

Interferon-stimulated gene 15 pathway is a novel mediator of endothelial dysfunction and aneurysms development in angiotensin II infused mice through increased oxidative stress

María González-Amor ^{1,2†}, Ana B. García-Redondo ^{1,2†‡}, Inmaculada Jorge ^{2,3}, Guillermo Zalba ⁴, Martina Becares ⁵, María J. Ruiz-Rodríguez^{2,6}, Cristina Rodríguez^{2,7,8}, Hugo Bermeo ¹, Raquel Rodríguez-Díez ^{1,2}, Francisco J. Ríos ⁹, Augusto C. Montezano ⁹, Jose Martínez-González^{2,8,10}, Jesús Vázquez^{2,3}, Juan Miguel Redondo^{2,6}, Rhian M. Touyz⁹, Susana Guerra⁵, Mercedes Salas ^{1,2}, and Ana M. Briones ^{1,2*}

¹Departamento de Farmacología, Instituto de Investigación Hospital La Paz, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, 28029 Madrid, Spain; ²CIBER de Enfermedades Cardiovasculares, ISCIII, Spain; ³Laboratorio de Proteómica Cardiovascular, Centro Nacional de Investigaciones Cardiovasculares, C. Melchor Fernández Almagro, 3, 28029 Madrid, Spain; ⁴Departamento de Bioquímica y Genética, Instituto de Investigación Sanitaria de Navarra, Facultad de Ciencias, Universidad de Navarra, C/ Irunlarrea, 1, Pamplona 31008 Navarra, Spain; ⁵Departamento de Medicina Preventiva y Microbiología, Instituto de Investigación Hospital La Paz, Universidad Autónoma de Madrid, C/Arzobispo Morcillo 4, 28029 Madrid, Spain; ⁶Grupo de Regulación Génica en Remodelado Cardiovascular e Inflamación, Centro Nacional de Investigaciones Cardiovasculares, C. Melchor Fernández Almagro, 3, 28029 Madrid, Spain; ⁷Institut de Recerca Hospital de la Santa Creu i Sant Pau, C/ Sant Quintí, 77, 08041 Barcelona, Spain; ⁸Instituto de Investigación Biomédica Sant Pau, Barcelona, Spain; ⁹Institute of Cardiovascular and Medical Sciences, University of Glasgow, 126 University Place Glasgow G12 8TA, Glasgow, UK; ¹⁰Instituto de Investigaciones Biomédicas de Barcelona-Consejo Superior de Investigaciones Científicas (IIBB-CSIC), C/ Rosselló, 161, 08036, Barcelona, Spain; and ¹¹Instituto de Investigación Biomédica Sant Pau, Barcelona, Spain

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Aims

Interferon-stimulated gene 15 (*ISG15*) encodes a ubiquitin-like protein that induces a reversible post-translational modification (ISGylation) and can also be secreted as a free form. *ISG15* plays an essential role as host-defence response to microbial infection; however, its contribution to vascular damage associated with hypertension is unknown.

Methods and results

Bioinformatics identified *ISG15* as a mediator of hypertension-associated vascular damage. *ISG15* expression positively correlated with systolic and diastolic blood pressure and carotid intima-media thickness in human peripheral blood mononuclear cells. Consistently, *Isg15* expression was enhanced in aorta from hypertension models and in angiotensin II (AngII)-treated vascular cells and macrophages. Proteomics revealed differential expression of proteins implicated in cardiovascular function, extracellular matrix and remodelling, and vascular redox state in aorta from AngII-infused *ISG15*^{−/−} mice. Moreover, *ISG15*^{−/−} mice were protected against AngII-induced hypertension, vascular stiffness, elastin remodelling, endothelial dysfunction, and expression of inflammatory and oxidative stress markers. Conversely, mice with excessive ISGylation (*USP18*^{C61A}) show enhanced AngII-induced hypertension, vascular fibrosis, inflammation and reactive oxygen species (ROS) generation along with elastin breaks, aortic dilation, and rupture. Accordingly, human and murine abdominal aortic aneurysms showed augmented *ISG15* expression. Mechanistically, *ISG15* induces vascular ROS production, while antioxidant treatment prevented *ISG15*-induced endothelial dysfunction and vascular remodelling.

Conclusion

ISG15 is a novel mediator of vascular damage in hypertension through oxidative stress and inflammation.

*Corresponding author. Tel: +34 91 4975399, E-mail: ana.briones@uam.es

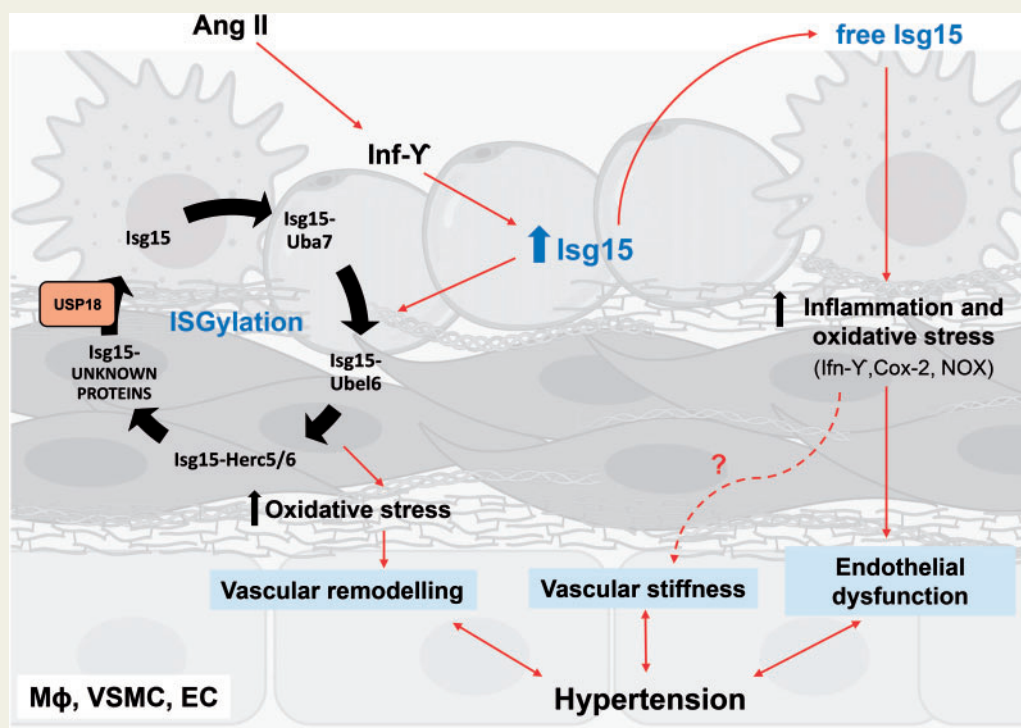
†The first two authors contributed equally to the study.

‡Present address. Departamento de Fisiología, Instituto de Investigación Hospital La Paz, Universidad Autónoma de Madrid, Madrid, Spain.

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Graphical Abstract



Keywords

ISG15 • Endothelial dysfunction • Vascular remodelling • Oxidative stress • Inflammation

1. Introduction

Hypertension is the main risk factor for cardiovascular diseases. Among the most important features of hypertension are enhanced vasoconstrictor responses, endothelial dysfunction, and vascular remodelling.^{1,2} Emerging evidence suggests that the innate and adaptive immune systems are key contributors to hypertension and cardiovascular damage. Thus, immune cells infiltrate blood vessels, kidney, heart, and brain to promote increases in blood pressure. Specifically, the participation of different subsets of T lymphocytes and proinflammatory cytokines such as IFN- γ or TNF α in hypertension and in endothelial dysfunction has been demonstrated.^{3–6} Thus, infusion of angiotensin II (AngII) in mice increases IFN- γ expression in aorta⁷ and perivascular adipose tissue⁸ provoking oxidative stress and endothelial dysfunction.^{3,8} Importantly, IFN- γ KO mice are protected against endothelial dysfunction induced by AngII.⁷

IFN-induced 15 kDa protein (ISG15) is a ubiquitin-like protein expressed mainly in immune cells, fibroblasts, epithelial-derived cell lines, and in several tumour cells.^{9–12} ISG15 is induced by IFNs, mainly type I (α and β) but also by type II IFN (IFN- γ), lipopolysaccharide, and TNF α .^{12–16} ISG15 protein can be found as a free molecule (intracellular and extracellular) or reversibly conjugated to lysine residues of *de novo* synthesized target proteins, a process known as *ISGylation*.^{12,16,17} ISGylation occurs in a manner similar to ubiquitination and is performed by ISG15-activating enzymes E1 (UBA7), ISG15-conjugating enzymes E2 (UBE2L6), and ISG15-ligases E3 (HERC5 in human, HERC6 in

mouse).^{12,16,17} As a reversible modification, ISG15 is removed from conjugated proteins by the ISG15-specific protease USP18.¹⁸

Protein ISGylation modulates viral infection, cancer progression, hypoxia response, or exosome secretion, among others.^{12,19,20} However, the role of free ISG15 is less known. In humans, intracellular free ISG15 promotes USP18-dependent regulation of IFN- α/β and prevention of IFN- α/β -dependent autoinflammation.²¹ Extracellular ISG15 induces IFN- γ secretion from peripheral blood mononuclear cells (PBMCs), especially from lymphocytes, and NK cells^{9,10,22–25} in an ISGylation-independent manner.¹⁰

At the cardiovascular level, the role of ISG15/USP18 is poorly known. ISG15 has been found in exosomes released by toll-like receptor 3-activated human brain microvascular endothelial cells in human immunodeficiency virus infection.²⁶ ISG15 has also been described as a mechanism of innate response in cardiomyocytes against viral infections, decreasing inflammatory cardiomyopathy, heart failure, and mortality.²⁷ Interestingly, cardiomyocyte-specific expression of constitutively active I κ B kinase 2, activated the ISG15 pathway, and elicited proteins ISGylation in an NF- κ B-dependent manner,²⁸ although the pathophysiological consequence of the ISG15 pathway was not determined. To the best of our knowledge, the role of ISG15 in hypertensive cardiovascular disease has never been described.

Because of the role of IFN- γ in hypertension, we hypothesized that, as an IFN-induced protein, ISG15 could be a novel mediator of vascular dysfunction and blood pressure elevation. This hypothesis was tested in

PBMCs and aortic segments from human subjects, arteries and macrophages from animal models of hypertension, and cultured vascular cells. We demonstrated the ability of AngII to increase vascular and macrophages ISG15 expression. We also demonstrated the beneficial effects of ISG15 deletion and the deleterious effects of excessive ISGylation in the functional, structural, and mechanical alterations of the vasculature associated with hypertension. Moreover, we uncover the role of inflammation and oxidative stress as the underlying mechanisms.

2. Methods

2.1 Bioinformatics analysis

A dataset of proteins related to hypertension was created from Public Health Genomics and Precision Health Knowledge Base (V5.2) 'Phenopedia' (<https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action>), using the term 'Hypertension'. The dataset was analysed by the Upstream Regulator Analysis of Ingenuity Pathways Analysis (IPA@, Qiagen) software, which identifies proteins included or not in the dataset, which may be potential database master regulators. This tool defines an overlap *P*-value to measure the enrichment of the different master regulators in the database. In addition, a protein–protein interaction network was created by introducing ISG15 into the list of proteins obtained from 'Phenopedia' by STRING (<https://string-db.org/>) V 11.0. Finally, the results were visualized using Cytoscape.

2.2 Human studies

2.2.1 Isolation of PBMCs and detection of superoxide production from patients

The study was performed in a group of 175 asymptomatic subjects in whom global risk assessment was performed at our institution in the course of a general health check-up after a 12-h overnight fast. In all subjects, absence of history of coronary disease, stroke, or peripheral arterial disease was recorded. Conventional cardiovascular risk factors, including arterial hypertension, obesity, smoking, and diabetes were defined as previously described.²⁹ Carotid ultrasonography was performed to determine intima-media thickness (IMT), as previously described.²⁹ Patients were recruited from May 2002 till June 2005. Characteristics of the studied population are summarized in [Supplementary material online, Table S1](#).

In all 175 subjects, PBMCs were isolated from venous blood samples with Lymphoprep with a high purity and immediately used for enzymatic and molecular measurements.

The superoxide anion production was measured by chemiluminescence in a plate reader luminometer (Luminoskan Ascent, Labsystem), in isolated 4×10^5 PBMCs stimulated with phorbol myristate acetate ($3.2 \mu\text{mol/L}$; Sigma-Aldrich, San Louis, MO, USA) in the presence of $5 \mu\text{mol/L}$ lucigenin. Luminescence measurements were recorded along with an interval of 1 h, and the value of the area under the curve was used to quantify chemiluminescence.

