPI for nuclei staining and mounted with ProLong™ Gold Antifade Mountant.

Total myosin light chain 2 (MLC) staining were performed on adjacent section of pMLC staining by immunofluorescence with HRP-tyramine signal amplification (TSA), using PerkinElmer TSA Plus fluorescence kits (NEL741001KT). Briefly, after deparaffinization, antigen retrieval and permeabilization, endogenous peroxidase was blocked in 0.3% hydrogen peroxide in PBS for 30 minutes, followed by incubation with Dako serum-free blocking solution for 1 hour. After that, sections were incubated in rabbit anti-MLC polyclonal primary antibody (1:300) (Cell Signaling Technology #3672) diluted in DAKO antibody dilution buffer overnight at 4°C. Sections were then washed with PBS for 3 times and incubated in biotinylated goat anti-rabbit (Vector BA-1000) secondary antibody for 1 hour, washed with PBS for three times and incubated in Avidin-biotinylated enzyme complex for 30 minutes. Sections were then washed with PBS for 3 times and incubated in TSA Plus fluorescein working solution (1:50) diluted in 1X amplification diluent for 3 minutes. After that, sections were stained for DAPI and mounted with ProLong™ Gold Antifade Mountant.

One cross section from the level of largest diameter of AAA for each animal was selected for staining. Matched IgG was used in place of the primary antibody as a negative control. Sections were viewed with a confocal microscope (Olympus FV1000-IX81) using 20X objective. Differential interference contrast (DIC) image was taken at the same field to see elastic lamina structures. Media lesion of AAA was defined as the area inward of external elastic lamina (EEL). Staining was analyzed with NIH ImageJ software by calculating the integrated optical density value of positive staining per media lesion area for PDE5, MLC, pMLC, PKGI or total section area for F4/80, Mac2, MMP9. Media lesion area was drawn with the freehand selection tool. The positive staining of each group was standardized to the averaged amount of staining in sham/vehicle group expressed as fold change.

Proximity ligation assay (PLA)

PLA is used to detect and quantify protein-protein interactions in situ. If cGMP physically interacts with PKGI, antibodies bound to each other of the molecules are in a close proximity (maximum 40nm) that oligonucleotides conjugated to the antibodies serve as guides to ligate additional oligonucleotides and form a circular template for DNA synthesis. Amplified DNA was detected with a fluorescent dye-labeled probe (signal amplification up to 1000 times). A single PLA florescence dot represent a site of colocalization between cGMP and PKGI. In this study PLA were performed using Duolink™ In Situ Red Starter Kit (Sigma, DUO92102-1KT) following the manufacturer's instructions. Briefly, aorta cross sections were deparaffinized, followed by heat treatment with citrate buffer for antigen retrieval. Then sections were permeabilized by 0.1% TritonX-100/PBS for 10min and then incubated with Duolink® Blocking Solution at 37°C for 1

hour. Following that, sections were incubated with rabbit anti-cGMP polyclonal primary antibody (Millipore Sigma 09-101)(1:200) and mouse anti-PKGIα/β monoclonal primary antibody (Santa Cruz sc-271765)(1:200) diluted in Duolink® Antibody Diluent at 4°C overnight. Sections were then incubated with anti-rabbit PLUS and anti-mouse MINUS PLA probes diluted (1:5) in the Duolink® Antibody Diluent at 37°C for 1 hour. PLUS and MINUS PLA probes are secondary antibodies coupled with oligonucleotides that target primary antibodies. Sections were then incubated with ligase diluted in 1X ligation buffer (1:40) at 37°C for 30 minutes. Ligase hybridize connector oligonucleotides when the PLA probes are in close proximity, which forms a closed circular DNA template. After that, sections were incubated with DNA polymerase diluted in 1X amplification buffer (1:80) at 37°C for 100 minutes. DNA polymerase amplify the closed circular DNA template by rolling-circle amplification using PLA probe as a primer. The generated concatemeric sequences are coupled to fluorochromes (labeled oligos) that hybridize to the complementary repeating sequences. After final washes, sections were mounted with Duolink® In Situ Mounting Media with DAPI.

