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Inflammation and Mechanical Stretch Promote Aortic Stiffening in Hypertension Through Activation of p38 MAP Kinase

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Abstract

Rationale—Aortic stiffening commonly occurs in hypertension and further elevates systolic pressure. Hypertension is also associated with vascular inflammation and increased mechanical stretch. The interplay between inflammation, mechanical stretch and aortic stiffening in hypertension remains undefined.

Objective—To determine the role of inflammation and mechanical stretch in aortic stiffening.

Methods and Results—Chronic angiotensin II infusion caused marked aortic adventitial collagen deposition, as quantified by Masson's Trichrome Blue staining and biochemically by hydroxyproline content, in wild-type (WT) but not in Recombination Activation Gene-1 deficient (RAG-1$^{-/-}$) mice. Aortic compliance, defined by ex-vivo measurements of stress-strain curves, was reduced by chronic angiotensin II infusion in WT mice ($p<0.01$) but not in RAG-1$^{-/-}$ mice ($p<0.05$). Adoptive transfer of T cells to RAG-1$^{-/-}$ mice restored aortic collagen deposition and stiffness to values observed in WT mice. Mice lacking the T cell derived cytokine IL-17a were also protected against aortic stiffening. In additional studies, we found that blood pressure normalization by treatment with hydralazine and hydrochlorothiazide prevented angiotensin II-induced vascular T cell infiltration, aortic stiffening and collagen deposition. Finally, we found that mechanical stretch induces expression of collagen 1α1, 3α1 and 5a1 in cultured aortic fibroblasts in a p38 MAP kinase-dependent fashion, and that inhibition of p38 prevented angiotensin II-induced aortic stiffening in vivo. IL-17a also induced collagen 3a1 expression via activation of p38 MAP kinase.

Conclusions—Our data define a pathway in which inflammation and mechanical stretch lead to vascular inflammation that promotes collagen deposition. The resultant increase in aortic stiffness likely further worsens systolic hypertension and its attendant end-organ damage.

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DISCLOSURES
None.

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Keywords

Inflammation; mechanical stretch; collagen deposition; aortic stiffening; vascular remodeling

INTRODUCTION

The capacitance property of the aorta normally blunts blood pressure elevation during systole and maintains diastolic pressure and tissue perfusion during diastole. Loss of this Windkessel function of the proximal aorta causes an increase in systolic pressure, a decline in diastolic pressure and an increase in pulse wave velocity.[1] The augmentation of systolic pressure caused by aortic stiffening increases the incidence of stroke, renal failure and myocardial infarction. Aortic stiffening is associated with aging, insulin resistance, diabetes, atherosclerosis and hypertriglyceridemia.[2–5] Importantly, hypertension per se causes aortic stiffening, leading to progressive elevation of systolic pressure. Thus, aortic stiffening not only contributes to hypertension but also portends cardiovascular morbidity and mortality.[6, 7]

The precise mechanisms underlying aortic stiffening remain undefined. Clinical studies suggest that inflammation and arterial stiffness are related.[8–11] Patients with inflammatory diseases such as lupus erythematosus, rheumatoid arthritis and psoriasis have increased pulse wave velocity.[12–14] Data from our laboratory and others have shown that T cells and T cell-derived cytokines are important in development of hypertension.[15, 16] We have previously found that Recombination Activation Gene-1 deficient (RAG-1$^{-/-}$) mice develop blunted hypertension in response to angiotensin II, DOCA-salt challenge and norepinephrine.[17] The RAG-1 gene encodes a gene responsible for recombining the variable regions of the T cell receptor and immunoglobulins and in its absence mice fail to develop either B cells or T cells. Adoptive transfer of T cells restores hypertension in RAG-1$^{-/-}$ mice, indicating a critical role of these cells. Recently, deletion of the RAG-1 gene in Dahl Salt-sensitive rats has been shown to lower blood pressure and to reduce renal injury upon salt feeding.[18] Other studies have shown that T cell-derived cytokines also contribute to hypertension, likely by promoting vascular dysfunction and renal injury.[16, 19, 20] One such cytokine is interleukin 17a (IL-17a), which is produced by a subset of pro-inflammatory CD4^{+} T cells referred to as T_H17 cells. Mice lacking IL-17a have blunted hypertension and reduced aortic production of reactive oxygen species (ROS) following angiotensin II infusion. Recent studies have also shown that administration of IL-17a to mice causes hypertension and reduces endothelium-dependent vasodilatation, at least in part by activating Rho kinase.[21] IL-17a also promotes collagen deposition and contributes to fibrosis in other tissues and conditions.[22–24]

In the present study we sought to examine mechanisms responsible for aortic stiffening in hypertension. In particular we examined the role of adaptive immunity mediated by T cells and their cytokines and the direct effects of mechanical stimulation by blood pressure lowering and by exposing aortic fibroblasts to hypertensive levels of stretch. We identify a novel pathway that promotes striking aortic adventitial collagen deposition and vascular stiffening.

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METHODS

Animals

Male wild type, RAG-1$^{-/-}$, CD4$^{-/-}$, CD8$^{-/-}$ and IL-17a$^{-/-}$ mice were studied at 3 months of age. Hypertension was induced by infusion of angiotensin II (490 ng/kg/min) via osmotic minipumps for two weeks unless otherwise indicated. For measurement of blood pressure, mice were implanted with telemetry units and ten days later osmotic minipumps were placed. Blood pressure was recorded for 10 minutes every hour from three days prior to osmotic minipump implantation until the end of angiotensin II infusion at Day 14. For studies of vascular stiffness, the descending thoracic aorta was mounted on cannulas at the in situ length in calcium-free buffer. Intraluminal pressure was increased in a step-wise fashion while video microscopy was used to follow outer and inner diameter. Diameters were recorded with every increment of 25 mmHg from 0 to 200 mmHg. Stress-strain relationships were calculated as described by Baumbach et al.[25] In some experiments, RAG-1$^{-/-}$ mice underwent adoptive transfer of T cells or their subsets as described previously.[26] The Institutional Animal Care and Use Committee approved all experimental protocols.

Flow cytometry

Single cell suspensions were prepared from aortas as previously described.[27] Leukocytes were further isolated using a Percoll gradient. The antibodies used were: FITC anti-CD45; PerCP anti-CD45; PE anti-CD3e; FITC anti-CD3e; APC anti-CD3; APC anti-CD4; PerCP anti-CD8a; PE-Cy7 anti-CD8a.

Measurement of aortic collagen and elastin

Aortic collagen was visualized by Masson's Trichrome staining and quantified by measurement of tissue hydroxyproline as described previously.[28] Elastin was visualized by Verhoeff Van Gieson staining and quantified as previously described, [29, 30] with minor modifications as outlined in the supplemental data section.

Cell culture

Low passage mouse aortic fibroblasts were studied at 90% confluence. The media was changed from 5% to 0.5% FBS for 24 hours prior to experiments. Cells were exposed to either cyclical stretch or to IL-17a (100 ng/mL) for 36 hours.

Real-time PCR and Western Blotting

RNA was extracted from aortas or cultured fibroblasts using the Qiagen RNeasy mini kit. PCR array was performed to screen for matrix and inflammatory genes and real-time PCR was employed to examine selected fibrotic genes. Western blotting was performed using specific rabbit anti-mouse monoclonal primary antibodies and goat anti-rabbit polyclonal secondary antibodies. Western blots were quantified by densitometry.

