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ORIGINAL RESEARCH

Progranulin Maintains Blood Pressure and Vascular Tone Dependent on EphrinA2 and Sortilin1 Receptors and Endothelial Nitric Oxide Synthase Activation

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BACKGROUND: The mechanisms determining vascular tone are still not completely understood, even though it is a significant factor in blood pressure management. Many circulating proteins have a significant impact on controlling vascular tone. Progranulin displays anti-inflammatory effects and has been extensively studied in neurodegenerative illnesses. We investigated whether progranulin sustains the vascular tone that helps regulate blood pressure.

METHODS AND RESULTS: We used male and female C57BL6/J wild type (progranulin$^{+/+}$) and B6(Cg)-Grn$^{tm1.1Aidi}$/J (progranulin$^{-/-}$) to understand the impact of progranulin on vascular contractility and blood pressure. We found that progranulin$^{-/-}$ mice display elevated blood pressure followed by hypercontractility to noradrenaline in mesenteric arteries, which is restored by supplementing the mice with recombinant progranulin. In ex vivo experiments, recombinant progranulin attenuated the vascular contractility to noradrenaline in male and female progranulin$^{+/+}$ arteries, which was blunted by blocking EphrinA2 or Sortilin1. To understand the mechanisms whereby progranulin evokes anticontractile effects, we inhibited endothelial factors. N(gamma)-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor) prevented the progranulin effects, whereas indomethacin (cyclooxygenase inhibitor) affected only the contractility in arteries incubated with vehicle, indicating that progranulin increases nitric oxide and decreases contractile prostanoids. Finally, recombinant progranulin induced endothelial nitric oxide synthase phosphorylation and nitric oxide production in isolated mesenteric endothelial cells.

CONCLUSIONS: Circulating progranulin regulates vascular tone and blood pressure via EphrinA2 and Sortilin1 receptors and endothelial nitric oxide synthase activation. Collectively, our data suggest that deficiency in progranulin is a cardiovascular risk factor and that progranulin might be a new therapeutic avenue to treat high blood pressure.

Key Words: blood pressure ■ nitric oxide ■ progranulin ■ vascular function

Hypertension, commonly known as high blood pressure (HBP), is a chronic medical condition that affects millions of people worldwide. It is a silent killer that often goes undetected for years and, if left untreated, can lead to serious health consequences such as heart disease, stroke, and kidney failure. HBP is still regarded as the primary risk factor for the global burden of illness despite substantial advancements in prevention and treatment,[1,2] and it equally affects people in economically developed and developing countries.[1,3] Patients with HBP are more likely to suffer from chronic renal disease, dementia, myocardial infarction, and stroke.[2,3] The central nervous system, kidneys, and vasculature have all been identified as potential

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RESEARCH PERSPECTIVE

What Is New?

- Progranulin displays vascular anticontractile effects dependent on EphrinA2 and Sortilin1 receptors and nitric oxide formation in male and female mice.
- Progranulin deficiency is associated with neurodegenerative diseases including frontotemporal dementia. Our study reveals that a lack of progranulin might be associated with vascular dysfunction and hypertension, which could be blunted by progranulin supplementation.

What Question Should Be Addressed Next?

- Future research is necessary to understand the role of progranulin as an intracellular signaling protein, the cellular origin of progranulin in models of hypertension, the crosstalk between EphrinA2 and Sortilin1 receptors, and by which molecular mechanisms progranulin regulates nitric oxide production.

Nonstandard Abbreviations and Acronyms

eNOS	endothelial nitric oxide synthase
HBP	high blood pressure
mMECs	mouse mesenteric endothelial cells
NOS	nitric oxide synthase

triggers for HBP.[3–7] In this work, our main goal was to comprehend how changes in the vasculature contribute to the development of HBP.

Vascular resistance is supported by 3 major factors: the viscosity of the blood and the length and diameter of the blood vessels.[1] High systemic vascular resistance, which can lead to HBP, is primarily caused by changes in vascular stiffness, inflammation, and tone.[2–6] Control of vascular tone is coordinated primarily by a balance between endothelium-derived relaxing factors such as nitric oxide, prostaglandins, endothelium-derived hyperpolarizing factor, and endothelium-derived contracting factors such as endothelin-1, thromboxane, and reactive oxygen species[2,3,7,8]; an unbalance between these factors triggers vascular hypercontractility, leading to elevated vascular resistance.

Obesity,[9,10] diabetes,[11] and lipodystrophy[12,13] are major causes of HBP. Interestingly, these conditions share a striking circulating increase of progranulin[14–18]; however, why progranulin is elevated in the plasma and if this increase can modulate the vascular tone and blood pressure is unknown. Progranulin is a highly conserved glycoprotein that is expressed and secreted by adipocytes, neurons, immune cells, and endothelial cells.[19–21] It plays a pivotal role in regulating wound healing, cell growth, and inflammation via autocrine, paracrine, or endocrine actions.[19–21] Studies have shown that progranulin has anti-inflammatory and antihypertrophic aspects in sepsis,[22] liver fibrosis,[23] diabetic nephropathy,[16,24] age-related cardiac disorders,[25] ischemia/reperfusion diseases,[26] and atherosclerosis.[27–29] Other studies have found that progranulin is associated with neurodegenerative diseases.[19,30,31] For instance, loss of progranulin causes neuronal ceroid lipofuscinosis[20,32] and frontotemporal dementia,[20,32] while reduced progranulin levels increase the risk of Parkinson disease and Alzheimer disease.[20] Finally, prior studies have shown a connection between progranulin and endothelial activity via regulating protein kinase B and endothelial nitric oxide synthase (eNOS), as well as blocking nuclear factor-κB.[27,33,34] Although a connection between endothelial activation and progranulin has been previously suggested, its association with vascular tone and blood pressure is still undetermined.

Herein, we used progranulin-deficient mice and recombinant progranulin combined with pharmacological interventions to better understand the contribution of circulating progranulin to vascular tone and blood pressure regulation and more specifically to test the hypothesis that progranulin maintains vascular function and blood pressure via modulating endothelial factors.

METHODS

Eleven- to 13-week-old male and female C57BL6/J wild type (progranulin$^{+/+}$) and B6(Cg)-Grn$^{tm1.1Aidi}$/J (progranulin$^{-/-}$) mice were used. All mice were fed with standard mouse chow, and tap water was provided ad libitum. Mice were housed in an American Association of Laboratory Animal Care–approved animal care facility in the Rangos Research Building at the Children's Hospital of Pittsburgh of the University of Pittsburgh. The Institutional Animal Care and Use Committee approved all protocols (Protocol Nos. 19065333 and 22061179). All experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals.

Mouse Models of HBP

To characterize the circulating levels of progranulin in HBP, we used 2 different models:

- *Angiotensin II–treated mice*[35]: Male mice were infused with vehicle or angiotensin II (490 ng/min per kg) for 14 days with ALZET osmotic minipumps (Alzet

Model 1002; Alzet Corp Durect, Cupertino, CA) while receiving regular drinking water.

- *Aldosterone-treated mice*[36]: Male mice were infused with vehicle or aldosterone (600 μg/kg per day) for 14 days with ALZET osmotic minipumps (Alzet Model 1002; Alzet Corp Durect) while receiving 1% saline in the drinking water.

Sodium Urine Collection and Measurement

Changes in blood pressure affect the urine volume and sodium excretion; therefore, we placed progranulin$^{+/+}$ and progranulin$^{-/-}$ mice placed in a metabolic cage for 24-hour urine collection. Briefly, mice were acclimated for 24 hours followed by an additional 24 hours for sample collection and sodium measurement. Urine was sent to Kansas State Veterinary Diagnostic Laboratory for sodium quantification.

Restoration of Circulating Progranulin

Mice were treated with recombinant progranulin ALZET osmotic minipumps (Alzet Model 1001; Alzet Corp Durect, Cupertino, CA) for 7 consecutive days (20 μg/day), as described elsewhere.[14]

Circulating Progranulin Levels

ELISA was used to detect plasmatic progranulin (R&D Systems, Minneapolis, MN).

Circulating Inflammatory Profile Via Proteome Profiler Mouse Cytokine

Plasma from progranulin$^{+/+}$ and progranulin$^{-/-}$ mice was collected and frozen at −80 °C until the day of the analysis. Plasma from 6 mice of each group was pooled, and the experiment was performed in duplicate. The proteome profiler mouse cytokine array kit (R&D Systems) was performed following the manufacturer's instructions. The intensity of each band generated in the assay was analyzed in ImageJ. Data were presented in fold changes in a heat map graph.

In Vivo Blood Pressure Measurement

Blood pressure was analyzed via radiotelemetry using an HD-X10 telemeter (Data Sciences International, St Paul, MN). Transmitters were implanted as described previously.[37,38] After 7 days of recovery from surgery, necessary for the mice to gain their initial body weight, data were recorded for 5 days as a baseline. Then, recombinant progranulin was continuously administered as described above. Systolic blood pressure, diastolic blood pressure, mean arterial pressure, and heart rate were analyzed.