One section from the level of largest diameter of AAA for each animal was used for each animal. PLA signal was visualized by confocal microscope FV1000-IX81 with 60X objective. Fluorescence images and DIC images were acquired in stack of 3.2μm intervals. PLA dots at media lesion area were counted. Total cell number in media lesion layer were counted by DAPI. 5 fields were acquired of each tissue section. Number of PLA dots per cell averaged from 5 fields were quantified for each animal. The fold change was calculated by normalizing PLA dots per cell of each animal in each group to the averaged PLA dots per cell in the sham/vehicle group.

Statistical analysis

Data of Fig.1B pass normality and constancy of variance; and was analyzed by unpaired Student's t-test. Data of Fig.1E reject normality and accept constancy of variance; and was analyzed by nonparametric paired Wilcoxon test. Data of Fig. 1G pass normality and reject constancy of variance; and was analyzed by parametric Welch ANOVA with Dunnett's T3 post-hoc test. Data of Fig. 1I, Fig. 2C, Fig. 2D, Fig. 3E pass normality and constancy of variance; and was analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. For Fig. 2E, elastase/vehicle group was compared to elastase/sildenafil group by nonparametric Mann-Whitney test as data reject normality and constancy of variance. Data of Fig. 3B pass normality and constancy of variance; and was analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test. One data point (0.422) in elastase/sildenafil group of Fig. 3B is an outlier based on the interquartile rule (1.5 times interquartile range) and was removed from statistics.

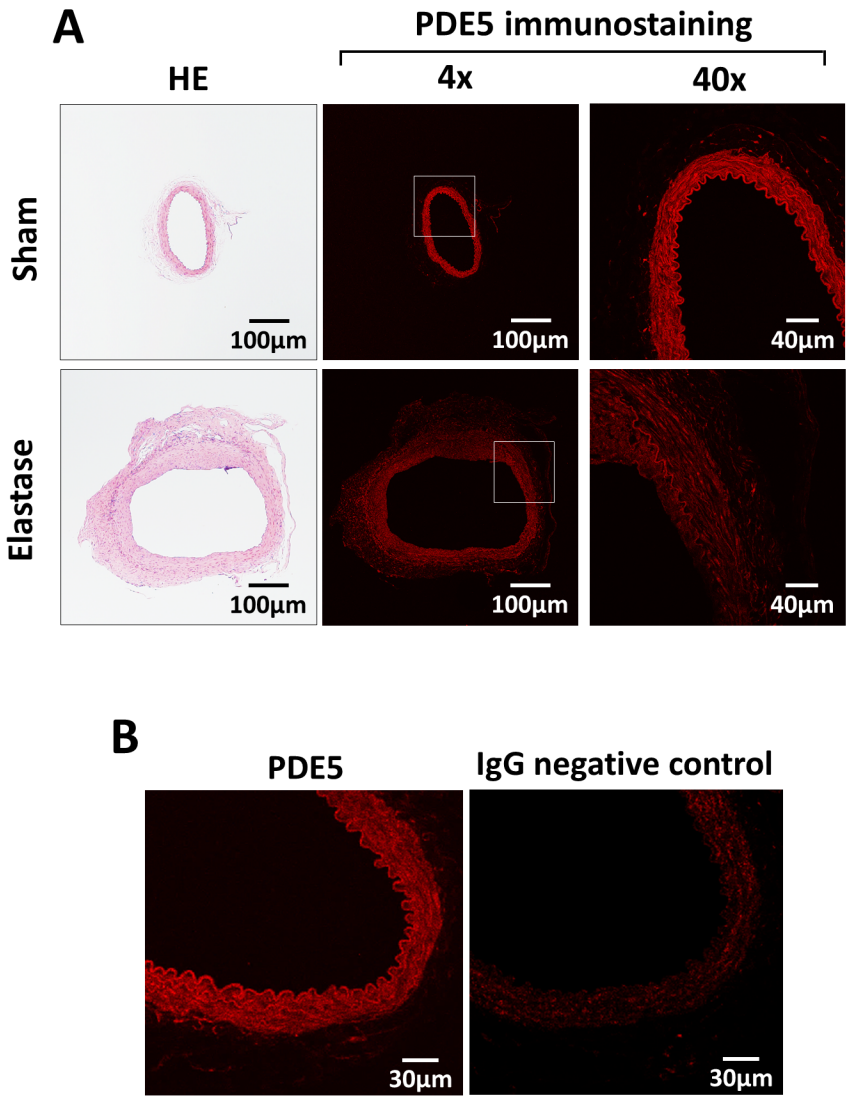

Figure S1. This is a supplementary to Fig. 1A. **A**, Left panels show Hematoxylin and eosin (HE) staining in sham abdominal aorta and abdominal aortic aneurysm. Middle panels show the whole cross section of PDE5 immunostaining on adjacent section. White box highlight the magnified area shown in right panels. **B**, Negative control of PDE5 immunostaining in sham abdominal aorta.

