Peripheral arteriopathy caused by Notch3 gain-of-function mutation involves ER and oxidative stress and blunting of NO⁄sGC⁄cGMP pathway

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Abstract

Notch3 mutations cause Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoenephalopathy (CADASIL), which predisposes to stroke and dementia. CADASIL is characterized by vascular dysfunction and granular osmiophilic material (GOM) accumulation in cerebral small vessels. Systemic vessels may also be impacted by Notch3 mutations. However vascular characteristics and pathophysiological processes remain elusive. We investigated mechanisms underlying the peripheral vasculopathy mediated by CADASIL-causing Notch3 gain-of-function mutation. We studied: i) small arteries and vascular smooth muscle cells (VSMC) from TgNotch3^{R169C} mice (CADASIL model), ii) VSMCs from peripheral arteries from CADASIL patients, and iii) post-mortem brains from CADASIL individuals. TgNotch3^{R169C} vessels exhibited GOM deposits, increased vasoreactivity and impaired vasorelaxation. Hypercontractile responses were normalized by fasudil (Rho kinase inhibitor) and 4-PBA (endoplasmic-reticulum (ER) stress inhibitor). Ca^{2+} transients and Ca^{2+} channel expression were increased in CADASIL VSMCs, with increased expression of Rho GEFs and ER stress proteins. Vasorelaxation mechanisms were impaired in CADASIL, evidenced by decreased eNOS phosphorylation and reduced cGMP levels, with associated increased guanylate cyclase (sGC) oxidation, decreased sGC activity and reduced levels of the vasodilator H_2O_2. In VSMCs from CADASIL patients, sGC oxidation was increased and cGMP levels decreased, effects normalized by fasudil and 4-PBA. Cerebral vessels in CADASIL patients exhibited significant oxidative damage. In conclusion, peripheral vascular dysfunction in CADASIL is associated with altered Ca^{2+} homeostasis, oxidative stress and blunted eNOS/sGC/cGMP signaling, processes involving Rho kinase and ER stress. We identify novel pathways underlying the peripheral

arteriopathy induced by Notch3 gain-of-function mutation, phenomena that may also be important in cerebral vessels.

Key words: Notch3, small arteries, vascular signalling, oxidation, vascular dementia.

Introduction

Notch proteins are cell membrane receptors that play a crucial role in cell-to-cell communication [1]. In mammals four Notch receptors and five ligands have been identified and although they share the same primary structure, Notch receptors are expressed in a cell-specific manner and mediate diverse cellular effects [2, 3]. Notch signalling induces transcription of target genes that influence cell differentiation, maturation, proliferation and apoptosis and is critically involved in the regulation of vascular smooth muscle cell (VSMC) function [4, 5]. In the vasculature, of the Notch receptor family, Notch3 is expressed predominantly in VSMCs, where it controls maintenance of cell phenotype and growth [5, 6]. Abnormal Notch3 signalling has been implicated in cardiovascular diseases associated with excessive VSMC proliferation and vascular remodeling such as pulmonary arterial hypertension [4].

Mutations of Notch3 are responsible for the monogenic inherited cerebral arteriopathy known as Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarctions and Leukoencephalopathy (CADASIL) that leads to premature stroke and vascular dementia [7]. Progressive degeneration of VSMCs, accumulation of abnormal protein (granular osmiophilic material (GOM)) around VSMCs, and cerebrovascular dysfunction are characteristic features of CADASIL [7, 8]. In the brain, these processes present as subcortical lacunes and white matter rarefaction due to

chronic ischaemia, and manifest clinically as premature stroke, mood and behaviour disturbances, cognitive decline, migraines and dementia [9-11].

Investigations of the pathogenesis and molecular mechanisms of CADASIL have been performed in large part using experimental models of CADASIL and cultured patient-derived VSMCs and induced pluripotent stem cells [12-14]. We recently demonstrated that isolated small peripheral arteries from CADASIL patients exhibit vascular dysfunction and structural remodelling with associated VSMC oxidative and endoplasmic reticulum (ER) stress and altered Rho kinase signalling (12). Proteomic analysis in VSMCs from a CADASIL patient showed increased expression of proteins involved in protein degradation/folding, cytoskeletal organisation, contraction and cell stress [15].