Indices of Autonomic Function

To analyze whether changes in autonomic response interfere with any changes in blood pressure, indices of the autonomic function were obtained on the last day of the recording baseline period. A classic pharmacological method consisting of a single intraperitoneal injection of the ganglionic blocker mecamylamine (5 mg/kg) or of the β-adrenergic receptor blocker propranolol (6 mg/kg) was used.[37–39] Injections were conducted >2 hours apart in random order. Changes in blood pressure response to pharmacological compounds within 60 minutes after injection were reported. Data were expressed as a percentage of the baseline value.

Histology

Progranulin$^{+/+}$ and progranulin$^{-/-}$ mice were euthanized for aortae harvest and perfused with cold PBS. Aortae were collected and placed in a 4% paraformaldehyde solution for histologic analysis. After 12 hours in paraformaldehyde, tissues were placed in 70% ethanol until the day of preparing the samples for histology. Aortae were embedded in paraffin, then samples were sectioned and stained with hematoxylin and eosin and Masson's trichrome to analyze the vascular remodeling and structure.

Vascular Function

Rings from second-order mesenteric resistance arteries were mounted in a wire myograph (Danysh MyoTechnology) for isometric tension recordings with PowerLab software (AD Instruments) as described before.[35,36,40] Briefly, rings (2 mm) were placed in tissue baths containing warmed (37 °C), aerated (95% O_2, 5% CO_2) Krebs Henseleit Solution (in mmol/L: 130 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 0.03 EDTA, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 1.18 KH_2PO_4, and 5.5 glucose). After 30 minutes of stabilization, curves of tension were performed to adjust the ideal tension for each segment followed by incubation with KCl (60 mmol/L).

- Protocol to study the effects of progranulin: Since noradrenaline is endogenously produced in mammals and is one of the most important regulators of vascular tone and blood pressure, concentration-response curves for noradrenaline were performed to study the role of progranulin on the vascular tone control. Mesenteric arteries were incubated with recombinant progranulin for 1 hour before noradrenaline curves in 3 different concentrations (100, 300, and 600 ng/mL).
- Protocol to study the endothelial factors: Concentration-response curve to noradrenaline was performed in the presence of cyclooxygenase 1 and 2 inhibitors (indomethacin, 10 μmol/L), NOS inhibitor (N[gamma]-nitro-L-arginine methyl ester [L-NAME],

100 μmol/L), or the combination of indomethacin and L-NAME (to indirectly analyze the endothelium-derived hyperpolarizing factor).
- Protocol to study the receptors of progranulin: Arteries were preincubated (30 minutes before recombinant progranulin incubation) with EphrinA2 antagonist (ALW-II-4127, 5 μmol/L)[41] or Sortilin1 inhibitor (AF38469, 40 μmol/L).[42]
- Protocol to study if deficiency in the progranulin affects vascular contractility: Concentration-response curve to noradrenaline was performed in mesenteric arteries from progranulin$^{+/+}$ and progranulin$^{-/-}$ mice.

Fresh Isolation of Endothelial Cells From Mesenteric Bed

We isolated endothelial cells from the mesenteric bed as described by our previous publications.[43,44] Male and female mice were euthanized, and their mesenteric beds were excised and pooled, washed in PBS, and diced into small pieces, which were incubated in DMEM (Gibco, Thermo Fisher Scientific), containing 10% FBS, 2 mg/mL of collagenase II and 40 mg/mL dispase-II at 37°C for 1 hour while shaking. The cell suspension was vigorously vortexed and meshed through 40-μm nylon cell strainers (Fisherbrand, Thermo Fisher Scientific). After centrifugation, the cell pellet was resuspended in 1× PBS with 0.5% BSA and 2 mmol/L EDTA. Endothelial cells were labeled with CD31-conjugated magnetic microbeads and sorted using magnetic separation LS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). RNA was isolated as described below, and the purity of endothelial cells was checked by evaluating smooth muscle cell (α-smooth muscle actin, αSMA) and endothelial cell (CD31 and eNOS) markers. Expression of EphrinA2 and Sortilin1 was evaluated in samples from male and female mice to analyze whether there might be any sex difference in progranulin receptors.

Culture of Endothelial Cells

Mouse mesenteric endothelial cells (mMECs) or human mesenteric endothelial cells were purchased from Cell Biologics (Chicago, IL) and were used to understand EphrinA2 and Sortilin1 are expressed in human and murine endothelial cells. Cells were maintained in Complete Mouse Endothelial Cell Medium (Cell Biologics) containing Endothelial Cell Medium Supplement Kit (Cell Biologics). Cells were used between passages 4 and 8.

Protocol of Endothelial Cell Treatment

Cells were treated with recombinant progranulin (600 ng/mL) at different times (0–60 minutes). To understand by which receptors progranulin induces endothelial cell activation. Human mesenteric endothelial cells were treated with recombinant progranulin with or without ALW-II-4127 (5 μmol/L) or AF38469 (40 μmol/L). Then, eNOS phosphorylation and nitric oxide levels were measured as described below. EphrinA2 and Sortilin1 protein expression was also analyzed in mMECs and human mesenteric endothelial cells.

Reverse Transcription Quantitative Polymerase Chain Reaction

mRNA from mesenteric beds and freshly isolated flow through and endothelial cells from mesenteric beds were extracted using RNeasy Mini Kit (Quiagen, Germantown, MD). Complementary DNA was generated by reverse transcription polymerase chain reaction with SuperScript III (Thermo Fisher, Waltham, MA). Reverse transcription was performed at 58 °C for 50 minutes; the enzyme was heat inactivated at 85 °C for 5 minutes, and quantitative reverse transcription polymerase chain reaction was performed with the PowerTrack SYBR Green Master Mix (Thermo Fisher). Sequences of genes are listed in Table 1. Experiments were performed in a 384-well QuantStudio 5 Real-Time PCR System (Thermo Fisher). Data were quantified by 2-delta-delta Ct and are presented by fold changes indicative of either upregulation or downregulation.

Table 1. List of Primers

Primer	Sequence	
αSMA	FW	TGCTGACAGAGGCACCACTGAA
	RV	CAGTTGTACGTCCAGAGGCATAG
CD31	FW	CCAAAGCCAGTAGCATCATGGTC
	RV	GGATGGTGAAGTTGGCTACAGG
eNOS	FW	CGCAAGAGGAAGGAGTCTAGCA
	RV	TCGAGCAAAGGCACAGAAGTGG
EphrinA2	FW	CAAAGTGCACGAGTTCCA
	RV	CTCCTGCCAGTACCAGAAGC
Sortilin1	FW	CTACTCCATCCTGGCAGCCAAT
	RV	CTACTCCATCCTGGCAGCCAAT
α-adrenergic 1a	FW	GGCTGGAGCATGGGTATATG
	RV	CTGCCATTCTTCCTCGTGAT
α-adrenergic 1b	FW	GGCATTGTAGTCGGAATGTTCATC
	RV	CTGTTGAAGTAGCCCAGCCAGA
α-adrenergic 1d	FW	GCTGGTTCCCCTTTTTCTTC
	RV	ATTGAAGTAGCCCAGCCAGA
GAPDH	FW	GAGAGGCCCTATCCCAACTC
	RV	TCAAGAGAGTAGGGAGGGCT

Primers were purchased from Integrated DNA Technologies. αSMA indicates alpha smooth muscle actin; eNOS, endothelial nitric oxide synthase; FW, forward; and RV, reverse.

Table 2. List of Antibodies

Antibody	Catalog number	Company	Concentration
Sortilin1	20681	Cell Signaling	1:1000
EphrinA2	sc-398832	Santa Cruz	1:500
Ser1177 eNOS	612393	BD Biosciences	1:1000
eNOS	610296	BD Biosciences	1:1000
β-actin	A3854	Sigma	1:20000

eNOS indicates endothelial nitric oxide synthase.

Western Blot

mMECs and human mesenteric endothelial cell samples were directly homogenized using 2× Laemmli Sample Buffer and supplemented with 2-mercaptoethanol (BioRad, Hercules, CA). Proteins were separated by electrophoresis on a polyacrylamide gradient gel (BioRad), and transferred to Immobilon-P poly (vinylidene fluoride) membranes. Nonspecific binding sites were blocked with 5% skim milk or 1% BSA in tris-buffered saline solution with Tween for 1 hour at 24 °C. Membranes were then incubated with specific antibodies overnight at 4 °C as described in Table 2. After incubation with secondary antibodies, the enhanced chemiluminescence luminol reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher) was used for antibody detection.

Nitric Oxide Measurement

Nitric oxide production was measured by a 4,5-diaminofluorescein diacetate (DAF-2 DA) probe. Briefly, mMECs were treated with recombinant progranulin (600 ng/mL) for 60 minutes, with or without preincubation of ALW-II-4127 (5 μmol/L) or AF38469 (40 μmol/L), then cells were washed with PBS and stained with 4,5-diaminofluorescein diacetate (5 μmol/L) for 30 minutes before analysis. Fluorescence intensity was analyzed in a fluorimeter (SpectraMax i3x Multi-Mode Microplate Reader; emission, 538 nm/excitation, 485 nm). Bradykinin (10 μmol/L)[45] was used as a positive control.