2.2.2 Abdominal aortic aneurysms patients

Cases were defined as those patients referred for elective open repair of abdominal aortic aneurysms (AAA) at the Angiology and Vascular Surgery Service of the Hospital de la Santa Creu i Sant Pau (HSCSP; Barcelona, Spain). Patients with infectious or inflammatory aneurysms, or pseudoaneurysms were excluded from the study. Inclusions were carried out between January 2012 and December 2015. Biopsies, devoid of

intraluminal thrombi if present, were systematically obtained from the anterolateral wall of the mid-infrarenal aorta at the level of the inferior mesenteric artery. Healthy non-atherosclerotic aortas from multiorgan donors were also taken from the same region of the infrarenal abdominal aorta.³⁰ Samples of control subjects had no post-mortem evidence of abdominal aortic aneurysm, atherosclerotic plaques, or other medical conditions that affect the study. Control patients included 88.2% male, 11.8% female, 64% smoker or ex-smoker, mean age 64 ± 4 years: AAA patients include 100% male, 67% smoker or ex-smoker, mean age: 71 ± 1 years ($P > 0.05$ vs. control). Samples were rapidly collected and stored at -80°C for subsequent RNA extraction.

2.3 Animal studies

Three- to four-month-old male mice on C57BL/6J genetic background were used. Wild-type mice (WT), ISG15 knockout mice (ISG15^{-/-}), and USP18^{C61A} knock-in mice, which have a mutation of the USP18 protein within the Cys at position 61 (substitution by alanine) that completely abolishes the isopeptidase activity leading to excessive ISGylation,³¹ were randomly untreated or infused with AngII (1.44 mg/kg/day, 2 weeks; Sigma-Aldrich) with subcutaneously implanted Alzet osmotic minipumps (model 2002, Durect Corp., Cupertino, CA, USA) implanted under isoflurane anaesthesia (2%). C57BL/6J mice were obtained from in house breeding pairs. ISG15^{-/-} mice and USP18^{C61A} transgenic mice were originally described elsewhere.^{31,32} USP18^{C61A} and WT littermate controls originated from the offspring of USP18^{C61A}/WT $\times$ USP18^{C61A}/WT mice. ISG15^{-/-} mice and WT littermate controls originated from the offspring of ISG15KO/WT $\times$ ISG15KO/WT mice. Once the initial stocks were generated, the maintenance of the colony was generated by successive cross-breeding of ISG15^{-/-} with ISG15^{-/-}; ISG15^{+/-} with ISG15^{+/-} or USP18^{C61A} with USP18^{C61A} mice, in house breeding pairs.

In another set of experiments, some USP18^{C61A} mice were treated with the antioxidant superoxide dismutase mimetic 4-hidroxi-2,2,6,6-tetrametilpiperidin-1-oxilo (tempol, 0.288 nmol/kg/day; Honeywell Fluka, Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. GA12207) and AngII. Six-month-old male Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) were also used. In another group of experiments, C57BL/6J mice were infused with AngII (1.44 mg/kg/day, 2 weeks) and treated or not with a combination of hydralazine plus hydrochlorothiazide (HH) (hydralazine: 20 mg/kg/day; hydrochlorothiazide: 6 mg/kg/day i.p.; Sigma-Aldrich Co. both compounds).

Three-month-old male apolipoprotein E-deficient mice (ApoE^{-/-}, B6.129P2-Apoetm1Unc/J, stock number 002052) were obtained from Jackson Laboratory. Mice were randomly distributed into AngII or saline-infused animals. AngII (1.44 mg/kg/day; 4 weeks; Sigma-Aldrich, St Louis, MO, USA) was infused via osmotic minipumps (model 1004, Alzet).

For the implantation of osmotic minipumps, mice were anaesthetized with isoflurane inhalation (2%). Anaesthetic depth was confirmed by loss of blink reflex and/or lack of response to tail pinch. The procedure takes ~ 15 min/mouse. Recovery after surgical procedures was carried out using aseptic techniques in a dedicated approved surgical area. Antibiotics (penicillin, 450 000 U/kg, intramuscular) and analgesics (buprenorphine, 0.05 mg/kg, subcutaneous) were given once immediately after surgery to prevent infection and discomfort. The animals were kept warm in a heating pad until awake after surgery and observed carefully by the investigators throughout the post-surgery period.

Mice were randomly allocated to treatment groups within genotypes and were labelled by an operator unaware of the nature of the experiments. Investigators for outcome assessments were not blinded to group allocation unless specified.

Blood pressure was measured by tail-cuff plethysmography. For this, animals were trained for 1 week prior to initial blood pressure measurements right before AngII pumps implantation. Then, blood pressure was measured at days 3, 7, and 14 during AngII infusion for the ISG15^{-/-} model and at days 3, 7, 10, and 14 during AngII infusion for the USP18^{C61A} model. Measurements were done always at the same time of the day from 8 to 10 am. At least six individual observations were performed and averaged for each animal.

At the end of the study, animals were euthanized by CO₂ or isoflurane (2%) narcosis.

2.3.1 Tissue preparation

Aorta and first-order branches of the mesenteric artery were dissected free of fat and connective tissue and placed in cold Krebs Henseleit Solution (KHS) (115 mmol/L NaCl, 25 mmol/L NaHCO₃, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄·7H₂O, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 11.1 mmol/L glucose, and 0.01 mmol/L Na₂EDTA) bubbled with a 95% O₂–5% CO₂ mixture. Vessels were divided into segments for analysis of vascular function, structure, and mechanics that was done on the same day. For histology, aortic segments were fixed in 4% paraformaldehyde. Aortic segments used for O₂^{•-} determination were placed in KHS containing 30% sucrose for 20 min, transferred to a cryomold containing Tissue Tek OCT embedding medium (Sakura Finetek, Europe, Netherlands) and then immediately frozen in liquid nitrogen for storage at –80°C until O₂^{•-} measurements. Remaining vascular segments were immediately frozen in liquid nitrogen and kept at –80°C until the day of gene expression studies.

For some experiments, aortic segments from WT mice were incubated with AngII (1 µmol/L, 6 h) in the presence and in the absence of an anti-IFNγ antibody (5 µg/mL; Thermo Fisher Scientific, Cat. No. 16731181) and kept at –80°C until the day of gene expression studies.

2.4 Intraperitoneal macrophage isolation

Intraperitoneal macrophages from control and AngII-infused mice were obtained in phosphate-buffered saline, and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 100 IU·mL⁻¹ penicillin, 100 µg·mL⁻¹ streptomycin, and 10 mmol·L⁻¹ L glutamine, for 24 h. The mRNA from macrophages was obtained and quantified.

2.5 Study approval

The study was carried out in accordance with the Declaration of Helsinki and all participants gave informed consent prior to the inclusion in the study. The ethics committees of Hospital de la Santa Creu i Sant Pau (13/049/1437) and Universidad de Navarra approved the protocol. PBMC samples were stored in the biobank of the Universidad de Navarra at the time of the study.

Animals were taken care of and used according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes and experiments were conducted in accordance with the ARRIVE guidelines. All mice and rats were bred at the conventional Animal Care Facility of the Faculty of Medicine, Universidad Autónoma de Madrid (UAM), and Institut de Recerca Hospital de la Santa Creu i Sant Pau-Programa ICCG. All animal care and experimental procedures were approved by the ethics committee of Research of the UAM and Dirección General de Medio Ambiente, Comunidad de Madrid, Spain (PROEX 345/14) and Institut de Recerca

Hospital de la Santa Creu i Sant Pau-Programa ICCG local ethics committee (Law 5/21 June 1995; Generalitat de Catalunya).

2.6 Cell culture

The human microvascular endothelial cells line (HMEC-1, ATCC[®], Middlesex, UK; CRL-3243TM) was used. Cells were cultured according to the manufacturer instructions. At 80% confluence, cells were serum-deprived for 24 h before stimulation. Human aortic endothelial cells (HAEC, ATCC[®] PCS-100-011TM) were also used. For functional studies, confluent cells were made quiescent by changing medium to DMEM with 0.5% FBS and penicillin/streptomycin (50 µg/mL) during 24 h. Before stimulation, HAEC were fully serum-deprived. Both types of endothelial cells were treated with AngII (0.1–100 nmol/L for 2–24 h; Sigma-Aldrich) or human recombinant ISG15 (rISG15, 10 ng/mL, Sino Biological, Life Technologies, Carlsbad, CA, EEUU, Cat. No. 12729-HNAE)³³ at times indicated in figure legends. Control cells were stimulated with vehicle.

Primary cultures of vascular smooth muscle cells (VSMC) were obtained from aorta of 3-month-old C57BL/6J mice. Aorta was cut into 1–2 mm pieces and incubated with collagenase type 2 solution (1.42 mg/mL) in a 37°C incubator (5% CO₂) for 45 min. Then, aortic explants were cultured for 4–5 days to allow VSMC to migrate from the artery. VSMC were detached with trypsin solution and cell suspension was centrifuged. Cells were grown in DMEM-medium (Sigma-Aldrich) supplemented with 20% FBS, 5 mmol/L glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. At 80% confluence, cells were serum-deprived for 24 h and then they were stimulated with IFN-γ (50 U/mL, 2 h; PeproTech, Rocky Hill, NJ, USA, Cat. No. 31505) and AngII (1–10 nmol/L for 3–24 h). Control cells were stimulated with vehicle.

2.7 qRT-PCR assay

Total RNA was isolated from PBMCs, vascular cells and macrophages, and mouse samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and from human tissues with TRIsureTM (Bioline, London, UK), according to the manufacturer's protocols. RNA (0.5–1 µg) was reverse-transcribed with SuperScriptTM VILOTM cDNA Synthesis Kit (Invitrogen) for PBMCs samples and with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) when cells and tissues were processed. Quantification of mRNA levels was performed by real-time PCR using specific primers and probes provided by the Assay-on-Demand system (Applied Biosystems) or PrimeTime[®] qPCR probe assays (Integrated DNA Technologies, IL, USA) ([Supplementary material online, Table S2](#)). Alternatively, forward and reverse primers were designed for real-time PCR using SYBR Green and the IQ SYBR Green supermix kit (Bio-Rad, Hercules, CA, USA) ([Supplementary material online, Table S2](#)). 18S or β-actin were used as housekeeping genes. To calculate the relative index of gene expression, we employed the 2^{-ΔΔCt} method.

2.8 Measurement of secreted ISG15

Secreted ISG15 was measured in aortic segments (2 mm in length) supernatant from WT mice infused or not with AngII (1.44 mg/kg/day, 2 weeks; Sigma-Aldrich). After incubation of the arteries for 6 h in 150 µL of Krebs-HEPES buffer (in mmol/L: 130 NaCl, 5.6 KCl, 2 CaCl₂, 0.24 MgCl₂, 8.3 HEPES, 11 glucose, pH = 7.4), ISG15 secretion was measured using CircuLex Mouse ISG15 ELISA Kit (MBL International, Woburn, MA, USA, Cat. No. CY-8091), following the manufacturer's instructions. Specificity of the kit was confirmed using arteries from

ISG15^{-/-} mice that showed no detectable values (data not shown). Values were normalized per total amount of protein.

2.9 Proteomics study

2.9.1 Protein digestion and peptide labelling and fractionation

Protein extract from aorta samples were prepared from homogenized tissue using ceramic beads (MagNa Lyser Green Beads instrument, Roche, Germany) in extraction buffer [50 mM Tris-HCl, 4% (w/v) SDS, 50 mM iodoacetamide, pH 8.5] and boiled 5 min, after which the sample is centrifuged and the supernatant collected. Protein concentration in each sample was measured using RC/DC Protein Assay (Bio-Rad) and stored at -80°C until tryptic digestion. Samples were subjected to tryptic digestion using filter-assisted sample preparation technology (FASP, Expedeaon, San Diego, CA, USA) according to previously published method.³⁴ Briefly, 100 µg of protein extract was diluted in urea sample solution (8 M urea in 100 mM Tris-HCl, pH 8.5) and loaded on filters. After centrifugation and a wash step with the same buffer, reversible oxidized protein thiol groups were reduced with dithiothreitol and then alkylated using methyl methanethiosulfonate, as described in the FASIOX method.³⁵ Proteins were digested overnight at 37°C using sequencing grade trypsin (Promega, Madison, WI, USA) in a 1:40 ratio (µg of trypsin:µg of protein) in 50 mmol/L ammonium bicarbonate, pH 8.8. Eluted peptides were desalted on Waters Oasis HLB C18 cartridges (Waters Corp, Milford, MA, USA).

The resulting peptides were labelled with iTRAQ 8plex reagents (AB Sciex, Framingham, MA, USA), according to the manufacturer's protocol. We performed two iTRAQ experiments and each one contained samples from eight individuals (two WT, two ISG15^{-/-}, two WT AngII, and two ISG15^{-/-} AngII). Labelled peptides were mixed, desalted, and separated into five fractions using high pH reversed-phase peptide fractionation (Thermo Fisher Scientific) by graded concentration of acetonitrile (ACN) prepared in triethylamine: (i) 12.5% ACN; (ii) 15% ACN; (iii) 17.5% ACN; (iv) 20% ACN; and (v) 50% ACN. Eluted fractions were dried and stored at -20°C until LC-MS/MS analysis.

