



# Endothelial NADPH Oxidase-2 Promotes Interstitial Cardiac Fibrosis and Diastolic Dysfunction Through Proinflammatory Effects and Endothelial-Mesenchymal Transition

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<b>Objectives</b>	This study sought to investigate the effect of endothelial dysfunction on the development of cardiac hypertrophy and fibrosis.
<b>Background</b>	Endothelial dysfunction accompanies cardiac hypertrophy and fibrosis, but its contribution to these conditions is unclear. Increased nicotinamide adenine dinucleotide phosphate oxidase-2 (NOX2) activation causes endothelial dysfunction.
<b>Methods</b>	Transgenic mice with endothelial-specific NOX2 overexpression (TG mice) and wild-type littermates received long-term angiotensin II (AngII) infusion (1.1 mg/kg/day, 2 weeks) to induce hypertrophy and fibrosis.
<b>Results</b>	TG mice had systolic hypertension and hypertrophy similar to those seen in wild-type mice but developed greater cardiac fibrosis and evidence of isolated left ventricular diastolic dysfunction ( $p < 0.05$ ). TG myocardium had more inflammatory cells and VCAM-1-positive vessels than did wild-type myocardium after AngII treatment (both $p < 0.05$ ). TG microvascular endothelial cells (ECs) treated with AngII recruited 2-fold more leukocytes than did wild-type ECs in an <i>in vitro</i> adhesion assay ( $p < 0.05$ ). However, inflammatory cell NOX2 per se was not essential for the profibrotic effects of AngII. TG showed a higher level of endothelial-mesenchymal transition (EMT) than did wild-type mice after AngII infusion. In cultured ECs treated with AngII, NOX2 enhanced EMT as assessed by the relative expression of fibroblast versus endothelial-specific markers.
<b>Conclusions</b>	AngII-induced endothelial NOX2 activation has profound profibrotic effects in the heart <i>in vivo</i> that lead to a diastolic dysfunction phenotype. Endothelial NOX2 enhances EMT and has proinflammatory effects. This may be an important mechanism underlying cardiac fibrosis and diastolic dysfunction during increased renin-angiotensin activation. (J Am Coll Cardiol 2014;63:2734–41) © 2014 by the American College of Cardiology Foundation

Endothelial dysfunction accompanies conditions such as hypertension that predispose to adverse cardiac remodeling and predicts future cardiovascular morbidity (1). As well as

modulating vascular function, the endothelium has direct effects on cardiac function (2–4). Pioneering work by Brutsaert (3) and co-workers demonstrated that cardiac endothelial cells (ECs) directly influence myocardial contraction, a concept later confirmed in humans *in vivo* (5).

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The importance of endothelial-cardiomyocyte crosstalk has been demonstrated in several disease settings (3,6). However, the role of endothelial dysfunction in the pathogenesis of adverse cardiac remodeling caused by increased renin-angiotensin activation remains unclear.

A major driver of endothelial dysfunction is the reactive oxygen species (ROS)-generating enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX2), which is activated by angiotensin II (AngII) and other agonists (7). NOX2 is expressed in endothelium, as well as cardiomyocytes, adventitial fibroblasts, inflammatory cells, and conduit vascular smooth muscle cells. Vascular NOX2 expression and activity are increased in hypertension, in which NOX2 modulates intracellular signaling pathways that promote vascular remodeling, contributes to vasodilator dysfunction through superoxide-mediated inactivation of nitric oxide, and may have proinflammatory effects (7).

To investigate the pathophysiological roles of endothelial NOX2, the authors generated a transgenic mouse model with endothelial-specific NOX2 overexpression (TG) (8). This model has a 2-fold increase in endothelial NOX2 protein levels and displays augmented NOX2 activation in response to AngII. Here, we used this model to investigate the effects of endothelial NOX2 activation on the development of cardiac hypertrophy, fibrosis, and dysfunction during chronic AngII elevation.

## Methods

Detailed methods are provided in the [Online Appendix](#).

Animal experiments complied with U.K. Home Office regulations. Male TG mice (8) were compared with wild-type littermates. Chimeric mice were generated by bone marrow (BM) transplantation (9). AngII (1.1 mg/kg/day) or vehicle was infused via osmotic minipumps. Blood pressure (BP) was measured by radiotelemetry or tail-cuff plethysmography (10). Left ventricular (LV) pressure-volume relationship measurements and echocardiography were performed as described (11). Cardiac fibrosis was quantified in Picosirius Red-stained paraffin sections, whereas immunostaining was performed in cryosections. LV homogenate NOX activity was measured using lucigenin-enhanced chemiluminescence (12).

In vitro leukocyte adhesion to coronary microvascular endothelial cells (CMECs) from TG or wild-type mouse hearts was studied in a flow chamber (13), with or without AngII (0.1  $\mu\text{mol/l}$ , 4 h). To assess endothelial-mesenchymal transition (EMT), cultured human aortic ECs were infected with Ad. $\beta\text{Gal}$  or Ad.NOX2 virus and were then treated with 0.1  $\mu\text{mol/l}$  AngII or vehicle for up to 5 days. Co-culture experiments with ECs and fibroblasts were performed in Transwell dishes (Corning B.V. Life Sciences, Amsterdam, the Netherlands).

Data are expressed as mean  $\pm$  SEM. Comparisons were made by repeated measures analysis of variance (ANOVA), 2-way ANOVA with Bonferroni post-hoc testing, 1-way ANOVA, or Student t test, as appropriate;  $p < 0.05$  was considered significant.