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Statistical analysis

Data in the manuscript are expressed as the mean ± SEM. Comparisons of blood pressure over time were made using one-way ANOVA for repeated measures, followed by a Student Newman Keuls post hoc test when significance was indicated. Compliance curves and stress-strain curves were also compared using ANOVA for repeated measures. To compare the effect of angiotensin II on parameters other than blood pressure, two-way ANOVA was employed, as indicated. The effects of adoptive transfer of pan T cells or antihypertensive treatment on collagen deposition or aortic inflammation were compared using one-way ANOVA. P values are reported in the figures.

A detailed description of the materials and methods can be found in the on-line supplement.

RESULTS

Effect of angiotensin II-induced hypertension on aortic collagen and elastin content: Role of T cells

Two matrix components that predominantly modulate tissue compliance are collagen and elastin. We performed initial studies to determine how hypertension affects these and to understand the role of T cells in this response. Using Masson's Trichrome staining, we observed that collagen is predominantly localized to the adventitia of the aorta of sham infused mice. During angiotensin II infusion, a striking accumulation of collagen in the aortic adventitia occurred. The thickness of this adventitial collagen deposition was often equivalent to the media (Figure 1A and 1B). By planimetry, adventitial collagen area was increased from 3.8×10^4 μm^2 to 13.6×10^4 μm^2 by angiotensin II treatment. To directly quantify aortic collagen, we measured hydroxyproline content, which was 19 ± 7 ng/μg of total protein in WT mice and was doubled by chronic angiotensin II infusion (Figure 1C). Thus, by both histochemical staining and biochemical analysis, we found that angiotensin II-induced hypertension is associated with a 2–3 fold increase of aortic collagen content, predominantly localized to the adventitia.

We and others have established a role of T cells in hypertension.[15,16] Moreover, the T cell derived cytokine IL-17 contributes to hypertension and has been shown to promote collagen deposition in experimental scleroderma.[17] We therefore examined the role of T cells in aortic stiffening. As in previous studies, we found that the hypertensive response to angiotensin II is blunted in RAG-$1^{-/-}$ mice (Supplemental figure I A and B). In addition, the increase in aortic collagen content caused by angiotensin II, as detected by either Masson's trichrome staining or by hydroxyproline assay, was blunted in RAG-$1^{-/-}$ mice (Figure 1A–C). Five weeks following adoptive transfer of pan T cells, flow cytometry documented a stable population of these cells in RAG-$1^{-/-}$ mice (Supplemental figure I C). In keeping with our prior studies, T cell adoptive transfer restored the hypertensive response to angiotensin II in RAG-$1^{-/-}$mice (Supplemental figure I D and I E). Importantly, adoptive transfer of T cells to RAG-$1^{-/-}$ mice also restored the aortic collagen deposition caused by angiotensin II to levels observed in WT mice (Figure 1A–C). Despite these fibrotic changes in the aorta, we did not observe collagen deposition in the mesenteric arteries of WT or RAG-$1^{-/-}$ mice treated with angiotensin II (Supplemental figure II).

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Van Gieson's Elastica staining did not reveal a qualitative difference in elastin between sham or angiotensin II-treat mice or in RAG-1$^{-/-}$ mice (Figure 1A). Angiotensin II-induced hypertension was associated with hypertrophy of medial cells, causing a greater separation of elastin laminae. Biochemical analysis also did not reveal differences in the absolute amount of elastin between sham and angiotensin II-treated WT or RAG-1$^{-/-}$ mice, or an effect of T cell adoptive transfer (Figure 1D). When normalized to total protein, relative elastin levels were decreased by angiotensin II in both mouse strains (Supplemental figure I F), but this could be attributed to the striking increase in total aortic protein associated with angiotensin II-induced aortic hypertrophy (Supplemental figure I G).

Angiotensin II-induced hypertension causes dramatic changes in the expression of the aortic matrix genes

In addition to collagen and elastin, numerous other matrix proteins can influence vascular stiffness. We therefore performed a PCR array to characterize expression of 84 aortic matrix genes related to fibrosis and remodeling. In response to angiotensin II infusion, 17 out 84 genes were significantly upregulated while no genes were down regulated (Supplemental Table I). Among these, collagen 1a1 and MMP-2 were upregulated by more than 8 fold and collagen 3a1 by 7 fold. MMP-11, thrombospondin-1, thrombospondin-2 and secreted acidic cysteine rich glycoprotein were upregulated by 4–5 fold. We also found more than 3 fold upregulation for collagen 5a1 and MMP-14. Surprisingly, the potent pro-fibrotic cytokine, TGFβ1, was not altered in angiotensin II-induced hypertension.

Hypertension reduces aortic compliance – Role of T cells

Additional studies were performed to measure compliance of isolated segments of the descending thoracic aorta. Chronic angiotensin II infusion caused marked stiffening of the thoracic aorta in WT mice as evidenced by a downward shift of the compliance curve (Figure 2A) and a leftward shift of the stress-strain relationship (Figure 2B). In contrast, in RAG-1$^{-/-}$ mice, chronic angiotensin II infusion had essentially no effect on aortic stiffness. Adoptive transfer of T cells to RAG-1$^{-/-}$ mice restored the increase in aortic stiffening caused by angiotensin II (Figure 2C and 2D). Analysis of the changes in vascular inner and outer diameter showed that these increased in parallel in vessels from sham treated mice as intraluminal pressure was increased. In contrast, following angiotensin II infusion, the increase in outer diameter was constrained in aortas from WT mice, but not in aortas from RAG-1$^{-/-}$ mice (Figure 2E and 2F).

To determine the relative contribution of T cell subtypes, we examined the effect of angiotensin II infusion in CD4$^{-/-}$ and CD8$^{-/-}$ mice. The increase in collagen caused by angiotensin II was blunted in mice lacking either T cell subtype, but was most striking in mice lacking CD8^{+} T cells as evidenced by both Masson's trichrome staining and by hydroxyproline assay (Supplemental Figure III A–C). In addition, CD4$^{-/-}$ and CD8$^{-/-}$ mice were both partially protected against the development of aortic stiffening caused by angiotensin II (Supplemental Figure III D–G). In additional studies, we performed adoptive transfer of CD4^{+} T cells or CD8^{+} T cells to RAG-1$^{-/-}$ mice and then infused angiotensin II three weeks later. Neither cell type alone increased aortic stiffness or collagen content

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significantly (Supplemental Figure IV). These data indicate that both cell types are required and likely interact to mediate hypertension-related aortic stiffening.

We have previously shown that that mice lacking IL-17a are protected against the development of hypertension, aortic inflammation and angiotensin II-induced vascular oxidative stress.[16] In the present study, we found that IL-17a$^{-/-}$ mice are also protected against aortic collagen deposition (Figure 3A–C) and aortic stiffening (Figure 3D and E) in response to chronic angiotensin II infusion.

We have previously shown that T cells play a role in models of hypertension other than angiotensin II infusion, including DOCA-salt hypertension and norepinephrine-induced hypertension.[15, 17] To determine if the reduced adventitial collagen deposition and aortic stiffening in RAG-1$^{-/-}$ mice are specific to angiotensin II, we also studied deoxycorticosterone acetate (DOCA)-salt-induced hypertension, which is characterized by low levels of circulating angiotensin II. As shown in supplemental figure V, while WT mice developed marked collagen deposition and aortic stiffening in response to DOCA-salt challenge, RAG-1$^{-/-}$ mice were protected from these changes. These data suggest that T cells contribute to aortic stiffening in forms of hypertension other than that caused by angiotensin II.