Statistical Analysis

For comparisons of multiple groups or repeated measures (blood pressure record), 1-way or 2-way ANOVA followed by the Tukey or Bonferroni posttest were used. Differences between 2 groups from independent samples (progranulin$^{+/+}$ versus progranulin$^{-/-}$) were determined using Student's *t* test, whereas in samples with size smaller than 6 replicates, the nonparametric Mann–Whitney test was used. The vascular function data are expressed as a percentage of KCl (60 mmol/L)-induced maximal response. The concentration–response curves were fitted by nonlinear regression analysis. Maximal response was determined and used to determine if there was difference between the groups. Analyses were performed using Prism 9.0 (GraphPad Software, La Jolla, CA). A difference was considered statistically significant when $P \leq 0.05$, and the precision of *P* is described in the legends of the figures.

RESULTS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

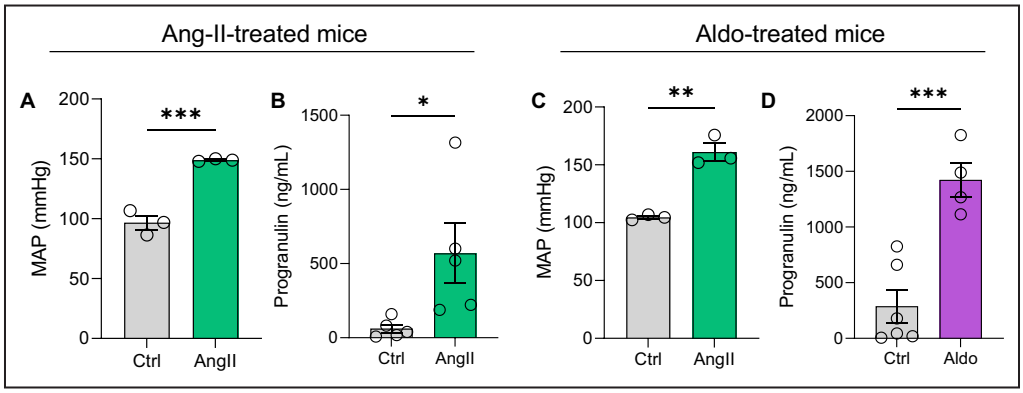

Figure 1. Hypertension is associated with high levels of circulating progranulin.
Mean arterial pressure (MAP) and progranulin plasma levels in angiotensin II (Ang-II)-treated mice (490 ng/kg for 14 days) (**A** and **B**) or aldosterone (Aldo)-treated mice (600 ng/kg for 14 days) (**C** and **D**). Data are presented as mean±standard error of the mean. N=3–6. *P<0.05 vs before treatment or Ctrl; **P<0.01 vs before treatment or Ctrl; ***P<0.001 vs Ctrl. The difference between before and after hypertensive treatments (angiotensin II or aldosterone) was determined by paired *t* test, whereas the difference of progranulin levels was calculated by the nonparametric Mann–Whitney test. Ctrl indicates control.

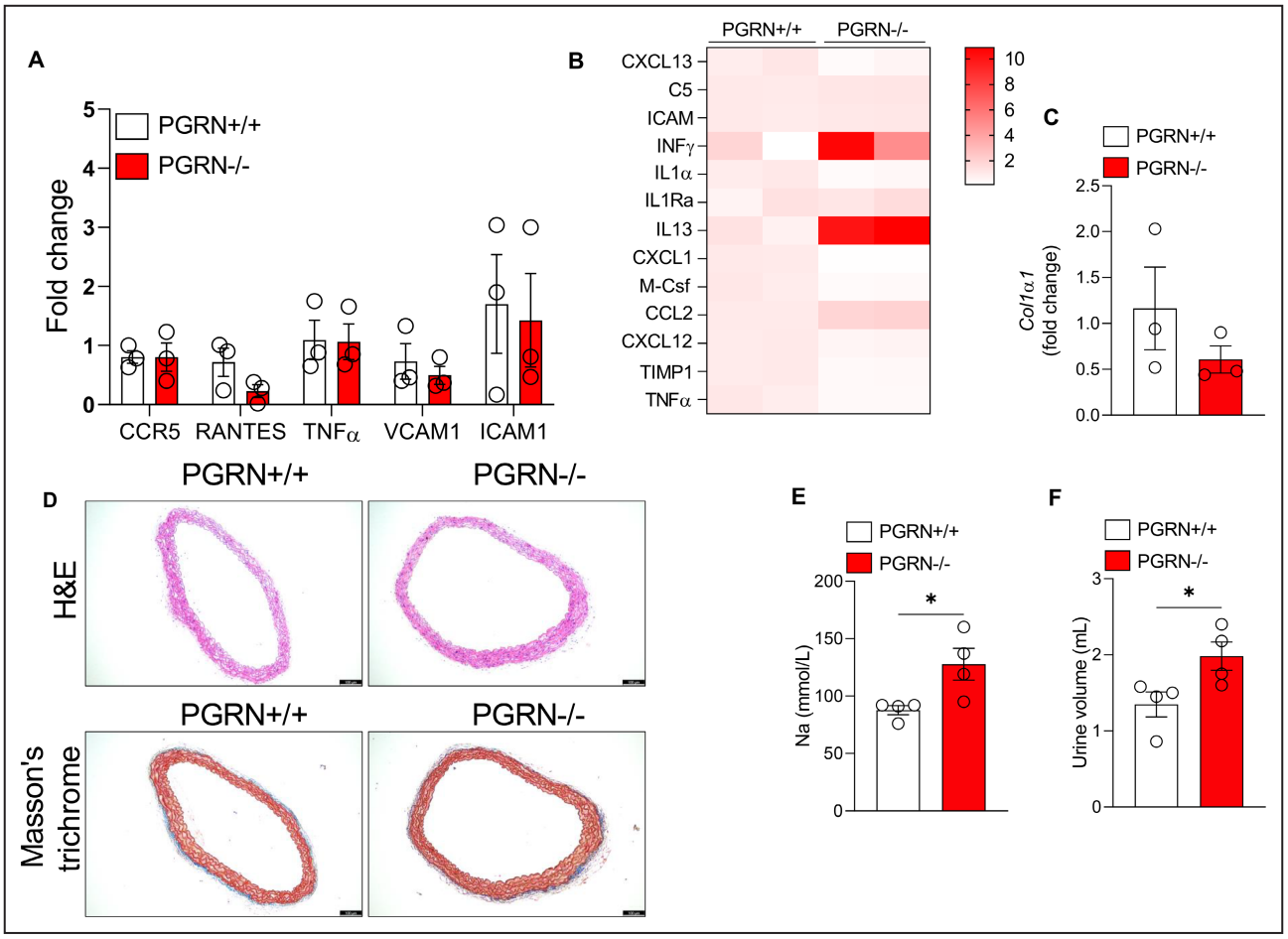

Figure 2. Deficiency in progranulin (PGRN) does not affect vascular inflammatory profile and aortic remodeling but increases circulating inflammatory markers and sodium and urine excretion.
Inflammatory profile in mesenteric beds, measured by reverse transcription quantitative polymerase chain reaction (**A**), and in plasma, measured by proteome profiler mouse cytokine array and presented as heat map (**B**), from wild-type ($PGRN^{+/+}$) and PGRN-deficient mice ($PGRN^{-/-}$). Collagen1α1 gene expression (**C**), hematoxylin and eosin, and Masson's trichrome stains (**D**) in thoracic aortae from $PGRN^{+/+}$ and $PGRN^{-/-}$ (representative from 3 different replicates per group). Twenty-four hours of urine sodium content (**E**) and volume (**F**) from $PGRN^{+/+}$ and $PGRN^{-/-}$. 11- to 13-week-old male mice were used. Scale bar=100μM. Data are presented as mean±standard error of the mean. N=3–4. *P<0.05 vs $PGRN^{+/+}$. The cytokine profile was analyzed in a pool of 6 samples from each group and is represented as fold changes and represented in a heatmap. *P<0.05 vs $PGRN^{+/+}$. The difference between $PGRN^{+/+}$ and $PGRN^{-/-}$ was determined by the nonparametric Mann–Whitney test. C5 indicates complement component C5a; CCL2, C-C motif chemokine ligand 2; CCR5, C-C motif chemokine receptor 5; CXCL1, C-X-C motif chemokine ligand 1; CXCL12, C-X-C motif chemokine ligand 12; CXCL13, C-X-C motif chemokine ligand 13; H&E, hematoxylin and eosin; ICAM, intercellular adhesion molecule 1; IL1α, interleukin 1α; IL13, interleukin 13; IL1Ra, interleukin 1 receptor antagonist; INFγ, interferon gamma; M-Csf, macrophage colony-stimulating factor; RANTES, regulated on activation, normal T cell expressed and secreted; TIMP1, tissue inhibitor of metalloproteinases 1; TNFα, tumor necrosis factor alpha; and VCAM1, vascular cell adhesion molecule 1.

Hypertension Is Associated With High Circulating Levels of Progranulin

Previous studies have shown that circulating progranulin is elevated in obesity,[9,10] diabetes,[11,46] and lipodystrophy,[12,13,47] major causes of cardiovascular diseases. Here, we observed that 2 different models of hypertension, angiotensin II–, or aldosterone-treated mice, displayed increases in circulating progranulin (Figure 1A through 1D). However, why progranulin is enhanced in hypertension is not fully known. In this study, we investigated the importance of progranulin in maintaining blood pressure and vascular tone by using a global progranulin-deficient mouse and progranulin treatment.