2.9.2 LC-MS/MS analysis and data acquisition

The tryptic peptide mixture was subjected to nanoLC-MS/MS. High-resolution analysis was performed on a nano-HPLC Easy nLC 1000 liquid chromatography coupled to a QExactive HF-Orbitrap (Thermo Fisher Scientific) mass spectrometer. Peptides were suspended in 0.1% formic acid, loaded onto a C18 reverse-phase nano-precolumn (Acclaim PepMap100, 75 µm internal diameter, 3 µm particle size, and 2 cm length, Thermo Fisher Scientific), and separated on an analytical C18 reverse-phase nano-column (75 µm I.D. and 50 cm, Acclaim PepMap100, Thermo Fisher Scientific), in a continuous gradient (9–30%B for 300 min, 30–90%B for 3 min, 90%B for 10 min, 90–2%B for 2 min, and 2%B for 30 min, where A is 0.1% formic acid in HPLC water and B is 90% ACN, 0.1% formic acid in HPLC grade water).

Spectra were acquired using full ion-scan mode over the mass-to-charge (m/z) range 390–1600 and 60 000 (Full Width at Half Maximum) FT-resolution. MS/MS was performed on the top 15 ions in each full MS scan in data-dependent acquisition mode with 45 s dynamic exclusion enabled. High collision energy dissociation induced fragmentation was set to 30% normalized collision energy. MS/MS scan resolution was set to 17 500 and the first mass in fragmentation spectrum range was fixed at 100 m/z. A total of 22 MS datasets, 4 from unfractionated material and

18 from the corresponding fractions, were registered with 125 h total acquisition time.

Proteins were identified in the raw files using the SEQUEST HT algorithm integrated into Proteome Discoverer 2.1 (Thermo Finnigan, Thermo Fisher Scientific). MS/MS scans were matched against a mouse protein database combined with human keratins and pig trypsin (UniProtKB/Swiss-Prot 2019_01 Release). For database searching, parameters were selected as follows: trypsin digestion with two maximum missed cleavages allowed, precursor mass tolerance of 800 ppm and a fragment mass tolerance of 0.02 ppm.³⁶ The N-terminal and lysine iTRAQ-8plex modifications were chosen as fixed modifications, whereas methionine oxidation, cysteine carbamidomethylation, and cysteine methylthiolation were chosen as variable modification. The false discovery rate (FDR) was calculated based on the results obtained by database searching against the corresponded inverted database using the refined method.³⁷ Quantitative information was extracted from the intensity of the iTRAQ reporter ions in MS/MS spectra.

For comparative analysis of protein abundance changes, we used the Weighted Scan-Peptide-Protein (WSPP) statistical model³⁸ under the SanXoT software package.³⁹ This model provides a standardized variable, Zq, defined as the mean-corrected log₂-ratio expressed in units of standard deviation at the protein level. For the analysis of coordinated protein changes we used the Systems Biology Triangle (SBT) statistical model,⁴⁰ which estimates functional category averages (Zc) from protein values by performing the protein-to-category integration. Proteins were annotated based on DAVID bioinformatics tool^{41,42} using Gene Ontology terms database. Data integration was calculated by the comparison of results from the ratio ISG15^{-/-} AngII/ISG15^{-/-} with respect to the ratio WT AngII/WT, both at Zq and Zc levels. Results of oxidized peptides abundance changes were tested for significance using the Kolmogorov-Smirnov test.

2.10 Masson's trichrome staining

Collagen was stained in paraffin-embedded aorta sections by Masson-Goldner staining following the instructions of the manufacturer (Merck, Darmstadt, Germany, Cat. No. 100485). The media thickness of each aorta was measured using ImageJ software (National Institute of Health, USA).

2.11 Pressure myography studies

The structural and mechanical properties of mesenteric arteries were studied using a pressure myograph (Danish Myo Tech, Model P100, J.P. Trading I/S, Aarhus, Denmark). After a pressure-diameter curve (3–120 mm Hg) arteries were set to 45 mmHg in 0Ca²⁺-KHS and then pressure-fixed with 4% paraformaldehyde in 0.2 mol/L phosphate buffer, pH 7.2–7.4 at 37°C for 60 min and kept in 4% paraformaldehyde at 4°C for confocal microscopy studies.

From internal and external diameters in passive conditions the following structural and mechanical parameters were calculated:

$$\text{Wall thickness (WT)} = (D_{e0Ca} - D_{i0Ca}) / 2.$$

Circumferential wall strain (ϵ) = $(D_{i0Ca} - D_{00Ca}) / D_{00Ca}$, where D_{00Ca} is the internal diameter at 3 mmHg and D_{i0Ca} is the observed internal diameter for a given intravascular pressure both measured in 0Ca²⁺ medium.

Circumferential wall stress (σ) = $(P \times D_{i0Ca}) / (2WT)$, where P is the intraluminal pressure (1 mmHg = 1.334×10^3 dynes·cm⁻²) and WT is wall thickness at each intraluminal pressure in 0Ca²⁺-KHS.

Arterial stiffness independent of geometry is determined by the Young's elastic modulus ($E = \text{stress/strain}$). The stress–strain relationship is non-linear; therefore, it is more appropriate to obtain a tangential or incremental elastic modulus (E_{inc}) by determining the slope of the stress–strain curve ($E_{\text{inc}} = \delta\sigma/\delta\epsilon$). E_{inc} was obtained by fitting the stress–strain data from each animal to an exponential curve using the equation: $\sigma = \sigma_{\text{orig}}e^{\beta\epsilon}$, where σ_{orig} is the stress at the original diameter (diameter at 3 mmHg). Taking derivatives on the equation presented earlier, we see that $E_{\text{inc}} = \beta\sigma$. For a given σ -value, E_{inc} is directly proportional to β . An increase in β implies an increase in E_{inc} , which means an increase in stiffness.

2.12 Organization of internal elastic lamina

The elastin organization within the internal elastic lamina was studied on pressure-fixed segments of small mesenteric arteries (SMA), using fluorescence confocal microscopy based on the autofluorescent properties of elastin (excitation wavelength 488 nm and emission wavelength 500–560 nm). Serial optical sections from the adventitia to the lumen (z step = 0.5 μm) were captured with a $\times 40$ oil objective (Zoom 4). From each stack of serial images, individual projections of the internal elastic lamina were reconstructed and mean fenestrae area was measured using Metamorph Image Analysis Software.

2.13 Vascular reactivity studies

Aorta and SMA were dissected and segments, 2 mm in length, were mounted in a wire myograph for isometric tension recording. After equilibration in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. Contractility of the segments was tested by an initial exposure to a high K^+ solution ($\text{K}^+\text{-KHS}$, 120 mmol/L). Concentration–response curves to acetylcholine (1 nmol/L–10 $\mu\text{mol/L}$) and the NO donor Diethylamine NONOate (DEA-NO, 1 nmol/L–10 $\mu\text{mol/L}$) were performed in phenylephrine precontracted arteries.

In other experimental set, aortic segments from male C57BL/6J mice (3-month-old) were incubated for 20 h [DMEM-low glucose medium (Sigma-Aldrich) supplemented with 4 mmol/L glutamine, 1% FBS, 100 U/mL of penicillin, and 100 $\mu\text{g/mL}$ of streptomycin] in an incubator at 37°C and 5% CO_2 in the absence or in the presence of mouse rISG15 (10 ng/mL; CircuLex MBL International Cat. No. CY-R2274). Some segments were co-incubated with the selective NADPH oxidase 1 inhibitor Nox1s (10 $\mu\text{mol/L}$; Calbiochem, Merck, Cat. No. 5327610001), the selective cyclooxygenase 2 inhibitor celecoxib (1 $\mu\text{mol/L}$; Pfizer, NY, USA, Cat. No. SC58635), or an anti-IFN γ antibody (5 $\mu\text{g/mL}$; Thermo Fisher Scientific). These drugs were added 30 min before incubation with rISG15. Vascular reactivity was studied in a wire myograph as described above. Phenylephrine responses (1 nmol/L–30 $\mu\text{mol/L}$) were also studied. Control arteries were incubated in the same culture conditions.

Vasodilator responses were expressed as a percentage of the previous tone generated by phenylephrine. Vasoconstrictor responses were expressed as a percentage of the tone generated by $\text{K}^+\text{-KHS}$.

2.14 In vivo ultrasound imaging

Aortic images were taken in isoflurane-anesthetized mice (2% isoflurane) by high-frequency ultrasound with a VEVO 2100 echography device (VisualSonics, Toronto, Canada) at 30 μm resolution. Maximal internal diameter was measured at systole using VEVO 2100 software, version

1.5.0. Measurements were taken before AngII administration to determine baseline diameters and were repeated at the indicated time points after AngII infusion.

2.15 Verhoeff–Van Gieson staining

Elastic fibres were stained with a modified Verhoeff–Van Gieson staining using the manufacturer's instructions. Images were obtained using an inverted microscope (Inverted microscope Axio Vert. A1, Zeiss) and a camera (AxioCam 105 colour, Zeiss) at $\times 40$ magnification. Finally, elastic lamina breaks were counted in the medial layer of six sections per mouse and the mean number of breaks was calculated.

2.16 Sirius red staining

Collagen deposition was assessed by Picrosirius Red staining (Sigma-Aldrich). Images were captured using an Olympus BX50 microscope at $\times 10$ magnification. The percentage of fibrosis was determined as the ratio between collagen area in the media and the total medial area. Investigators for outcome assessment were blinded to group allocation.

2.17 In situ detection of vascular $\text{O}_2^{\cdot-}$ production

Incubation with the oxidative fluorescent dye dihydroethidium (DHE, Sigma-Aldrich) was used to evaluate $\text{O}_2^{\cdot-}$ production *in situ*. OCT-frozen aorta sections were equilibrated for 30 min at 37°C in Krebs-HEPES buffer. Fresh buffer containing DHE (2 $\mu\text{mol/L}$) was topically applied onto each tissue section, incubated for 30 min in a light-protected humidified chamber at 37°C, and viewed with a fluorescent laser scanning confocal microscope (Leica TCS SP5 equipped with $\times 63$ objective; Leica Microsystems S.L.U.). Fluorescence was detected with a 568 nm long-pass filter by using the same imaging settings for all experimental conditions. For quantification, three to four rings per animal were sampled and averaged. The mean fluorescence densities in the target region excluding elastic lamina were calculated. To minimize laser fluctuations from 1 day to another, data were expressed as % of signal in control arteries visualized every day.

2.18 NADPH oxidase activity assay

The $\text{O}_2^{\cdot-}$ production generated by NADPH oxidase was determined by a chemiluminescence assay. Briefly, endothelial cells were rinsed with PBS and harvested in phosphate buffer (50 mmol/L KH_2PO_4 , 1 mmol/L EGTA, 150 mmol/L sucrose, pH 7.4). The reaction was performed in cell homogenates starting by the addition of a lucigenin (5 $\mu\text{mol/L}$) and NADPH (100 $\mu\text{mol/L}$; Sigma-Aldrich) mixture to the protein sample in a final volume of 250 μL . Chemiluminescence was determined every 2.4 s for 3 min in a microtitre plate luminometer (Enspire Perkin Elmer). Basal activity in the absence of NADPH was subtracted from each reading and normalized to protein concentration.

2.19 Superoxide measurement by electron paramagnetic resonance

Superoxide production in endothelial cells homogenate was measured by electron paramagnetic resonance (EPR) in samples containing 10 μg of protein and CMH (1 mmol/L; Enzo Life Sciences, Exeter, UK; ALX-430-117) in a total volume of 100 μL of Krebs-HEPES buffer containing deferoxamine (25 $\mu\text{mol/L}$) and DETC (5 $\mu\text{mol/L}$). After homogenization, EPR samples were placed in 50 μL glass capillaries and measurements

were performed by Bruker BioSpin's e-scan EPR (Bruker® BioSpin Corp., Southborough, MA, USA) equipped with a super-high Q microwave cavity at room temperature. The EPR instrument settings for experiments were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 656 ms; time constant, 656 ms; 512 points resolution and receiver gain, 1×10^5 . Results were normalized by protein content.