Aortic stiffening is dependent on blood pressure elevation

In clinical studies, aortic stiffening is commonly associated with hypertension; however, the causal relationship remains unclear. To address the possible role of pressure elevation in the genesis of aortic stiffening, we normalized blood pressure in angiotensin II-infused mice by adding hydralazine (320 mg/L) and hydrochlorothiazide (60 mg/L) to the drinking water. As shown in Figure 4A and 4B, concurrent hydralazine and hydrochlorothiazide treatment completely prevented the elevation of blood pressure. This treatment also protected against adventitial collagen deposition (Figure 4C–E). More importantly, the shifts in compliance and stress-strain curves were prevented by this treatment regimen (Figure 4F and 4G). Interestingly, we found that hydralazine and hydrochlorothiazide also abrogated T cell infiltration and inflammation in the aortas of angiotensin II-infused mice (Figure 4H–L). Collectively, these data suggest that aortic stiffening and vascular inflammation are at least in part due to mechanical effects of hypertension.

To determine whether normalization of blood pressure in established hypertension could reverse collagen deposition and aortic stiffening, hydralazine and hydrochlorothiazide treatment was administered from day 15 to day 28 during a 4-week angiotensin II infusion. Although this successfully normalized blood pressure during the last two weeks of angiotensin II infusion, it failed to reverse adventitial collagen deposition and aortic stiffening (Supplemental Figure VI). These data suggest that hypertension might cause irreversible large vessel fibrosis and remodeling.

Aortic fibroblasts express collagen in response to IL-17a and hypertensive mechanical stretch

The above experiments suggest that both inflammation and mechanical forces contribute to aortic stiffening. To differentiate between the direct effect of mechanical stretch and the

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inflammatory cytokine IL-17a, we performed additional studies using cell culture. Because we found aortic collagen deposition in hypertension occurs largely in the adventitia, we studied aortic fibroblasts, which represent the predominant cell type of the adventitia. We focused these studies on the collagen subtypes identified in the real-time PCR array. Fibroblasts were exposed to either 5% or 10% stretch, mimicking levels of mechanical stretch observed in the setting of normal and elevated blood pressures, or were exposed to IL-17a (100 ng/mL) for 36 hours. We found that while IL-17a had no effect on the mRNA expression of collagen 1a1, 10% mechanical stretch doubled expression of this subtype beyond that observed with 5% stretch (Table 1). Collagen 3a1 was increased by more than 4-fold by IL-17a and by more than 3-fold in response to 10% stretch. Collagen 5a1 mRNA expression was increased approximately 2-fold by IL-17a and by 10% stretch, but was not further increased by the combination of this cytokine and stretch. The addition of angiotensin II had minimal effect on these responses except for collagen 3a1, where it doubled the effect of 10% stretch. These experiments show that the hypertensive milieu, which includes increased vascular stretch, inflammatory cytokines such as IL-17a and angiotensin II interact to modulate aortic fibroblast collagen production.

Activation of p38 MAPK is required for collagen expression and aortic collagen deposition induced by stretch and inflammation in vitro and in vivo

p38 MAPK has been implicated in the induction of collagen expression and fibrosis in different hypertensive models.[31–34] Interestingly, both 10% stretch and IL-17a at 100 ng/mL induced p38MAP kinase phosphorylation in aortic fibroblasts (Figure 5A–C). Moreover, inhibition of p38 MAP kinase with SB203580 prevented the increase in mRNA for collagens 1a1, 3a1 and 5a1 caused by stretch (Figure 5D–5F). SB203580 also inhibited IL-17a-induced expression of collagen 3a1 but not collagen 5a1 (Figure 5G–I). We also found that angiotensin II-induced hypertension increased p38 MAPK phosphorylation in thoracic aortas, and that this was prevented by the normalization of blood pressure (Figure 6A–C). To gain insight into the role of p38 MAPK in vivo, we treated mice with SB203580 (10 mg/kg/day, i.p.) concurrently with angiotensin II. SB203580 reduced the hypertension caused by angiotensin II (Figure 6D and E). More importantly, SB203580 prevented collagen deposition (Figure 6F–H) and prevented the shift in aortic compliance and stress strain relationships caused by angiotensin II (Figure 6I and J). These data show that p38 MAP kinase is responsive to stretch and inflammation, and thus plays a central role in mediating collagen expression in cultured fibroblasts and in inducing aortic stiffness in vivo.

DISCUSSION

In the present study, we identified novel mechanisms of aortic stiffening associated with hypertension as delineated in the scheme shown in Figure 7. Our data indicate that elevation of blood pressure and the attendent increase in vascular stretch leads to an inflammatory process characterized by infiltration of T cells and activation of p38 MAP kinase. These events lead to a striking deposition of collagen in the aortic adventitia, with scattered amounts of collagen in the media. This is associated with a marked alteration in aortic complaince, characterized by a leftward shift in the stress strain relationship and a downward relationship between aortic distending pressure and diameter. In vivo, this

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increase in aortic stiffness leads to a loss of the Windkessel or capacitance function of the aorta, increasing systolic pressure and promoting hypertension-related end-organ damage.

Interestingly, we found that T cells and in particular the T cell-derived cytokine IL-17a contribute to aortic stiffening. We have previously shown that mice lacking IL-17a are protected against hypertension.[16] More recently, IL-17 has been shown to increase Rho kinase-mediated phosphorylation of the endothelial nitric oxide synthase, to promote endothelial dysfunction and to elevate blood pressure when administered parenteraly.[21] In keeping with a role of inflammation in collagen deposition, we found that RAG-1$^{-/-}$ mice and IL-17a$^{-/-}$ mice are protected against aortic stiffening and collagen depostion during angiotensin II infusion, and that adoptive transfer of T cells to RAG-1$^{-/-}$ mice restores these abnormalities. We cannot exclude a role of cytokines other than IL-17a. As an example, TNFα often acts synergistically with IL-17 to promote inflammatory responses. Recent studies showed that the TNFα antagoinist etanercept reduces pulse wave velocity and aortic stiffness in patients with rheumatoid arthritis.[35, 36] We have also found that mice treated with the TNFα antagonist etanercept and mice lacking IL-17a are protected against these vascular alterations.[15] Thus, it is possible that this and other cytokines act in concert with IL-17 to promote aortic stiffening.

It is likely that both CD4$^+$ and CD8$^+$ T cells contribute to aortic stiffening, as mice lacking either one alone had an intermediate phenotype. In particular, mice lacking CD8$^+$ T cells had a reduction in aortic collagen accumulation caused by angiotensin II. Likewise, adoptive transfer of only CD4$^+$ cells or only CD8$^+$ T cells to RAG-1$^{-/-}$ mice failed to restore aortic stiffening in response to angiotensin II. While CD8$^+$ T cells are generally considered cytotoxic, it is now clear that CD8$^+$ T cells produce substantial amounts of cytokines and contribute to the inflammatory millieu.[37, 38] In preliminary studies, we have found that angiotensin II infusion induces IL-17a production in several T cell subtypes in vivo.