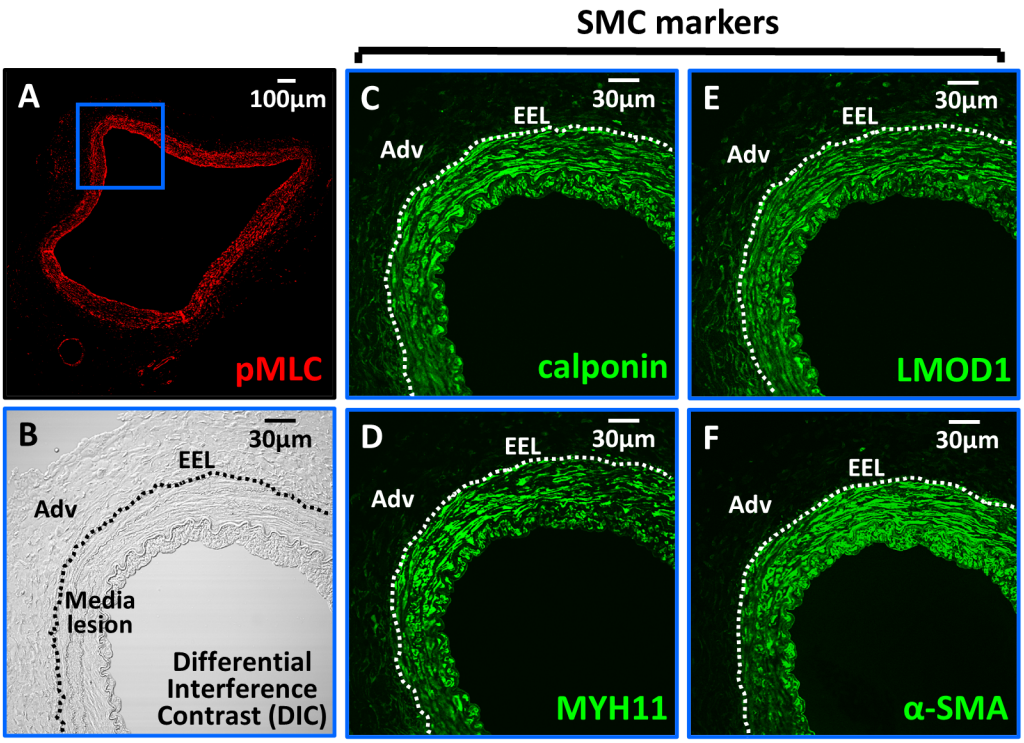

Figure S2. The media lesion area of abdominal aortic aneurysm (AAA) (the area inward of external elastic lamella) were dominated by smooth muscle cells (SMCs). **A**, Cross section of AAA from elastase/vehicle mouse. Blue box highlight the magnified area shown in panel B-F. **B**, Differential interference contrast (DIC) image show the external elastic lamella (EEL), dashed line. Adv, adventitia. **C-F**, SMC markers calponin, myosin heavy chain 11 (MYH11), leiomodin 1 (LMOD1), α-smooth muscle actin (α-SMA) were stained on AAA sections from the same level.

