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GSDMD Deficiency Protects Against Aortic Rupture

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Running Title: GSDMD and Aortic Disease

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ABSTRACT

Objective:
Aortic ruptures are fatal consequences of aortic aneurysms with macrophage accumulation being a hallmark at the site of ruptures. Pyroptosis is critical in macrophage-mediated inflammation. This study determined effects of pyroptosis on aortic dilation and rupture using GSDMD deficient mice.

Approach and Results:
In an initial study, male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice in C57BL/6J background (8 – 10 weeks old) were infected with adeno-associated viral vectors encoding mouse PCSK9D377Y gain-of-function mutation and fed a Western diet to induce hypercholesterolemia. After two weeks of AAV infection, angiotensin II (AngII, 1 μg/kg/min) was infused. During the 4 weeks of AngII infusion, 5 of 13 *Gsdmd*$^{+/+}$ mice died of aortic rupture, whereas no aortic rupture occurred in *Gsdmd*$^{-/-}$ mice. In surviving mice, no differences in either ascending or abdominal aortic dilation were observed between *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice. To determine whether protection of GSDMD deficiency against aortic rupture is specific to AngII infusion, we subsequently examined aortic pathologies in mice administered beta-aminopropionitrile (BAPN). BAPN (0.5% wt/vol) was administered in drinking water to male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice (4 weeks old) for 4 weeks. Six of 13 *Gsdmd*$^{+/+}$ mice died of aortic rupture, whereas no aortic rupture occurred in *Gsdmd*$^{-/-}$ mice. In mice survived, no differences of diameters in the ascending, arch, or abdominal aortic regions were observed between *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice.

Conclusions:
GSDMD deficiency protects against AngII or BAPN-induced aortic ruptures in mice.

Highlights

1. GSDMD deficiency protects against angiotensin II-induced aortic rupture in hypercholesterolemic mice.
2. GSDMD deficiency protects against beta-aminopropionitrile (BAPN)-induced aortic dissection and rupture in C57BL/6J mice.

Non-standard abbreviations:

Abbreviation	Full Term
AAV	adeno-associated viral vectors
AngII	angiotensin II
AAAs	abdominal aortic aneurysms
BAPN	beta-aminopropionitrile
GSDMD	gasdermin D

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INTRODUCTION

Aortic aneurysms have high risk for aortic dissection and rupture that lead to high mortality.[1] Most studies report aortic diameters to evaluate the disease severity. However, rupture of smaller aortic aneurysms also occurs in humans,[2-4] suggesting aneurysm size is a limited predictor of aortic rupture.

Inflammation is profound in dissected and ruptured abdominal aortic aneurysms (AAAs).[1, 5] Inflammatory lesions often contain dead cells. Pyroptosis, distinct from apoptosis, is a lytic form of programmed cell death.[6, 7] This process is dependent on the cleavage of gasdermin D (GSDMD) by inflammatory caspase-1 or -11.[8-11] Pyroptosis and production of interleukin (IL)-1β/IL-18 are the prominent outcomes of inflammasome activation in response to pathogenic molecules as well as danger signals and tissue damage.[12, 13] Inflammasomes contribute to aortic aneurysms.[14, 15] However, aortic aneurysm studies of inflammasomes focused on IL-1β have yielded conflicting results.[16-20] Of note, the clinical trial of AAAs on an IL-1β antibody (NCT02007252) was terminated prematurely due to lack of efficacy and futility. Whether GSDMD-mediated pyroptosis contributes to aortic aneurysms, dissection, and rupture remains unknown.

In this study, we determined effects of genetic GSDMD deficiency on aortic aneurysms, dissection, and rupture in two mouse models. One was angiotensin II (AngII) infusion to hypercholesterolemic mice; the other was administration of beta-aminopropionitrile (BAPN) in young normolipidemic mice.

RESULTS AND DISCUSSION

GSDMD deficiency prevents AngII-induced aortic rupture

To induce hypercholesterolemia, which augments AngII-induced aortic aneurysms,[21] we injected AAVs encoding mouse PCSK9D377Y to male *Gsdmd*$^{+/+}$ or *Gsdmd*$^{-/-}$ mice and fed them a Western diet for 2 weeks prior to AngII infusion.[22] This study only used male mice because female mice have low incidence of AngII-induced aortic aneurysms.[23] Plasma total cholesterol concentrations were increased greatly within 2 weeks in both male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice (Figure 1A). During AngII infusion, 5 of 13 mice died due to aortic rupture in *Gsdmd*$^{+/+}$ mice, but no death occurred in *Gsdmd*$^{-/-}$ mice (Figure 1B). After 4 weeks of AngII infusion, survived mice were terminated, aortas were dissected and cleaned to measure surface area of the ascending and arch region (representing ascending and arch dilation) and outer width of the abdominal region (representing abdominal aortic dilation) since AngII induces dilations in both ascending and abdominal aortic regions (Figure I in the online-only Data Supplement). We did not detect differences on surface area of the ascending aorta and maximal outer diameters of suprarenal aorta between *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice (Figure 1C and D). These findings suggest that depletion of GSDMD prevents aortic ruptures, but does not affect aortic dilation.

