

Drebrin regulates angiotensin II-induced aortic remodelling

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Aims The actin-binding protein Drebrin is up-regulated in response to arterial injury and reduces smooth muscle cell (SMC) migration and proliferation through its interaction with the actin cytoskeleton. We, therefore, tested the hypothesis that SMC Drebrin inhibits angiotensin II-induced remodelling of the proximal aorta.

Methods and results Angiotensin II was administered via osmotic minipumps at 1000 ng/kg/min continuously for 28 days in SM22-Cre⁺/*Dbn*^{fllox/fllox} (SMC-*Dbn*^{-/-}) and control mice. Blood pressure responses to angiotensin II were assessed by telemetry. After angiotensin II infusion, we assessed remodelling in the proximal ascending aorta by echocardiography and planimetry of histological cross sections. Although the degree of hypertension was equivalent in SMC-*Dbn*^{-/-} and control mice, SMC-*Dbn*^{-/-} mice nonetheless exhibited 60% more proximal aortic medial thickening and two-fold more outward aortic remodelling than control mice in response to angiotensin II. Proximal aortas demonstrated greater cellular proliferation and matrix deposition in SMC-*Dbn*^{-/-} mice than in control mice, as evidenced by a higher prevalence of proliferating cell nuclear antigen-positive nuclei and higher levels of collagen I. Compared with control mouse aortas, SMC-*Dbn*^{-/-} aortas demonstrated greater angiotensin II-induced NADPH oxidase activation and inflammation, evidenced by higher levels of Ser-536-phosphorylated NFκB p65 subunits and higher levels of vascular cell adhesion molecule-1, matrix metalloproteinase-9, and adventitial macrophages.

Conclusions We conclude that SMC Drebrin deficiency augments angiotensin II-induced inflammation and adverse aortic remodelling.

Keywords Drebrin • Smooth muscle • Aorta • Angiotensin II

1. Introduction

Drebrin, ‘developmentally regulated brain protein’, is an actin-binding protein which has been shown to stabilize actin filaments and link the actin cytoskeleton to the microtubular network.^{1,2} In mammals, a single Drebrin gene (*Dbn1*) transcript undergoes alternative splicing to produce two isoforms: adult (Drebrin A) and embryonic (Drebrin E).³ Drebrin A is expressed in neurons, where it stabilizes actin filaments, thereby contributing to memory.^{3,4} Outside of the nervous system, Drebrin E expression has been reported in a variety of cell types.^{5,6} We previously found that Drebrin is abundantly expressed in smooth muscle cells (SMCs) and is up-regulated in response to arterial injury.⁷ Using a

model of global Drebrin haploinsufficiency, we previously found that Drebrin inhibits neointimal hyperplasia and outward remodelling (luminal diameter expansion) in response to wire-mediated carotid endothelial denudation. Consistent with a role in vascular inflammation, SMC Drebrin is up-regulated in atherosclerotic human arteries.⁷ On a cellular level, Drebrin reduces SMC migration and proliferation through stabilization of actin filaments.⁷

Hypertension is a highly prevalent chronic condition that results in vascular injury and remodelling, and is a major risk factor for the development of multiple cardiovascular diseases. Hypertensive arterial remodelling encompasses thickening of the tunica media; this process involves not only SMCs but also endothelial cells, inflammatory cells, and

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fibroblasts.^{8–10} An important contributor to hypertensive arterial remodelling is angiotensin II,^{8,11} which promotes hypertension and adverse arterial remodelling in a manner that varies by region of the arterial tree.^{11,12} Whereas angiotensin II-induced medial thickening involves SMC hypertrophy in the descending thoracic and abdominal aorta, it involves SMC hyperplasia in the ascending aorta.¹² Furthermore, angiotensin II promotes medial expansion independently of blood pressure (BP).¹² Angiotensin II signalling also contributes to the formation of thoracic and abdominal aortic aneurysms characterized by irreversible increases in luminal diameter, medial degeneration, extracellular matrix disruption, and inflammation.¹³ Because our previous studies demonstrated that Drebrin reduces SMC activation,⁷ we asked whether Drebrin expression in SMCs reduces remodelling in the ascending aorta in a model of angiotensin II-induced hypertension.

2. Methods

Detailed methods are available in the [Supplementary material](#).

3. Results

3.1 Angiotensin II-induced hypertension in SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mice

We previously generated mice heterozygous for the presence of a trapping cassette upstream of floxed Drebrin exons 3–8.⁷ Germline removal of the trapping cassette was achieved by mating *Dbn*^{+/-} mice heterozygous for the trapping cassette to mice expressing Flp recombinase in the C57BL/6 background (*Figure 1A*). *Dbn*^{flox/+} mice were mated with congenic C57BL/6 mice that express Cre recombinase under the control of the endogenous SM22 promoter¹⁴ to generate SM22-Cre^{+/+}/*Dbn*^{flox/+} mice which were then crossed to *Dbn*^{flox/flox} mice to generate SM22-Cre^{+/+}/*Dbn*^{flox/flox} mice (SMC-*Dbn*^{-/-}), which had SMC-specific loss of Drebrin. Immunoblotting confirmed that SMCs of the aorta's tunica media lacked Drebrin protein in SMC-*Dbn*^{-/-} mice but expressed wild-type (WT) levels of Drebrin in SM22-Cre^{+/+}/*Dbn*^{+/+} (SMC-Cre) and *Dbn*^{flox/flox} mice (*Figure 1B*). Further immunoblotting showed that Drebrin expression was unchanged in brain tissue of SMC-*Dbn*^{-/-} mice ([Supplementary material online, Figure S1A](#)). Because SM22 expression has been reported in multiple myogenic lineages during embryogenesis,¹⁵ we immunoblotted adult heart and skeletal muscle extracts for Drebrin but found none in either *Dbn*^{flox/flox} or SMC-*Dbn*^{-/-} mice ([Supplementary material online, Figure S1A](#)). Consistent with the known expression of SM22 in activated fibroblasts,¹⁶ we also observed that Drebrin expression was deleted in activated adventitial fibroblasts from SMC-*Dbn*^{-/-} mice *in vitro* ([Supplementary material online, Figure S1B](#)).

To determine whether SMC Drebrin reduces angiotensin II-induced aortic remodelling, we used osmotic minipumps to infuse angiotensin II (1000 ng/kg/min) subcutaneously for 28 days.¹⁷ In a pilot experiment, we found no difference in systolic BP between SMC-Cre and *Dbn*^{flox/flox} mice assessed by tail cuff plethysmography¹⁸ during a 28-day course of angiotensin II administration ([Supplementary material online, Figure S2](#)). Consequently, for subsequent studies involving radiotelemetry BP measurements, our control mice were *Dbn*^{flox/flox}, which express WT levels of SMC Drebrin (*Figure 1B*). To measure BP continuously by radiotelemetry in conscious, unrestrained SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mice, a pressure-sensing catheter was inserted via the left common carotid artery of anaesthetized mice as previously reported.¹⁷ Ten days after catheter insertion, an osmotic minipump was implanted to infuse

angiotensin II (1000 ng/kg/min) continuously for 28 days.¹⁷ SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mice were equivalent with regard to systolic BP, diastolic BP, and heart rate over the course of angiotensin II infusion ($n = 7$ per group, *Figure 1C* and *D*). Expression of the AT_{1A} angiotensin II receptor (AT_{1A}-R), which is responsible for angiotensin II-induced hypertension,¹⁰ was equivalent in SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} aortas by qRT-PCR ([Supplementary material online, Figure S3](#)).

