ONLINE SUPPLEMENT

METHODS-

Generation of knockout mice

All targeted alleles and transgenes were maintained on an FvB genetic background. To achieve VSMC specific deletion of Jak2, mice carrying loxP sites placed around the first coding exon of the Jak2 allele as previously described,[1] were crossed with mice expressing Cre recombinase under the control of the SM22α promoter.[2] Males of genotype SM22αCreJak2fl/+ were crossed with Jak2fl/fl females, which resulted in mice whose VSMCs are devoid of Jak2 (SM22αCre(+)Jak2fl/fl). Genotypes of all offspring were analyzed by PCR using primers 5'-GCTAAACATGCTTCATCGTCGGTC and 5'-CAGATTACGTATATCCTGGCAGCG in the Cre coding region and 5'-ATTCTGAGATTCAGGTCTGAGC and 5'-CTCACAACCATCTGTATCTCAC in the Jak2 coding region. The null Jak2 allele was identified using primers 5'-GTCTATACACCACCACTCCTG and 5'-GAGCTGGAAAGATAGGTCAGC. To analyze the expression of VSMC-specific SM22αCre, mice expressing SM22αCre(+)Jak2fl/fl were crossed with Rosa26 β-galactosidase reporter mice and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining was performed.

Blood Pressure Measurements

Three month old male littermates of genotypes SM22αCre(-)Jak2fl/fl (Control) and SM22αCre(+)Jak2fl/fl (VSMC Jak2 null) were anesthetized by isoflurane, and surgically prepared for telemetry using full sterile technique. Briefly, mice were fitted with telemetry probes (PA-C10, DSI Company, MN) placed in the left carotid artery and the transmitter body placed subcutaneously on the right flank, close to the right hind limb and allowed at least 10 days of recovery. Baseline blood pressure measurements were made over 3 days. Alzet Model 1004 micro-osmotic pumps (Alzet Corp) were then placed subcutaneously into the mice for infusion of 1,000 ng/kg/min Ang II.[3] Radio telemetry recordings were then performed over the ensuing four week period.

Aortic Contraction/Relaxation Studies

2-mm abdominal aorta ring segments were mounted on a wire myograph (Model 610M; Danish Myo Technology A/S, Aarhus N, Denmark) in Krebs-bicarbonate buffer equilibrated with 95% O_2, 5% CO_2 at 37°C. The rings were allowed to equilibrate for 45 minutes, stimulated with different pharmacological agents and changes in contraction/relaxation were recorded.

NO Measurements

Aortic rings were incubated with 7 μM fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM) aerated with 95% O_2-5% CO_2 at 37°C for 45 minutes. Samples for basal accumulation of NO were taken. The rings were then treated with Ang II (10^{-7} M) or Ach (10^{-6} M) for 30 minutes, removed, dried with filter paper, and weighed. Fluorescence was measured at an excitation wavelength of 495 nm and an emission wavelength of 520 nm and normalized to tissue weight.

Histology
Tissue samples were prepared for histology as previously described,[4] and stained with hematoxylin and eosin or Masson's trichrome. Immunohistological detection of anti-smooth muscle α-actin (CM001B) was carried out using the Rat on Mouse AP-Polymer Kit (Biocare Medical) according to the manufacturer's instructions. Anti-Jak2 (ab39636 Abcam) immunohistochemistry was performed as previously described.[4]

VSMC culture
VSMCs were isolated and cultured according to procedures previously described [5] with a few modifications. Aortic VSMCs were isolated using 1 mg/ml collagenase (type II, Worthington Biomedical Corporation), and cultured in Dulbecco's modified eagle medium (Mediatech, Inc Manassas, Va) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were harvested for passaging at confluency with trypsin-EDTA (Mediatech, Inc Manassas, Va). The cells were used between passages 3 and 8.

H_2O_2 detection
VSMC were serum starved for 24 hours, washed twice with Krebs-Ringer phosphate buffer, and H_2O_2 production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188) according to the manufacturer's instructions (Invitrogen). To identify the H_2O_2-specific signal, control samples were co-incubated with 500 units/ml catalase. Triplicate readings were taken in a 96-well plate and H_2O_2 levels were calculated in terms of catalase-inhabitable signal and were then normalized to cellular protein as measured by the Bio-Rad *DC* Protein assay.