## Results

**Effects of endothelial NOX2 overexpression on angiotensin II-induced hypertension and cardiac hypertrophy.** Long-term AngII infusion for 14 days caused similar systolic hypertension in TG and wild-type mice ([Online Fig. 1A](#)). LV NADPH oxidase activity increased in both AngII-treated groups, with a significantly greater rise in TG mice ([Online Figs. 1B](#) and [Online Fig. 2](#)). AngII infusion induced a similar increase in left ventricle/body weight ratio or cardiomyocyte cross-sectional area in TG and wild-type mice ([Online Figs. 1C](#) and [1D](#)).

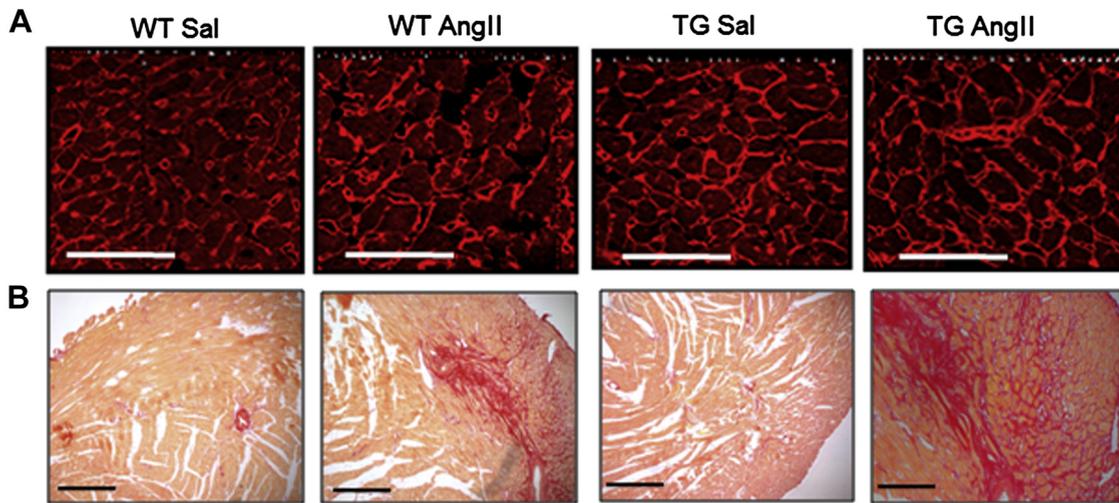
**Endothelial NOX2 overexpression potentiates angiotensin II-induced cardiac fibrosis.** The

extent of AngII-induced fibrosis was significantly ( $\sim 2$ -fold) greater in TG than in wild-type hearts ([Online Fig. 1E](#)). TG hearts also had significantly higher procollagen I messenger ribonucleic acid (mRNA) expression than did wild-type hearts ([Online Fig. 1F](#)). Representative examples of the fibrosis and myocyte hypertrophy are shown in [Figure 1](#).

**Left ventricular diastolic dysfunction with preserved systolic function in endothelial NOX2 TG mice.** TG hearts had significantly lower echocardiographic LV end-diastolic dimensions than did wild-type hearts after long-term AngII infusion ([Online Fig. 3](#)). However, there were no significant differences in indices such as interventricular septal thickness or ejection fraction. More detailed assessment was undertaken by pressure-volume analysis. Systolic function assessed by end-systolic elastance or LV  $\text{dP/dt}_{\text{max}}$ /end-diastolic volume relation was similar in AngII-treated wild-type and TG groups ([Online Figs. 4A](#) and [4B](#)). LV end-diastolic volume increased in wild-type mice after AngII but decreased in TG mice ([Online Fig. 4C](#)). LV end-systolic volume tended to increase in wild-type mice after AngII treatment but fell significantly in TG mice ([Online Fig. 4D](#)). As a result, both stroke volume and stroke work were significantly lower in AngII-treated TG hearts versus wild-type hearts ([Online Fig. 4E](#), [Table 1](#)). Isovolumic LV relaxation time-constant and LV end-diastolic pressure were similar in wild-type and TG groups ([Table 1](#)). However, LV diastolic stiffness assessed by the end-diastolic pressure volume relationship was significantly higher in AngII-treated TG hearts versus wild-type hearts ([Online Fig. 4F](#)). Although there was no difference in systolic BP, arterial elastance was significantly higher in AngII-treated TG hearts ([Table 1](#)). [Online Figures 4G](#) and [4H](#) show representative steady-state pressure-volume loops and the

### Abbreviations and Acronyms

AngII	= angiotensin II
BM	= bone marrow
BP	= blood pressure
CMEC	= coronary microvascular endothelial cell
EC	= endothelial cell
EMT	= endothelial-mesenchymal transition
LV	= left ventricular
mRNA	= messenger ribonucleic acid
NOX	= nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
ROS	= reactive oxygen species
TG	= transgenic
VCAM	= vascular cell adhesion molecule



**Figure 1** Representative Myocardial Sections Showing Cardiac Hypertrophy and Fibrosis

(A) Cardiomyocyte borders outlined using wheat-germ agglutinin. Scale bars, 50  $\mu\text{m}$ . (B) Picosirius Red–stained sections. Scale bars, 100  $\mu\text{m}$ . AngII = angiotensin II; Sal = saline; TG = transgenic; WT = wild-type.

response to preload reduction in wild-type and TG hearts. These findings indicate that TG hearts were smaller and stiffer than were wild-type hearts after long-term AngII treatment, thus indicating a phenotype of isolated diastolic LV dysfunction with preserved systolic function.