Because Drebrin stabilizes F-actin in SMCs,⁷ and because stabilization of F-actin can affect SMC contractility,¹⁹ we sought to determine whether Drebrin affects angiotensin II-induced SMC contractility. To address this issue, we used infrarenal aortic rings from SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mice. Tension generated by these rings was assessed using a calibrated force transducer, as previously described.²⁰ Tension generated by SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mouse aortic rings was equivalent not only at baseline and in response to KCl but also in response to angiotensin II ([Supplementary material online, Figure S4](#)). Although angiotensin II elicits contraction of infrarenal aortic rings primarily via the AT_{1B} receptor,²⁰ signalling downstream of this receptor largely mirrors that of the AT_{1A}-R, which mediates angiotensin II-induced hypertension.¹⁷ Thus, just as it does not affect angiotensin II-induced hypertension, Drebrin does not affect angiotensin II-induced aortic contractility. Furthermore, unlike the Drebrin-binding protein Homer,⁷ the AT_{1A}-R does not co-immunoprecipitate with Drebrin when co-expressed in HEK-293 cells ([Supplementary material online, Figure S5](#)).

3.2 SMC Drebrin regulates angiotensin II-induced remodelling in the ascending aorta

After 28 days of saline or angiotensin II infusion, mice were sacrificed and their aortas were harvested. There were no aneurysms (defined as a focal dilatation of more than 1.5 times of the normal outer aortic diameter)²¹ in the thoracic or abdominal aortas of SMC-*Dbn*^{-/-}, *Dbn*^{flox/flox} or SMC-Cre⁺ mice (data not shown). We assessed vascular remodelling in the proximal ascending aorta, because in response to angiotensin II this aortic segment develops medial thickening resulting from SMC hyperplasia,¹² which is reduced by Drebrin activity.⁷ Aortic dimensions were equivalent in saline-infused SMC-*Dbn*^{-/-}, *Dbn*^{flox/flox}, and SMC-Cre⁺ mice (*Figure 2A–D*). Thus, like global Drebrin deficiency,⁷ SMC-specific Drebrin deficiency did not affect aortic anatomy in unstressed mice. Assessed histologically, angiotensin II induced insignificant medial thickening and no luminal expansion in the proximal aortas of *Dbn*^{flox/flox} and SMC-Cre⁺ mice. However, angiotensin II induced significant medial thickening and luminal expansion in SMC-*Dbn*^{-/-} mice: 61 ± 2% and 102 ± 9% greater than control mouse aortas with regard to medial thickness and lumen area, respectively (*Figure 2A–C*). Consistent with their greater outward remodelling, proximal aortas from angiotensin II-infused SMC-*Dbn*^{-/-} mice demonstrated 2.9 ± 0.2-fold more elastic lamina breaks than aortas from angiotensin II-infused control mice (*Figure 2E*).

Ex vivo measurements of aortic lumen area accorded with ascending aortic diameter measurements made *in vivo* with echocardiography (*Figure 3A*); however, the lumen area increment for SMC-*Dbn*^{-/-} aortas was approximately 20% less when calculated from echocardiography-derived diameters than from direct histological measurement. Angiotensin II-induced left ventricular hypertrophy (*Figure 3B*) and myocardial contractility assessed as fractional shortening (*Figure 3C*) were equivalent in SMC-*Dbn*^{-/-} and control mice—concordant with the equivalence of angiotensin II-induced hypertension in SMC-*Dbn*^{-/-} and control mice.

Whereas angiotensin II-induced medial thickening involves SMC hyperplasia in the ascending aorta, angiotensin II-induced medial thickening

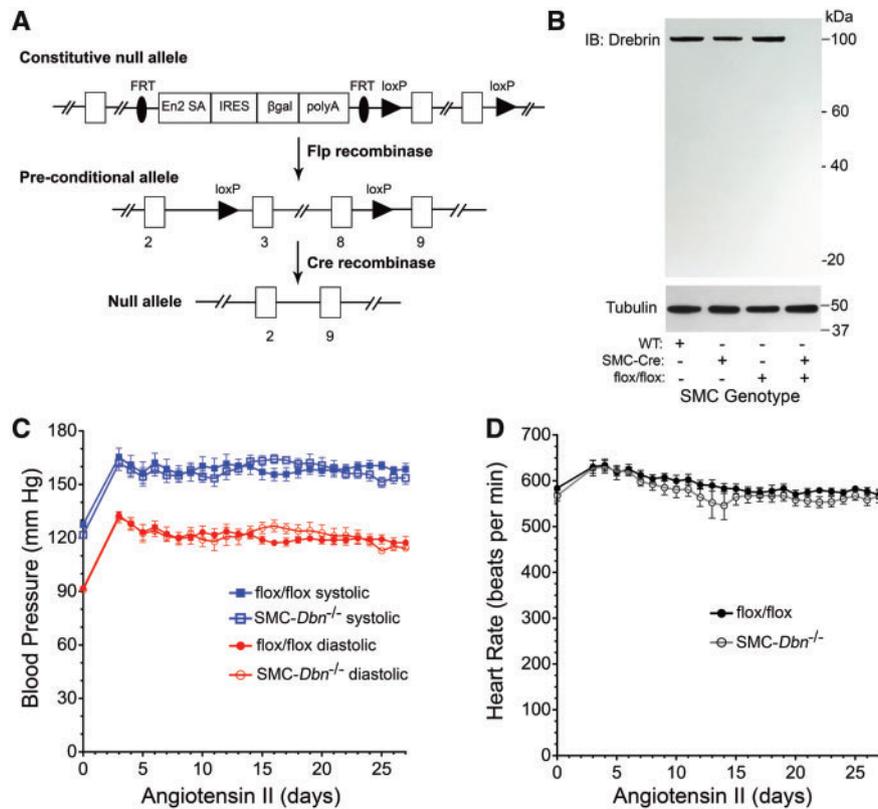


Figure 1 Angiotensin II produces equivalent hypertension in SMC-*Dbn*^{-/-} and *Dbn*^{lox/lox} mice. (A) The *Dbn* gene trapping cassette upstream of floxed Drebrin exons 3–8 is diagrammed (top); excision of the trapping cassette by Flp recombinase produces the floxed *Dbn* allele (middle); excision of the floxed exons 3–8 by Cre recombinase produces the null allele (bottom). (B) Aortas from WT, SMC-Cre, *Dbn*^{lox/lox}, and SMC-*Dbn*^{-/-} mice were stripped of endothelium and adventitia, solubilized, and immunoblotted sequentially for Drebrin and then tubulin. The immunoblot shown is representative of three experiments performed with independent aortas. (C) Blood pressure in ambulatory mice was measured continuously by radiotelemetry using pressure-sensing catheters implanted in the common carotid artery. Ten days after pressure-sensing catheter insertion, osmotic minipumps were implanted to infuse angiotensin II (1000 ng/kg/min) continuously for 28 days. Shown are means \pm S.E. for systolic blood pressure (blue) and diastolic blood pressure (red) over the course of angiotensin II infusion for SMC-*Dbn*^{-/-} ($n = 7$) and *Dbn*^{lox/lox} ($n = 7$) mice. (D) Heart rate in ambulatory mice was measured continuously by telemetry as in (C). Shown are means \pm S.E. for heart rate (b.p.m.) over the course of angiotensin II infusion for SMC-*Dbn*^{-/-} ($n = 7$) and *Dbn*^{lox/lox} ($n = 7$) mice.