2.20 Western blot

Cultured endothelial cells were homogenized in lysis buffer [(in mmol/L) sodium pyrophosphate 50, NaF 50, NaCl 5, EDTA 5, EGTA 5, HEPES 10, Na₃VO₄ 2, PMSF 50, Triton 100 0.5%, and leupeptin/aprotinin/pepstatin 1 mg/mL]. Proteins (10 µg) were separated by electrophoresis on 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with anti-NOX1 (SAB4200097, Sigma, rabbit, 1:1000), anti-NOX4 (ab133303, Abcam, Cambridge, UK, rabbit, 1:1000), anti-NOX5 (kindly provided by Dr David Harrison, rabbit, 1:1000), and beta-actin (A2228, Sigma, mouse, 1:1000) overnight at 4°C. Next, membranes were washed with TBS-T and incubated with secondary fluorescence-coupled antibodies goat-anti-mouse-IRDye 680 or goat-anti-rabbit-IRDye 800 (LI-COR, Cambridge, UK) 1 h, at room temperature in the dark and visualized by an infrared laser scanner (Odyssey Clx, LI-COR). Western blotting images were quantified using the software Image Studio™ Lite free version (LI-COR). Protein expression levels were normalized to loading controls and expressed as percentage of the control.

2.21 Statistics

All data are expressed as mean values $\pm$ standard mean error and *n* represents the number of animals, different cell cultures or patients studied. Data are reported as dot plots that represent different biological replicates. When this is not possible, the number of animals in each group is reported in figure legends. Statistical analysis was done by GraphPad Prism Software (v7.04). Data distribution (by Shapiro–Wilk normality test) was used to choose the appropriate statistics test. Results were analysed by the Mann–Whitney non-parametric or Student's *t*-tests when appropriate (two-tailed) or one-way or two-way ANOVA followed by Bonferroni's, Tukey's, or Sidak's multiple comparison tests. For survival analysis, a log-rank (Mantel–Cox test) was calculated. Statistical analysis for the human PBMC study was performed by SPSS 15.0. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT or systolic blood pressure as dependent variables, including in the model the traditional risk factors and those variables that were significant in the univariate analysis. Statistical analysis for proteomic studies was performed using the refined method³⁷ for peptide identification, with a 1% FDR criterion used to ascertain true identification. Statistics for protein quantification was performed according to the WSPP model³⁸ under the SanXoT software package.³⁹ For the analysis of coordinated protein changes we used the SBT statistical model.⁴⁰ A FDR <0.05 was considered significant. Results of oxidized peptides abundance changes were tested for significance using the Kolmogorov–Smirnov test. The exclusion of data from the analysis was done by the ROUT method with GraphPad Prism Software. A *P*-value <0.05 was considered significant.

3. Results

3.1 Interaction network analysis provides evidence that ISGylation and ISG15 might be associated with hypertension and vascular damage

To identify the common proteins involved in hypertension, we searched for the term 'Hypertension' in Phenopedia, which is an online encyclopaedia of Human Genome Epidemiology that provides information about genes related to human disease. As shown in [Supplementary material online, Table S3](#), 2206 proteins were retrieved. We then applied Upstream Regulator Analysis tool from Ingenuity Pathways Analysis software. Among the Upstream Regulators, we found cytokines like TNF, IL-1 β , TGF β 1, IFN- γ , or IL-6, and transcription factors such as STAT3 and NF- κ B as the most important master regulators for the dataset (*Upstream Regulator For Hypertension* in [Supplementary material online, Table S4](#)). IFN- γ was connected with 932 proteins from the dataset, among which 410 were directly connected to IFN- γ (*IFN γ Network* in [Supplementary material online, Table S4](#)). The ISGylation-related protein UBA7 and the de-ISGylation protein USP18 also appear among the master regulators (*Upstream Regulator For Hypertension* in [Supplementary material online, Table S4](#)), evidencing a possible role for ISGylation in hypertension. In detail, UBA7 and USP18 directly interacted with several inflammatory cytokines such as TNF, IFN- γ , or IL-6, the anti-inflammatory cytokine IL-10, proteins related to IFN signalling pathways such as interferon-induced helicase C domain-containing protein 1 (IFIH1), suppressor of cytokine signalling (SOCS) 1/3, and with the transcription factor nuclear factor of activated T cells (NFAT) (*Figure 1A and B and UBA7 and USP18_Network* in [Supplementary material online, Table S4](#)).

We then explored the specific relationship of ISG15 with hypertension by creating a protein–protein interaction network with the 2206 hypertension-related proteins and ISG15 by STRING/Cytoscape. We found that ISG15 could be related with 60 proteins in the network (*Figure 1C*). Some of these proteins, such as NF- κ B1, CCL2, TNF, IFN- γ , STAT3, or TLR4, are closely related to endothelial dysfunction, vascular remodelling, or both (*Figure 1C and Supplementary material online, Table S5*), suggesting that ISG15 could be a new hypothetical mediator of vascular damage associated with hypertension.

3.2 ISG15 expression in PBMCs correlates with systolic and diastolic blood pressure and vascular remodelling in patients

We then tested a possible relationship between ISG15, blood pressure, and vascular damage in PBMCs from a population of 175 asymptomatic patients in which left carotid IMT, a marker of vascular remodelling, was also measured.

Univariate analysis showed a positive correlation between ISG15 mRNA and systolic and diastolic blood pressure (*Figure 1D and Supplementary material online, Table S6*) and between ISG15 and carotid IMT (*Figure 1E and Supplementary material online, Table S6*). Importantly, the association between carotid IMT ([Supplementary material online, Table S7](#)) or systolic blood pressure ([Supplementary material online, Table S8](#)) and ISG15 mRNA levels remained significant after adjusting for traditional risk factors.

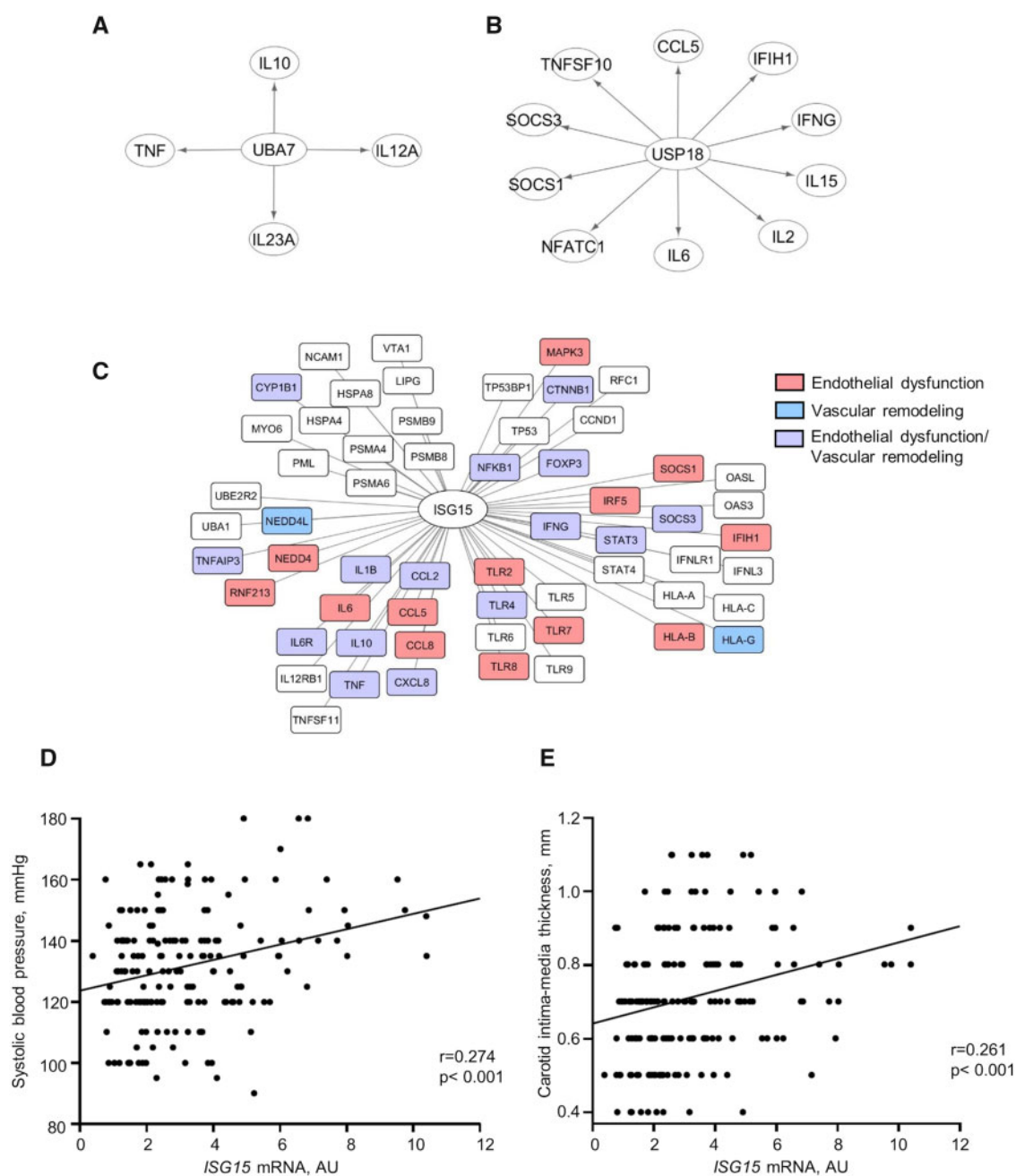


Figure 1 ISG15 is a potential mediator of vascular damage. Interactions between UBA7 (A) or USP18 (B) with other proteins involved in hypertension, obtained with Ingenuity Pathways Analysis software. Protein–protein interaction network between hypertension-related proteins and ISG15 by STRING/Cytoscape (C). Correlation between *ISG15* mRNA and systolic blood pressure (D) or carotid intima-media thickness (E) in human peripheral blood mononuclear cells of asymptomatic patients. AU, arbitrary units.

3.3 AngII induces ISG15 at the vascular level and in macrophages

In cultured mice VSMC IFN- γ increased *Isg15* mRNA expression (Figure 2A) demonstrating the ability of type II IFN to stimulate *Isg15* expression at the vascular level. As described previously,⁷ AngII infusion increased *Ifng* expression in aorta (Figure 2B). Moreover, acute ex vivo

incubation of aorta with AngII also increased *Ifng* (data not shown) and *Isg15* mRNA expression that was prevented by co-incubation with an anti-IFN- γ antibody (Figure 2C). Hypertensive AngII-infused mice showed increased aorta *Isg15* mRNA expression and protein secretion (Figure 2D and E). *Isg15* transcript was also increased in aorta from adult 6-month-old SHR (Figure 2F). Co-treatment of AngII-infused mice with