Our data also indicate that the mechanical effect of increased blood pressure also contributes to aortic stiffening. Prevention of hypertension with hydralazine and hydrochlorothiazide prevented collagen deposition and the increase in aortic stiffness caused by chronic angiotensin II infusion. These data do not separate mechanical effects from inflammation, because blood pressure lowering also prevented aortic leukocyte iniltration. In keeping with this finding, there are numerous mechanisms by which mechanical stretch could affect vascular inflammation. As examples, increased cyclical strain, similar to that observed in hypertension, activates the NADPH oxidase and NF-κB, increases expression intracellular adhesion molecule-1 (ICAM1), and enhances adhesion of monocytes to the endothelium.[39,40]

Substantial research has focused on the role of TGFβ in tissue fibrosis and collagen deposition. In several preliminary studies, we found that mRNA for TGFβ1 is not increased in aortas from angiotensin II-treated mice nor in cultured fibroblasts exposed to stretch. This does not exclude the possibility that TGFβ activity is increased at a post-translational level in response to hypertension. Latent TGFβ is processed to its active form by components of the inflammatory milieu, including matrix metalloproteinases, reactive oxygen species, thrombospondin-1 and integrins.[41,42] Related to this, our array analysis of the genes affected

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by hypertension provides insight. This analysis showed that hypertension led to a 9-fold increase in MMP-2 and a 5-fold increase in thrombospondin-1. These could contribute to post-translational activation of latent TGFβ in the absence of changes in its expression.

In an effort to separate the direct effects of stretch and inflammation, we exposed cultured murine aortic fibrobasts to either cyclical stetch or IL-17a. We selected fibroblasts because these represent the predominant cell in the adventital region where collagen deposition occurs. Our data indicate that both of these stimuli induce mRNA of various collagen subtypes, all of which could contribute to aortic stiffening. Our data also indicate that p38 MAPK likely plays an important role in collagen expression both in cultured fibroblasts and in vivo. Several studies has shown that inhibition of p38 blocks the pro-fibrotic effects of mechanical stretch both in vitro and in vivo. Park et al have shown that inhibition of p38 MAPK reduces cardiac and renal fibrosis in double transgenic rats harboring the human renin and angiotensinogen genes.[32] Similarly p38 inhibition decreases cyclical stretch-induced collagen expression in isolated smooth muscle cells from WKY and SHR rats.[34] Of note, p38 MAPK has been shown to bypass TGFβ signaling by transactivation of Smad-2/3 in mouse models of Marfan's Syndrome and systemic inhibition of p38 blocks these effects. In addition, increased activation of p38 MAPK due to loss of MAPK phosphatase-1 activity induces expression of microRNA-21 (miR-21),[43] a critical regulator of TGF-β signaling involved in renal and myocaridal fibrosis.[44,45,46] MiR-21 inhibits Smad-7 and augments TGF-β signalling, enhancing its pro-fibrotic effects.[47] Interestingly, miR-21 also contributes to renal fibrosis by regulating MMP9/TIMP-1. Thus, p38 MAPK plays a pivital role in the interaction between vascular inflammation and mechanical stretch.

It would be desirable to differentiate the causal relationship bewteen aortic stiffening and hypertension in vivo. Recent data from the Framingham study indicate that increases in pulse wave velocity precede the development of overt hypertension in the general population.[48] Our study does not refute this, but suggests that once hypertension is initiated, aortic stiffening is markedly exagerated. In keeping with this, in mice subjected to transverse aortic constriction, adventitial collagen deposition is observed proximal to the constriction, where pressures are elevated, but not distal to the constriction where pressures are normal.[49] Likewise, a recent finding suggest that mild hypertension in children predisposes to increasesd pulse wave velocity in adulthood.[50] No matter the cause, stiffening of the proximal aorta leads to the loss of Windkessel effect, further elevating systolic blood pressure. Therefore, in the long run, aortic stiffening and hypertension likely promote one another, ultimately leading to progressive elevations in systolic blood pressure.

In the present study, the absolute amount of elastin present in the aorta was not affected by angiotensin II infusion, while the relative amount of elastin, expressed as a percent of total protein was reduced by about 50%. This relative loss of elastin might also contribute to the reduced aortic compliance observed in angiotensin II-induced hypertension. Loss of aortic elastin leads to aortic stiffening and hypertension in mice that are haploinsufficient for the elastin gene.[51] It is also possible that while total elastin is not changed, elastin damage might occur in the setting of angiotensin II-induced hypertension. Of note, MMP3, which was increased by 2.5 fold by angiotensin II infusion in our gene array analysis, has elastase activity, and could contribute to elastin fragmentation in hypertension.

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The striking deposition of collagen in the adventitial layer might not only promote vascular stiffness, but could also increase compression of vascular smooth muscle cells during systole. Our in vitro studies showed that when intraluminal pressure is increased in a normal aorta, there is relatively equal expansion of both the lumen and the outer vascular media, thus preserving medial thickness. In contrast, increasing intraluminal pressure in aortas from angiotensin II-treated mice led to an increase in the lumen that was substantially greater than that of the outer media, leading to compression of the media. This could lead to a condition in which vascular smooth muscle cells undergo cyclic compression during each cardiac cycle. The impact of this on vascular smooth muscle function remains to be defined.

In summary, this study provides new insight into mechansims of aortic stiffening in hypertension as depicted in Figure 7. An important component of this process is a striking deposition of collagen in the adventitia. Our data indicate that aortic stiffening is mediated by both mechanical factors assoicated with elevated pressures and by T cells and their release of inflammatory cytokines. The p38 MAP kinase likely plays an important signalling role in response to these stimuli. Adventitial collagen deposition and aortic stiffening can not only be considered a form of end-organ damage in hypertension but can also further elevate systolic pressure, leading to progression of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

RAG-1	Recombination activating gene-1
DOCA	Deoxycorticosterone acetate
IL-17a	Interleukin 17a
ROS	Reactive oxygen species
T_H17 cells	T helper cells producing interleukin 17
CD	Cluster of differentiation
FBS	Fetal bovine serum
ANOVA	Analysis of variance
WT	Wild-type

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MMP	Matrix metalloproteinase
TGFβ1	Transforming growth factor beta1
HCTZ	Hydrochlorothiazide
Hyd	Hydralazine

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Novelty and Significance

What Is Known?

- Aortic stiffening occurs in hypertension in humans and increases systolic pressure.
- Mild hypertension in childhood predisposes to aortic stiffening in adults. Conversely, adults with stiff aortas subsequently develop worse hypertension.
- Hypertension increases mechanical stretch of the aorta and induces vascular inflammation.

What New Information Does This Article Contribute?

- Angiotensin II and Deoxycorticosterone Acetate (DOCA)-salt hypertension causes formation of a “rind” of collagen that surrounds the aorta, which causes marked aortic stiffening.
- Inflammation and mechanical stretch seem to promote the production of collagen in the adventitial cells of the aorta, and this seems dependent on the p38 MAP kinase.

The normal aorta is compliant and able to expand with each heartbeat, accommodating a portion of the stroke volume in a capacitance fashion. In a variety of common conditions like aging, diabetes, obesity, atherosclerosis and smoking, the aorta becomes stiff such that the stroke volume from each heartbeat is rapidly ejected to the distal tissues. This raises systolic blood pressure and predisposes to stroke, renal failure and heart failure. The exact causes of aortic stiffening are unknown. In this study we show that inflammation, in the form of T cells and cytokines like interleukin 17 act in concert with the increased mechanical stretch caused by elevated blood pressure to induce aortic collagen production and aortic stiffening. We found that the MAP kinase p38 plays a central role in signaling these processes. Efforts to decrease aortic mechanical stretch and to reduce vascular inflammation might be effective in preventing aortic stiffening.