in the descending thoracic and abdominal aorta involves SMC hypertrophy.¹² For this reason, we examined the descending thoracic aorta and peripheral arteries to determine if Drebrin's effects on remodelling were specific to the proximal aorta. SMC-specific loss of Drebrin did not affect angiotensin II-induced medial thickening in the distal thoracic aorta (Supplementary material online, Figure S6) or in bronchial arteries (Supplementary material online, Figure S7). Thus, the effects of Drebrin on aortic remodelling are specific to the proximal ascending aorta, where angiotensin II-induced medial thickening involves SMC hyperplasia.¹²

3.3 SMC Drebrin limits SMC proliferation and extracellular matrix deposition

Angiotensin II-induced medial expansion in the ascending aorta involves both SMC proliferation and the deposition of extracellular matrix.^{12,22} As assessed by the prevalence of medial SMCs staining for proliferating cell nuclear antigen (PCNA), there was approximately two-fold more SMC proliferation in ascending aortas from SMC-*Dbn*^{-/-} than from

Dbn^{lox/lox} mice treated with angiotensin II (Figure 4A and B). Nuclear density was equivalent in control and SMC-*Dbn*^{-/-} aortas: 2838 ± 101 vs. 2745 ± 90 nuclei per sq mm (SMC-*Dbn*^{-/-} vs. *Dbn*^{lox/lox}). However, despite having a nuclear density equivalent to *Dbn*^{lox/lox} aortas, SMC-*Dbn*^{-/-} aortas exhibited greater medial area—and therefore, a greater number of total SMCs—than *Dbn*^{lox/lox} aortas; thus, together with our morphometry data, our nuclear density data accord with the increased SMC proliferation observed in SMC-*Dbn*^{-/-} proximal aortas. Congruently,²³ ERK1/2 activation (judged by phosphorylation) was also greater in SMC-*Dbn*^{-/-} aortas, even though total ERK1/2 expression was equivalent in SMC-*Dbn*^{-/-} and *Dbn*^{lox/lox} aortas—as shown by both immunostaining (Figure 4C and D) and immunoblotting (Figure 4E and F).

To quantitate extracellular matrix in the ascending aortas of angiotensin II-infused mice, we immunostained for collagen I. Collagen I levels were 2.1 ± 0.2 -fold higher in SMC-*Dbn*^{-/-} than in *Dbn*^{lox/lox} aortas (Figure 4C and D). This increase in collagen I protein expression in SMC-*Dbn*^{-/-} aortas correlated with collagen I mRNA levels assessed by quantitative RT-PCR (Figure 4G). Congruently, Picosirius red staining of ascending aortas from angiotensin II-infused mice showed 2.3 ± 0.6 -fold more

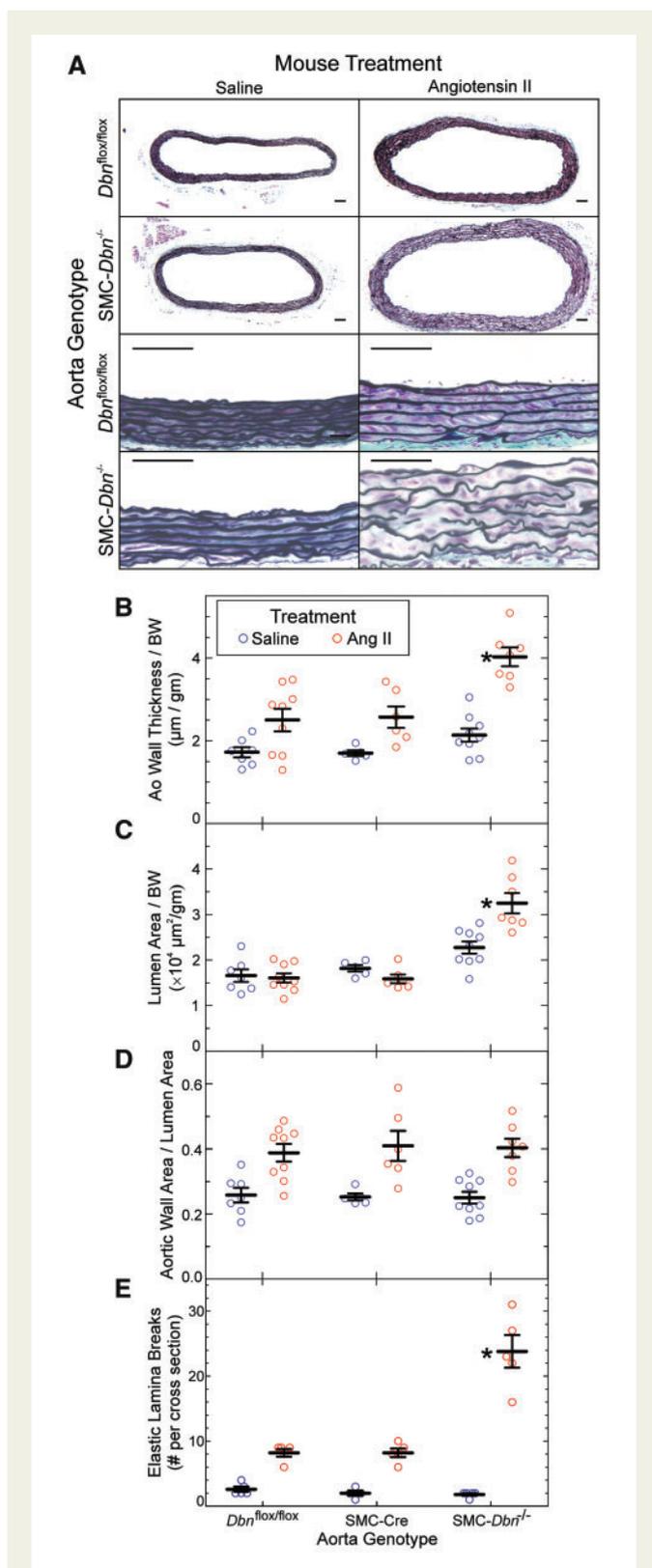


Figure 2 SMC Drebrin regulates angiotensin II-induced aortic remodelling. Congenic *Dbn^{flox/flox}*, SMC-Cre, and SMC-*Dbn^{-/-}* mice were treated with either saline or angiotensin II (Ang II, 1000 ng/kg/min) subcutaneously via osmotic minipump for 28 days and then euthanized. After perfusion fixation, the proximal ascending aorta was embedded in paraffin, sectioned, and stained with a modified connective tissue stain. (A) Representative cross-sections from the indicated mice are shown

collagen in SMC-*Dbn^{-/-}* than in *Dbn^{flox/flox}* aortas (Supplementary material online, Figure S8). Thus, excess angiotensin II-induced medial thickening in SMC-*Dbn^{-/-}* ascending aortas involves not only increased SMC hyperplasia but also increased extracellular collagen deposition.