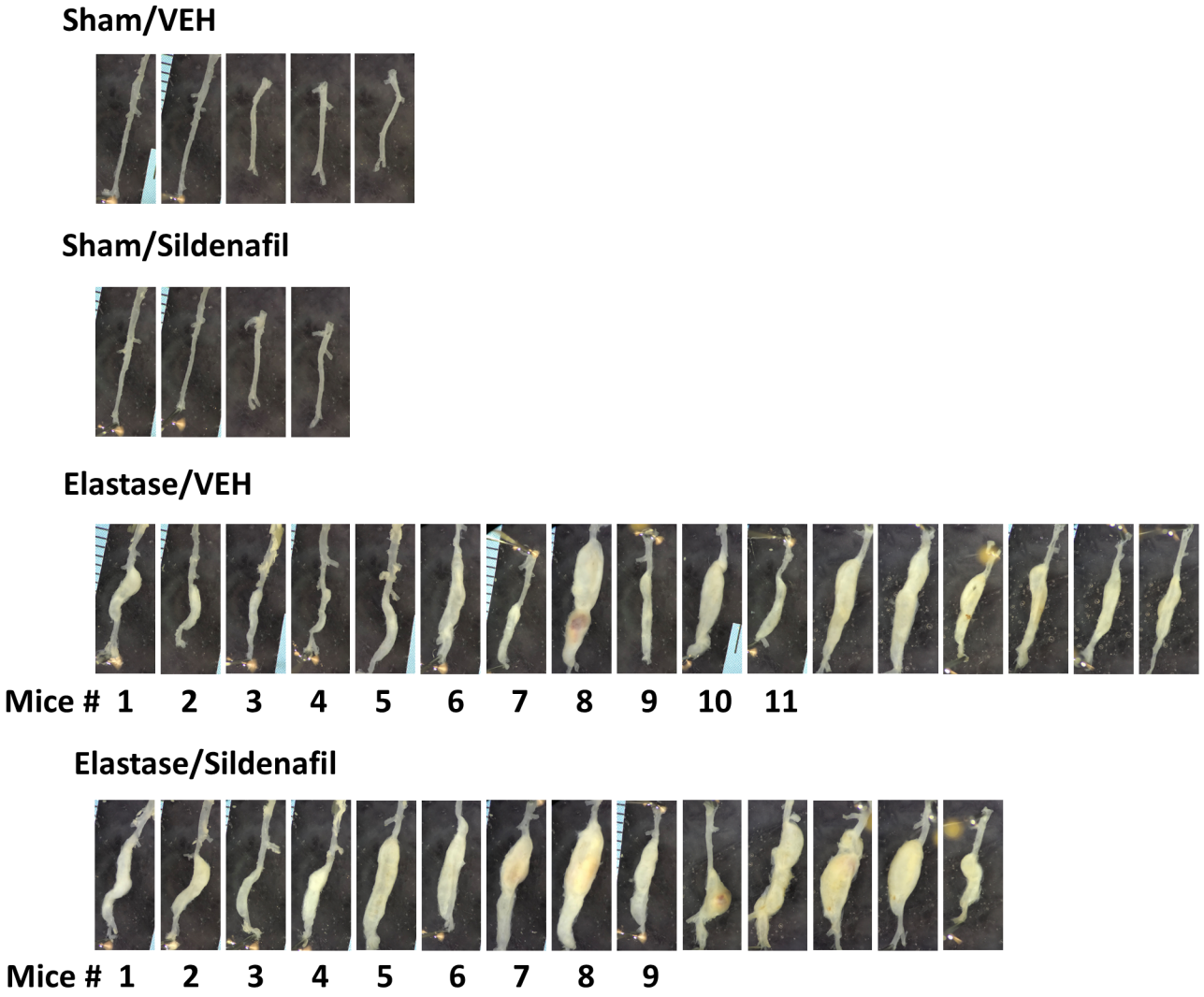

Figure S3. Images of all sham or abdominal aortic aneurysm samples shown in Fig. 1G. Mice #1-11 in the elastase/vehicle group and Mice #1-9 in the elastase/sildenafil group were included for histology and immunostaining analysis.