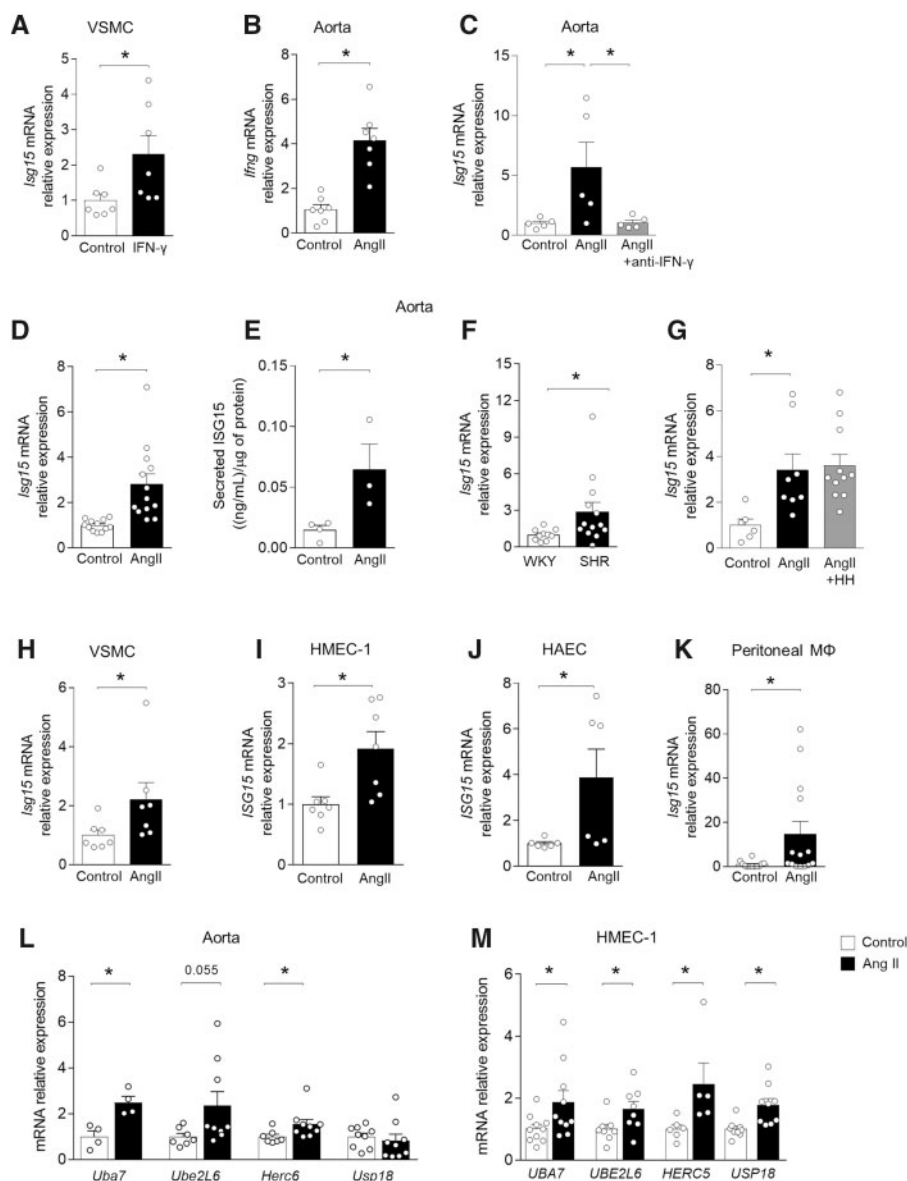


Figure 2 Angiotensin II (AngII) induces ISG15 expression at the vascular level and in macrophages. (A) *Isg15* mRNA levels in vascular smooth muscle cells (VSMC) from C57BL/6 mice incubated or not with IFN- γ (50 U/mL, 2 h). (B) *Irfng* mRNA levels in aorta from mice untreated (control) and treated with AngII (1.44 mg/kg/day, 2 weeks). (C) *Isg15* mRNA levels in aorta from C57BL/6 mice incubated or not with AngII (1 μ mol/L 6 h) in the absence or presence of anti-IFN- γ antibody (5 μ g/mL). *Isg15* mRNA levels (D) and secreted ISG15 protein (E) in aorta from mice untreated (Control) and treated with AngII. (F) *Isg15* mRNA levels in aorta from 6-month-old normotensive (Wistar Kyoto, WKY) and spontaneously hypertensive (SHR) rats. (G) *Isg15* mRNA levels in aorta from mice untreated (Control) and treated with AngII or AngII and hydralazine plus hydrochlorothiazide (HH, hydralazine: 20 mg/kg/day; hydrochlorothiazide: 6 mg/kg/day i.p.). *ISG15* mRNA levels in VSMC (H), human microvascular endothelial cells (HMEC-1) (I), and human aortic endothelial cells (HAEC) (J) incubated with AngII (1 nmol/L, 4 h). *Isg15* mRNA levels in peritoneal macrophages from untreated mice (Control) and treated with AngII (K). mRNA levels of ISGylation enzymes (*UBA7*, *Ube2L6*, and *HERC5/Herc6*) and de-ISGylation enzyme *USP18* in aorta from Control and AngII-infused mice (L) and HMEC-1 treated or not with AngII (1 nmol/L, 4 h) (M). * $P < 0.05$ by Student's *t*-test or one-way ANOVA.

HH significantly decreased blood pressure (Control: 97 ± 1 ; AngII: 131 ± 17 ; AngII + HH: 105 ± 7 mmHg; P -value < 0.05 Control vs. AngII; P -value < 0.05 AngII vs. AngII + HH, $n = 6-11$), but it did not modify the increase in vascular *Isg15* mRNA expression induced by AngII (Figure 2G).

We then evaluated the ability of AngII to increase *ISG15* expression in different vascular cells. AngII increased *ISG15* mRNA expression in

cultured mouse VSMC (Figure 2H), HMEC-1 (Figure 2I), and HAEC (Figure 2J). In addition, AngII infusion increased *Isg15* expression in peritoneal macrophages (Figure 2K).

AngII also increased the expression of ISGylation enzymes (*UBA7*, *Ube2L6*, and *HERC5/Herc6*) in aorta (Figure 2L) and endothelial cells (Figure 2M). Regarding the de-ISGylation enzyme *USP18*, AngII increased its mRNA levels in HMEC-1 but not in aorta (Figure 2L and M).

3.4 ISG15 deletion modifies abundance of proteins involved in cardiovascular remodelling, extracellular matrix, and cardiovascular function

We performed a multiplexed quantitative proteomics approach to explore changes in protein abundance in aorta from untreated or AngII-infused WT and ISG15^{-/-} mice. A total of 1538 proteins were quantified (FDR < 0.01), of which 52 proteins were differentially expressed between ISG15^{-/-} and WT mice in response to AngII (FDR < 0.05; the complete list of quantitative results is presented in [Supplementary material online, Table S9](#)). Using the SBT model⁴⁰ and a database of 428 GO terms constructed from the DAVID repository, we detected a set of co-ordinated protein abundance changes at the functional category level ([Supplementary material online, Table S10](#)). To simplify interpretation, we manually grouped the categories into functional clusters. The cluster *cardiovascular remodelling* included proteins that belong to 13 GO terms related to extracellular matrix, blood vessel development, or remodelling among others (Figure 3A and [Supplementary material online, Table S11](#)). Both *cardiovascular remodelling* and *extracellular matrix* clusters were significantly decreased (FDR < 0.05) in the absence of ISG15 (Figure 3D). The cluster *cardiovascular function* comprised proteins that belong to five related GO terms (Figure 3B and [Supplementary material online, Table S11](#)) and was significantly increased in the absence of ISG15 (FDR < 0.05) (Figure 3D). Since ISG15 is an immune response protein, we also analysed the *immune system* cluster, represented with 14 GO terms (Figure 3C). A tendency towards a decrease ($P = 0.076$) was observed in the absence of ISG15 (Figure 3D and [Supplementary material online, Table S11](#)). All together, these results provide evidence that ISG15 might play a relevant role in the vascular functional and structural alterations associated with hypertension.

3.5 ISG15 plays a role in AngII-induced hypertension, vascular stiffness, and endothelial dysfunction

We aimed to address the role of ISG15 in hypertension. Similar systolic blood pressure was detected in WT and ISG15^{-/-} mice (Figure 4A). However, ISG15 deletion partially prevented the increase in systolic blood pressure induced by AngII infusion (Figure 4A). Vascular structure of aorta and SMA was comparable in untreated WT and ISG15^{-/-} mice (Figure 4B–D), while AngII infusion similarly increased arterial wall thickness and decreased lumen diameter (Figure 4B–D). In WT SMA, AngII increased vascular stiffness (reflected by the leftward shift of the stress–strain curve and the increased β value) and altered 3D elastin structure in the internal elastic lamina with smaller fenestrae (Figure 4E and F), and these effects were completely prevented by ISG15 deletion (Figure 4E and F).

Regarding the role of ISG15 in vascular function, endothelium-dependent relaxation to acetylcholine was similar in aorta and in SMA from untreated WT and ISG15^{-/-} mice (Figure 4G and I). AngII impaired endothelium-dependent relaxation in aorta and SMA from WT mice but not from ISG15^{-/-} mice that were protected (Figure 4G and I). No effect of AngII or genotype was observed in the vascular response to exogenous added NO (Figure 4H and J), thus excluding that the observed differences in acetylcholine responses are due to changes in VSMC sensitivity to NO.

3.6 ISGylation plays a role in AngII-induced hypertension and vascular remodelling

To confirm the role of ISG15 in hypertension and vascular injury, we used a gain of function approach evaluating the effect of AngII in transgenic USP18^{C61A} mice that selectively lack functional protease activity leading to excessive ISGylation.³¹ AngII infusion induced lethal aortic dissection in 11 of the 27 USP18^{C61A} mice, but only in 1 of 15 in the WT animals (Figure 5A). No changes in systolic blood pressure were found between untreated WT and USP18^{C61A} mice. However, AngII increased systolic blood pressure more in surviving USP18^{C61A} than in WT mice (Figure 5B). *In vivo* ultrasound imaging showed that AngII infusion increased the diameter of both ascending and abdominal aorta, and this increase was higher in ascending aorta from USP18^{C61A} than WT mice (Figure 5C). No lethal aortic dissection or changes in ascending or in abdominal aorta were found in untreated WT or USP18^{C61A} mice (Figure 5C). Histological analysis of elastin fibres fragmentation showed that AngII produced a higher increase in the number of elastic lamina breaks in surviving USP18^{C61A} than in WT mice (Figure 5D). AngII increased collagen deposition in WT mice (Figure 5E). Moreover, USP18^{C61A} mice showed augmented collagen deposition even in basal conditions that was not modified by AngII (Figure 5E). AngII did not modified mRNA levels of transforming growth factor and connective tissue growth factor in arteries from WT mice (Figure 5F). However, increased gene expression of these profibrotic factors were observed in arteries from USP18^{C61A} mice compared to WT mice, both in the absence and in the presence of AngII, (Figure 5F). AngII increased fibronectin expression only in WT mice and no differences in fibronectin expression were found among genotypes (Figure 5F).

To determine the role of ISG15 in aorta dilation we measured the levels of ISG15 and USP18 transcripts in aneurysms from ApoE^{-/-} plus AngII-infused mice and from healthy donors ($n = 17$) and abdominal aortic aneurysm patients ($n = 84$). As shown in [Supplementary material online, Figure S1](#), ISG15 expression was significantly enhanced in murine and human aneurysms. Moreover, USP18 expression was enhanced in human aneurysms. A positive correlation was found between the expression of ISG15 and USP18 in human samples ([Supplementary material online, Figure S1B](#)).

No differences in the structural and mechanical properties of SMA were observed between WT and USP18 mice either under basal or after AngII infusion (data not shown). Similarly, endothelium-dependent and -independent relaxation was unchanged in arteries from untreated and AngII-infused mice from both genotypes (Figure 5G–J).

Together, these findings demonstrate that ISGylation participates in AngII-induced hypertension and vascular remodelling, specifically of large arteries.

3.7 ISG15 plays a role in AngII-induced vascular inflammation and oxidative stress

Secreted ISG15 is an IFN γ -inducing molecule from lymphocytes^{9,22–24} and NK cells.^{10,25} Moreover, IFN γ produces reactive oxygen species (ROS) that mediate the endothelial dysfunction induced by AngII.^{7,8} We have previously demonstrated the participation of both oxidative stress and inflammatory mediators, such as cyclooxygenase-2 (COX-2), in endothelial dysfunction, vascular remodelling, and arterial stiffness in hypertension.^{43–45} Then, we questioned whether inflammation and oxidative stress could be potential underlying mechanisms responsible for the role of ISG15 in hypertension and vascular damage. Our proteomics study