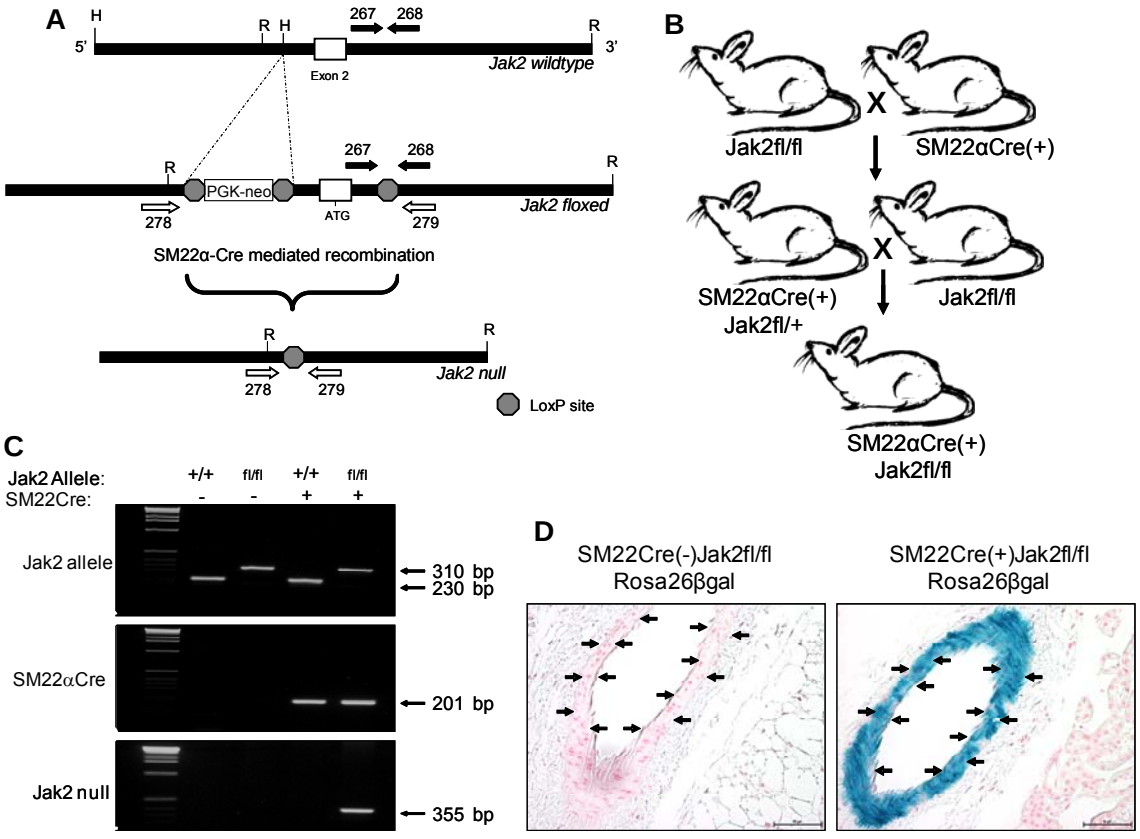
Rho kinase activity
VSMC were cultured in 10 cm dishes to 80% confluency and serum-starved for 24 hours. Cells were treated Ang II (10^{-7} M) for 0, 20, 40, or 60 minutes, washed and cell lysates were obtained. Rho kinase activity was determined using the CycLex Rho-kinase Assay Kit (MBL International, Nagano, Japan), according to the manufacturer's instructions. Some lysates were immunoprecipitated using MYPT1 antibody (SC-25618, Santa Cruz), followed by western blotting using phospho-MYPT1 antibody (SC-17556, Santa Cruz), as previously described.[6]

Calcium imaging
VSMC plated at a density of $5x10^4$ cells/mL in 35-mm glass-bottom culture dishes (MatTek Corporation) were serum-starved for 24 hours, washed in Krebs-Ringer buffer and loaded with 5 μM fura-2 (Invitrogen) for 90 minutes at room temperature. Cells were then transferred to a microscope chamber and imaged with a cooled charge-coupled device camera (Photometrics SenSys; Roper Scientific, Tucson, AZ) fitted to a fluorescence microscope (UM-2; Nikon, Tokyo, Japan). Ratio measurements were performed every 5 seconds during baseline and drug application.