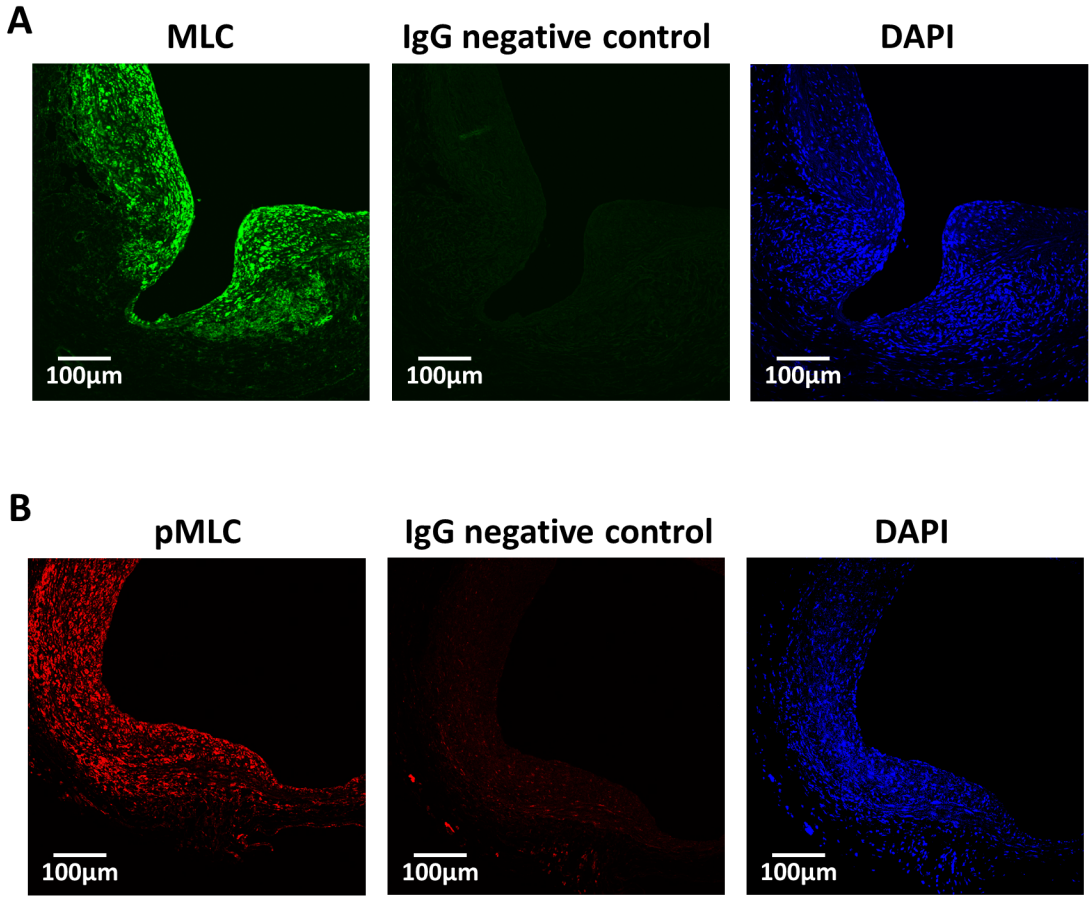

Figure S4. This is a supplementary to Fig. 2A and 2B, showing negative control of myosin light chain 2 (MLC) or myosin light chain 2 phosphorylated at Ser19 (pMLC) immunostaining in mouse AAA.