**Endothelial NOX2 enhances myocardial inflammatory cell infiltration.** To investigate mechanisms underlying the increased fibrosis in TG, the authors assessed myocardial inflammatory cell infiltration. TG hearts had significantly more CD45<sup>+</sup> inflammatory cells and Mac3<sup>+</sup> cells (macrophages) after AngII infusion than did wild-type hearts

(Figs. 2A and 2B, Online Fig. 5). AngII treatment increased connective tissue growth factor and osteopontin mRNA levels, but levels were similar in wild-type and TG groups (Online Fig. 6). There was no difference between groups in transforming growth factor- $\beta$ , tumor necrosis factor- $\alpha$ , monocyte chemoattractant protein-1, or endothelin-1 mRNA levels.

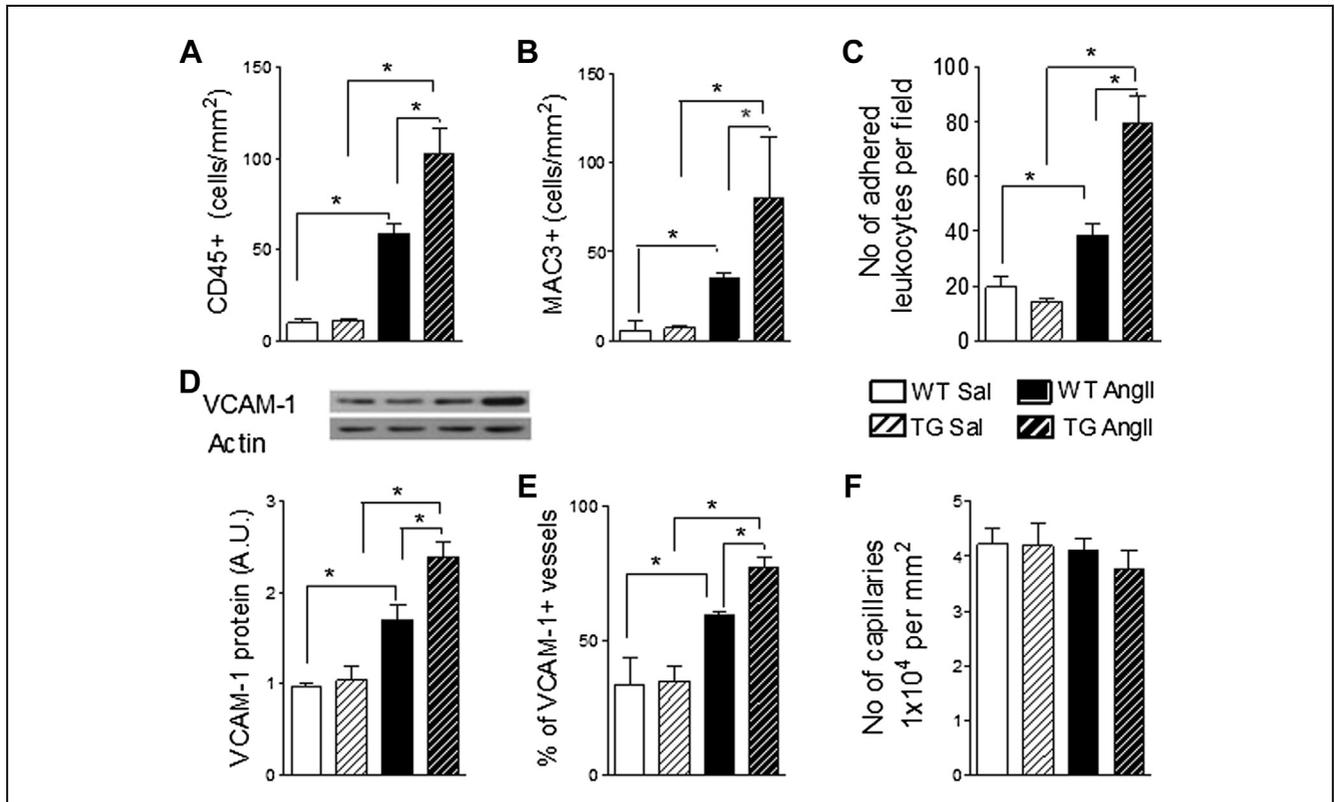
To confirm that the effects of endothelial NOX2 on inflammatory infiltration and fibrosis were ROS dependent, mice were treated with the antioxidant *N*-acetylcysteine concurrent with AngII infusion. BP and cardiac hypertrophy