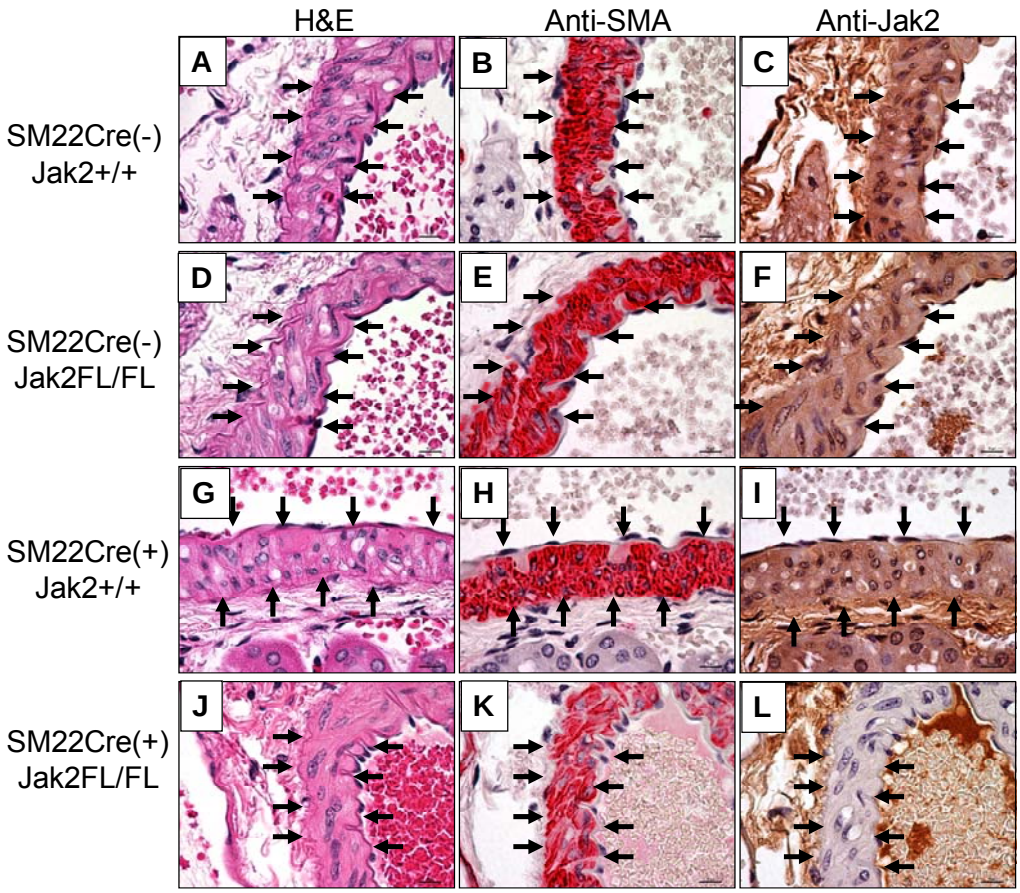
Statistics

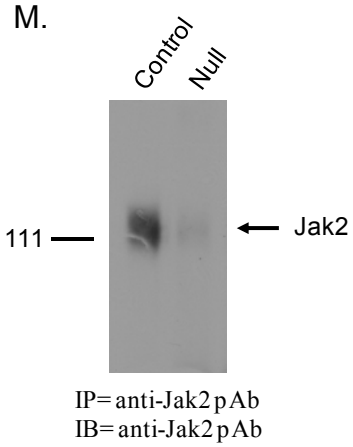
All results were expressed as means +/- SEM. Comparison of genotypes and treatments was performed by unpaired/paired Student's *t*-test, analysis of variance followed by the Bonferroni *t*-test, or by Friedman's test. p values of less than 0.05 were considered significant.