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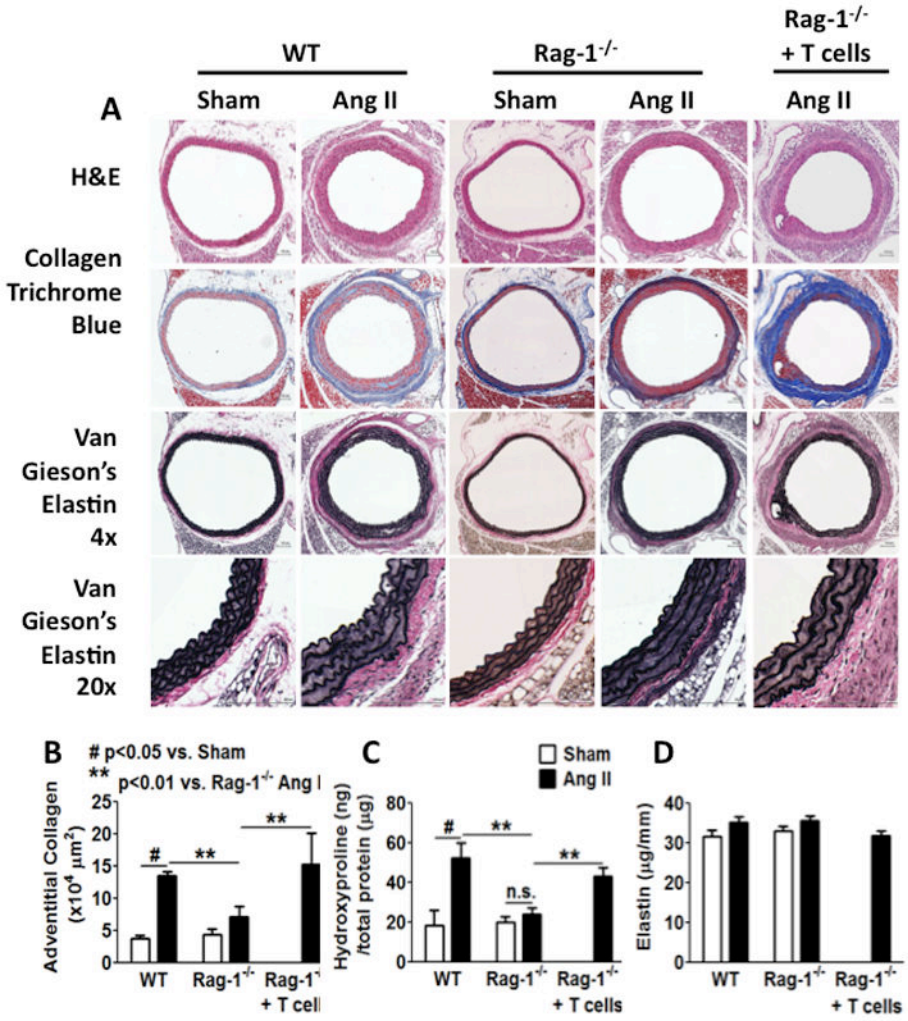

Figure 1. Role of T cells in aortic collagen deposition in hypertension

A, Effects of angiotensin II-induced hypertension on vascular collagen and elastin in WT and RAG-1$^{-/-}$ mice. Perfusion-fixed sections of the thoracic aortas were sectioned (6 mm) and stained with Hematoxylin and Eosin, Masson's trichome and Van Gieson's Elastica staining to highlight collagen (blue) and elastin (black). Scale bar indicates 100 μm. B, Adventitial collagen area was quantified by planimetry. C, Aortic collagen quantification by hydroxyproline assay. Isolated thoracic aortas were digested in 6 N HCl at 105 °C for 48 hours before hydroxyproline was quantified. D, Elastin was quantified biochemically by initial separation in 0.1 N NaOH at 90 °C for 30 min and subsequently by ninhydrin assay. Ang II, angiotensin II. Data were analyzed using two-way ANOVA, n=6–9.

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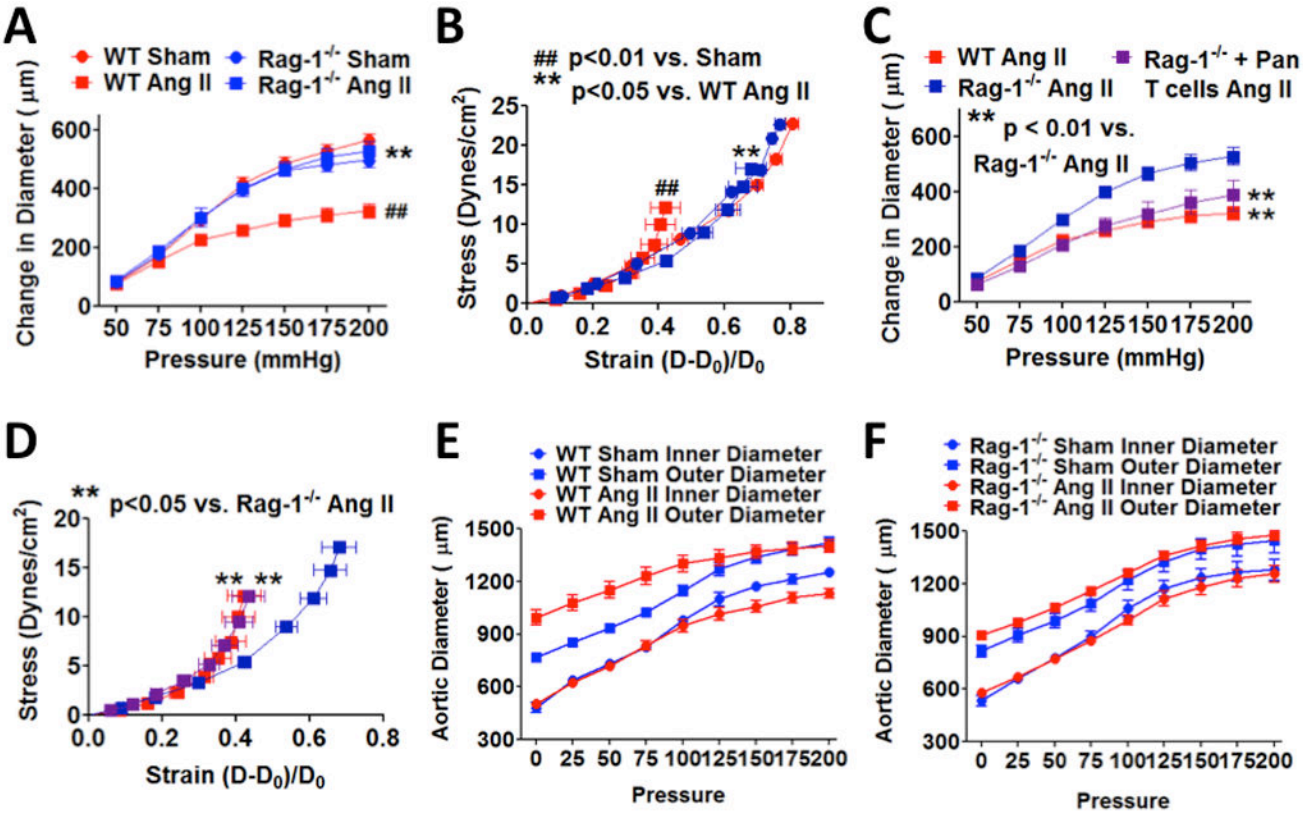

Figure 2. T cells mediate angiotensin II-induced aortic stiffening

Freshly-isolated aortas were mounted on a myograph system in Ca^{2+}-free buffer to determine pressure-diameter relationships. A–D, compliance curves and stress-strain relationships were constructed from inner diameter and outer diameter. E and F, inner and outer diameter of WT and RAG-$1^{-/-}$ mice measured at 25 mmHg step changes in pressure from 0–200 mmHg. Ang II, angiotensin II. Data were analyzed using one-way ANOVA with repeated measures, n=6–9.