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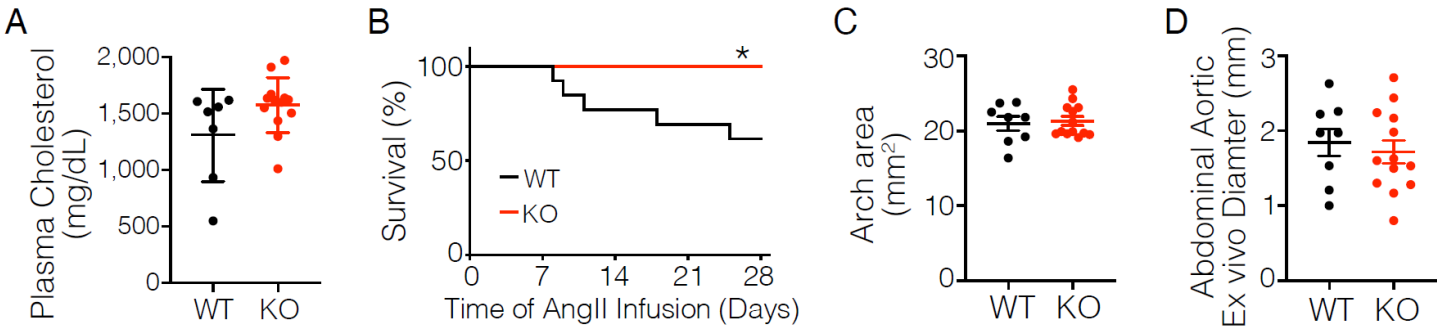

Figure 1. Deficiency of GSDMD prevents aortic rupture in AngII-infused mice. Male *Gsdmd*$^{+/+}$ (WT) and *Gsdmd*$^{-/-}$ (KO) mice in C57BL/6J background were injected intraperitoneally with AAVs encoding mouse PCSK9D377Y, fed a Western diet, and infused with AngII. (**A**). Plasma total cholesterol concentrations were measured by an enzymatic assay kit. (**B**). Survival rate of mice were analyzed by LogRank analysis. * P < 0.05. (**C**). Surface area was measured using an *en face* method. (**D**). Maximal outer width was measured using an *ex vivo* method.

Deficiency of GSDMD prevents BAPN-induced aortic rupture

Lysyl oxidase (LOX) and LOX-like (LOXL) contribute to cross-linking of collagen and elastin of the aorta. BAPN inhibits LOX and LOXL enzymatic activity.[24, 25] In young mice at the age of 3 – 4 weeks, BAPN induces aortic dissection and rupture.[26] We administered BAPN to male *Gsdmd*$^{+/+}$ or *Gsdmd*$^{-/-}$ mice when they were 4 weeks old. Six of 13 *Gsdmd*$^{+/+}$ mice died of descending aortic rupture as demonstrated by necropsy (Figure 2A), but no *Gsdmd*$^{-/-}$ mice died of aortic rupture. In mice survived after 4 weeks of BAPN administration, aortic pathologies were found in the ascending, arch, and descending thoracic regions (Figure II in the online-only Data Supplement). Ascending, arch, and descending aortic dilations were not different between *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice (Figure 2B). No apparent dilation was noted in abdominal aortas of both *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice (Figure 2C). These findings are consistent with the AngII-infused mouse model that GSDMD deficiency prevents aortic rupture but not aortic dilation.