**Table 1** Cardiac Function Assessed by LV Pressure-Volume Loops in WT and TG Mice

	WT Saline	WT AngII	TG Saline	TG AngII
Heart rate, beats/min	552 $\pm$ 9	509 $\pm$ 17	543 $\pm$ 14	543 $\pm$ 12
LVEDP, mm Hg	7.5 $\pm$ 0.7	5.0 $\pm$ 0.8	6.9 $\pm$ 1.1	8.1 $\pm$ 2.3
dP/dt <sub>max</sub> , mm Hg/min	7,804 $\pm$ 459	8,348 $\pm$ 365	7,497 $\pm$ 529	8,098 $\pm$ 435
dP/dt <sub>min</sub> , mm Hg/min	-6,497 $\pm$ 406	-6,654 $\pm$ 417	-6,600 $\pm$ 544	-6,843 $\pm$ 604
Stroke volume, $\mu\text{l}$	16.7 $\pm$ 0.9	22.8 $\pm$ 1.7*	15.4 $\pm$ 1	16.3 $\pm$ 1.6*
End-systolic volume, $\mu\text{l}$	17.8 $\pm$ 1.1	21.9 $\pm$ 3.6	17.4 $\pm$ 1.4	13.7 $\pm$ 0.9†
End-diastolic volume, $\mu\text{l}$	29.5 $\pm$ 1.7	39.9 $\pm$ 3.7*	29.0 $\pm$ 1.4	25.3 $\pm$ 1.5†
Ejection fraction, %	53 $\pm$ 1	57 $\pm$ 5	52 $\pm$ 4	60 $\pm$ 4
Cardiac output, ml/min/g	105.5 $\pm$ 8.1	112.0 $\pm$ 11.5	91.2 $\pm$ 5.0	82.3 $\pm$ 8.2‡
Stroke work, mm Hg/ $\mu\text{l}$ /g	14,472 $\pm$ 1,280	17,216 $\pm$ 1,888	12,365 $\pm$ 865	11,569 $\pm$ 1,324‡
Ea, mm Hg/ $\mu\text{l}$	5.7 $\pm$ 0.2	4.6 $\pm$ 0.4	5.9 $\pm$ 0.4	7.0 $\pm$ 1.0§
Tau-Weiss, ms	7.4 $\pm$ 0.4	6.2 $\pm$ 0.6	7.4 $\pm$ 0.7	7.8 $\pm$ 1.2
dP/dt <sub>max</sub> /EDV	261.9 $\pm$ 39.6	258.8 $\pm$ 32.0	273.9 $\pm$ 73.0	334.8 $\pm$ 71.1
Ees	4.28 $\pm$ 0.38	8.91 $\pm$ 0.69	4.78 $\pm$ 0.40	8.91 $\pm$ 1.28
EDPVR	0.22 $\pm$ 0.03	0.25 $\pm$ 0.04	0.20 $\pm$ 0.02	0.46 $\pm$ 0.11*‡
Number	10	10	9	8

\*p < 0.01 for effect of AngII within the group. †p < 0.001 for comparison of the TG AngII group with the WT AngII group. ‡p < 0.05 for comparison of the TG AngII group with the WT AngII group. §p < 0.01 for comparison of the TG AngII group with the WT AngII group. ||p < 0.001 for effect of AngII within the group.

AngII = angiotensin II; Ea = arterial elastance; EDV = end-diastolic volume; Ees = end-systolic elastance; EDPVR = end-diastolic pressure volume relation; TG = transgenic; WT = wild-type.



**Figure 2** Inflammatory Cell Infiltration In Vivo and Endothelial-Leukocyte Interaction In Vitro

(A, B) Mean number of CD45<sup>+</sup> and Mac3<sup>+</sup> cells/field, respectively. (C) Number of leukocytes adherent to wild-type (WT) or transgenic (TG) endothelial cells with or without angiotensin II (AngII) treatment (0.1  $\mu$ M, 4 h) in an in vitro assay. (D) Vascular cellular adhesion molecule-1 (VCAM-1) protein levels in WT and TG endothelial cells treated with AngII. Representative Western blots (top), mean data (bottom). (E) Number of VCAM-1-positive vessels in myocardial sections from WT and TG. (F) Capillary density in myocardial sections from WT and TG (capillaries/mm<sup>2</sup>). \*p < 0.05 by 2-way analysis of variance; n = 6 to 8/group. Sal = saline.

were similar in wild-type and TG groups treated with *N*-acetylcysteine (Online Fig. 7). *N*-Acetylcysteine markedly reduced CD45<sup>+</sup> cell number in both wild-type and TG AngII-treated groups and prevented the development of fibrosis (Online Fig. 7).

**NOX2 enhances endothelial activation and leukocyte-endothelial interaction.** We assessed the interaction between inflammatory cells and NOX2-overexpressing ECs in an in vitro adhesion assay under flow conditions. Leukocyte attachment to TG and wild-type CMECs was low at baseline, but after AngII treatment (0.1  $\mu$ M, 4 h) significantly more leukocytes attached to TG CMECs (Fig. 2C). TG CMECs had significantly higher vascular cell adhesion molecule-1 (VCAM-1) protein expression than did wild-type CMECs after AngII treatment (Fig. 2D). In line with this, LV sections of TG hearts treated with long-term AngII infusion had more VCAM-1-positive blood vessels than did wild-type hearts (Fig. 2E, Online Fig. 8). There was no difference in capillary density between TG and wild-type hearts (Fig. 2F).

**Inflammatory cell NOX2 is not essential for angiotensin II-induced cardiac fibrosis.** Because NOX2 is abundantly expressed in inflammatory cells, the authors investigated

whether NOX2 in these cells was required for the profibrotic response to AngII. We used BM transplantation to create chimeric mice with different NOX2 genotypes in BM and resident tissue cells: 1) wild-type recipient mice with wild-type BM (WT:WT); 2) wild-type recipients with BM from global NOX2 knockout mice (KO:WT); 3) global NOX2 knockout mice with wild-type BM (WT:KO); 4) NOX2 TG with TG BM (TG:TG); 5) NOX2 TG with wild-type BM (WT:TG); and 6) wild-type mice with TG BM (TG:WT). Successful alteration of NOX2 genotype after BM transplantation was confirmed by assessing ROS production in recipient BM and peripheral blood mononuclear cells (Online Fig. 9A).