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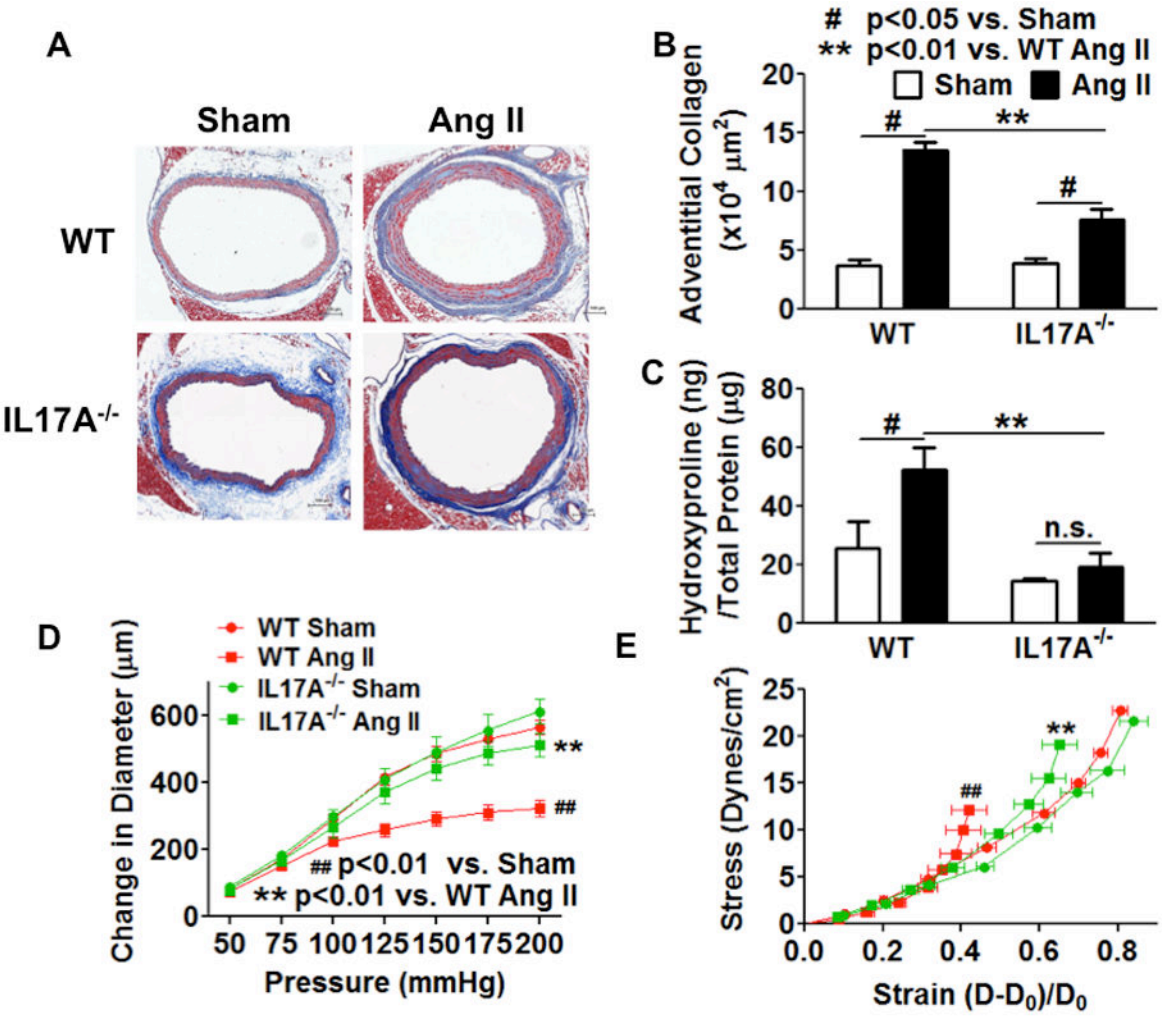

Figure 3. Role of IL-17a in aortic stiffening

IL-17a deficient mice were infused with chronic angiotensin II (490ng/kg/min) for 14 days. A and B, the thoracic aortas were fix-perfused for Masson's trichrome staining and quantified for adventitia collagen staining. Scale bar indicates 100 μm. C, segments of the thoracic aorta were digested in 6N HCl at 105 °C for 48 hours and measured for hydroxyproline concentration. Data analyzed using two-way ANOVA. D and E, compliance curves and stress-strain curves for IL-17a$^{-/-}$ mice. Ang II, angiotensin II. Data analyzed using one-way ANOVA with repeated measures, n=6–8.

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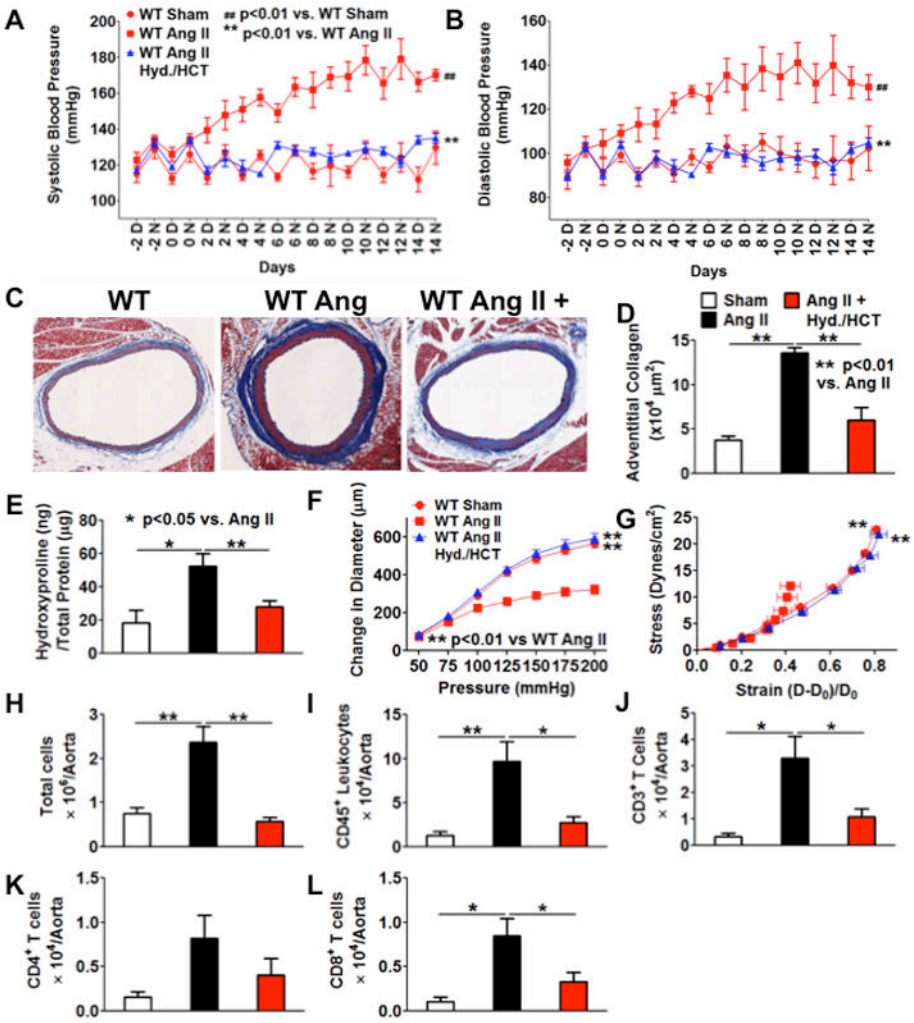

Figure 4. Antihypertensive treatment (antiHBP) prevents collagen deposition, aortic stiffening and T cell infiltration

Mice were treated with hydralazine and hydrochlorothiazide (320 mg/L and 60 mg/L in the drinking water) concurrently with angiotensin II infusion. Hyd, Hydralazine, HCTZ, hydrochlorothiazide. A and B, telemetry recording of blood pressures. D, day; N, night. The effects of blood pressure normalization on collagen depostion (C–E), aortic stiffening (F and G) and aorfic inflammatory cell infiltration (H–L) are shown. Scale bar indicates 100 μm. Ang II, angiotensin II. Data for blood pressure and aortic stiffness were analyzed using one-way ANOVA with repeated measurements (n=8). Collagen deposition and flow cytometry were analyzed using one-way ANOVA (n=8).