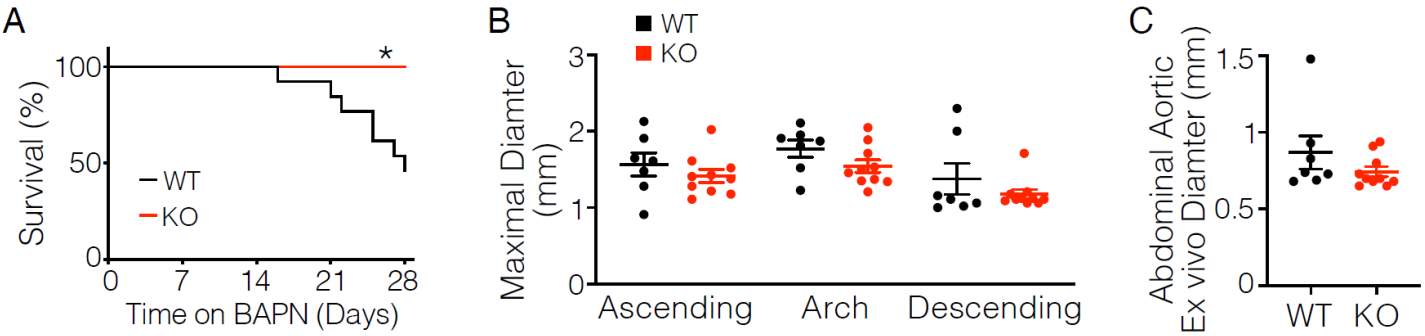

Figure 2. Deficiency of GSDMD prevents aortic rupture in mice administered BAPN. Male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice in C57BL/6J background were studied. (**A**). Survival rate of mice were analyzed by LogRank analysis. * P < 0.05. (**B**). Maximal diameter was measured on in situ images. (**D**). Maximal outer width was measured using an ex vivo method.

In summary, deficiency of GSDMD, the executioner of macrophage pyroptosis, protects against aortic ruptures in two aortic aneurysm animal models: mice infused with AngII or administered BAPN. Macrophage accumulation, accompanied by elastin fragmentation, is a hallmark of aortic aneurysmal tissues.[27, 28] Pyroptotic macrophages release cellular content in an uncontrolled manner, which could promote MMPs release into extracellular matrix, contributing to elastin fragmentation. Our data

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provide solid evidence that inhibition of macrophage pyroptosis by GSDMD deficiency is effective in aortic rupture prevention. Further studies are warranted to determine the molecular and cellular mechanisms by which depletion of GSDMD prevents aortic ruptures.

MATERIALS AND METHODS

Animals

Gsdmd$^{+/+}$ and *Gsdmd*$^{-/-}$ mice on a C57BL/6J background were bred in-house.[29] All animal experiments reported in this manuscript were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Adeno-associated viral (AAV) vectors

AAV vectors (serotype 8) driven by a hepatocyte-specific thyroxine-binding globulin (TBG) promoter were produced by the Vector Core in the Gene Therapy Program at the University of Pennsylvania. These AAV vectors contained inserts expressing mouse PCSK9D377Y mutation (equivalent to human PCSK9D374Y gain-of-function mutation).[22] For AngII infusion study, male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice at the age of 8 – 10 weeks were injected intraperitoneally with AAV vectors (2 x 10^{11} genome copies/mouse), and fed a Western diet (TD.88137, Envigo) for 2 weeks to induce hypercholesterolemia.

Osmotic mini pump implantation and angiotensin II (AngII) infusion

AngII (1 µg/kg/min; Cat# H-1706; Bachem) was infused subcutaneously via mini osmotic pumps (Alzet Model # 2004; Durect Corp.) for 4 weeks, preceded by 2 weeks of Western diet feeding. Western diet continued during AngII infusion. Surgical procedure is described in our previous publication.[30]

Administration of beta-aminopropionitrile (BAPN)

Male *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice at the age of 4 weeks were administered BAPN (0.5% wt/vol; Cat # A3134; Sigma) in drinking water for 4 weeks. Fresh drinking water containing BAPN was changed twice a week.

Plasma cholesterol measurements

Mouse blood were collected in the presence of EDTA (final concentration: 1.8 mg/ml) by cardiac bleeding via right ventricle. Plasma total cholesterol concentrations were measured using an enzymatic commercial kit (Cat # 999-02601; Wako Chemicals USA).

Statistical analysis

Data are represented as means ± standard error of means (SEM). SigmaPlot version 14.0 (SYSTAT Software Inc.) was used for statistical analyses. Survival Log-Rank analysis was used to compare the cumulative survival rate. Incidence of aortic rupture was analyzed by Fisher exact test. Aortic diameters were analyzed by Student's t-test if data passed normality and equal variance tests. If either normality or equal variance test failed, data were analyzed by Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant.

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SOURCES OF FUNDING

Congqing Wu is K99 awardee (K99HL145117) of the NIH/NHLBI. The authors' research work was supported by the NIH/NHLBI under award numbers K99HL145117 and R01HL139748, and a start-up funding of the University of Kentucky to HSL. The content in this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

DISCLOSURES

The authors have filed a patent application (treatment for aortopathy targeting GSDMD) in June 2020.