3.4 SMC Drebrin inhibits angiotensin II-induced pro-inflammatory signalling

Outward remodelling results largely from aortic inflammation,²⁴ including inflammation associated with angiotensin II, which activates reactive oxygen species production and pro-inflammatory NFκB signalling not only in aortas but also in cultured SMCs.²⁵ In light of Drebrin's effects on angiotensin II-induced aortic remodelling, we sought to determine whether Drebrin affects angiotensin II-induced aortic inflammation. To determine the extent to which NFκB was activated in our aortas, we employed two read-outs: (i) NFκB p65 Ser536 phosphorylation, which augments NFκB transcriptional activity and is effected by IκB kinase-β and other kinases,^{26,27} and (ii) expression of vascular cell adhesion molecule-1 (VCAM-1, CD106), an integrin-binding protein that facilitates adhesion of monocytes, and that is encoded by an NFκB-dependent gene.²⁸ Aortas of angiotensin II-infused SMC-*Dbn^{-/-}* mice demonstrated 1.5 ± 0.1 to 2.4 ± 1.0-fold greater levels of NFκB phospho-p65(Ser536) than aortas of angiotensin II-infused *Dbn^{flox/flox}* mice, assessed either by immunofluorescence (Figure 5A and C) or by immunoblotting (Figure 5E), respectively, even though levels of total NFκB p65 protein were equivalent in aortas from both mouse lines. Consistent with this evidence of increased NFκB activation, medial SMCs of SMC-*Dbn^{-/-}* aortas also demonstrated a 1.3 ± 0.1-fold increase in VCAM-1 protein expression (Figure 5A and C).

As further evidence that angiotensin II promoted greater inflammation in SMC-*Dbn^{-/-}* than in *Dbn^{flox/flox}* aortas, matrix metalloproteinase-9 protein (MMP-9) levels were 1.7 ± 0.2 to 2.2 ± 0.5-fold higher in SMC-*Dbn^{-/-}* aortas by immunofluorescence (Figure 5B and C) and immunoblotting (Figure 5F), respectively. MMP-9 activity assessed by gelatin zymography was also 4.4 ± 1.1-fold greater in *Dbn^{-/-}* than in *Dbn^{flox/flox}* SMCs (Supplementary material online, Figure S9). The expression of MMP-9 is driven by both NFκB, which is hyperactivated in *Dbn^{-/-}* SMCs (Figure 5A, C, E), and AP-1,²⁹ which would be expected to be hyperactive in SMC-*Dbn^{-/-}* aortas because of their higher degree of ERK1/2 activation (Figure 4C–F). As demonstrated in several systems, MMP-9 contributes to outward arterial remodelling,^{30,31} as we observed in SMC-*Dbn^{-/-}* aortas.

(original magnification × 100, top), and corresponding 630× images of the same aortas are also shown (bottom). Scale bars = 50 μm. (B) Average aortic wall thickness was calculated for each cross section from the area of the tunica media. Aortic thickness was normalized to body weight; quotients were plotted as the means ± S.E. from 5 to 9 mice in each group. Compared with saline-treated and cognate angiotensin II-treated controls: **P* < 0.01. (C) The lumen area for each aorta cross section was normalized to body weight, and quotients were plotted as in B. Compared with saline-treated and cognate angiotensin II-treated controls: **P* < 0.01. (D) For each aortic section, the area of the tunica media was normalized to area of the lumen; quotients were plotted as in B. (E) The number of discontinuities, or 'breaks' in the elastic laminae were counted for each aortic cross section stained with modified connective tissue stain (A), and plotted as means ± S.E. from five mice in each group. Compared with saline-treated and cognate angiotensin II-treated controls: **P* < 0.01. (All comparisons used two-way ANOVA with Tukey *post hoc* test).

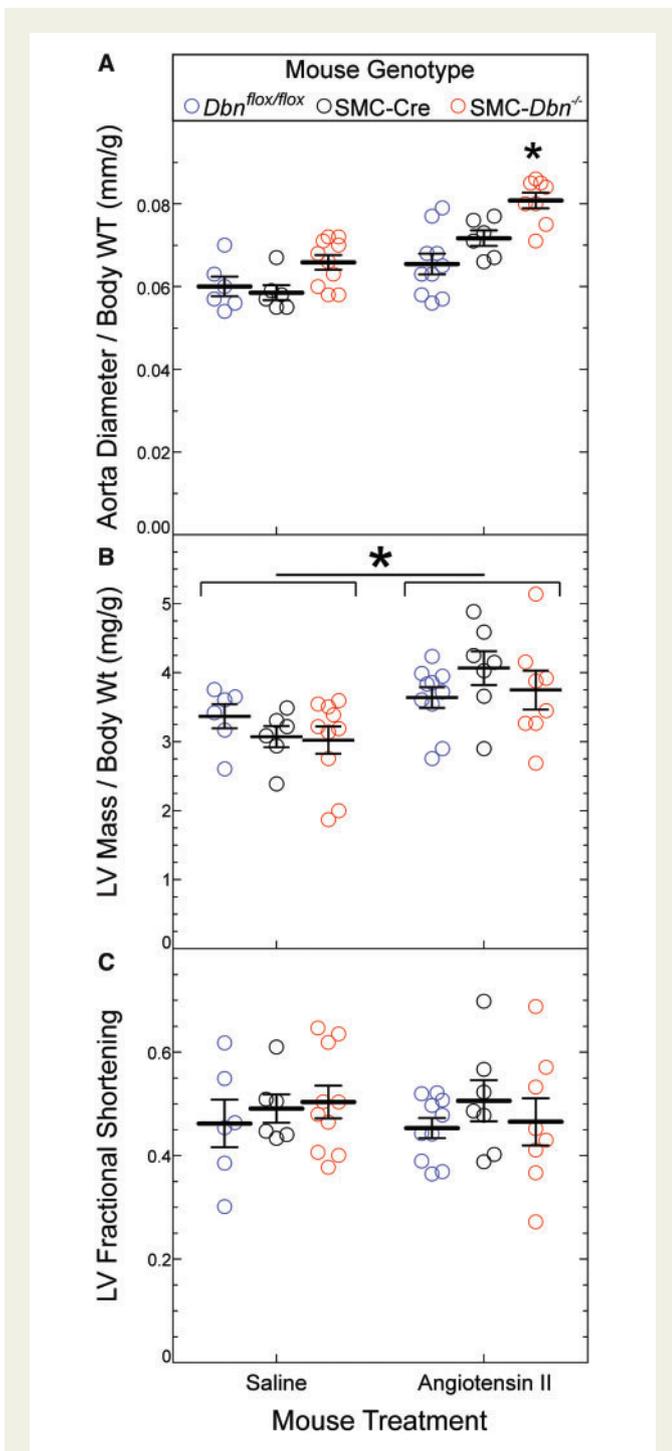


Figure 3 SMC Drebrin regulates aortic remodelling but not cardiac hypertrophy or contractility in response to angiotensin II. Non-anaesthetized mice from Figure 2 were subjected to echocardiography 1 day prior to sacrifice. (A) Aortic root diameter was normalized to body weight, and plotted as the means \pm S.E. from 6 to 10 mice in each group. Compared with cognate angiotensin II-treated controls: $*P < 0.05$ (two-way ANOVA with Tukey *post hoc* test). (B) Left ventricular mass was calculated from echocardiographic measurements of left ventricular wall thickness and normalized to body weight. Shown are means \pm S.E. from 6 to 10 mice of each group. Compared with cognate saline-treated groups: $*P < 0.05$ (two-way ANOVA with Tukey *post hoc* test). (C) Left ventricular fractional shortening is plotted as the means \pm S.E. from 6 to 10 mice in each group.