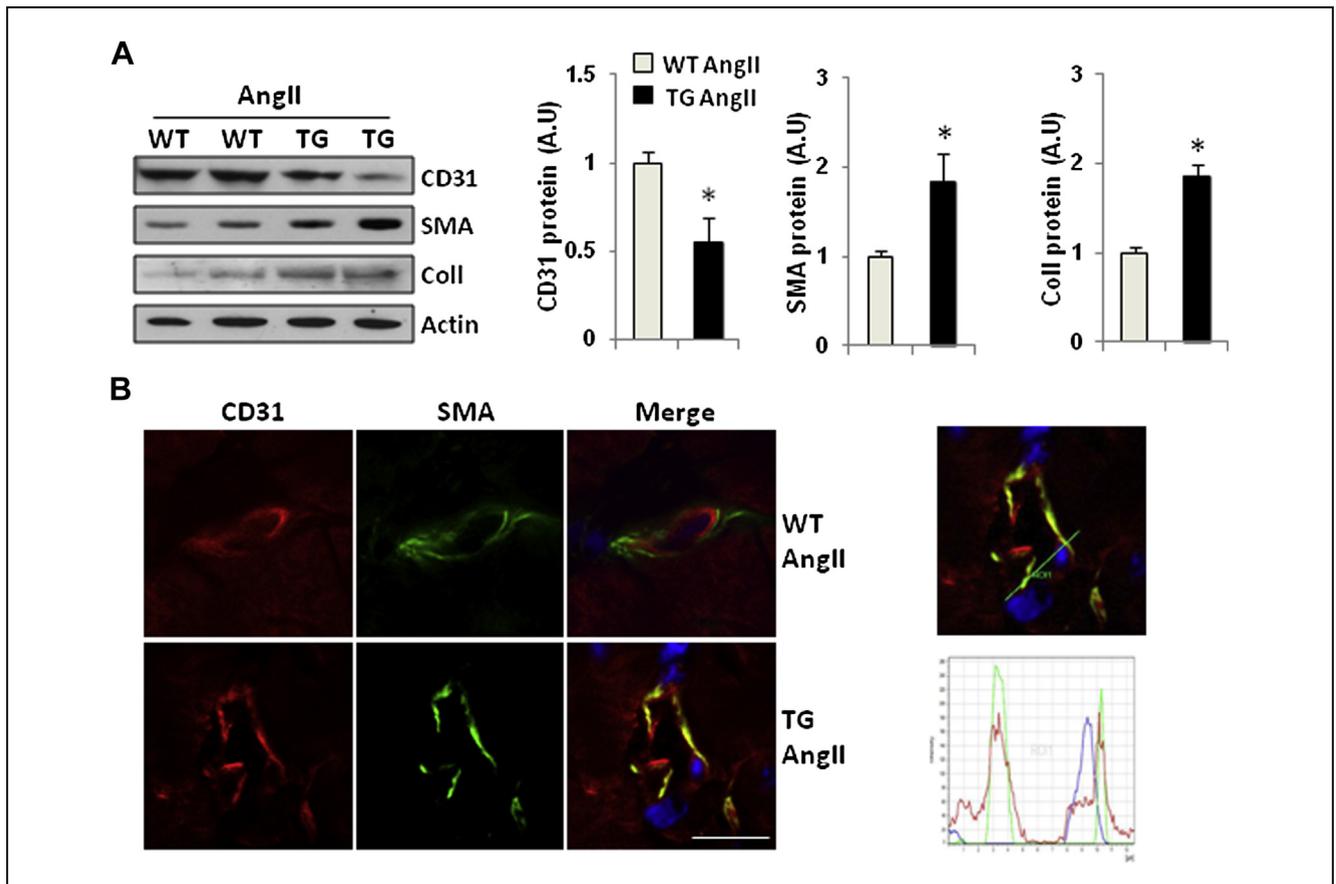
AngII infusion caused similar increases in BP and hypertrophy among groups (Online Figs. 9B and 9C). AngII-stimulated cardiac fibrosis was significantly lower in global NOX2 knockout versus wild-type mice, as reported previously (12) (Online Fig. 10). WT:WT and KO:WT mice both had fibrosis similar to wild-type mice that did not undergo BM transplantation (Online Figs. 10A and 10B). In contrast, WT:KO mice had significantly less fibrosis, similar to knockout mice that did not undergo BM transplantation (Online Figs. 10A and B). Thus, knockout BM

cells did not significantly alter AngII-induced fibrosis. Fibrosis in NOX2 TG: TG mice was similar to that in WT: TG mice, whereas transplantation of TG BM to wild-type mice did not increase the level of fibrosis to that in the TG: TG group (Online Fig. 10A right-hand panels, Online Fig. 10B). Thus, TG BM cells did not significantly alter AngII-induced fibrosis. Taken together, these results indicate that NOX2 in BM cells is dispensable for the profibrotic effects of AngII, and it is NOX2 in the resident tissue cells that is essential. Furthermore, endothelium-targeted NOX2 overexpression does not significantly alter the effects of BM cells as compared with wild-type groups.

**NOX2 promotes endothelial-mesenchymal transition.** Studies indicate that EMT is an important contributor to cardiac fibrosis during chronic pressure overload (14). The authors therefore investigated whether enhancing NOX2 activity in ECs affected EMT. Hearts of AngII-treated TG mice had significantly higher levels of fibroblast-specific  $\alpha$ -smooth muscle actin and collagen-1 than wild-type, whereas levels of endothelial-specific CD31 were