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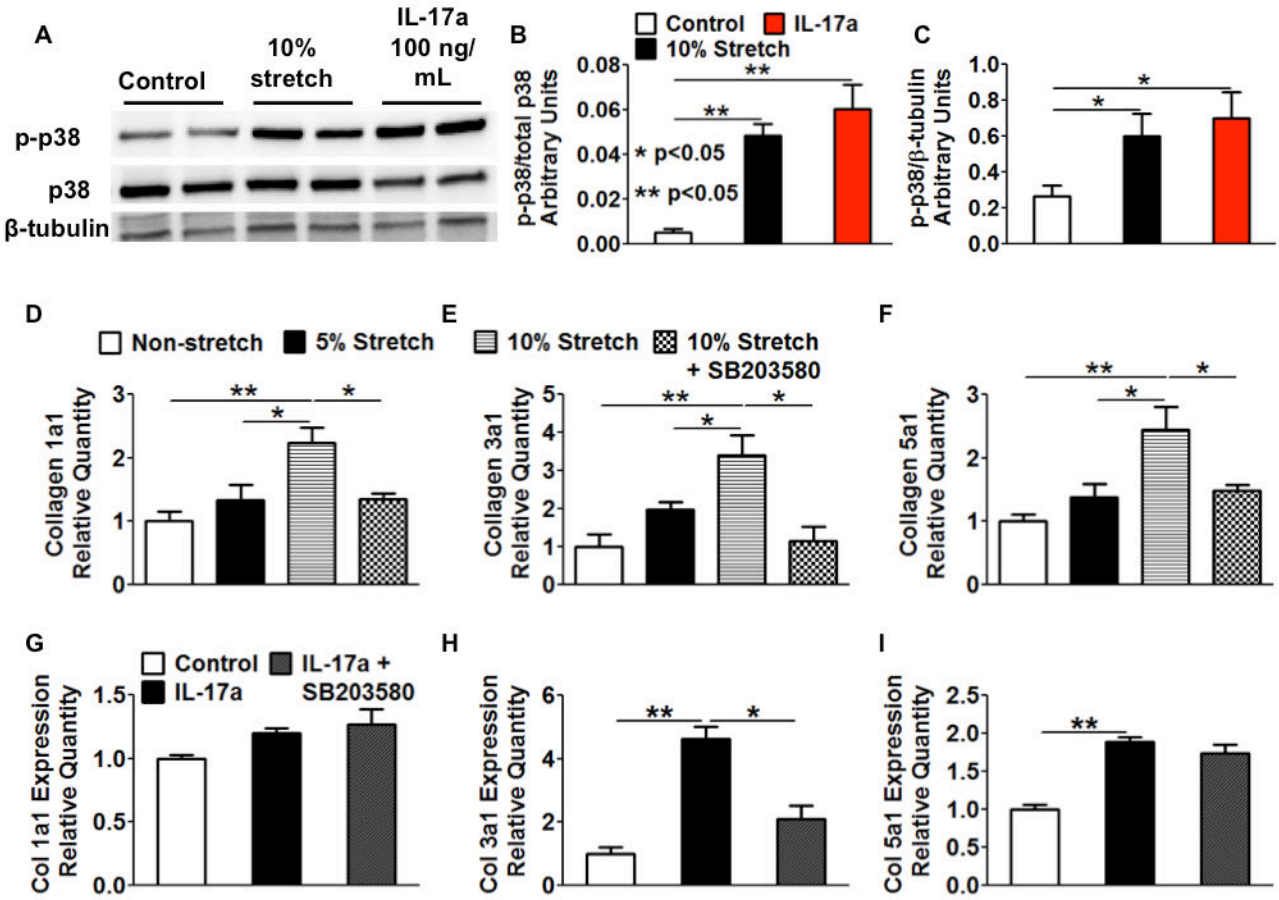

Figure 5. Effect of cyclical stretch and IL-17a on aortic fibroblasts and role of p38 MAP kinase

Mouse aortic fibrobasts were exposed to various degrees of cyclical stretch or the T cell cytokine IL-17a (100 ng/mL) in culture for 36 hours. A–C, both hypertensive mechanical stretch and IL-17a activated p38 MAPK in cultured mouse aortic fibroblasts. D–F, The effects of p38 inhibitor SB203580 (10 ng/mL) on mechanical stretch-induced expression of collagen subtypes in fibroblasts. G–I, The effects of p38 inhibition on IL-17a-induced collagen expression. Data analyzed using one-way ANOVA, n=3–6.