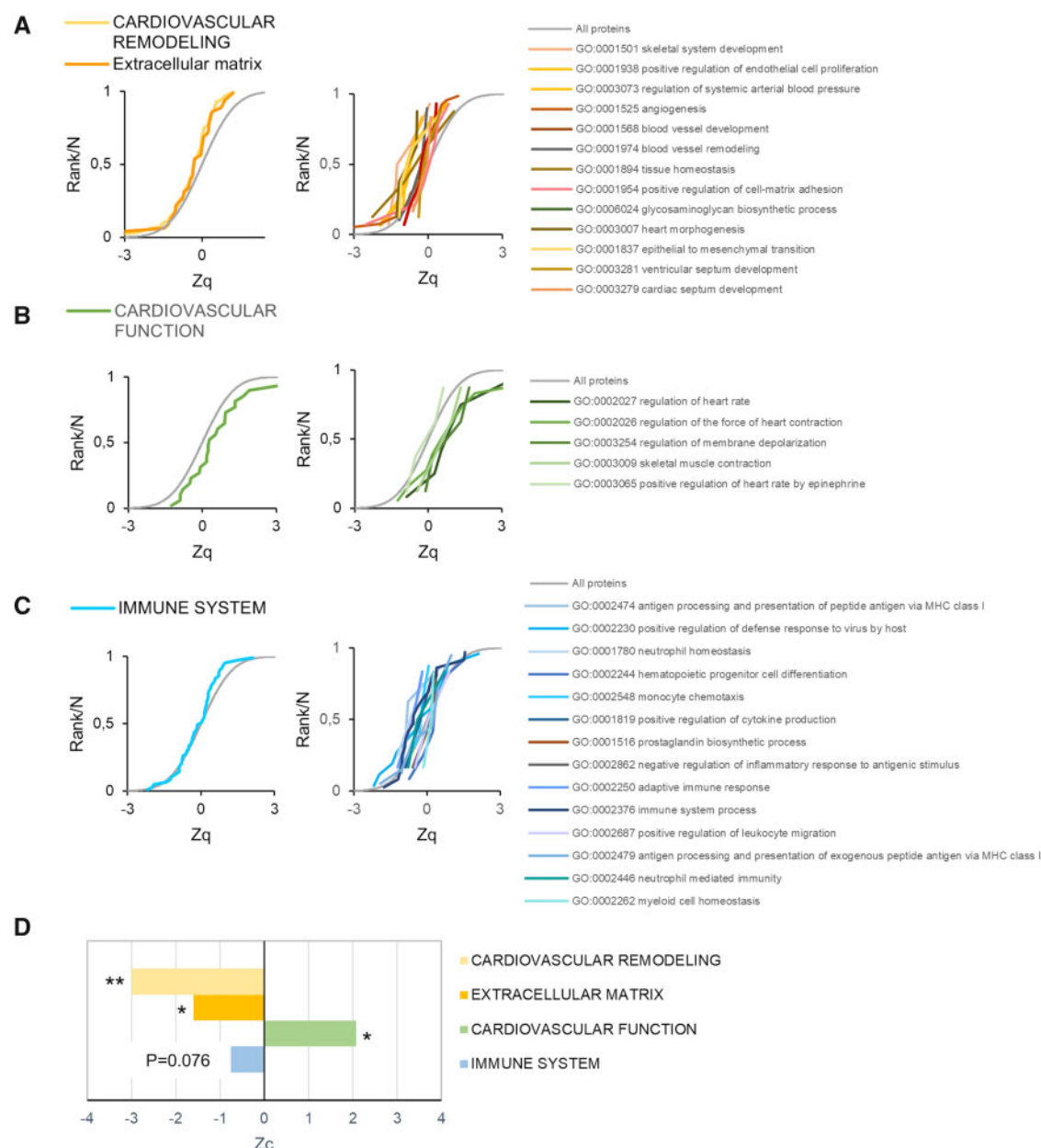


Figure 3 Quantitative proteomics demonstrates that ISG15 promotes a coordinated alteration of *cardiovascular remodelling*, *extracellular matrix* and *cardiovascular function* categories. Aortic tissue samples from WT and ISG15^{-/-} treated or not with Angiotensin II (AngII) were subjected to quantitative proteomics using multiplexed isobaric labelling and LC-MS/MS. The quantitative data were analysed using the SBT model to detect coordinated protein changes in functional categories. The distributions of quantitative protein values (Zq) are plotted for four clusters (A) *cardiovascular remodelling* and *extracellular matrix*, (B) *cardiovascular function* and (C) *immune system*. Panels on the left display the cumulative distribution of Zq from proteins belonging to each cluster. Panels on the right display the protein values belonging to the related GO terms which compose each cluster. Protein values (Zq) are log₂ fold changes in AngII-treated ISG15^{-/-} compared to AngII-treated WT, normalized with respect to untreated samples, expressed in units of standard deviation. (D) Standardized log₂ fold changes (Zc) of the four category clusters (*FDR < 0.05; **FDR < 0.01). The complete set of proteins belonging to each cluster is listed in [Supplementary material online, Table S11](#).

revealed a significant coordinated decrease of proteins belonging to a cluster of six GO categories related to *Vascular redox state* in AngII-infused ISG15^{-/-} compared to WT mice (FDR < 0.05; [Figure 6A](#) and [Supplementary material online, Table S11](#)). To further explore this

finding, we studied the aortic thiol redoxome by analysing relative abundance changes in the levels of oxidized cysteines (Cys) in the different experimental groups using the FASIOX method.³⁵ AngII induced a very clear global increase ($P < 0.002$) in the abundance of oxidized

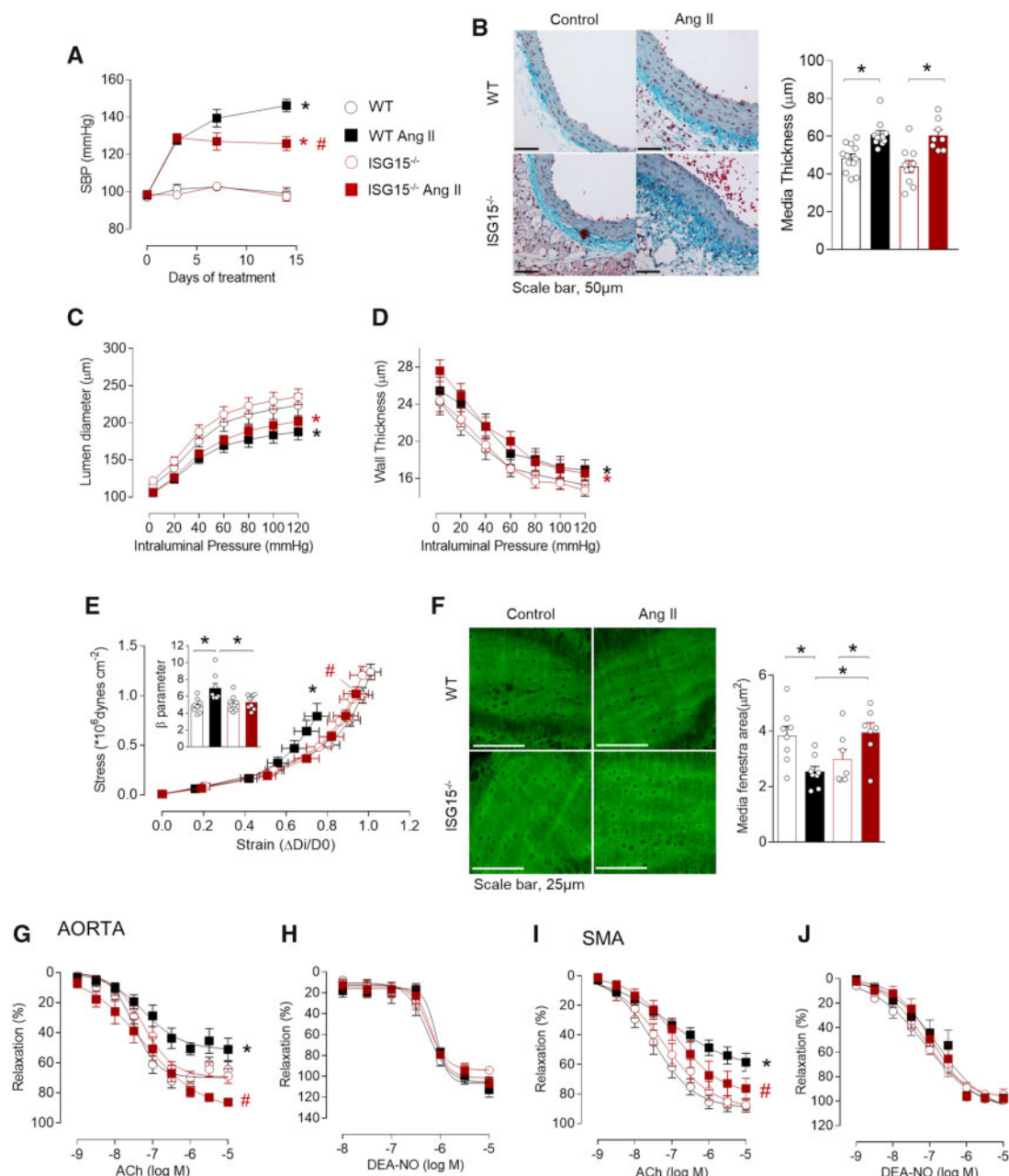


Figure 4 ISG15 participates in Angiotensin II (AngII)-induced hypertension and vascular damage. (A) Systolic blood pressure (SBP) measured by tail-cuff plethysmography (WT and WT AngII: $n = 12$, ISG15^{-/-}: $n = 10$, ISG15^{-/-} AngII: $n = 11$); $*P < 0.05$ vs. untreated, $^{\#}P < 0.05$ vs. WT AngII by two-way ANOVA. (B) Aortic Masson stain and media thickness quantification; $*P < 0.05$ by one-way ANOVA. Structural (C and D) and mechanical parameters (E) in small mesenteric arteries (SMA) ($n = 7-12$); $*P < 0.05$ vs. untreated by two-way ANOVA or $*P < 0.05$ by one-way ANOVA. (F) Quantification of internal elastic lamina structure of SMA; $*P < 0.05$ by one-way ANOVA. Concentration-response curves to acetylcholine (ACh; G and I) and diethylamine NONOate (DEA-NO; H and J) of aorta or SMA ($n = 7-12$); $*P < 0.05$ vs. untreated, $^{\#}P < 0.05$ vs. WT AngII by two-way ANOVA.

Cys-containing peptides in the aorta from WT but not from ISG15^{-/-} mice (Figure 6B and Supplementary material online, Table S12). These results were validated by direct measurements of $O_2^{\bullet-}$. As shown in Figure 6C, ISG15^{-/-} mice were protected against the increase in vascular $O_2^{\bullet-}$ production induced by AngII, both in the media and adventitial layers.

Regarding inflammatory mediators, no differences in mRNA levels of *Ifng* and *Ccl2* were found in aorta from untreated WT and ISG15^{-/-} mice (Figure 6D). However, unexpectedly, an increase in *Ptgs2* and *Cd3e* and a decrease in the macrophages marker *Adgre1* mRNA levels were observed in arteries from untreated-ISG15^{-/-} mice, indicating that ISG15 might modulate basal immune and inflammatory status. AngII increased

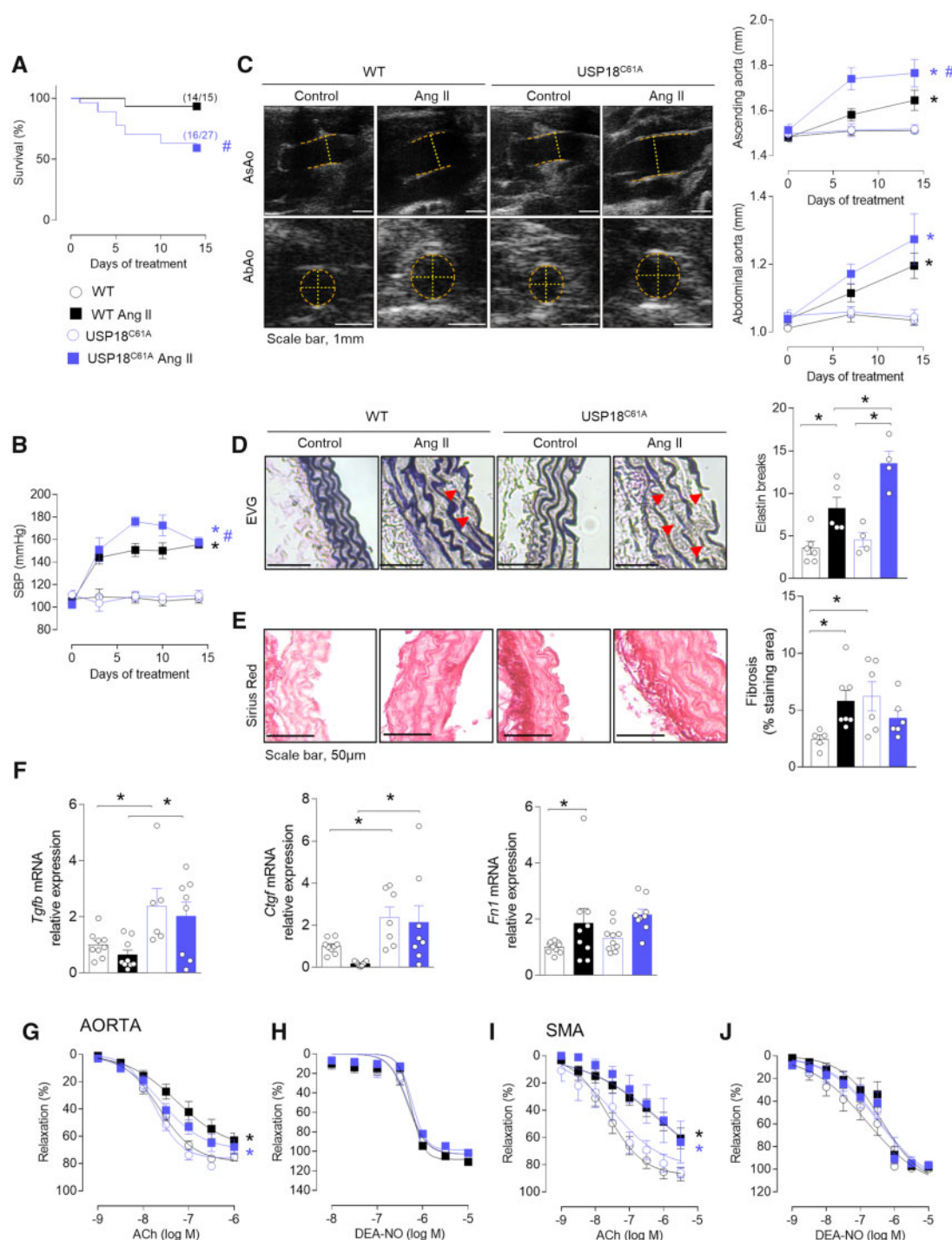


Figure 5 ISGylation participates in angiotensin II (AngII)-induced hypertension and vascular damage. (A) Survival curve of AngII-treated WT and USP18^{C61A} mice ($n = 15-27$); $\#P < 0.05$ vs. WT AngII by log-rank (Mantel-Cox) test. (B) Systolic blood pressure (SBP) measured by tail-cuff plethysmography (WT and WT AngII: $n = 9$, USP18^{C61A} and USP18^{C61A} AngII: $n = 11$); $*P < 0.05$ vs. untreated, $\#P < 0.05$ vs. WT AngII by two-way ANOVA. (C) Representative ultrasound images and quantification of maximal diameter in ascending aorta (AsAo) or abdominal aorta (AbAo) (WT and WT AngII: $n = 12$, USP18^{C61A}: $n = 8$, USP18^{C61A} AngII: $n = 9$); $*P < 0.05$ vs. untreated, $\#P < 0.05$ vs. WT AngII by two-way ANOVA. (D) Representative aortic elastic Van Gieson (EVG) staining and quantification; $*P < 0.05$ by one-way ANOVA. (E) Representative aortic Sirius red staining and quantification; $*P < 0.05$ by one-way ANOVA. (F) Aortic mRNA expression of fibrotic markers; $*P < 0.05$ by one-way ANOVA. (G-J) Concentration-response curves to acetylcholine (ACh) and diethylamine NONOate (DEA-NO) of aorta and SMA ($n = 6-9$); $*P < 0.05$ vs. untreated by two-way ANOVA.