Furthermore, increased MMP-9 expression may be responsible for the approximately three-fold increase in the prevalence of elastic lamina breaks in SMC-*Dbn*^{-/-} aortas (Figure 2), as suggested by aneurysm studies in *Mmp9*^{-/-} mice.³¹ Finally, consistent with our finding of increased VCAM-1 expression, CD68⁺ macrophages were 1.3 ± 0.2 -fold more prevalent in SMC-*Dbn*^{-/-} than in *Dbn*^{flox/flox} aortas (Figure 5B and D). These excess adventitial macrophages may also augment degradation of elastic laminae in SMC-*Dbn*^{-/-} aortas (Figure 2), by secreting MMP-9³¹ or cathepsin K.³² Unlike monocyte/macrophages, mast cells were not more prevalent in SMC-*Dbn*^{-/-} than in *Dbn*^{flox/flox} aortas (Supplementary material online, Figure S10).

3.5 SMC Drebrin limits reactive oxygen species production

In response to angiotensin II, SMC-*Dbn*^{-/-} aortas thus far have demonstrated greater SMC proliferation and inflammatory signalling than *Dbn*^{flox/flox} aortas. Because SMC activation and proliferation are regulated, in part, by SMC NADPH oxidases,³³ we assessed aortic SMC NADPH oxidase activity by CellROX[®] Orange and dihydroethidium staining (Figure 6A–D). Production of reactive oxygen species in ascending aortas of angiotensin II-infused mice was 1.7 ± 0.1 -fold higher in SMC-*Dbn*^{-/-} than in *Dbn*^{flox/flox} mice (Figure 6A and B). Thus, Drebrin activity in SMCs appears to reduce NADPH oxidase activity.

NADPH oxidase activity augments pro-inflammatory signalling pathways³⁴ which, in a positive-feedback manner, further enhance expression of NADPH oxidases.³⁵ For this reason, we used qRT-PCR to quantitate aortic medial expression levels of NOX1 and NOX4, the primary NADPH oxidases in the murine aorta.³⁶ Consistent with their higher level of NADPH oxidase activity, angiotensin II-treated SMC-*Dbn*^{-/-} aortas expressed 3.2 ± 1.7 -fold more NOX1 mRNA than angiotensin II-treated *Dbn*^{flox/flox} aortas, even though they expressed equivalent levels of NOX4 (Figure 6E). Concordantly, NOX1 protein expression was 2.5 ± 0.7 -fold greater in SMC-*Dbn*^{-/-} aortas than in *Dbn*^{flox/flox} aortas from angiotensin II-infused mice (Figure 6F).

In SMC-*Dbn*^{-/-} aortas, could their higher NADPH oxidase activity be responsible for their greater NF κ B activation, assessed (Figure 5) as NF κ B p65 Ser536 phosphorylation and VCAM-1 expression? To address this question, we used primary SMCs from SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} mice. In response to tumour necrosis factor (TNF), a potent activator of NF κ B, SMC-*Dbn*^{-/-} SMCs demonstrated 1.9 ± 0.6 -fold greater NF κ B p65 Ser536 phosphorylation than *Dbn*^{flox/flox} SMCs (Figure 6G and H). Thus, *in vitro* as *in vivo*, Drebrin activity in SMCs attenuated NF κ B activation. However, when we inhibited NADPH oxidase with the cell-permeant NOX inhibitor VAS3947,³⁷ TNF-induced NF κ B p65 Ser536 phosphorylation was abolished in both SMC-*Dbn*^{-/-} and *Dbn*^{flox/flox} SMCs. Together, these data support a model in which the excess NADPH oxidase activity of SMC-*Dbn*^{-/-} SMCs augments NF κ B activation³⁴ and subsequent aortic inflammation.

4. Discussion

We found that mice lacking Drebrin specifically in SMCs developed increased angiotensin II-induced medial hypertrophy and outward remodelling in the ascending aorta. Aortic medial expansion in response to angiotensin II involves SMC hyperplasia or hypertrophy in a manner that varies by region of the aorta.¹² We previously observed that Drebrin inhibits SMC proliferation, both *in vivo*, in response to carotid endothelial denudation, and *in vitro*, in response to foetal bovine serum through its