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Figure I. Ex vivo images of aortas from *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice infused with AngII.

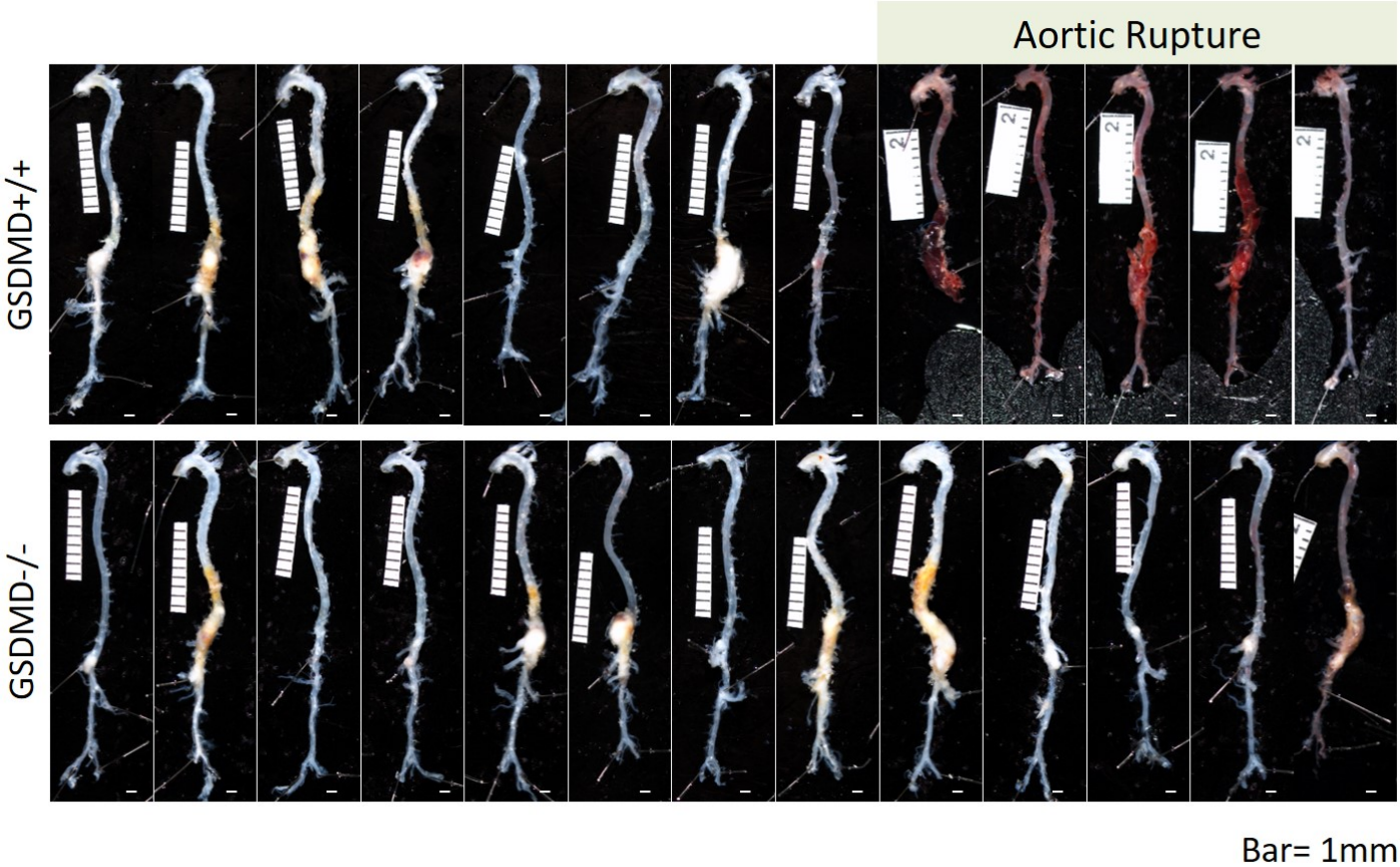
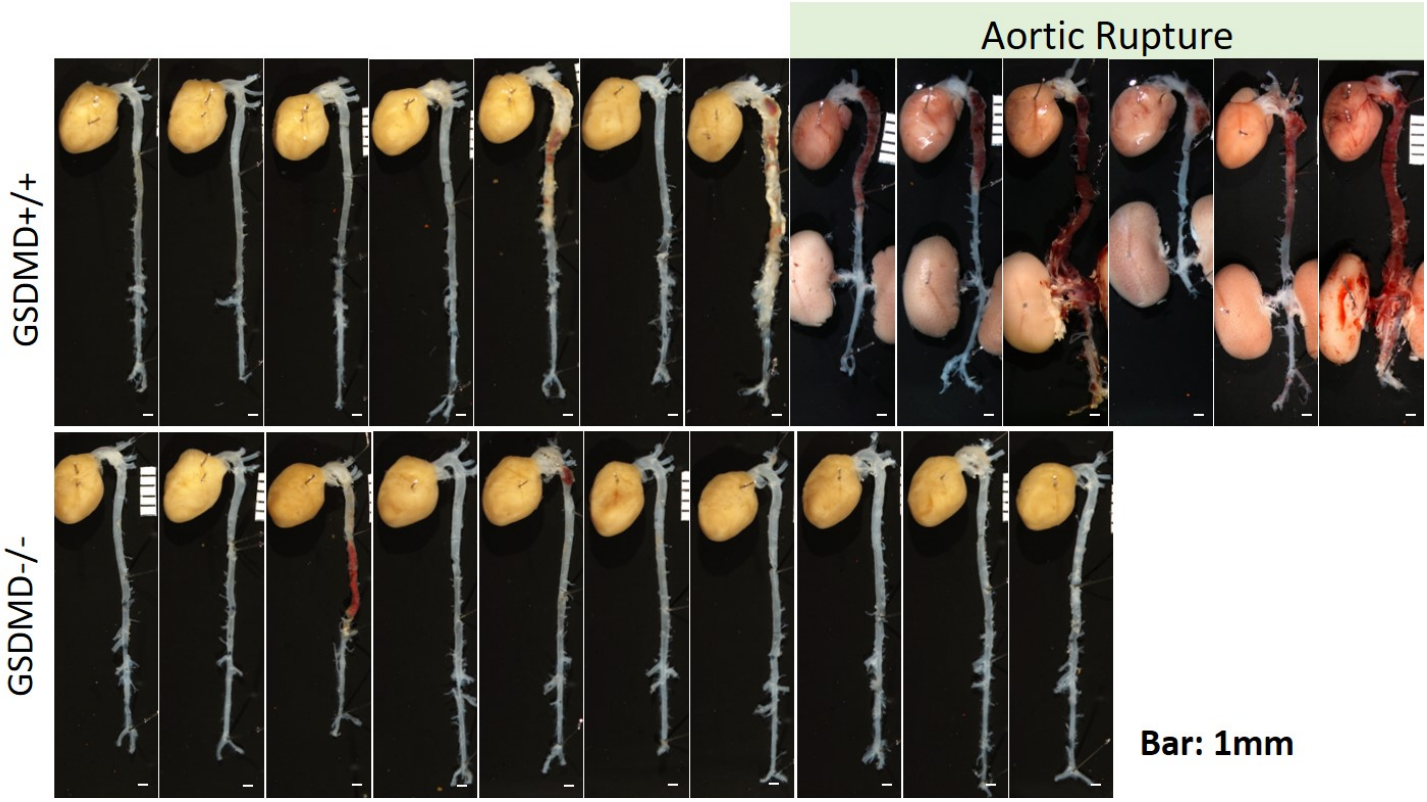


Figure II. Ex vivo images of aortas from *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice administered BAPN.



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Major Resources Tables

Mouse Models (in vivo studies)

Mouse Model	Vendor or Source	Sex	Age
Gsdmd$^{+/+}$	In house breeding	Male	AngII infusion: 8 – 10 weeks old
Gsdmd$^{-/-}$			BAPN administration: 4 weeks old

Mouse Housing Conditions

	Mouse Housing Conditions	Note
Set Temperature range	22 °C	
Set Humidity range	50%	
Light : Dark Cycle	14 hours : 10 hours	
Water	Reverse Osmosis Water	ad libitum
Standard Feed	Teklad Irradiated Global 18% Protein Rodent Diet (Envigo); Diet # 2918	ad libitum
Standard Bedding	P.J. Murphy Coarse SaniChip	
Experimental Feed	Envigo; Diet #TD.88137 (AngII study) or Diet # 2918 (BAPN study)	ad libitum
SPF	Yes	

Major Reagents/Kits

Reagents	Vendor or Source	Catalog #
Angiotensin II	Bachem	4006473
Beta-aminopropionitrile (BAPN)	Sigma	A3134
Cholesterol E for plasma total cholesterol concentrations	Fujifilm Wako Diagnostics U.S.A.	999-02601