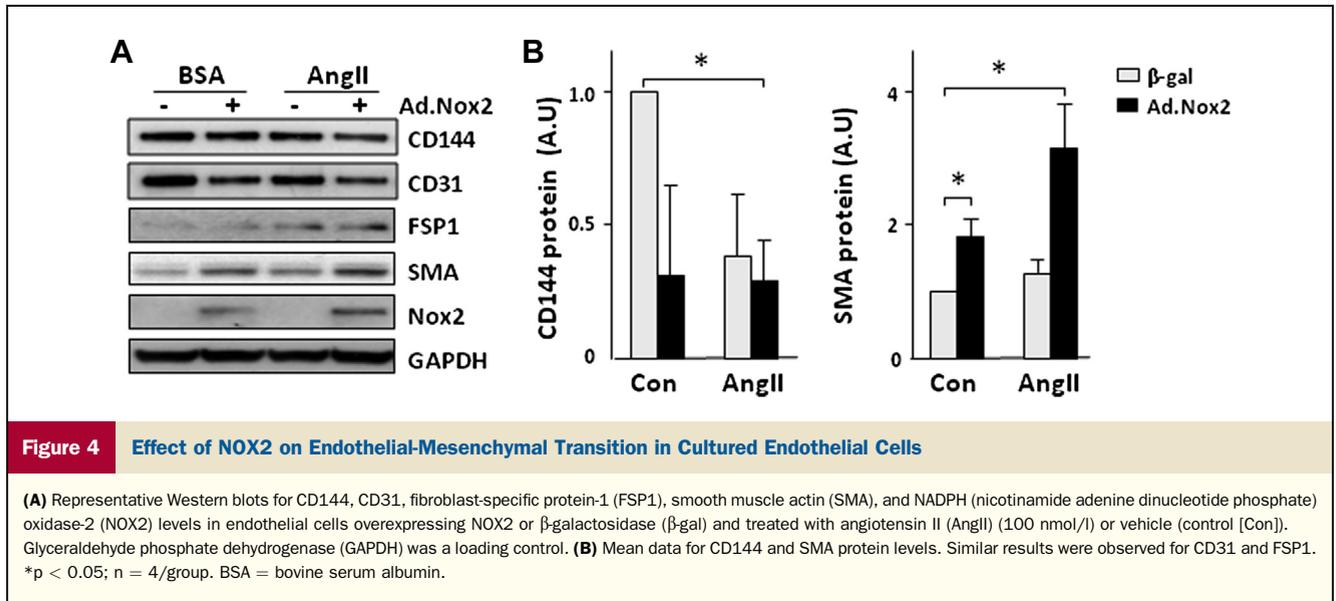
significantly lower (Fig. 3A). Myocardial sections of AngII-treated TG mice showed increased evidence of EMT, as indicated by co-localization of endothelial-specific and mesenchymal markers, compared with wild-type mice (Fig. 3B).

To assess the effects of endothelial NOX2 further, the authors studied cultured human aortic ECs with adenovirally mediated overexpression of NOX2 or  $\beta$ -galactosidase control and treated the ECs with AngII. AngII caused a modest increase in fibroblast-specific markers (fibroblast-specific protein-1,  $\alpha$ -smooth muscle actin) and a concomitant decrease in endothelial-specific markers (CD31, CD144) in control ECs. This transition from endothelial- to fibroblast-specific expression was enhanced in NOX2-overexpressing cells (Fig. 4). NOX2 overexpression per se caused a small switch from endothelial to fibroblast-specific expression in the absence of AngII. Online Figure 11A shows representative examples of coexpression of endothelial-specific and mesenchymal markers in NOX2-overexpressing cells after AngII treatment.



**Figure 3** Enhanced Endothelial-Mesenchymal Transition in AngII-Treated TG Hearts

(A) Representative Western blots and mean data for CD31, smooth muscle actin (SMA), and collagen 1 (Coll) levels in cardiac homogenates from angiotensin II (AngII)-treated wild-type (WT) or transgenic (TG) mice. Actin was used as a loading control. \*p < 0.05; n = 4/group. (B) Myocardial sections co-stained for CD31 (red) and SMA (green) and imaged by confocal microscopy. Yellow indicates co-localization of endothelial and mesenchymal markers. Scale bar, 10  $\mu$ m. Panels to the right show pixel-by-pixel fluorescence intensities of the 2 fluorophores along the line indicated in the top panel (TG AngII) and confirm a significant overlapping pattern between CD31 and SMA that is indicative of endothelial-mesenchymal transition.



Cellular morphology was significantly different after 5 days of AngII treatment, with NOX2-overexpressing cells showing a more elongated fibroblast phenotype (Online Fig. 11B).

**Paracrine effects of endothelial cells on fibroblasts?** It is feasible that NOX2 elevation in ECs affects fibroblasts through the direct effects of ROS or other released factors. To assess this possibility, we performed experiments in which human aortic ECs overexpressing NOX2 or  $\beta$ -galactosidase were co-cultured with fibroblasts (see the supplemental Methods section in the Online Appendix). Experiments were performed with and without AngII. No difference was found between the groups in the fibroblast expression of procollagen I mRNA or in markers of myofibroblast transformation (Online Fig. 12).

## Discussion

We investigated the specific effects of endothelial NOX2 activation on AngII-induced cardiac remodeling and took advantage of a mouse model with endothelium-targeted overexpression of NOX2 (8). The 2-fold increase in endothelial NOX2 protein in this model has no basal effects but enhances AngII-stimulated increases in NOX2 activity. In the present study, the authors found that in vivo AngII-induced endothelial NOX2 activation has the following effects: 1) it enhances the development of cardiac fibrosis independent of cardiomyocyte hypertrophy and leads to a phenotype of LV diastolic dysfunction with preserved systolic function; 2) it promotes myocardial inflammatory cell infiltration through increased endothelial expression of VCAM-1 (i.e., endothelial activation), although NOX2 in inflammatory cells is dispensable for the profibrotic effects; and 3) it enhances EMT, which contributes to the cardiac fibrosis. Collectively, these data suggest that NOX2-driven

endothelial dysfunction and activation may comprise an important mechanism that promotes cardiac fibrosis and LV diastolic dysfunction in pathological settings of renin-angiotensin system activation.