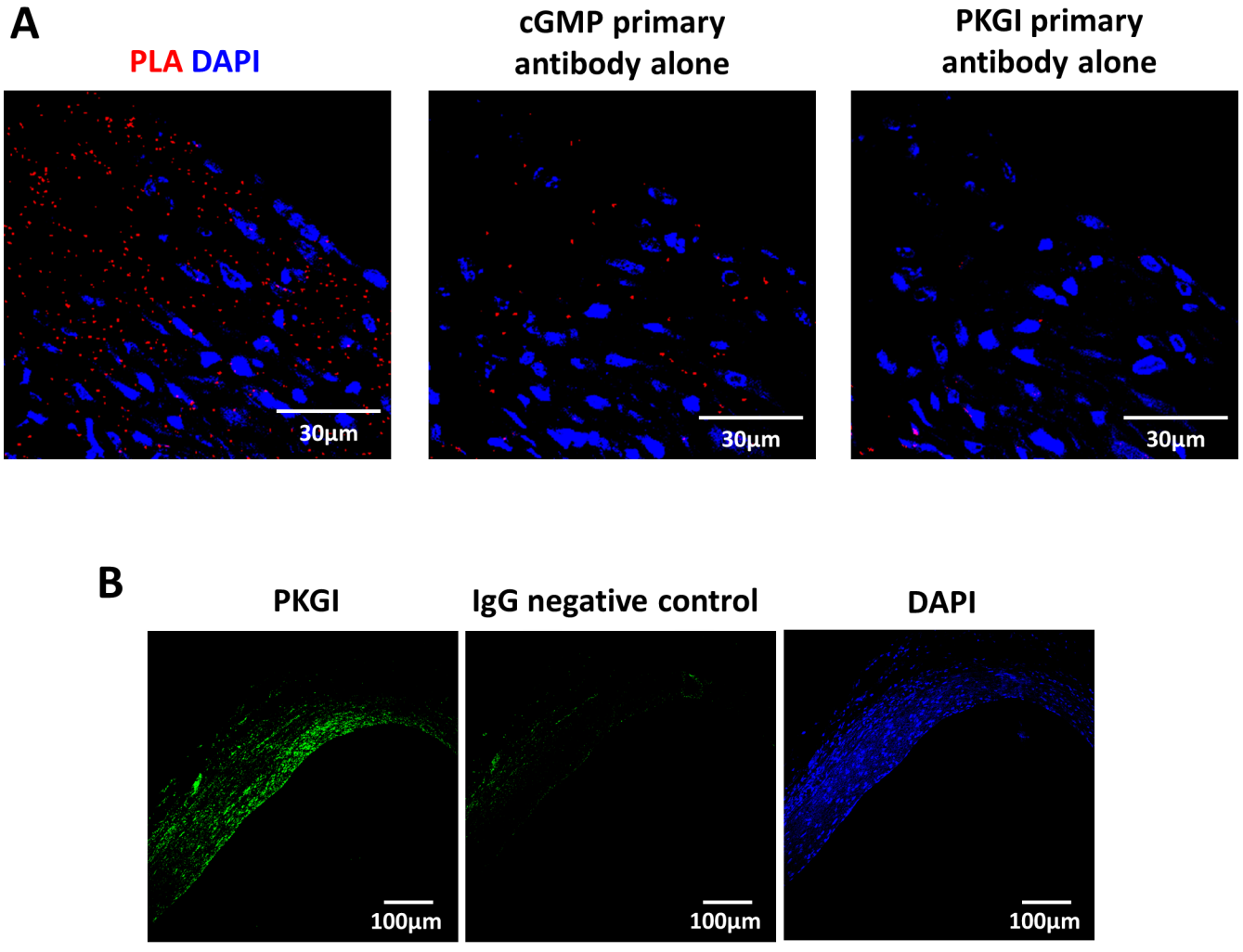

Figure S5. Negative controls of PLA (A) and cGMP-dependent protein kinase I (PKGI) immunostaining (B) that used cGMP primary antibody alone or PKGI antibody alone in mouse AAA.