Progranulin Deficiency Does Not Affect Vascular Inflammation and Structure but Increases Circulating Inflammatory Markers and Sodium Excretion

Progranulin seems to have anti-inflammatory effects in different cells and organs and to modulate cell growth[19,21]; therefore, we investigated whether lack of progranulin might affect the inflammatory profile,

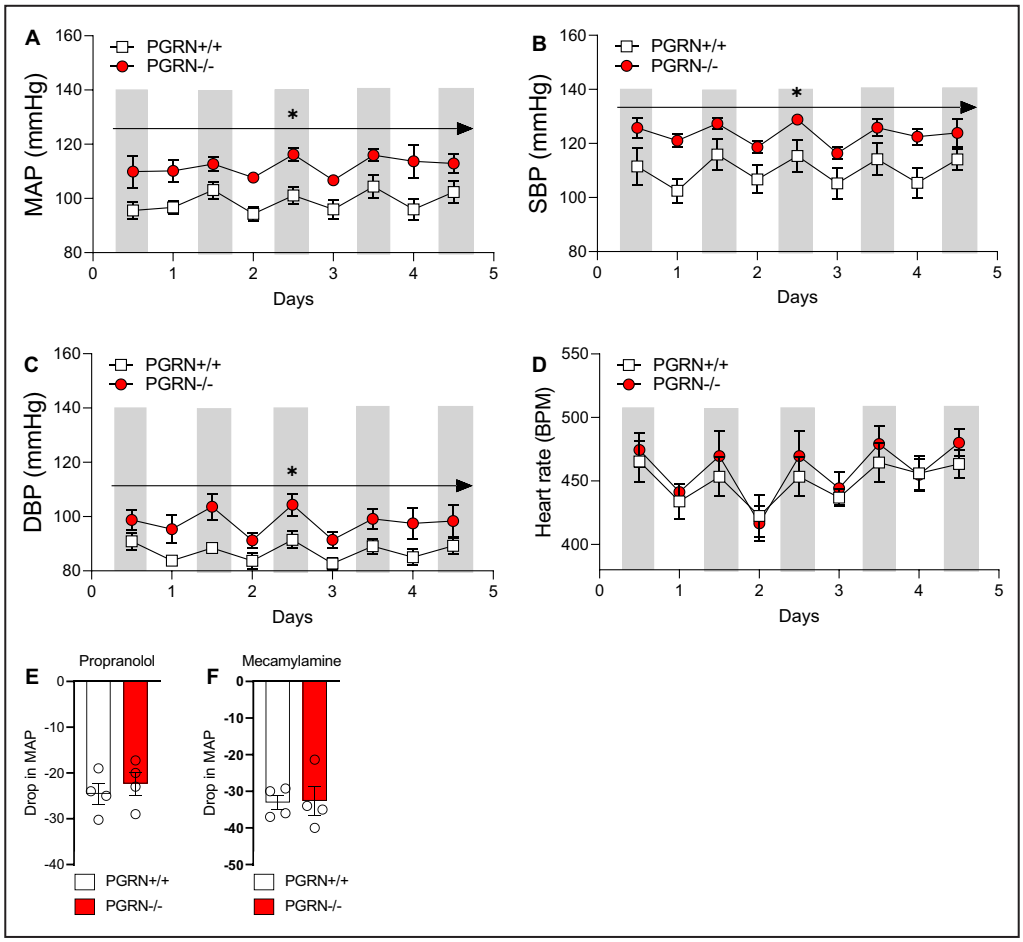

Figure 3. Deficiency in progranulin (PGRN) triggers high blood pressure.
Mean arterial pressure (MAP), systolic blood pressure, diastolic blood pressure, and heart rate measured via radiotelemetry in male (11- to 13-week-old) wild-type ($PGRN^{+/+}$) and PGRN-deficient mice ($PGRN^{-/-}$) (**A** through **D**). Effects of propranolol, 6 mg/kg (**E**) and mecamylamine, 5 mg/kg (**F**) on MAP. Gray bars represent nighttime in telemetry. Data are presented as mean±standard error of the mean. N=4. *P<0.05 vs $PGRN^{+/+}$. Blood pressure data were analyzed by repeated measures via 2-way ANOVA followed by the Bonferroni posttest, whereas the effects of propranolol and mecamylamine were calculated by the nonparametric Mann–Whitney test.

vascular fibrosis and hypertrophy, and sodium excretion. We observed that progranulin$^{-/-}$ mice do not present inflammation in mesenteric beds, but they display increases in circulating C-Cmotif ligand 2 (inflammatory chemokine), interleukin-13 (cytokine involved in allergic inflammation), and interferon-γ (inflammatory soluble cytokine), with no changes in aortic remodeling (fibrosis, hypertrophy, and collagen amount) (Figure 2A through 2D). We also found that progranulin$^{-/-}$ mice excrete more sodium and a larger amount of urine compared with progranulin$^{+/+}$ (Figure 2E and 2F), which are characteristics of hypertension.[48–50]

Progranulin Deficiency Elevates Blood Pressure and Increases Vascular Contractility

Via radiotelemetry, we found that lack of progranulin increases the mean arterial pressure, systolic blood pressure, and diastolic blood pressure with no effects on heart rate (Figure 3A through 3D). Changes in blood pressure do not seem to be dependent on sympathetic modulation because propranolol and mecamylamine affected similarly the blood pressure in progranulin$^{+/+}$ and progranulin$^{-/-}$ (Figure 3E and 3F). Analysis of vascular contractility revealed that progranulin$^{-/-}$ mice did not show changes in KCl-induced contractility (Figure 4A and 4D), but they presented higher vascular contractility compared with progranulin$^{+/+}$ independent of sex since mesenteric arteries from male and female mice responded equally (Figure 4B and 4D). Furthermore, an increase in contractility in males and females for noradrenaline (an adrenergic agonist) was not associated with changes in the expression of α-adrenergic receptors since no difference in α1a, α1b, and α1d was found (Figure 4C and 4F). However, changes in sensitivity of adrenergic receptors have been previously reported in hypertension[51,52]; therefore, we cannot eliminate the