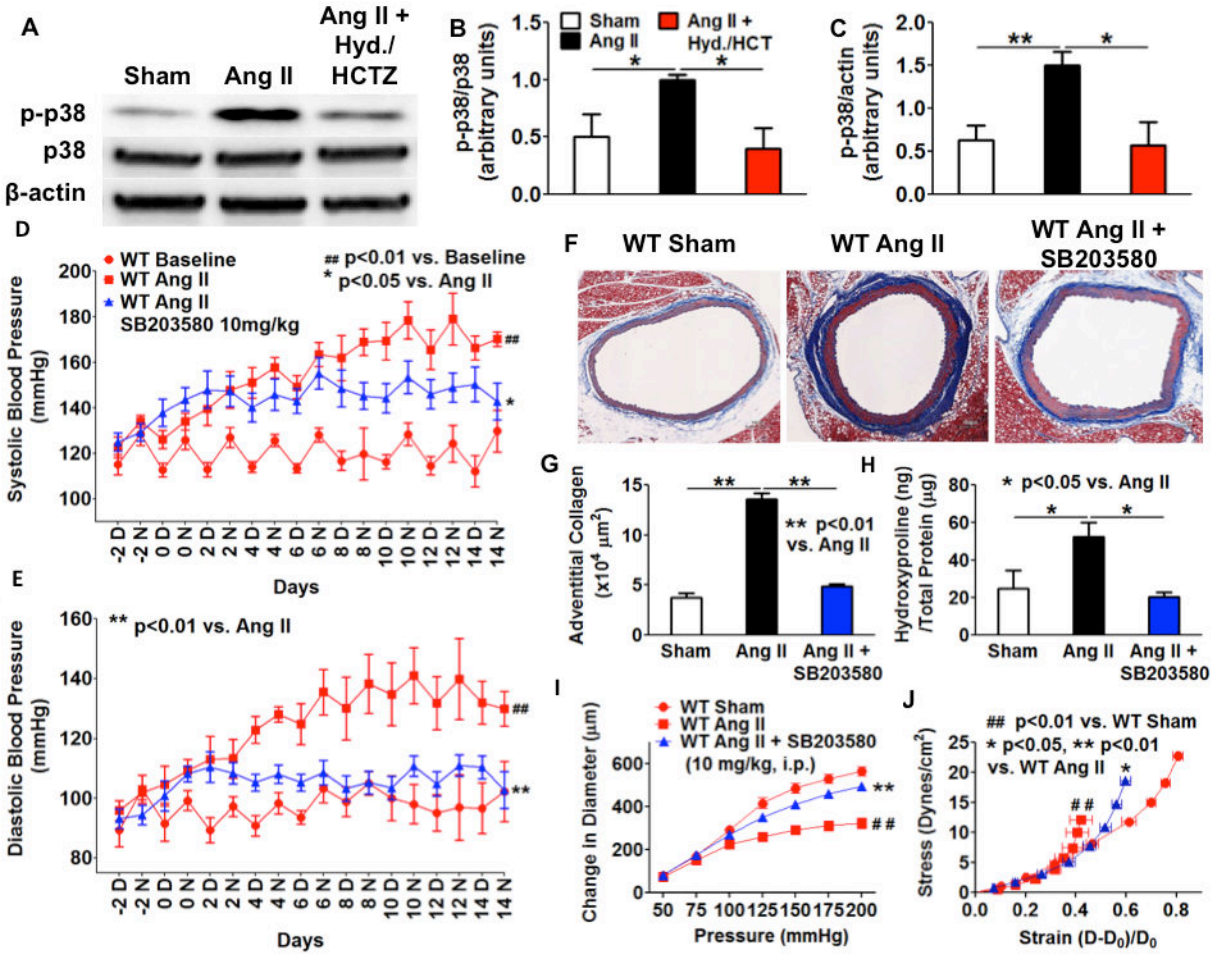

Figure 6. P38 MAP kinase mediates collagen deposition and aortic stiffening in angiotensin II-induced hypertension

A–C, Normalization of blood pressure prevented the activation of p38 MAP kinase in angiotensin II-infused mouse aortas. Data analyzed with one-way ANOVA (n=4–6). D and E, Effect of SB203580 on blood pressure. D, day; N, night. F–H, Effect of SB203580 on aortic collagen deposition. Data analyzed using one-way ANOVA (n=6–8). Mice received intraperitoneal injections of SB203580 (10 mg/kg/day) during angiotensin II infusion. Scale bar indicates 100 μm. I and J, Effect of SB203580 on aortic stiffening. Ang II, angiotensin II. Blood pressure and aortic stiffness were analyzed using one-way ANOVA with repeated measures (n=6–8).

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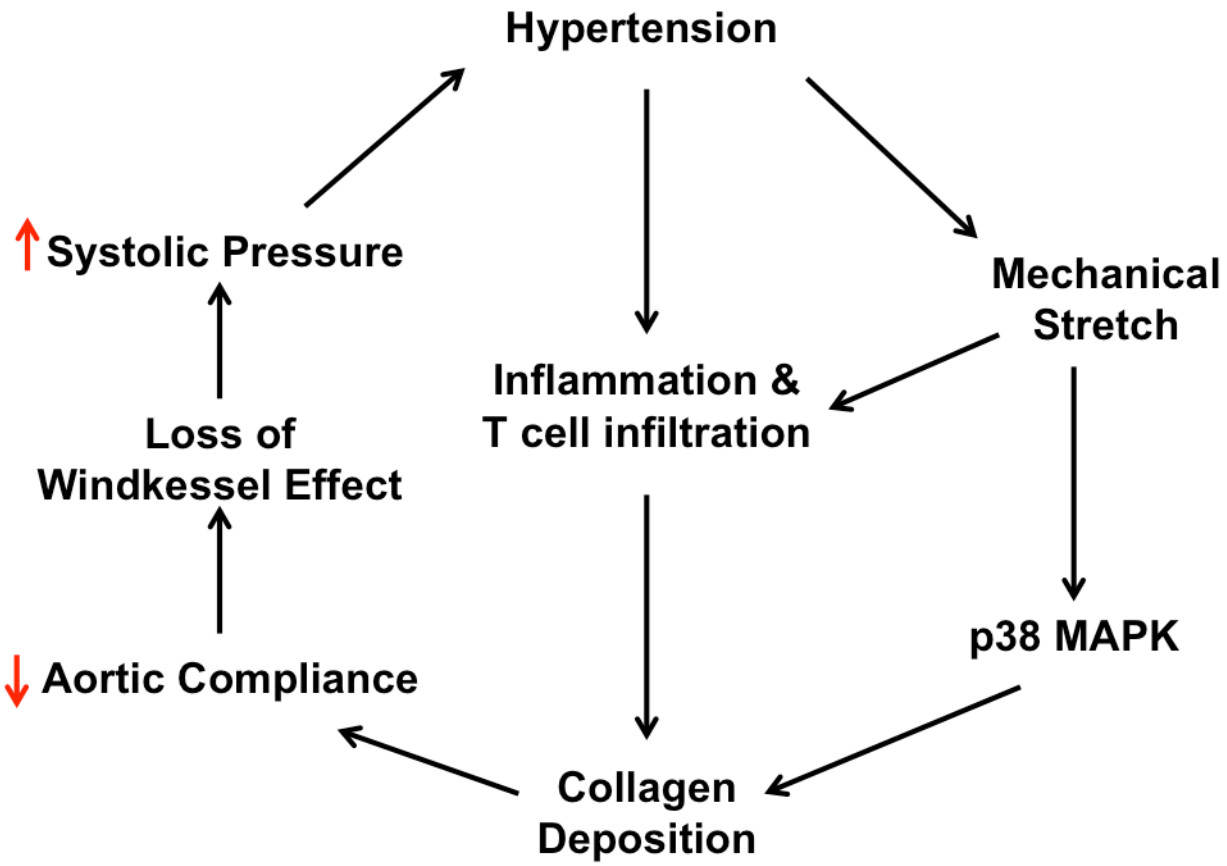

Figure 7. Pathway showing interactions of mechanical stretch and inflammation in aortic stiffening

Increased mechanical stretch promotes perivascular T cell infiltration and activates p38 MAP kinase, leading to adventitial collagen deposition. This reduces aortic compliance and ultimately leads to loss of windkessel effect in large capacitance arteries such as the aorta.

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Table 1

Effects of IL-17a, angiotensin II and mechanical stretch on collagen expression by mouse aortic fibroblasts

Treatment				Angiotensin II (100 nM)		
	Col 1a1	Col 3a1	Col 5a1	Col 1a1	Col 3a1	Col 5a1
Control	1.00±0.05	1.00±0.34	1.00±0.12	1.03±0.02	1.59±0.08	1.24±0.25
IL-17a (100 ng/mL)	1.20±0.07	4.63±0.66 **	1.89±0.10 *	1.12±0.12	4.28±0.33 **	1.74±0.20
5% Stretch	1.33±0.25	1.98±0.20	1.39±0.21	NA	NA	NA
10% Stretch	2.24±0.24**	3.40±0.53 ** #	2.45±0.37 **	2.80±0.34 **	7.50±1.43 **	2.68±0.62**
IL-17a (100 ng/mL) + 10% Stretch	2.16±0.13* *	3.67±0.41 **	2.32±0.43 **	NA	NA	NA

Primary mouse aortic fibroblasts were exposed to 5% or 10% cyclic stretch or exposed to IL-17a (100 ng/mL) in the presence or absence of angiotensin II (100 nM) for 36 hours. Relative quantity of mRNA was calculated based on the ΔΔCt value normalized to control. Data are expressed as mean ± SE. The effects of IL17a and mechanical stretch are analyzed with one-way ANOVA, while the effect of angiotensin II by two-way ANOVA (n=3–6),

*p<0.05,

**p<0.01 vs. control;

#p<0.05 vs. angiotensin II. NA, not assessed.