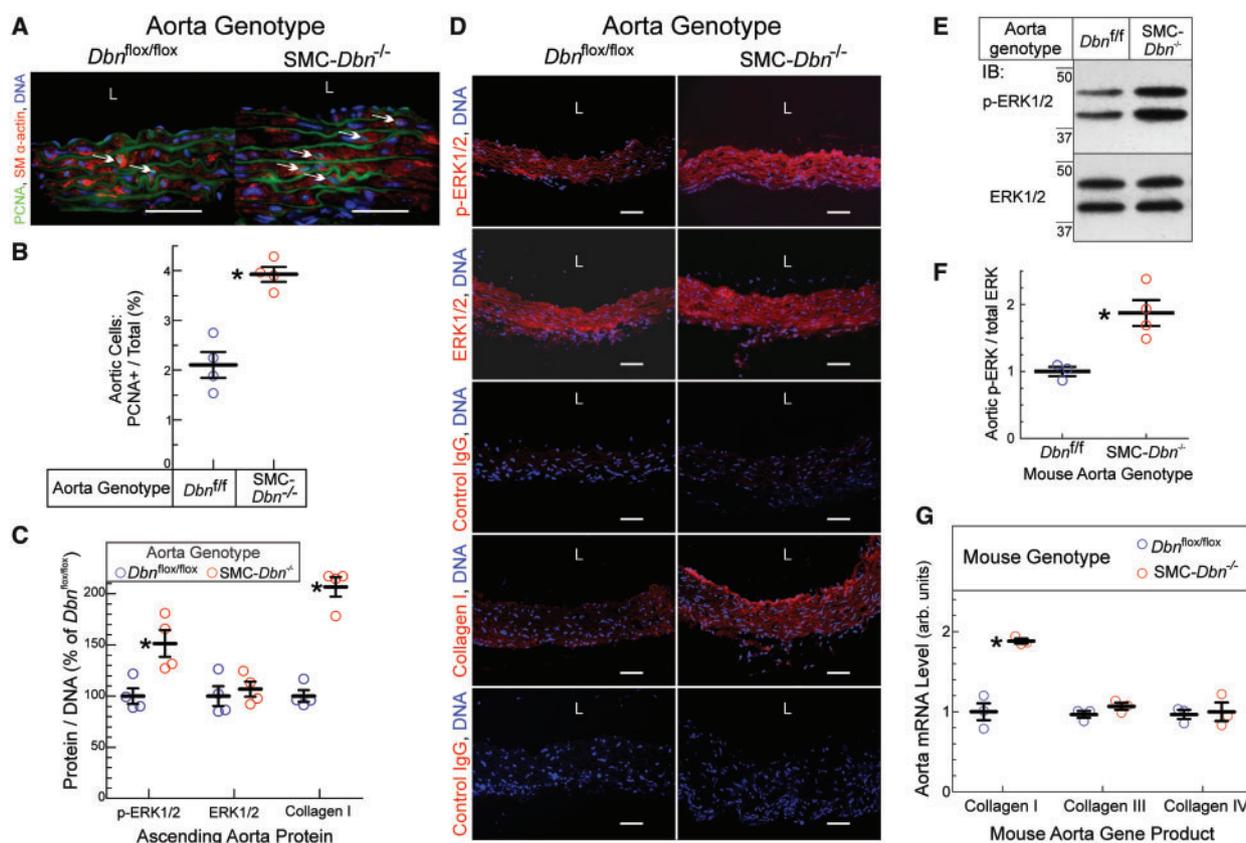


Figure 4 SMC Drebrin limits angiotensin II-induced aortic SMC proliferation and extracellular matrix deposition. *Dbn^{flx/flx}* and *SMC-Dbn^{-/-}* mice were exposed to angiotensin II for 28 days, as in Figure 2, but the aortas were embedded in OCT and frozen. (A) Proximal aortic sections were immunostained for PCNA (green), smooth muscle α -actin (red), and counterstained with Hoechst 33342 (blue, DNA). Arrows designate PCNA-positive nuclei. Scale bars = 50 μ m. (B) PCNA-positive medial cells were counted manually and normalized to the number of Hoechst 33342-stained nuclei by an observer unaware of specimen genotype. The percentage of PCNA-positive cells is plotted as the means \pm SE from four independent aortas in each genotype group. Compared with *Dbn^{flx/flx}*; * $P < 0.01$ (t test). (C and D) Serial aortic frozen sections were immunostained for the indicated (phospho)protein (or with isotype control IgG) and counterstained with Hoechst 33342. Immunofluorescence intensities for phospho-ERK1/2, total ERK1/2, and Collagen I in the arterial media were normalized to corresponding DNA fluorescence intensities; the resulting ratios were plotted as the means \pm S.E. from four specimens of each group. Compared with *Dbn^{flx/flx}*; * $P < 0.01$ (two-way ANOVA with Sidak *post hoc* test). Samples from a single staining session are shown and represent four independent samples from each group with equivalent results. Scale bar = 50 μ m; 'L' denotes lumen. (E) Aortas from *Dbn^{flx/flx}* and *SMC-Dbn^{-/-}* mice infused with angiotensin II were stripped of endothelium and adventitia, and then solubilized. Thirty μ g of aortic protein were immunoblotted for phospho-ERK1/2 and total ERK1/2. (F) Immunoblot band densities for phospho-ERK1/2 were normalized to cognate total ERK1/2 band densities; the ratios were plotted as means \pm SE from 3 to 4 aortas of each genotype. Compared with *Dbn^{flx/flx}*; * $P < 0.05$ (t test). (G) Aortas from *Dbn^{flx/flx}* and *SMC-Dbn^{-/-}* mice infused with angiotensin II were stripped of endothelium and adventitia, and then RNA was isolated. This RNA was subjected to qRT-PCR for GAPDH as well as for the α chains of collagens 1, 3, and 4 as described in Methods section. Threshold count values were normalized to cognate GAPDH values, and plotted as arbitrary units (with mean \pm S.E.) from four aortas in each genetic group. Compared with *Dbn^{flx/flx}*; * $P < 0.05$ (two-way ANOVA with Sidak *post hoc* test).

effects on stabilizing actin filaments.⁷ In this study, we found that loss of SMC Drebrin results in increased SMC proliferation, extracellular matrix deposition, and pro-inflammatory signalling in response to angiotensin II in the proximal aorta where angiotensin II-induced medial thickening involves SMC hyperplasia,¹² but has no effect on the degree of angiotensin II-induced hypertension or medial thickening in the descending thoracic aorta and peripheral arteries, where angiotensin II-induced remodelling involves SMC hypertrophy. Together, these findings suggest that Drebrin influences the phenotype of 'synthetic/proliferative' SMCs.

The complex effects of angiotensin II on aortic remodelling include its role in promoting oxidative stress, inducing SMC proliferation through mitogen-activated protein kinases, and enhancing pro-inflammatory

NF κ B signalling.^{25,38,39} Excessive NF κ B signalling has previously been implicated in the development of pathologic aortic dilation.^{40,41} Increased NF κ B activation in *SMC-Dbn^{-/-}* aortas can be explained by Drebrin's role in limiting up-regulation of NOX1 expression in response to chronic angiotensin II treatment (Supplementary material online, Figure S11). Increased NF κ B activation in *SMC-Dbn^{-/-}* aortas may also be explained by Drebrin's role in stabilizing actin filaments,⁷ because NF κ B signalling is regulated in part by the actin cytoskeleton: loss of smooth muscle α -actin or pharmacological disruption of actin filaments increases NF κ B activation.^{41,42}

SMC NADPH oxidase activity and inflammatory signalling are constrained not only by Drebrin but also by other F-actin-stabilizing