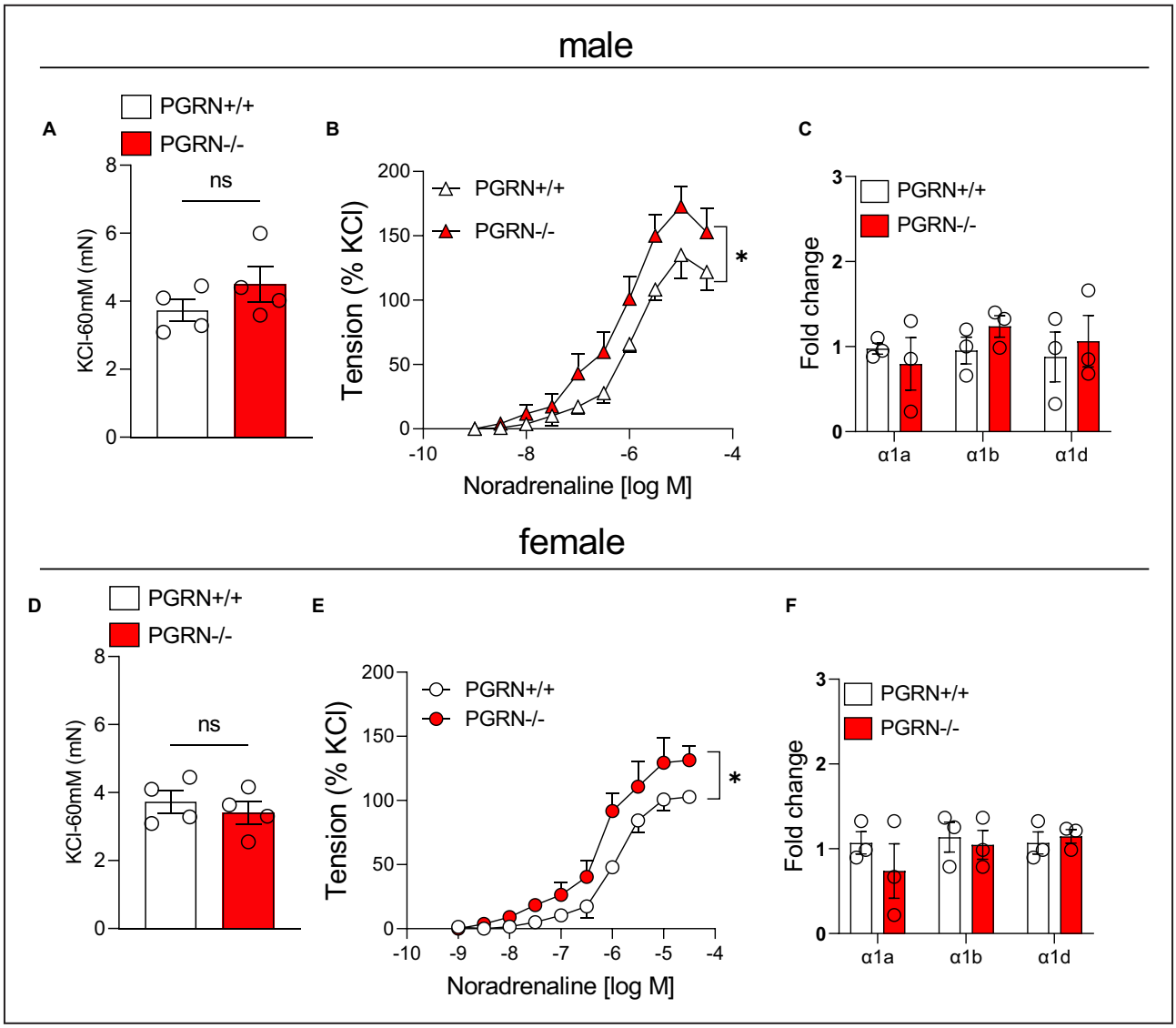

Figure 4. Deficiency in progranulin (PGRN) triggers vascular dysfunction.
KCl (60 mmol/L) response in mesenteric arteries (second order) from male (**A**) and female (**D**) wild-type ($PGRN^{+/+}$) and PGRN-deficient mice ($PGRN^{-/-}$). Concentration-response curves to noradrenaline in mesenteric arteries from male (**B**) and female (**E**) $PGRN^{+/+}$ and $PGRN^{-/-}$ mice. α1-adrenergic gene expression in mesenteric beds from male (**C**) and female (**F**) $PGRN^{+/+}$ and $PGRN^{-/-}$ mice. Data are presented as mean±standard error of the mean. N=3–4. *P<0.05 vs $PGRN^{+/+}$. For all analyses, the nonparametric Mann–Whitney test was used.

chances of loss or gain of receptor activity including the α- and β-adrenergic receptors families.

Progranulin Replacement Restores Blood Pressure and Vascular Contractility

To understand whether circulating progranulin maintains blood pressure and vascular function, we sought to restore progranulin levels by treating $progranulin^{-/-}$ mice with recombinant progranulin (20 μg/day for 7 days, via osmotic minipump).[14] Treatment with recombinant progranulin increased the progranulin levels in $progranulin^{-/-}$, but not restored as $progranulin^{+/+}$ mice, furthermore in $progranulin^{+/+}$ mice treatment with recombinant progranulin slightly elevated progranulin levels (Figure 5A). In addition, recombinant progranulin treatment decreased the vascular contractility in male and female $progranulin^{-/-}$ mice (Figure 5B and 4C), as well as recovered the mean arterial pressure, systolic blood pressure, and diastolic blood pressure, with no significant effects in $progranulin^{+/+}$. No effect was observed in heart rate (Figure 5D through 5G). These data suggest that circulating progranulin helps maintain physiological blood pressure levels and that arteries and blood pressure from $progranulin^{-/-}$ mice are more sensitive to recombinant progranulin treatment.

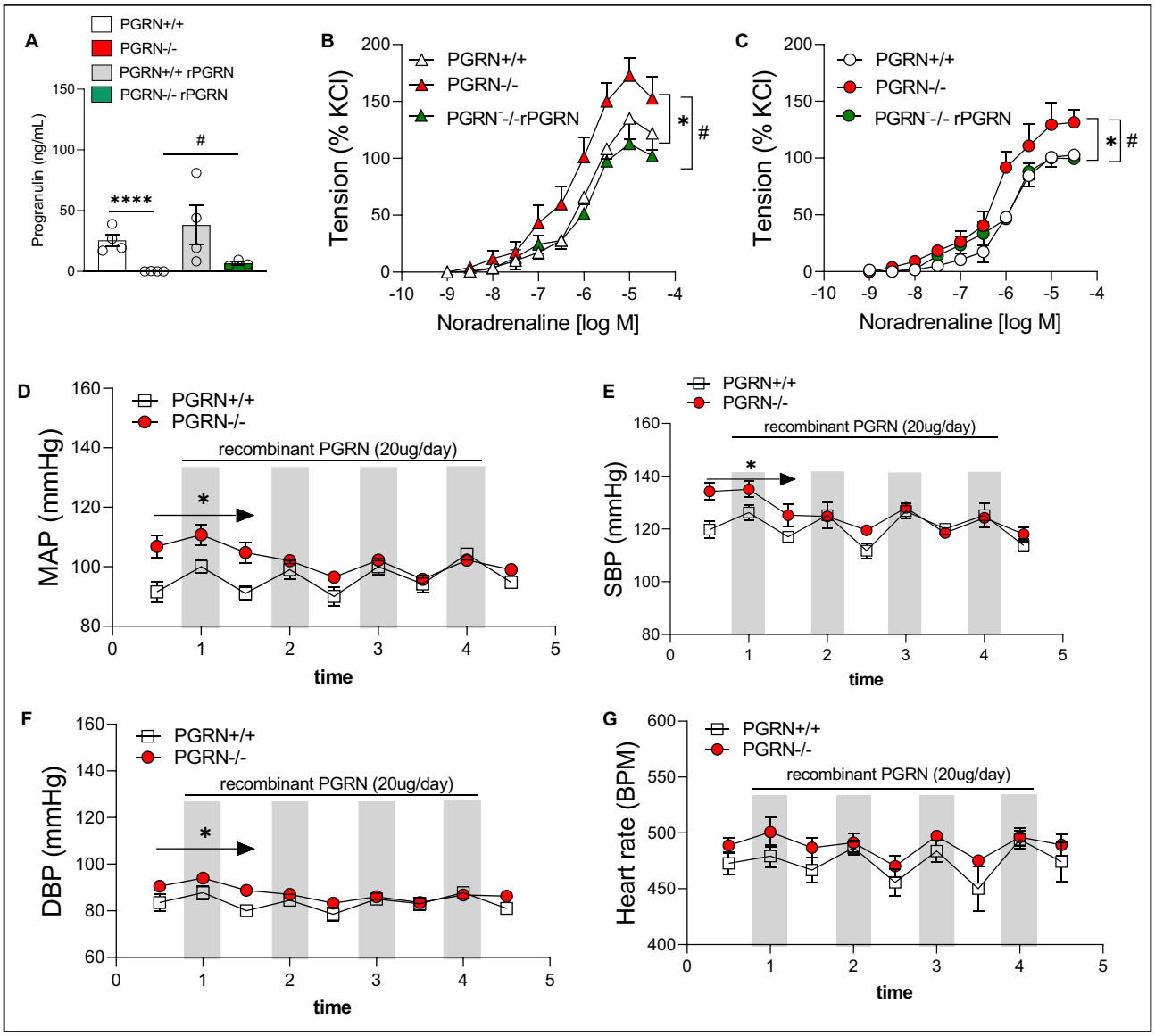

Figure 5. Progranulin (PGRN) treatment restores vascular function and blood pressure in PGRN-deficient mice.
Circulating PGRN levels (**A**) and concentration-response curves to noradrenaline in mesenteric arteries from male (**B**) and female (**C**) wild-type (PGRN+/+) and PGRN-deficient mice ($PGRN^{-/-}$) treated or not with recombinant progranulin (20 μg/day for 7 days). Mean arterial pressure (MAP) (**D**), systolic blood pressure (**E**), diastolic blood pressure (**F**), and heart rate (**G**) measured via radiotelemetry in male (11- to 13-week-old) $PGRN^{+/+}$ and $PGRN^{-/-}$ (**A** through **D**) treated with recombinant progranulin (20 μg/day for 7 days). Gray bars represent nighttime in telemetry. Data are presented as mean±standard error of the mean. N=3–4. *P<0.05 vs $PGRN^{+/+}$ and #P<0.05 vs $PGRN^{-/-}$. The difference in circulating PGRN levels was determined by 2-way ANOVA. For maximal response of vascular reactivity studies, 1-way ANOVA was used followed by Tukey's posttest, whereas blood pressure data were analyzed by repeated measures via 2-way ANOVA followed by the Bonferroni posttest.

Progranulin Decreases Vascular Contractility in Male and Female Mice Dependent on EphrinA2 and Sortilin1

To study the mechanisms whereby progranulin exerts its anticontractility effects, we treated mesenteric arteries from male and female control mice ($progranulin^{+/+}$) for 1 hour before noradrenaline concentration-response curves. We found that 600 ng/mL of progranulin, but not 100 or 300 ng/mL, decreased vascular contractility in male and female mice (Figure 6A and 6B).

Next, we evaluated by which receptor progranulin exerts its anticontractile effect via pharmacologically blocking 2 known receptors for progranulin: EphrinA2 and Sortilin1. Antagonism of EphrinA2, with ALW-II-4127 (5 μmol/L), or Sortilin1, with AF38469, (40 μmol/L) prevented the recombinant progranulin effects in mesenteric arteries from male and female mice (Figure 7A through 7D).