SUPPLEMENTAL DATA-

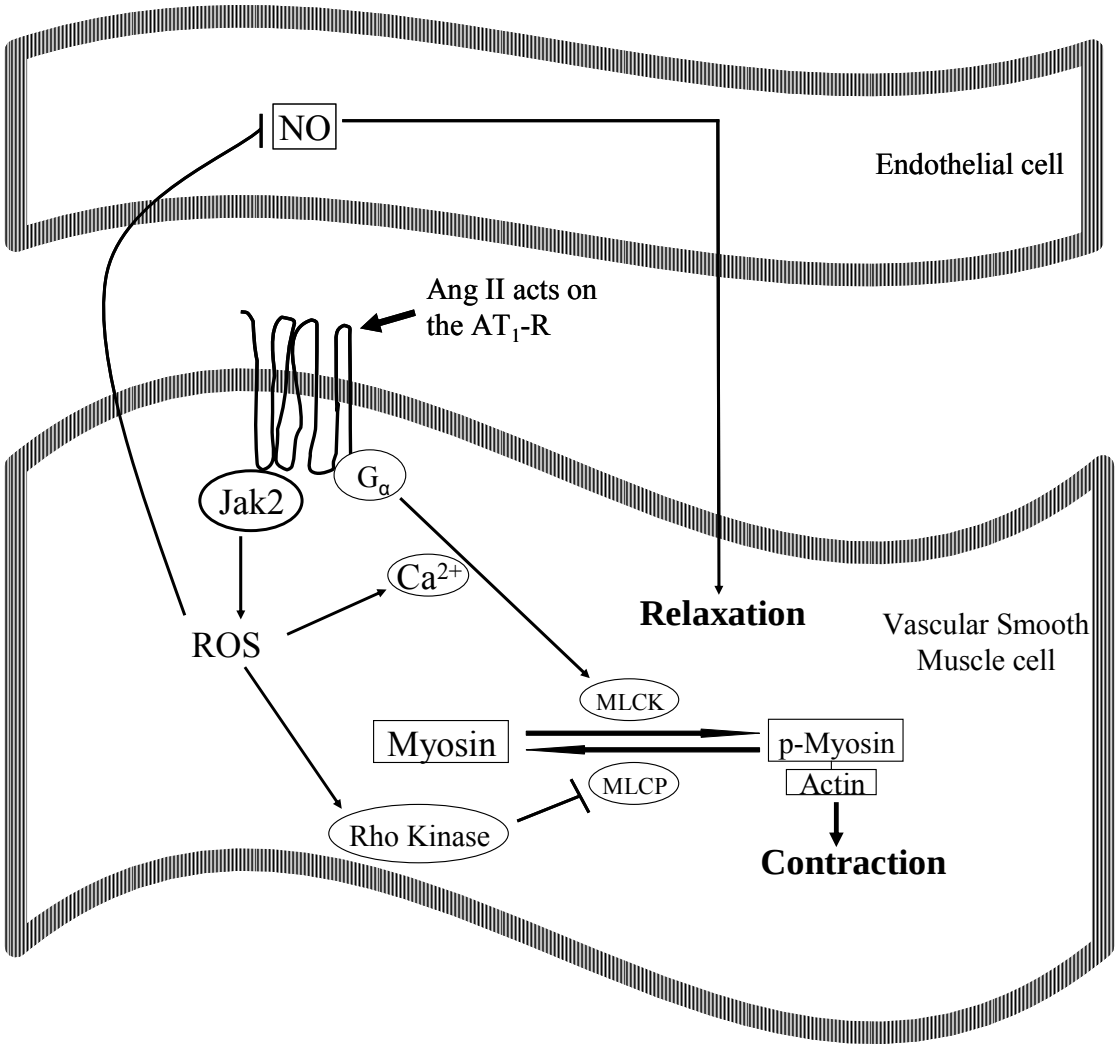


Supplemental Figure 1. Generation of mice with vascular smooth muscle cell specific deletion of Jak2. (A) Cre-mediated deletion of the Jak2 gene; arrows indicate location of primers. (B) Breeding strategy to convert the floxed Jak2 allele into a vascular smooth muscle cell specific null Jak2 mutation. (C) Results of a PCR assay to verify presence of a floxed Jak2 allele (top; primers 267 and 268), the SM22αCre specific amplicon (middle) and the null Jak2 allele in vascular smooth muscle cells (bottom; primers 278 and 279). D, X-Gal staining of kidney tissue sections derived from Rosa26 β-gal mice with and without the SM22αCre transgene. Data represent three independent experiments for each genotype.