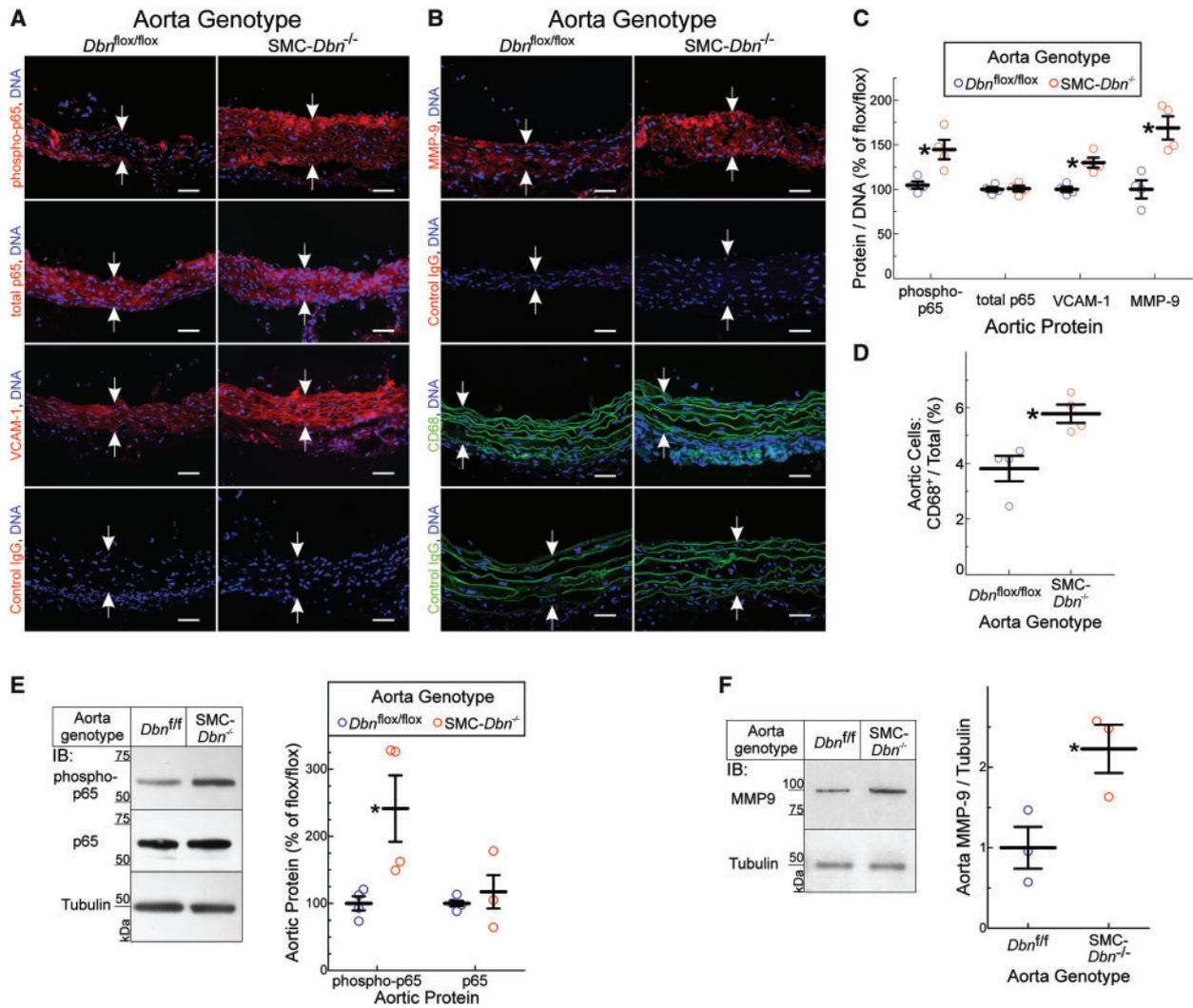


Figure 5 SMC Drebrin inhibits angiotensin II-induced aortic inflammation. (A) Serial sections of the aortas of *Dbn^{fllox/fllox}* and *SMC-Dbn^{-/-}* mice used in Figure 4 were immunostained with rabbit IgG specific for the indicated proteins (or non-immune rabbit IgG), as described for Figure 4. Samples from a single staining session are shown and represent four independent samples from each group with equivalent results. Scale bar = 50 μ m. Arrows designate the internal (upper arrow) and external elastic laminae. ('Phospho-p65' denotes the NF κ B p65 subunit phosphorylated on Ser536). (B) Serial sections of aortas from A were immunostained for MMP-9 or for macrophages with CD68 (or with isotype control IgG) and counterstained with Hoechst 33342 (blue, DNA). Samples from a single staining session are shown and represent four independent samples from each group with equivalent results. Scale bar = 50 μ m. (C) Immunofluorescence in the aortic media was normalized to DNA fluorescence; these ratios were divided by the value obtained for *Dbn^{fllox/fllox}* samples in the same staining batch, to obtain '% of flox/flox'. Plotted are the means \pm S.E. from four independent aortas from each group. Compared with *Dbn^{fllox/fllox}*: * $P < 0.01$ (two-way ANOVA with Sidak *post hoc* test). (D) CD68-positive cells were counted manually and normalized to the number of Hoechst 33342-stained nuclei, by an observer unaware of specimen genotype. A minimum of 500 nuclei per aorta were counted. The percentage of CD68-positive cells is plotted as the mean \pm S.E. from four independent aortas in each genotype group. Compared with *Dbn^{fllox/fllox}*: * $P < 0.01$ (t test). (E) Aortas from *Dbn^{fllox/fllox}* and *SMC-Dbn^{-/-}* mice infused with angiotensin II (Figure 4) were stripped of endothelium and adventitia, and then solubilized. Thirty μ g of aortic medial protein were immunoblotted sequentially for phospho-p65, total p65, and then tubulin. Band densities for phospho-p65 and total p65 were normalized to cognate tubulin band densities; these ratios for *SMC-Dbn^{-/-}* aortas were divided by mean values obtained for *Dbn^{fllox/fllox}* aortas, to obtain '% of flox/flox', plotted as means \pm SE from 3 to 4 aortas from each genotype. Compared with *Dbn^{fllox/fllox}*: * $P < 0.05$ (two-way ANOVA with Sidak *post hoc* test). (F) Thirty μ g of aortic protein aliquots from E were immunoblotted sequentially for MMP-9 and then tubulin. Immunoblot band densities for MMP-9 were normalized to cognate tubulin band densities; the ratios were plotted as means \pm SE from three aortas of each genotype. Compared with *Dbn^{fllox/fllox}*: * $P < 0.05$ (t test).

proteins. Like deficiency of Drebrin, deficiency of SM22 increases production of reactive oxygen species and NF κ B signalling.³⁴ In further parallels with Drebrin, SM22 also represses MMP-9 expression, through inhibition of ERK signalling and a reduction in AP-1-dependent

transactivation of MMP-9 promoter activity.²⁹ Because our *SMC-Dbn^{-/-}* mice harbour a knock-in of Cre recombinase that inactivates one native *Sm22* allele, our *SMC-Dbn^{-/-}* mice are haploinsufficient for SM22. Nonetheless, our data demonstrate that the phenotype of *SMC-Dbn^{-/-}*