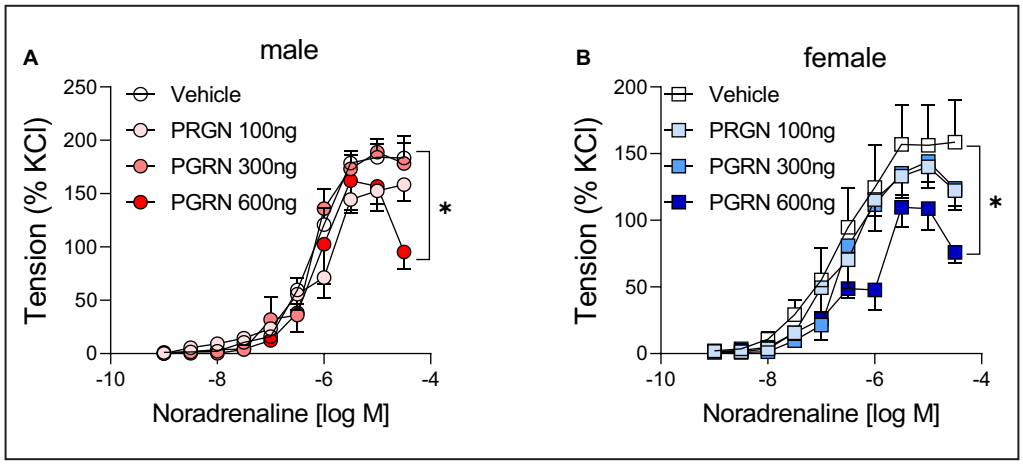

Figure 6. Progranulin (PGRN) incubation attenuates vascular contractility.
Effects of PGRN (100, 300, and 600 ng/mL for 1 hour) on concentration-response curves to noradrenaline in mesenteric arteries (second order) from male (**A**) and female (**B**) C57BL6/J (11- to 13-week-old) mice. Data are presented as mean±standard error of the mean. N=4–6. *P<0.05 vs vehicle. For maximal response of vascular reactivity studies, 1-way ANOVA was used followed by Tukey's posttest to determine the effects of incubation with recombinant progranulin.

Progranulin Reduces Vascular Contractility Via Nitric Oxide Production

To study the molecular mechanisms whereby progranulin reduces adrenergic contractility in male and female mice, we used different pharmacological tools to block endothelial-derived factor formation including nitric oxide, prostaglandin production, and endothelium-derived hyperpolarizing factor. We found that L-NAME (NOS inhibitor) blunted the difference in noradrenaline response caused by progranulin incubation in mesenteric arteries from male and female mice (Figure 8A and 8D), whereas indomethacin (cyclooxygenase inhibitor) only affected the response in arteries incubated with the vehicle but did not impact the progranulin effects in both sexes (Figure 8B and 8E). Furthermore, the L-NAME and indomethacin combination did not change any response in mesenteric arteries from male and female mice (Figure 8C and 8F). These findings suggest that progranulin exerts its anticontractile effects via modulating nitric oxide formation and attenuating contractile prostaglandins.

Finally, we established a protocol of fresh isolation of endothelial cells from mesenteric beds by using $CD31^+$ microbeads (Figure 9A) to measure the expression of EphrinA2 and Sortilin1 expression in endothelial cells from male and female mice and to analyze any sex difference in progranulin receptor expression. We first characterized the purity of endothelial cells by measuring the gene expression of αSMA (marker of smooth muscle cells) and CD31 and eNOS (markers of endothelial cells) in flow through and endothelial cells, we found a striking reduction in αSMA along with a clear increase in CD31 and eNOS in endothelial cells versus flow through (Figure 9B). Analysis of the expression of EphrinA2 and Sortilin1 in freshly isolated endothelial cells from male and female mice revealed no difference between the sexes (Figure 9C).

In this study, we particularly focused on understanding whether progranulin induces nitric oxide formation in mesenteric endothelial cells. Thus, we first analyzed if mesenteric endothelial cells from mice and humans express EphrinA2 and Sortilin1; as demonstrated in Figure 9D, both receptors are well expressed in mouse and human cells. Next, we analyzed whether recombinant progranulin induces eNOS activation and nitric oxide formation in mMECs. Progranulin triggered eNOS activation (phosphorylation of Ser^{1177} residue) and elevated nitric oxide production, which were prevented by blocking EphrinA2 and Sortilin1 (Figure 9E and 9F). Bradykinin was used as a positive control of nitric oxide production. Therefore, we can hypothesize that progranulin modifies the vascular tone and blood pressure by controlling the synthesis of nitric oxide and prostanoids, which seem to be dependent on EphrinA2 and Sortilin1.

DISCUSSION

Although progranulin is extensively researched in neurodegenerative disorders,[19,20,53] little is known about its significance in vascular biology and blood pressure regulation. Our study is the first to extensively examine how circulating progranulin affects blood pressure regulation and vascular tone. As a result, we were able to show that, under physiological conditions, circulating progranulin aids in regulating vascular resistance and

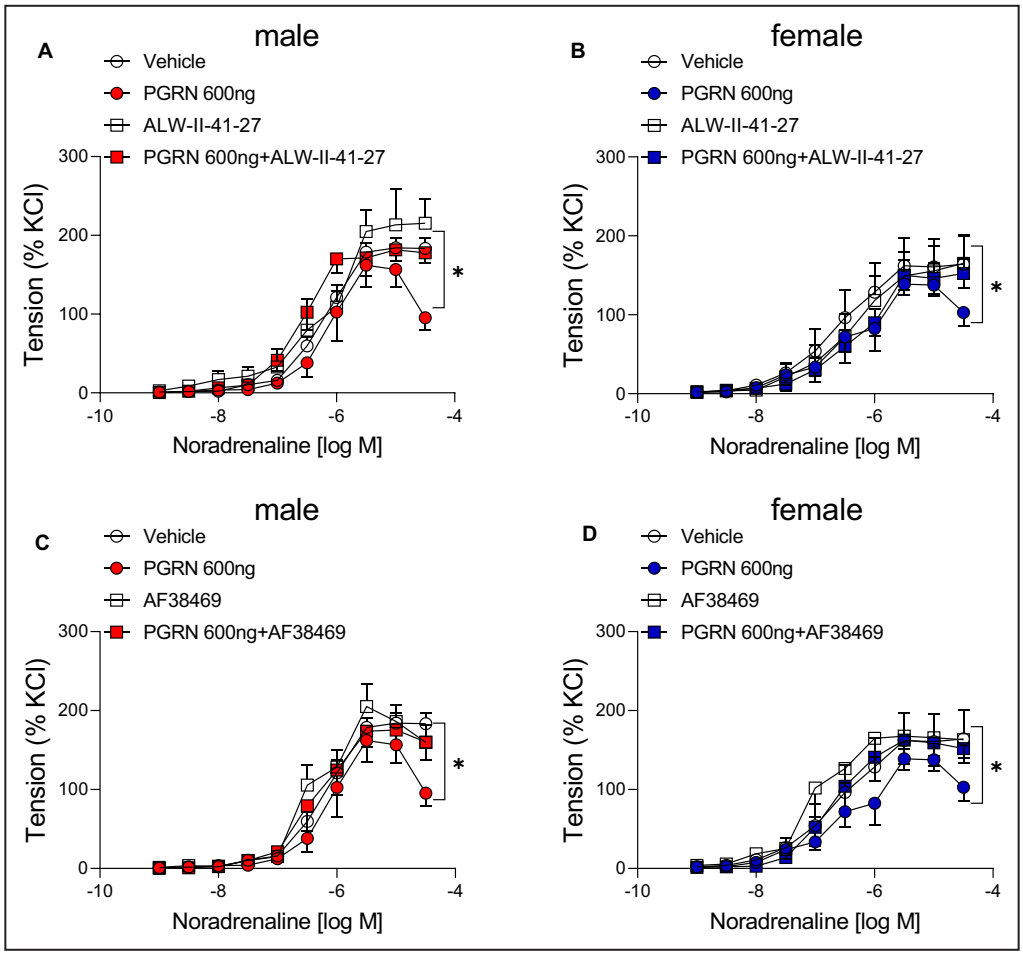

Figure 7. Progranulin (PGRN) incubation attenuates vascular contractility dependent on EphrinA2 and Sortilin1.
Concentration-response curves to noradrenaline in mesenteric arteries (second order) from male (**A** and **C**) and female (**B** and **D**) C57BL6/J (11- to 13-week-old) mice in presence of PGRN (600 ng/mL for 1 hour) with or without EphrinA2 antagonist (ALW-II-4127, 5 μmol/L) (**A** and **B**) or Sortilin1 inhibitor (AF38469, 40 μmol/L) (**C** and **D**). Data are presented as mean±standard error of the mean. N=4–6. *P<0.05 vs arteries treated with recombinant progranulin. For maximal response of vascular reactivity studies, 2-way ANOVA was used followed by Tukey's posttest to determine the effects of antagonists and recombinant progranulin.

possibly blood pressure maintenance. Moreover, high levels of progranulin associated with HBP may act as a defensive mechanism to help lower blood pressure. Finally, it appears that these protective processes depend on EphrinA2, Sortilin1, and nitric oxide. According to these findings, individuals with low progranulin levels may be more susceptible to cardiovascular events, whereas chronic progranulin replacement treatment is not only safe but also advantageous for the cardiovascular system in individuals with a deficiency in progranulin. Moreover, the therapy of HBP with progranulin may be an interesting therapeutic route because it likely modulates nitric oxide formation.