**Endothelium-cardiomyocyte crosstalk.** The cardiac endothelium forms a strategic interface between circulating blood and myocardial tissue. Crosstalk between cardiac ECs and cardiomyocytes plays important roles in normal cardiac development (3,4). The cardiac endothelium also influences contractile function in the adult heart (e.g., through paracrine factors such as nitric oxide and endothelin) (2). Reciprocal signaling between cardiomyocytes and the myocardial microvasculature has been found to be important during chronic pressure overload. For example, signaling from ECs to cardiomyocytes through the neuregulin-1-ErbB2 axis promotes cardiomyocyte survival during pressure overload (15), and angiogenic factors released by cardiomyocytes in the chronically overloaded heart regulate myocardial capillary density (11,16). Although these study results support the potential for endothelial dysfunction to affect cardiomyocyte growth, survival, and contractile function, the effects on cardiac fibrosis in the heart under stress are unclear.

**Proinflammatory effects of endothelial NOX2.** Here, we demonstrate that endothelial dysfunction has a profound ROS-dependent impact on the development of cardiac fibrosis, independent of cardiomyocyte hypertrophy. One mechanism underlying these effects may be the proinflammatory properties of activated endothelium. The healthy endothelium is anti-inflammatory and antithrombotic, but in pathological settings such as renin-angiotensin system activation, it becomes activated and promotes increased interaction with circulating inflammatory cells (17). Previous studies suggested that NOX2 is involved in cytokine- and AngII-induced EC activation and the expression of adhesion molecules such as VCAM-1 (18,19), as well as in

enhanced monocyte binding to ECs under oscillatory shear stress (20). Here, we found that NOX2-overexpressing CMECs had a larger increase in VCAM-1 expression after AngII stimulation than did wild-type CMECs, which were associated with greater endothelial-leukocyte adhesion in an *in vitro* flow assay. Consistent with a similar proinflammatory action *in vivo*, TG myocardium had more VCAM-1–positive blood vessels and inflammatory cells after AngII treatment than did wild-type myocardium. These results clearly indicate that AngII-induced endothelial NOX2 activation augments an inflammatory response in the heart *in vivo*. Whether other cell types (e.g., vascular smooth muscle cells, pericytes) are also involved is an interesting question.

In addition to ECs, NOX2 is abundant in inflammatory cells, where it is suggested to contribute to oxidative stress (e.g., in atherosclerosis) (21). NOX2 activation in inflammatory cells is involved in AngII-induced vascular hypertrophy (22). It was therefore possible that NOX2 activation in infiltrating inflammatory cells (as well as the endothelium) might be important in the development of cardiac fibrosis. To dissect the specific contribution of inflammatory cell NOX2 activation, we generated chimeric mice in which the BM consisted of NOX2-competent or NOX2-deficient cells. This series of studies demonstrated that NOX2 activation in infiltrating inflammatory cells is dispensable for the development of AngII-induced fibrosis and that it is NOX2 in recipient heart cells that is essential for the profibrotic effects. This result suggests that although NOX2 may be important for certain functions of inflammatory cells (e.g., phagocytosis, low-density lipoprotein oxidation in atherosclerosis [21]), it is not essential for all functions and is dispensable in the setting of AngII-induced cardiac disease.

**Role of endothelial-mesenchymal transition.** Another mechanism by which endothelial dysfunction may promote cardiac fibrosis is through EMT. The transition of epithelial cells and ECs to a mesenchymal phenotype is well recognized to be important in cardiac development. In a landmark study, Zeisberg et al. (14) showed that EMT makes a significant contribution to pathological cardiac fibrosis during chronic pressure overload. EC production of endothelin-1 has also been reported to contribute to EMT in the diabetic heart (23). We therefore investigated whether endothelial NOX2 activation affects EMT. We found evidence of significantly increased EMT in TG hearts after long-term AngII treatment as compared with wild-type hearts. More definitive evidence that endothelial NOX2 promotes EMT was obtained in cultured ECs in which NOX2 significantly enhanced the transition from endothelial to fibroblast phenotype after AngII treatment. These results strongly suggest that a NOX2-dependent increase in EMT may be a crucial mechanism that enhances cardiac fibrosis in response to long-term AngII treatment.

We also tested the possibility that paracrine effects of ECs on fibroblasts (e.g., through the direct effects of ROS released by ECs) may contribute to fibrosis. *In vitro* co-culture

experiments with ECs and fibroblasts did not provide evidence in support of this possibility. However, a possible contribution of such a mechanism *in vivo* cannot be excluded.

**Isolated diastolic dysfunction.** A striking functional consequence of endothelial NOX2 overexpression on the response to chronically elevated AngII was a phenotype of LV diastolic dysfunction with preserved systolic function, thus resulting in smaller and stiffer hearts and reduced stroke volumes in TG mice compared with wild-type mice. This finding was documented in TG mice by the gold standard method of *in vivo* pressure-volume analysis, which demonstrated significantly increased LV diastolic passive stiffness in the AngII-treated TG group versus the wild-type group, whereas load-independent indices of systolic function were similar in the two groups.