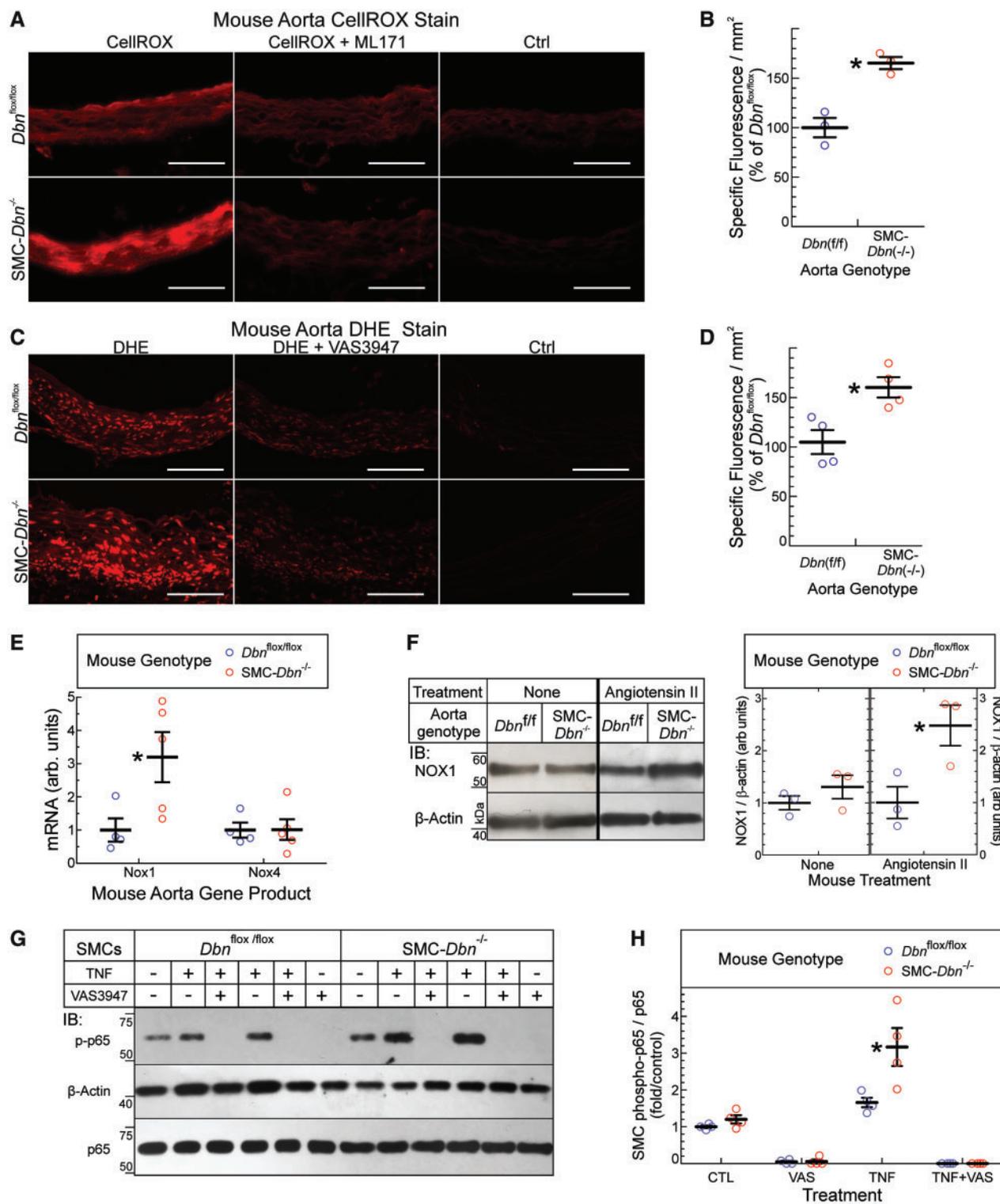


Figure 6 SMC Drebrin limits reactive oxygen species production. (A) Frozen sections of the proximal aorta from *Dbn^{flox/flox}* and SMC-*Dbn^{-/-}* mice used in Figure 4 were incubated without (control, 'Ctrl') or with CellROX[®] Orange in the absence (total signal) or presence (non-specific, NOX-independent signal) of the NOX1 inhibitor ML171 (see Methods section). Shown are fluorescence photomicrographs from single aortas of each genotype. Scale bars = 100 μm. (B) Specific CellROX[®] fluorescence was calculated as the fluorescence (red pixels/mm²) in aortic slices incubated with CellROX[®] Orange minus that obtained from slices incubated with CellROX[®] Orange plus ML171 (see Methods); values from three distinct aortas of each genotype were plotted, along with means ± SE. Compared with *Dbn^{flox/flox}*. **P* < 0.01 (t test). (C) Serial sections from A were incubated with dihydroethidium in the absence (total signal) or presence (non-specific, NOX-independent signal) of the NOX inhibitor VAS3947 (see Methods section); scale bars = 100 μm. (D) Specific ethidium fluorescence was calculated and plotted as for B, for four distinct aortas of each genotype. Compared with *Dbn^{flox/flox}*. **P* < 0.01 (t test). (E) From the aortic tunica media of angiotensin II-infused SMC-*Dbn^{-/-}* and *Dbn^{flox/flox}* mice (from Figure 4), RNA was subjected to qRT-PCR for GAPDH as well as for NOX1 and

mice is not due to SM22 haploinsufficiency: our SMC-Cre (*Sm22^{-/+}*) mice were equivalent to *Dbn^{fllox/fllox}* mice with regard to aortic medial thickening and luminal expansion (Figures 2 and 3). Furthermore, SM22 haploinsufficient mice also phenocopy WT mice with regard to arterial neointimal hyperplasia evoked by endothelial denudation.³⁴

In addition to limiting up-regulation of NOX1 in response to angiotensin II, mechanisms through which Drebrin limits SMC NADPH oxidase activity may also involve endocytosis, a process that is inhibited by Drebrin⁴³ but required for NOX1 activation in SMCs stimulated by TNF.⁴⁴ Drebrin inhibits endocytosis via clathrin-coated pits and caveolae by associating with the actin-binding protein cortactin, and thereby preventing cortactin from binding to dynamin-2.⁴³ By binding to dynamin-2, cortactin scaffolds a ternary complex of cortactin/dynamin-2/actin-related protein 3 (ARP3); this ternary complex promotes endocytosis through a process that involves growth of actin filaments.⁴⁵ Cortactin also mediates the interaction of the p47^{phox} subunit of the NADPH oxidase complex with the actin cytoskeleton^{46,47}—an interaction that is required for angiotensin II-induced NADPH oxidase activity.⁴⁷ Thus, by associating with cortactin, Drebrin may inhibit NADPH oxidase activity by inhibiting both endocytosis and the assembly of the NADPH oxidase complex.

Angiotensin II-induced aortic remodelling involves SMC hyperplasia in the ascending aorta.¹² Interestingly, deletion of floxed angiotensin II type 1A (AT_{1A}) receptor alleles with an SM22-Cre transgene does not affect medial thickening of the ascending aorta.^{10,48} Instead, medial thickening of the ascending aorta is substantially reduced by deletion of floxed AT_{1A} receptor alleles with S100A4-driven Cre, specifically in fibroblasts—which populate not only the aortic adventitia but also the ascending aortic media of angiotensin II-treated mice, as demonstrated by lineage-tracing studies.¹⁰ In the aortic media, these fibroblast-derived cells express smooth muscle α -actin, and thus assume a SMC-like phenotype.¹⁰ Thus, medial expansion in the ascending aorta of angiotensin II-treated mice involves migration of fibroblasts from the adventitia to the media.¹⁰ As adventitial fibroblasts migrate into the ascending aortic tunica media and trans-differentiate into SMC-like cells expressing SM22 in our SM22-Cre⁺ knock-in mice, we would expect these cells to express Cre and thus to delete their floxed *Dbn* alleles. Indeed, we observed that Drebrin expression is deleted in activated adventitial fibroblasts from SMC-*Dbn^{-/-}* mice *in vitro* (Supplementary material online, Figure S1B). Therefore, Drebrin may reduce angiotensin II-induced SMC proliferation and medial expansion in the ascending aorta by affecting trans-differentiating adventitial fibroblasts. It is also possible that Drebrin affects the response of medial SMCs to the migrating, trans-differentiating adventitial fibroblasts.

We have found that Drebrin suppresses angiotensin II-promoted aortic inflammation and remodelling. These findings raise the possibility that

Drebrin activity may constrain aortic aneurysm formation, because angiotensin II signalling contributes to the formation of thoracic and abdominal aortic aneurysms.^{49,50} We did not observe thoracic or abdominal aneurysms in our study, likely because the incidence of aneurysm formation in C57BL/6 mice treated chronically with angiotensin II is low in the absence of a hypercholesterolaemic background or lysyl oxidase inhibition.^{49,50} Whether Drebrin deficiency would promote aortic aneurysm formation in these models remains to be determined.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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Figure 6 Continued

NOX4; data processing paralleled that of Figure 4. Compared with *Dbn^{fllox/fllox}*: **P* < 0.05 (two-way ANOVA with Sidak *post hoc* test). (F) Aortas from *Dbn^{fllox/fllox}* and SMC-*Dbn^{-/-}* mice infused with saline or angiotensin II were processed for immunoblotting as in Figure 5; shown are sequential immunoblots for NOX1 and β -actin. Band densities for NOX1 were normalized to cognate β -actin band densities and expressed as arbitrary units, plotted as means \pm SE from three aortas from each genotype. Compared with *Dbn^{fllox/fllox}*: **P* < 0.05. (G) Quiescent *Dbn^{fllox/fllox}* and SMC-*Dbn^{-/-}* SMCs were incubated in the absence ('-') or presence of mouse TNF (5 ng/mL) and in the absence or presence of the NOX inhibitor VAS3947 (10 μ mol/L) for 15 min (37°C) as indicated, and then solubilized. Equal volumes of SMC lysates were run on parallel SDS-PAGE and immunoblotted in parallel for phospho-p65 or total p65, and then stripped and reprobed for β -actin (shown for the p-p65 panel). Results are from a single experiment, representative of four performed. (H) Band densities for phospho-p65 and total p65 were normalized to cognate β -actin bands; phospho-p65/actin ratios were then normalized to cognate total p65/actin ratios. Subsequently, these ratios were normalized to those of untreated *Dbn^{fllox/fllox}* SMCs, and plotted as the means \pm SE from four independent experiments. Compared with *Dbn^{fllox/fllox}*: **P* < 0.05 (two-way ANOVA with Sidak *post hoc* test).

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