Although PGRN effects have been studied in other diseases, little is known about how it may affect cardiovascular physiology or pathophysiology. For instance, progranulin-deficient mice exhibit worse cardiorenal phenotype in a hyperhomocysteinemia diet,[54] greater renal injury in the diabetes model,[16] and accelerated heart hypertrophy on an age-dependent basis.[25] More recently, Gerrits et al[53] revealed that the neurovascular unit is severely affected in progranulin-associated frontotemporal dementia. Although these studies implicate that lack of progranulin induces cardiovascular and renal dysfunction or predisposes a worse phenotype in different conditions, the role of progranulin deficiency in controlling blood pressure is not well explored. Previous findings reported that progranulin is elevated in plasma from patients with hypertension,[55,56] but why and which cells are producing this progranulin are fully unclear. In our 2 models of hypertension, circulating progranulin was strikingly high. However, we found that aldosterone plus saline treatment produced a further increase in progranulin compared with

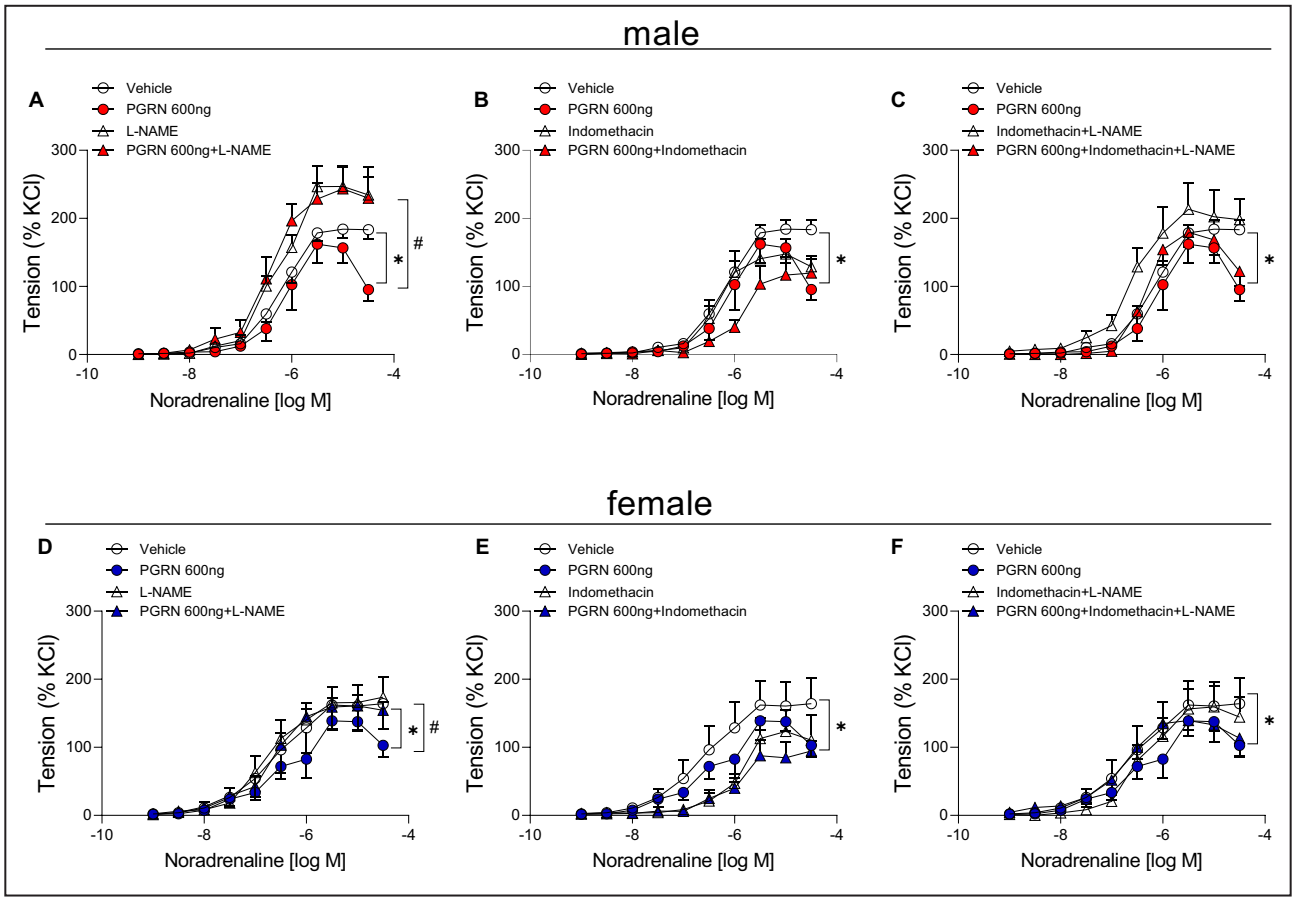

Figure 8. Progranulin (PGRN) incubation attenuates vascular contractility dependent on nitric oxide.
Concentration-response curves to noradrenaline in mesenteric arteries (second order) from male (**A** through **C**) and female (**D** through **F**) C57BL6/J (11- to 13-week-old) mice in presence of PGRN (600 ng/mL for 1 hour) with or without nitric oxide synthase inhibitor (N[gamma]-nitro-L-arginine methyl ester [L-NAME], 100 μmol/L) (**A** and **D**), cyclooxygenase inhibitor (indomethacin, 10 μmol/L) (**B** and **D**), or L-NAME+ indomethacin (**C** and **F**). Data are presented as mean±standard error of the mean. N=4–6. **P*<0.05 vs arteries treated with recombinant progranulin; #*P*<0.05 vs L-NAME. Two-way ANOVA was used followed by Tukey's posttest to determine the effects of inhibitors and recombinant progranulin.

angiotensin II treatment indicating that there might be a renal contribution to such discrepancy, which could be mediated by volume-dependent hypertension mechanisms, since aldosterone and salt is a model of increased blood volume. Further studies are necessary to evaluate this inconsistency between the hypertension models, as well as to understand which cells are mostly producing progranulin in hypertension.

We also described here that progranulin maintains the blood pressure and vascular tone by observing that global progranulin-deficient mice display elevated blood pressure and increased vascular contractility followed by a large amount of sodium excretion and circulating inflammatory profile. Our data differ from previous findings, where the authors found, via echocardiography,[25,54] that deficiency in progranulin does not affect blood pressure. It is possible that our blood pressure recorded via telemetry was more sensitive to detect any small changes in blood pressure. Interestingly, we could restore the blood pressure in progranulin$^{-/-}$ mice by returning circulating progranulin for 7 consecutive days. Surprisingly, recombinant progranulin treatment (20 μg/day) did not achieve progranulin levels as the control group but only increased it. We postulate that progranulin might be degraded, cleaved by proteases, or internalized by different cells,[19–21,57] thus reducing the circulating availability. Further investigation is needed to dissect this event.

A study published by Kazama et al in 2017[58] revealed that isolated superior rat mesenteric artery rings incubated with progranulin (10–100 ng/mL) show an increased sensitivity to acetylcholine, suggesting that progranulin is physiologically relevant to keep the vascular tone. Aligned with these findings, we observed that deficiency in progranulin triggers vascular dysfunction, characterized by the elevated response to noradrenaline, which is blunted by supplementing progranulin$^{-/-}$ mice with recombinant progranulin.

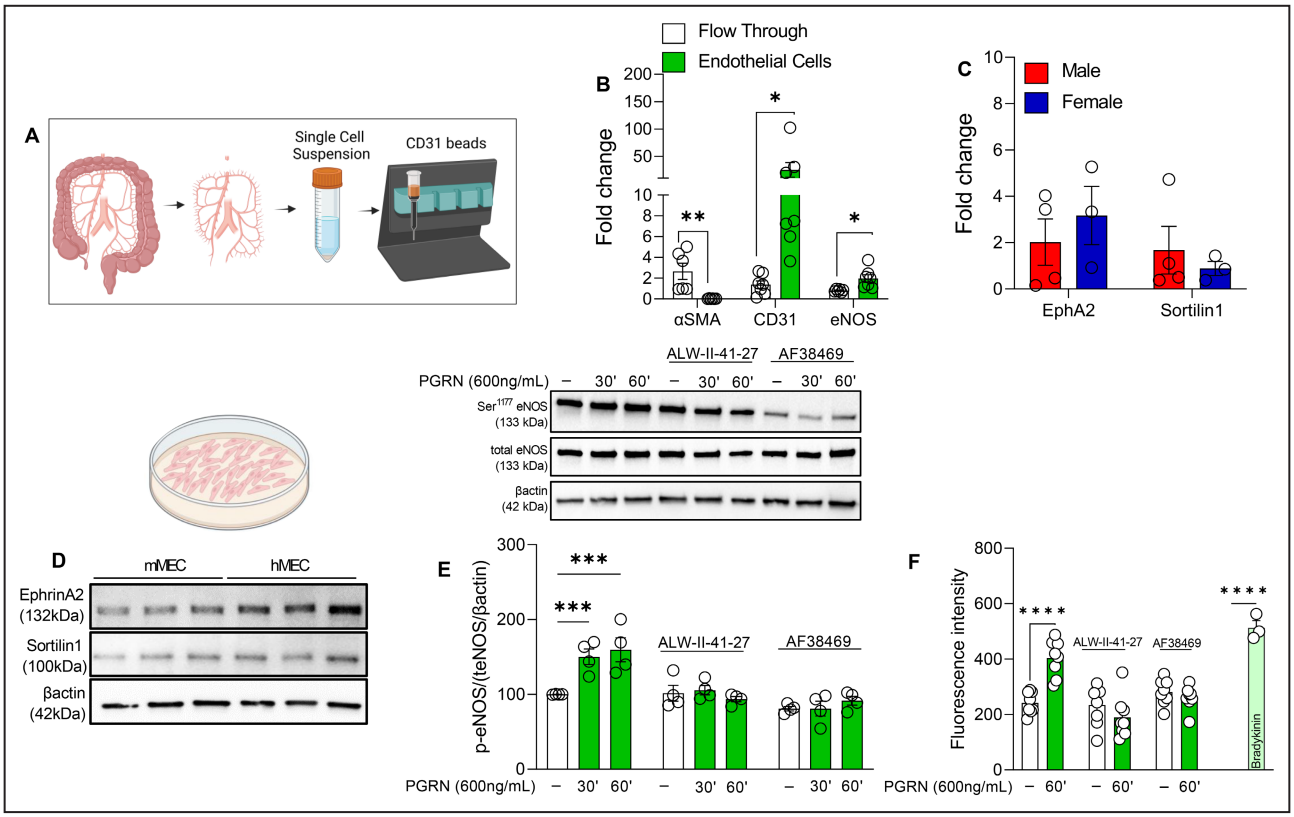

Figure 9. Progranulin (PGRN) activates endothelial nitric oxide synthase (eNOS) in mesenteric endothelial cells via EphrinA2 and Sortilin1.