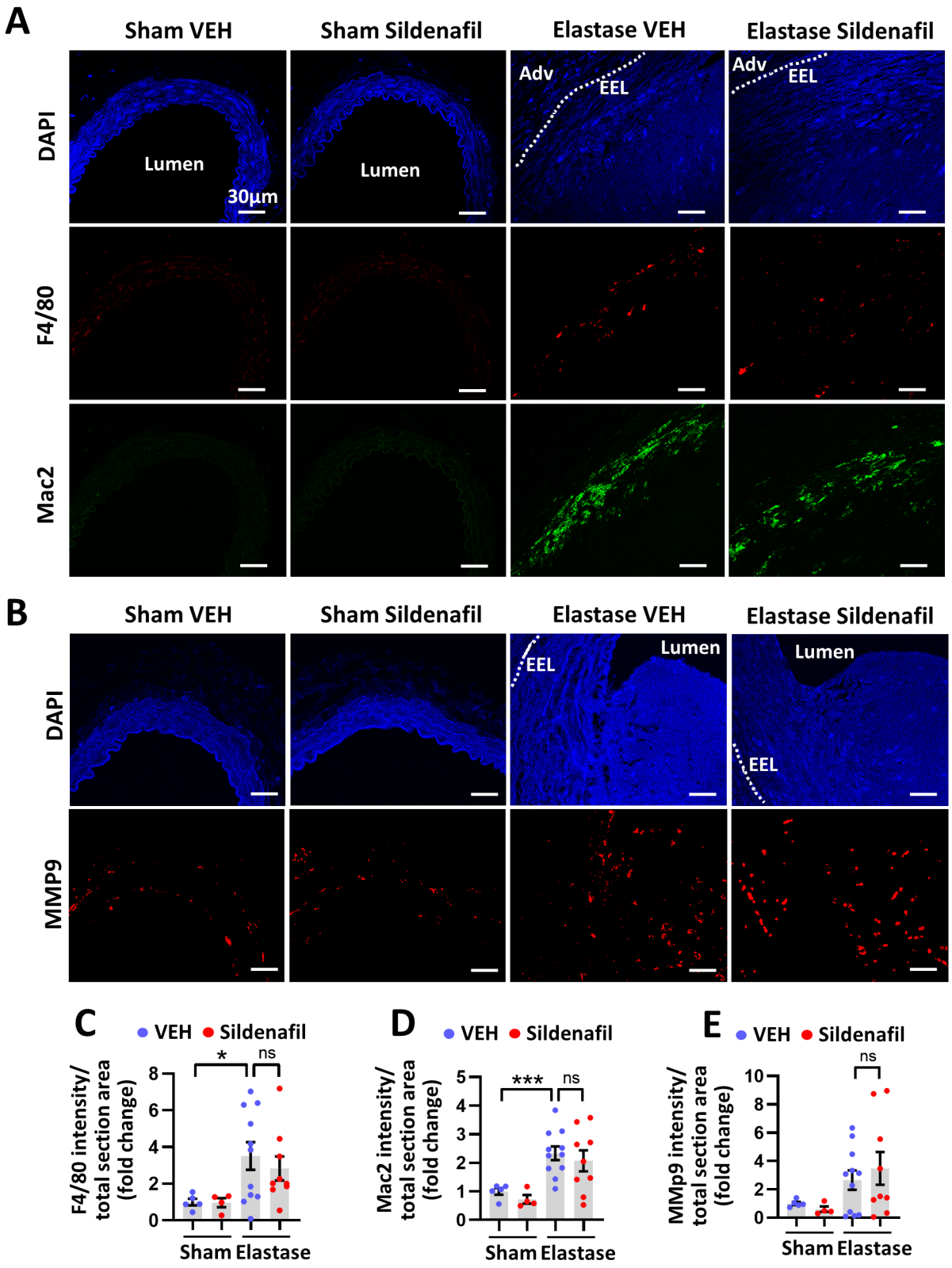

Figure S6. Legend on the next page.

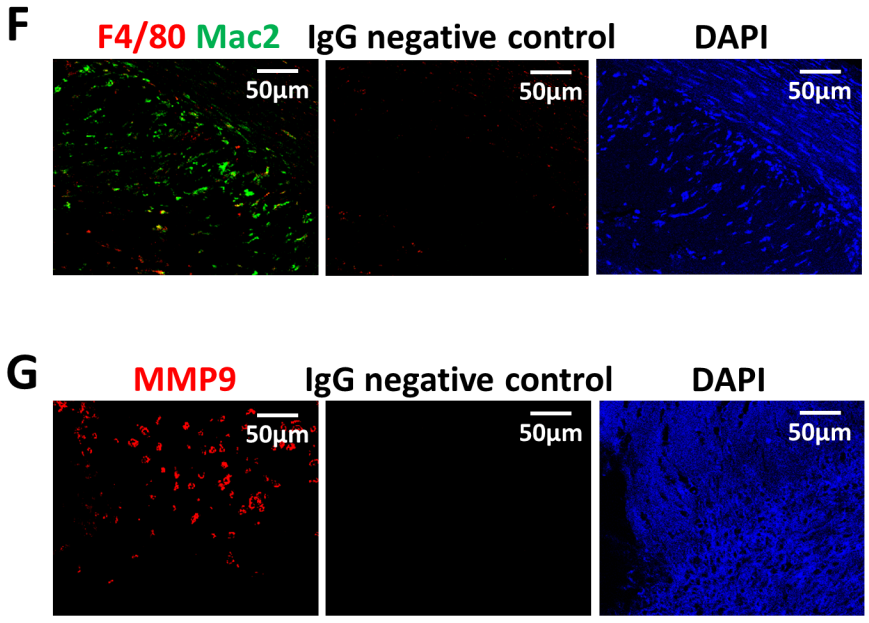

Figure S6. Immunostaining of macrophage marker F4/80, Mac2 (**A**) and MMP9 (**B**) in sham or abdominal aortic aneurysm. Scale bar, 30 μm. **C-E**, Staining intensity of F4/80 (C) or Mac2 (D) or MMP9 (E) in total section area. Data (C, D) analyzed by parametric Welch ANOVA with Dunnett's T3 post-hoc test (data pass normality and reject constancy of variance). The probability of a type II error for C between elastase vehicle and elastase sildenafil is 0.795. The probability of a type II error for D between elastase vehicle and elastase sildenafil is 0.952. Data (E) analyzed by parametric one-way ANOVA with Holm-Sidak's post-hoc test (data pass normality and constancy of variance). The probability of a type II error for E between elastase vehicle and elastase sildenafil is 0.705. **F-G**, Negative control of F4/80 and Mac2 (F), and MMP9 (G) immunostaining in mouse AAA.