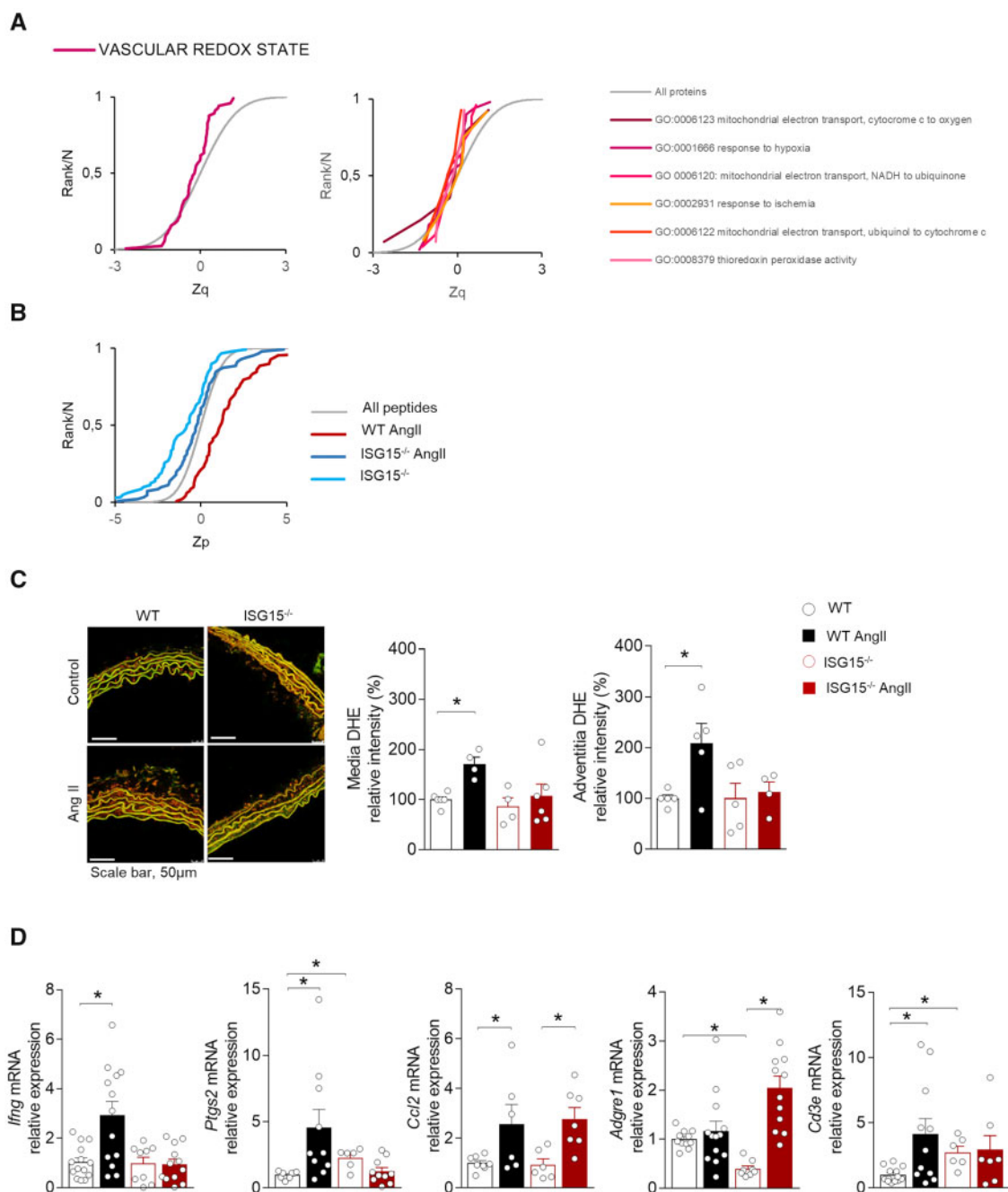


Figure 6 ISG15 participates in angiotensin II (AngII)-induced inflammation and oxidative stress. (A) Quantitative proteomics analysis of proteins related to the *vascular redox state* cluster. Results were presented as in Figure 3. The functional category abundance change value for this cluster was $Z_c = -1.83$ ($\log_2$ fold change in ISG15^{-/-} AngII with respect to WT AngII, normalized with respect to untreated samples, in units of standard deviation), which is statistically significant at FDR < 0.05. The complete set of proteins belonging to this cluster is listed in [Supplementary material online, Table S11](#). (B) Quantitative redox proteomics shows increased abundance of peptides containing reversibly oxidized Cys sites in AngII-treated WT mice in comparison with ISG15^{-/-} mice. Shown are the cumulative distributions of Zp, the standardized $\log_2$ ratio of oxidized-Cys-containing peptides in WT AngII, ISG15^{-/-} AngII and ISG15^{-/-}, with respect to WT. The graph also shows the distribution of Zp for all the peptides of the experiment (in grey). The oxidized-Cys-containing peptides of the WT AngII sample is significantly increased with respect to the WT sample ($P < 0.002$, Kolmogorov–Smirnov test). (C) Representative dihydroethidium (DHE) fluorescence and quantification in media and adventitia layers. (D) Aortic mRNA expression of *Ifng*, *Ptgs2*, *Ccl2*, *Adgre1*, and *Cd3e*. * $P < 0.05$ by one-way ANOVA.

the expression of the proinflammatory markers *Ifng*, *Ptgs2*, *Ccl2*, and *Cd3e* in arteries from WT mice (Figure 6D). However, AngII failed to increase *Ifng*, *Ptgs2*, and *Cd3e* expression in arteries from ISG15^{-/-} mice,

suggesting that ISG15 is involved in AngII-induced vascular inflammation. In agreement, aortas from USP18^{C61A} mice showed a clear inflammatory profile as demonstrated by an increased expression of *Ifng*, *Ptgs2*, *Ccl2*,

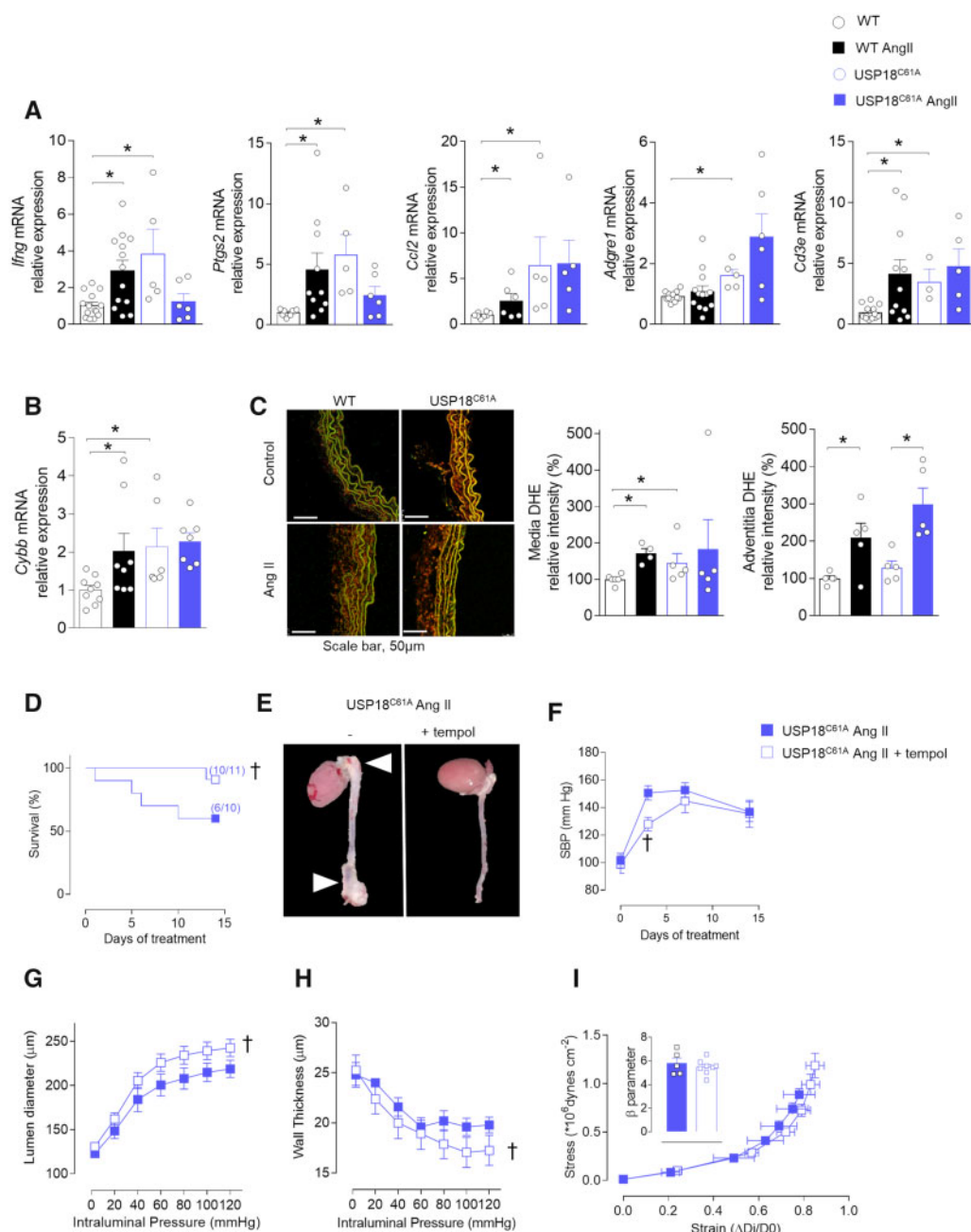


Figure 7 ISGylation increases inflammation and reactive oxygen species generation that participate of vascular damage. (A) Aortic mRNA expression of *Ifng*, *Ptgs2*, *Ccl2*, *Adgre1*, and *Cd3e*; * $P < 0.05$ by one-way ANOVA. (B) Aortic mRNA expression of *Cybb*; * $P < 0.05$ by one-way ANOVA. (C) Representative dihydroethidium (DHE) fluorescence and quantification in media and adventitia layers. * $P < 0.05$ by one-way ANOVA. Because experiments were run simultaneously, data from WT and WT-angiotensin II (AngII) from (A) and (C) are the same as Figure 6D and C, respectively. Representative images for DHE staining are different. (D) Survival curve of AngII-infused USP18^{C61A} mice treated or not with the antioxidant tempol (0.288 nmol/kg/day) ($n = 10-11$); † $P < 0.05$ vs. USP18^{C61A} AngII by log-rank (Mantel-Cox) test. (E) Representative images of aorta from USP18^{C61A}-AngII mice treated or not with tempol (F) systolic blood pressure (SBP) measured by tail-cuff plethysmography ($n = 8-12$); † $P < 0.05$ vs. USP18^{C61A} AngII by two-way ANOVA. Structural (G and H) and mechanical (I) parameters in small mesenteric arteries ($n = 4-8$); † $P < 0.05$ vs. USP18^{C61A} AngII by two-way ANOVA.

Adgre1, and *Cd3e*, which was not further modified by AngII (Figure 7A). Moreover, AngII increased Nox2 gene expression in arteries from WT mice; and augmented expression of Nox2 gene was also found in

USP18^{C61A} mice in basal conditions that was not modified by AngII (Figure 7B). Accordingly, basal vascular O₂^{•-} production was greater in the media of arteries from untreated USP18^{C61A} mice, but not in the

adventitia (Figure 7C) and interestingly, AngII infusion increased $O_2^{\bullet-}$ production in the adventitia but not in the media from USP18^{C61A} mice (Figure 7C).