**Study limitations.** We found no significant difference between the groups in parameters of active LV relaxation (e.g., the isovolumic relaxation time-constant), and this finding suggested that cardiomyocyte relaxation was probably similar in the two groups. This contractile dysfunction phenotype is consistent with, and fully explainable by, the increased fibrosis observed in the TG group independent of differences in cardiomyocyte hypertrophy. The use of an endothelium-targeted transgenic model allowed the authors to study the role of endothelial dysfunction and activation independent of other changes that occur in the remodeling heart during increased renin-angiotensin system activation. As mentioned earlier, NOX2 is also expressed in cardiomyocytes, and previous studies in cultured cells and *in vivo* models showed that cardiomyocyte NOX2 activation may contribute to the development of AngII-induced cardiac hypertrophy (12). No difference was found in the extent of hypertrophy between wild-type and TG groups in the current study, consistent with the finding that NOX2 was overexpressed specifically in ECs. Therefore, the phenotype of diastolic LV dysfunction with preserved systolic function found in the current model was the consequence specifically of endothelial dysfunction and activation.

## Conclusions

The clinical syndrome of heart failure with preserved ejection fraction is currently receiving significant attention because of the high prevalence of the condition, controversy regarding its etiology and pathogenesis, and the lack of effective therapies (24). Although simple extrapolations cannot be made from the current study in a murine model to the clinical setting, the occurrence of a contractile dysfunction phenotype that apparently closely resembles the cardiac dysfunction found in human heart failure with preserved ejection fraction is of great interest. It is notable that previous human studies suggested that inflammation contributes to enhanced fibrosis in such patients (25), consistent with the findings of increased inflammatory cell infiltration, fibrosis, and diastolic dysfunction in our study. The current results raise the intriguing possibility that endothelial

dysfunction and activation, as well as downstream pathways such as inflammation and EMT, may be important mechanisms contributing to the development of heart failure with preserved ejection fraction. If so, this could provide new therapeutic options to tackle this prevalent condition.

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## REFERENCES

1. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation* 2001;104:2673–8.
2. Shah AM. Paracrine modulation of heart cell function by endothelial cells. *Cardiovasc Res* 1996;31:847–67.
3. Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol Rev* 2003;83:59–115.
4. Hsieh PC, Davis ME, Lisowski LK, Lee RT. Endothelial-cardiomyocyte interactions in cardiac development and repair. *Annu Rev Physiol* 2006;68:51–66.
5. Paulus WJ, Vantrimpont PJ, Shah AM. Paracrine coronary endothelial control of left ventricular function in humans. *Circulation* 1995;92:2119–26.
6. MacCarthy PA, Grieve DJ, Li JM, Dunster C, Kelly FJ, Shah AM. Impaired endothelial regulation of ventricular relaxation in cardiac hypertrophy: role of reactive oxygen species and NADPH oxidase. *Circulation* 2001;104:2967–74.
7. Lassegue B, San Martin A, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* 2012;110:1364–90.
8. Murdoch CE, Alom-Ruiz SP, Wang M, et al. Role of endothelial Nox2 NADPH oxidase in angiotensin II-induced hypertension and vasomotor dysfunction. *Basic Res Cardiol* 2011;106:527–38.
9. Foteinos G, Hu Y, Xiao Q, Metzler B, Xu Q. Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice. *Circulation* 2008;117:1856–63.
10. Ray R, Murdoch CE, Wang M, et al. Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo. *Arterioscler Thromb Vasc Biol* 2011;31:1368–76.
11. Zhang M, Brewer AC, Schroder K, et al. NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. *Proc Natl Acad Sci U S A* 2010;107:18121–6.
12. Bendall JK, Cave AC, Heymes C, Gall N, Shah AM. Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 2002;105:293–6.
13. Burns SO, Killock DJ, Moulding DA, et al. A congenital activating mutant of WASp causes altered plasma membrane topography and adhesion under flow in lymphocytes. *Blood* 2010;115:5355–65.
14. Zeisberg EM, Tarnavski O, Zeisberg M, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med* 2007;13:952–61.
15. Lemmens K, Segers VF, Demolder M, De Keulenaer GW. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte cross-talk. *J Biol Chem* 2006;281:19469–77.
16. Shiojima I, Sato K, Izumiya Y, et al. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest* 2005;115:2108–18.
17. Frey RS, Ushio-Fukai M, Malik AB. NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. *Antiox Redox Signal* 2009;11:791–810.
18. Wang HD, Xu S, Johns DG, et al. Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ Res* 2001;88:947–53.
19. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. *Mol Cell Biol* 2005;25:2320–30.
20. Hwang J, Saha A, Boo YC, et al. Oscillatory shear stress stimulates endothelial production of O<sub>2</sub><sup>-</sup> from p47phox-dependent NAD(P)H oxidases, leading to monocyte adhesion. *J Biol Chem* 2003;278:47291–8.
21. Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004;24:23–8.
22. Liu J, Yang F, Yang XP, Jankowski M, Pagano PJ. NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. *Arterioscler Thromb Vasc Biol* 2003;23:776–82.
23. Widyantoro B, Emoto N, Nakayama K, et al. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. *Circulation* 2010;121:2407–18.
24. Lam CS, Brutsaert DL. Endothelial dysfunction: a pathophysiologic factor in heart failure with preserved ejection fraction. *J Am Coll Cardiol* 2012;60:1787–9.
25. Westermann D, Lindner D, Kasner M, et al. Cardiac inflammation contributes to changes in the extracellular matrix in patients with heart failure and normal ejection fraction. *Circ Heart Fail* 2011;4:44–52.

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**Key Words:** angiotensin II ■ diastolic dysfunction ■ endothelial-mesenchymal transition ■ endothelium ■ NADPH (nicotinamide adenine dinucleotide phosphate) oxidase.

## ▶ APPENDIX

For a supplemental Methods section, and figures and tables, please see the online version of this article.