Supplemental Figure 2. Generation of mice lacking Jak2 protein within vascular smooth muscle cells. Histological analysis of serial sections from four genotypes; SM22αCre(-)Jak2+/+ (A-C), SM22αCre(-)Jak2fl/fl (D-F), SM22αCre(+)Jak2+/+ (G-I) and SM22Cre(+)Jak2fl/fl (J-L). The first three genotypes are genetic controls whereas the last is the VSMC Jak2 specific knockout. The sections were stained with hematoxylin and eosin (H&E) for general morphological analysis (Fig. 1A, 1D, 1G and 1J), IHC with anti-smooth muscle α-actin (anti-SMA) to highlight the location of the VSMC layer (Fig. 1B, 1E, 1H and 1K), and IHC with an anti-Jak2 antibody to demonstrate presence or absence of the Jak2 protein in each section (Fig. 1C, 1F, 1I and 1L). The VSMC layers are further highlighted by arrows. We found that there was no detectable expression of Jak2 in the VSMC of the conditional knockout mice (Fig. 1L), when compared to the three control genotypes (Fig. 1C, 1F and 1I). (M) VSMC were obtained from the aortas of control and null mice, cultured *ex vivo*, and lysed. The levels of Jak2 within the VSMC were then determined by immuno-precipitating and western blotting the lysates with anti-Jak2 antibody.



Supplemental Figure 3. Proposed model showing the role of Jak2 in the regulation of vascular tone. The binding of Ang II couples the AT_1 receptor to Jak2 signaling and subsequent generation of reactive oxygen species in the VSMC. Elevated reactive oxygen species leads to the scavenging of nitric oxide in the endothelial cell, increased intracellular Ca^{2+} levels in the VSMC, and increased Rho-kinase activity in the VSMC. Collectively, these actions contribute to increased vasoconstriction and hypertension.

Supplemental Table 1: Mean Arterial Pressure and Heart Rate of Vascular Smooth Muscle Cell Jak2 Null and Control Mice

Mean Arterial Pressure (mmHg)									
Time (Days)	-3	-1	3	13	15	19	22	26	28
Dark Period									
Control	115.6 ± 6.6	114.3 ± 5.5	126.3 ± 8.8	139.4 ± 12.4	142.4 ± 11.9	149.4 ± 9.8	145.2 ± 8.6	148.4 ± 7.1	137.6 ± 9.7
Null	109.5 ± 6.0	105.8 ± 6.0*	124.4 ± 4.4	119.6 ± 6.7**	113.7 ± 8.8**	123.3 ± 7.1**	119.6 ± 7.2**	116.5 ± 5.9**	107.5 ± 6.0**
Light Period									
Control	112.2 ± 6.1	105.3 ± 7.9	123.1 ± 11.2	142.8 ± 10.0	141.8 ± 9.6	140.2 ± 12.1	131.6 ± 9.6	140.9 ± 6.8	135.0 ± 9.1
Null	99.4 ± 6.6*	99.6 ± 5.6	118.5 ± 8.1**	119.5 ± 5.9**	114.8 ± 6.6**	123.8 ± 7.0**	112.0 ± 8.2**	114.5 ± 5.7**	111.3 ± 7.0**

Heart Rate (beats/min)									
Dark Period									
Control	480.9 ± 14.7	489.6 ± 12.6	475.5 ± 13.5	500.1 ± 16.6	503.6 ± 14.4	516.5 ± 17.7	537.3 ± 14.9	534.4 ± 15.8	528.2 ± 13.6
Null	504.7 ± 15.4	460.8 ± 14.2	480.7 ± 14.1	512.6 ± 10.2	510.4 ± 12.9	508.9 ± 15.7	513.4 ± 13.6	522.3 ± 13.3	514.6 ± 15.1
Light Period									
Control	473.8 ± 9.6	440.5 ± 12.5	460.8 ± 15.6	503.3 ± 16.4	485.9 ± 13.7	500.7 ± 14.4	479.9 ± 14.2	487.5 ± 13.4	477.2 ± 14.7
Null	477.2 ± 14.7	426.0 ± 12.4	433.6 ± 9.0	527.1 ± 14.3	517.3 ± 15.1	500.7 ± 18.9	505.8 ± 14.5	477.4 ± 11.5	496.7 ± 13.8

Ang II infusion started on Day 0.

*****p<0.05 versus control, **p<0.01 versus control.

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