Because ISG15 deletion protected from endothelial dysfunction as well as the enhanced ROS generation and the upregulation of *Ptgs2* promoted by AngII, we tested the ability of ISG15 to induce ROS generation and *PTGS2* expression in endothelial cells. As shown in [Supplementary material online, Figure S2A](#), in HMEC-1, recombinant ISG15 (rISG15) enhanced *PTGS2* mRNA expression as early as 3 h after exposure. rISG15 also increased NADPH oxidase (NOX)1 and NOX5 subunits mRNA expression as well as NADPH oxidase activity ([Supplementary material online, Figure S2B–D](#)). These results were confirmed in HAEC where rISG15 increased NADPH oxidase activity, $O_2^{\bullet-}$ generation measured by EPR and NOX1/4/5 protein expression ([Supplementary material online, Figure S2E–I](#)).

Interestingly, no correlation between *ISG15* mRNA and $O_2^{\bullet-}$ production was found in human PBMCs ([Supplementary material online, Table S6](#)), suggesting that ISG15 might influence redox biology in some specific cell types such as vascular endothelial cells but not in immune cells. Altogether, these findings uncover a new role for ISG15 in vascular inflammation and ROS production.

3.8 Oxidative stress and inflammation mediate ISG15-induced endothelial dysfunction

In aortic segments from WT animals, rISG15 significantly impaired endothelium-dependent relaxation, without modifying DEA-NO relaxation or contractile responses to phenylephrine ([Supplementary material online, Figure S3](#)). The selective COX-2 inhibitor celecoxib and the selective NOX1 inhibitor NoxA1ds prevented ISG15-induced endothelial dysfunction ([Supplementary material online, Figure S3A](#)). Similar results were obtained with an anti-IFN- γ antibody ([Supplementary material online, Figure S3D](#)), suggesting that ISG15 induces endothelial dysfunction through IFN- γ generation that might facilitate vascular inflammation and oxidative stress.

3.9 Oxidative stress mediate ISGylation-induced vascular remodelling

To confirm the potential role of oxidative stress in the vascular damage associated with ISGylation, AngII-infused USP18^{C61A} mice were co-treated with the antioxidant tempol. As shown in [Figure 7D and E](#), tempol treatment significantly improved survival and reduced aneurysm formation. Moreover, tempol induced a significant delay in the rise of systolic blood pressure at the beginning of the treatment ([Figure 7F](#)).

Vascular remodelling ([Figure 7G and H](#)), but not vascular stiffness ([Figure 7I](#)), of SMA was also improved by tempol in AngII-treated USP18^{C61A} mice as shown by the increased lumen diameter ([Figure 7G](#)), and reduced wall thickness ([Figure 7H](#)).

These data demonstrate an essential role of oxidative stress in ISGylation-dependent vascular remodelling.

4. Discussion

Clinical and experimental evidence demonstrate increased presence of different proinflammatory cytokines including IFN- γ or TNF α in plasma and tissue of hypertensive individuals.⁶ More importantly, they have a role in hypertension, endothelial dysfunction and cardiac damage

induced by prohypertensive stimuli such as AngII.^{3,7,8,46} Our unbiased bioinformatics study identified TNF α and IFN- γ as the first and fourth master regulators, respectively, of proteins involved in hypertension. This highlights the need to identify their downstream mediators.

Our study demonstrates for the first time that ISG15 might be a novel mediator of hypertension and vascular damage in patients, as *ISG15* mRNA in PBMCs positively correlated not only with systolic/diastolic blood pressure, but also with carotid IMT, a surrogate marker of vascular remodelling. Importantly, these correlations remained significant after adjustment for traditional risks factors such as age, gender, smoking, body mass index, systolic blood pressure, glucose, and total cholesterol. We also found that AngII is a novel stimulus for *ISG15* expression in macrophages, isolated VSMC, and endothelial cells and in vascular tissue from AngII-induced hypertension in mice. Specifically, we observed *ISG15* expression as early as 4–6 h after AngII stimulation, in agreement with effects induced by IFN stimulation.⁴⁷ Moreover, we detected an increase in ISG15 secretion in arteries from AngII-infused mice and augmented expression of conjugating enzymes by AngII both *in vitro* and *in vivo*. Of note, adult hypertensive SHR also showed augmented vascular *lsg15* expression, but the treatment of AngII-infused mice with HH did not affect *lsg15* expression, despite of a near normalization of blood pressure, suggesting that hypertension-associated *lsg15* expression does not depend exclusively on augmented blood pressure.

The effects of ISG15 on the cardiovascular system are poorly explored and the only evidence comes from studies mostly performed in the context of viral infections reporting beneficial effects of the ISG15 pathway in viral cardiomyopathy.^{27,28,48} Our bioinformatics analysis suggested that the ISG15 pathway might have a role in the development of hypertension, endothelial dysfunction, and vascular remodelling, through interaction with different proteins involved in inflammation, IFN signaling, or activation of transcription factors. This was confirmed experimentally by analysing changes in the proteome of aorta from AngII-infused WT and ISG15^{-/-} mice that showed differential expression in proteins involved in cardiovascular function and remodelling, and extracellular matrix. Notably, we did not find significant changes in proteins belonging to the immune system albeit a tendency to decrease was observed in arteries from ISG15^{-/-} mice likely because the limited number of proteins detected, further suggesting a direct involvement of ISG15 pathway in vascular responses.

By using a multidisciplinary approach including ISG15 KO mice that lack free and conjugated ISG15, USP18^{C61A} mice that show excessive irreversibly ISGylation, and exogenously added rISG15, we demonstrated the involvement of the ISG15 pathway in hypertension-induced vascular damage. Thus, we found that upon AngII infusion, USP18^{C61A} mice develop aortic dilation, lethal dissection, and elastin degradation. Moreover, aorta from the classical model of aneurysms in AngII-infused ApoE^{-/-} mice and human aortic aneurysms showed enhanced ISG15 expression, suggesting that the ISG15 system is a novel mechanism involved in vascular remodelling of large arteries. In addition, ISG15^{-/-} mice were protected against AngII-induced vascular stiffness, elastin remodelling, and endothelium dysfunction and conversely, rISG15 induces endothelial dysfunction in healthy arteries, pointing to the vascular endothelium as the main target for ISG15 actions. In fact, vascular contractile responses were unmodified by rISG15 addition or by ISG15 deletion or excessive ISGylation (not shown). One limitation of our study is that we cannot definitively conclude on the specific contributions of free and conjugated ISG15, and this warrants further investigation. However, the fact that upon AngII infusion, USP18^{C61A} mice develop aortic dilation, aneurysm formation, and lethal dissection, suggests that ISGylation, rather than

free ISG15, might be the mechanism involved in vascular remodelling. In fact, the development of abdominal aneurysms in the absence of ApoE or low density lipoprotein receptor (LDLR) deficiency with only 2 weeks of AngII infusion is rare, which highlights the importance of ISGylation in vascular homeostasis. On the other hand, ISG15^{-/-} mice are protected against AngII-induced vascular stiffness and endothelial dysfunction, an effect not modified in USP18^{C61A} mice, pointing to free extracellular ISG15 as potential mediator. In agreement, exogenously added rISG15-induced endothelial dysfunction in healthy arteries. Importantly, vascular changes induced by ISG15 might affect hypertension development as ISG15^{-/-} mice showed an attenuated hypertensive response to AngII and the opposite was observed in USP18^{C61A} mice. Alternatively, differences in blood pressure might contribute to ISG15-induced vascular damage. In this sense, the enhanced hypertensive response in USP18^{C61A} mice might trigger aortic dissection and mortality, which was observed during the first days of AngII infusion.

Our proteomics analysis also revealed that the proteins implicated in the vascular redox state are decreased in AngII-infused ISG15^{-/-} mice. This prompted us to investigate oxidative stress as a putative underlying mechanism responsible for the effects of ISG15 pathway in vascular damage. We found less O₂^{•-} production and lower amounts of oxidized Cys-containing peptides in arteries from AngII-infused ISG15^{-/-} mice compared to WT mice. Moreover, in endothelial cells, rISG15 increased O₂^{•-} generation and the activity and expression of the NADPH oxidase enzyme. Finally, even in the absence of AngII, arteries from USP18^{C61A} mice showed increased O₂^{•-} formation and Nox2 expression. Together, these results demonstrate a novel role for ISG15 system as a ROS-generating stimuli at the vascular level. More importantly, this increased oxidative stress milieu had functional consequences. Thus, selective NOX1 inhibition prevented the rISG15-induced endothelial dysfunction and antioxidant therapy with tempol reduced the AngII-induced aneurysm development, mortality, and SMA vascular remodelling found in USP18^{C61A} mice. In addition, tempol treatment partially prevented AngII-induced hypertension during the first days of treatment.

Earlier studies defined ISG15 as an IFN-γ inducing molecule in immune cells such as T lymphocytes or NK cells^{9,10,22–24} independently from ISGylation.¹⁰ We did not find differences in markers of macrophage infiltration between AngII-treated WT and ISG15^{-/-} mice. However, in aorta from WT mice, AngII increased the expression of *Ifng*, *Ptgs2*, and the lymphocytes marker *Cd3*, an effect not observed in ISG15^{-/-} mice. Importantly, selective inhibition of COX-2 and IFN-γ prevented the rISG15-induced endothelial dysfunction thus confirming a role of inflammation in ISG15-induced vascular damage. Of note, arteries from USP18^{C61A} mice showed a clear proinflammatory phenotype even in the absence of AngII, as shown by the increased expression of *Ifng*, *Ptgs2*, and the chemoattractant protein *Cd2*, and enhanced infiltration of macrophages and lymphocytes, that was not further augmented by AngII. The reasons for the vascular inflammation are unknown but probably, the basal proinflammatory state predisposes USP18^{C61A} mice to the actions of AngII thus inducing the observed vascular phenotype. Importantly, no clear phenotypic differences were observed between untreated WT and ISG15^{-/-} mice, suggesting that ISG15 activation is needed to mediate its vascular deleterious effects.

Insight into the molecular function of ISG15 requires identification of ISG15 substrates and/or interaction partners at the organismal level. Mass spectrometry studies have identified hundreds of proteins that can be conjugated with ISG15, including proteins induced by type I interferon or involved in IFN signalling such as Janus Kinase 1/2 (JAK1/2) and STAT1.¹⁵ Of note, AngII activates the JAK–STAT pathway in the

cardiovascular system mediating several of its deleterious effects.⁴⁹ Until now, and unlike other post-translational modifications such as ubiquitination, the real relevance of ISGylation is still unknown, being more related to the stability and activity of the proteins. Because ISGylated proteins have been found in different biological processes such as metabolism, cell cycle, cell proliferation and differentiation, cell structure and motility, muscular contraction, immune response, intracellular protein traffic, protein translation, ubiquitination, autophagy, or exosome secretion^{12,20} and there is a paucity of information of the biological functions for these interactions, future studies are warranted to address this issue. However, our proteomics study provides a comprehensive overview of cellular protein dynamics regulated by ISG15, and this included proteins involved in cardiovascular remodelling and function, extracellular matrix, and vascular oxidative stress.

In conclusion, our study provides evidence of a previously unrecognized role for ISG15 in the vascular system. We found that hypertension increases the expression of the ISG15 pathway at the vascular level and in macrophages. ISG15 deletion reduces vascular oxidative stress and inflammation whereas excessive ISGylation induced by USP18 mutation augments oxidative stress and inflammation, even in the absence of AngII. By inducing inflammation and ROS generation, ISG15 contributes to the development of hypertension, endothelial dysfunction, vascular stiffness and remodelling, and aneurysm development, although the specific contribution of vascular vs. immune cells in these effects needs to be determined. Moreover, we uncover a possible role for this pathway in human pathology as *ISG15* expression correlated with vascular remodelling and aneurysms. For these reasons, we propose ISG15 as a novel mediator of vascular damage in hypertension.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

Due to the implementation of multiple approaches that require different expertise, sharing of first author position was agreed with first author when starting the project. A.B.G.-R., A.C.M., F.J.R., S.G., and A.M.B. designed research studies. M.G.-A., A.B.G.-R., M.B., R.R.-D., I.J., M.J.R.-R., G.Z., and C.R. conducted experiments. M.G.-A., A.B.G.-R., G.Z., R.R.-D., H.B., J.V., I.J., A.M.B., J.M.R., S.G., and M.S. analysed data. M.G.-A., A.B.G.-R., R.M.T., S.G., M.S., and A.M.B. wrote the manuscript. All authors approved the manuscript.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Translational perspective

Recent evidence from randomized clinical trials demonstrate the effectiveness of specific anti-inflammatory treatments in cardiovascular prevention. In this study, we have identified a new inflammatory mediator involved in vascular damage in experimental and human hypertension and aneurysms. We found that interferon-stimulated gene 15 (ISG15) is increased at the vascular level in animal models of hypertension and aneurysms. More importantly, ISG15 correlates with human hypertension, vascular remodelling, and aneurysms presence. Underlying mechanisms responsible for vascular damage induced by ISG15 include oxidative and inflammation. Our results further support the role of inflammation in vascular damage in different cardiovascular pathologies.