A, Schematic of endothelial cell isolation from mesenteric bed. **B**, Smooth muscle cell (α smooth muscle actin [α SMA]) and endothelial cell markers (CD31 and eNOS) in freshly isolated endothelial cells and flow through from mesenteric bed measured by reverse transcription quantitative polymerase chain reaction. **C**, EphrinA2 and Sortilin1 gene expression in freshly isolated endothelial cells from male and female mice. **D**, Expression of EphrinA2 and Sortilin1 in mouse mesenteric endothelial cells (mMECs) and human mesenteric endothelial cells (hMECs). **E**, Effects of recombinant progranulin (600 ng/mL for 30 and 60 minutes) on eNOS phosphorylation (Ser^{1177}) in mMECs. **F**, Effects of recombinant progranulin on nitric oxide formation measured by 4,5-diaminofluorescein diacetate (DAF-2 DA). Bradykinin (10 μmol/L) was used as a positive control. Experiments were performed in the presence or absence of EphrinA2 antagonist (ALW-II-4127, 5 μmol/L) or Sortilin1 inhibitor (AF38469, 40 μmol/L). Data are presented as mean±standard error of the mean. N=3–8. **P*<0.05 vs flow through; ***P*<0.01 vs flow through; ****P*<0.001 vs vehicle in experiments of mMECs treated with recombinant progranulin (–); *****P*<0.0001 vs vehicle in experiments of mMECs treated with recombinant progranulin (–). The difference between flow through and endothelial cells and nitric oxide production was determined by *t* test, whereas eNOS levels were determined by the nonparametric Mann–Whitney test.

Furthermore, we found that 600 ng/mL (6 times more progranulin than shown previously[58]), attenuates noradrenaline response in mesenteric arteries from male and female mice. Therefore, we can suggest that circulating progranulin maintains vascular tone and blood pressure, and, in hypertension models, circulating progranulin is likely elevated as a compensatory mechanism to promote a decrease in vascular resistance and subsequently to reduce the blood pressure.

Several progranulin receptors have been identified, including EphrhinA2[59] and Sortilin1.[20] EphrhinA2 belongs to a family of receptor tyrosine kinases, which is crucial for migration and vascular and epithelial development.[59,60] Studies have shown that progranulin can bind to EphrinA2 and induce protein kinase B activation and angiogenesis.[59] While Sortilin1 is a sorting receptor that can reduce progranulin bioavailability by capturing and transporting it to the lysosome for destruction,[61] not all progranulin proceeds along the lysosome pathway; instead, some may move to an unknown additional cellular compartment.[61] Here, we found that blocking EphrinA2 or Sortilin1 blunts the anticontractile action brought on by progranulin. These findings indicate that a possible crosstalk between EphrinA2 and Sortilin1 may exist and that further research is necessary to unravel such communication. Perhaps Sortilin1 might regulate some intracellular pathways, which can interfere with EphrinA2 receptor activation, for example, protein kinase B pathway[59] or even a direct modulation of intracellular protein–tyrosine kinase domain

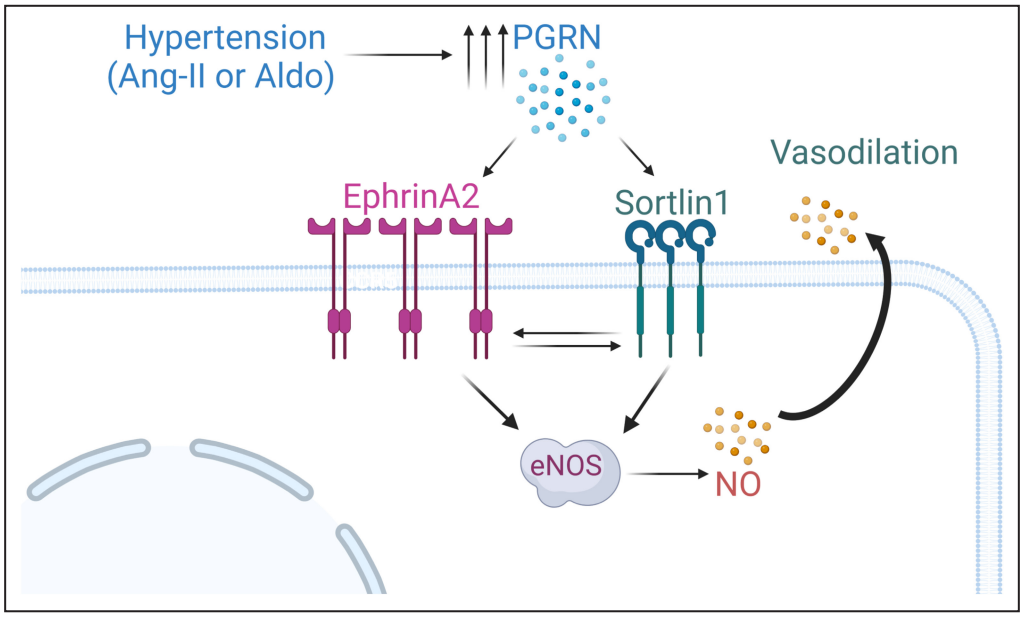

Figure 10. Summary of our findings.
Progranulin (PGRN) activates endothelial nitric oxide synthase (eNOS) in endothelial cells via EphrinA2 and Sortilin1 regulating vascular tone and blood pressure. Aldo indicates aldosterone; Ang-II, angiotensin II; and NO, nitric oxide.

by Sortilin1 pathway. Since we found that progranulin affects vascular contractility in male and female mice, we investigated whether EphrinA2 or Sortilin1 expression is similar in male and female mesenteric endothelial cells; we discovered that both male and female mesenteric endothelial cells express these 2 progranulin receptors equally.

Endothelial cells are responsive to progranulin effects.[34,59] Progranulin can directly protect the vascular endothelium against atherosclerotic environment by eNOS and nuclear factor-κB,[34] induce capillary morphogenesis and GRN autoregulation, and influence the growth and development of blood vessels.[57,59,62] We found that the anticontractile effect of progranulin is mediated by nitric oxide formation since blocking NOS blunted the difference between arteries incubated or not with recombinant progranulin. Moreover, such an effect seems to be sex independent because arteries from male and female mice responded similarly. Also, we discovered that progranulin may alter vasoconstrictor prostanoids. This conclusion may be drawn from the observation that only naive arteries, not progranulin-treated arteries, responded to cyclooxygenase inhibitors. To better understand the methods through which progranulin modifies the cyclooxygenase pathway, more research is required. Since double inhibition (NOS and cyclooxygenase) did not affect any progranulin response, our data further imply that endothelium-derived hyperpolarizing factor does not appear to be involved in the effects of progranulin.

As mentioned previously, progranulin can modulate nitric oxide production in the endothelium.[34] To confirm whether progranulin induces nitric formation via EphrinA2 or Sortilin1 we took advantage of isolated mesenteric endothelial cells. First, we demonstrated that mesenteric endothelial cells from mice and humans express both receptors, and then we observed that progranulin leads to eNOS activation and nitric oxide formation via EphrinA2 or Sortilin1 activation. Thus, we can suggest that progranulin modulates the vascular tone via EphrinA2 or Sortilin1 and eNOS activation.

In conclusion, this work offers the first proof that progranulin aids in controlling blood pressure and vascular tone through the production of nitric oxide and EphrinA2 or Sortilin1. Moreover, we have shown that progranulin exerts an anticontractile effect in both male and female mice via similar mechanisms (Figure 10). Together, these findings suggest that progranulin deficiency may contribute to vascular dysfunction and hypertension or predispose patients to a more severe cardiovascular outcome. Therefore, progranulin may provide a novel treatment strategy for lowering blood pressure and restoring vascular tone. However, further research is required to comprehend (1) the relationship between EphrinA2 and Sortilin1, (2) the effects of progranulin supplementation in established high blood pressure, and (3) the toxicological effects of progranulin especially on hepatic integrity.

ARTICLE INFORMATION

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Disclosures

None.

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