Vascular remodeling in CADASIL mice is related to increased deposition of extracellular matrix (ECM) proteins [14, 16, 17], VSMC proliferation and cellular mitochondrial dysfunction [12, 18-20]. Studies in a CADASIL mouse model, TgNotch3^{R169C} mice, demonstrated cerebrovascular dysfunction and thickening, with associated increased deposition of extracellular matrix proteins, upregulation of voltage-dependent potassium (Kv1) channels, blunted membrane depolarization and reduced myogenic tone [14, 21].

Although the genetic cause of CADASIL is known and the clinical features of CADASIL are well defined, understanding the molecular and cellular processes underlying the vasculopathy induced by the Notch3 mutations still remain incompletely understood. This is attributed to the wide genetic diversity of Notch3 mutations, but also to the incomplete understanding of Notch3 function in blood vessels. Our previous findings identified important interplay between vascular Notch3, ER stress and Rho kinase, in part through Nox5-derived reactive oxygen species (ROS), in CADASIL

patients [12]. Here we have further interrogated molecular and cellular mechanisms whereby a Notch3 gain-of-function mutation alters vascular function of peripheral small arteries, using a mouse model harbouring one of more than 200 distinct Notch3 mutations already described as associated with CADASIL [22].

Methods

Study approval

Ethics approval for the use of human blood vessel samples was obtained from the West of Scotland Research Ethics Service (WS/12/0294). Written informed consent was obtained for all study participants in accordance with the Declaration of Helsinki. Human brain samples were from biobanked tissue from NHSGGC Biorepository and Pathology Service Tissue Resource. Ethical approval for use of surplus tissue from diagnostic blocks was obtained (REC 16/WS/0207). Experiments were approved by the University of Glasgow Animal Welfare and Ethics Review Board. All experimental protocols on mice were performed in accordance with the United Kingdom Animals Scientific Procedures Act 1986 (Licence No. 70/9021) and with ARRIVE Guidelines. All animal studies were conducted in the Institute of Cardiovascular and Medical Sciences at the University of Glasgow, UK.

Subject recruitment

Patients with genetically confirmed CADASIL were recruited from the Neurovascular Genetics clinic, Queen Elizabeth University Hospital, Glasgow. Healthy controls were volunteers at the hospital. Under local anaesthetic, all subjects underwent a gluteal biopsy from which intact small arteries (<400 μm diameter) were dissected from subcutaneous fat. VSMCs were isolated for primary cell culture, as previously

described [23, 24] and summarized below. Identical protocols were used for CADASIL patients and control studies.

Human brain samples from patients with CADASIL

Human brain samples were from biobanked tissue from NHSGGC Biorepository and Pathology Service Tissue Resource. At routine diagnostic autopsy, whole brains were immersion fixed ion 10% formal saline for a minimum of 2 weeks prior to dissection, standardized anatomical sampling, tissue processing and embedding in paraffin as previously described [25]. From diagnostic blocks, sections of 5 μm were dissected. Samples where blood vessels were visible were chosen. We studied four brains from CADASIL patients and three brains from individuals who had died from various causes (Supplemental Table 1).

Mouse model of CADASIL

The transgenic (Tg) mouse lines, TgNotch3^{WT} and TgNotch3^{R169C}, have been previously characterized and described. Briefly, TgNotch3^{WT} and TgNotch3^{R169C} mice (on an FVB background) express rat wild-type Notch3 and the CADASIL-causing Notch3(R169C) mutant protein, respectively, to a similar degree (approximately four-fold) compared with levels of endogenous Notch3 in non-transgenic mice [22]. The transgene is integrated on the X chromosome, and random inactivation of 1 X chromosome in females results in mosaic expression of the mutant protein in TgNotch3^{R169C} female mice (Unpublished data). The mutation studied in our mice is commonly seen in patients with CADASIL [26], however it is unclear whether similar X-linked mosaic processes occur in humans. It has been suggested that men have more severe disease than women, but underlying reasons remain unclear [10, 27]. In our investigation, to avoid the confounding effects of mosaic expression in females, we only studied male mice. Mice were housed in individual cages in a room with

controlled humidity and temperature (22°C - 24°C), and in light/dark cycles of 12 hours with free access to food and tap water. They were studied at 24 weeks of age, at which stage features consistent with CADASIL are well established and they exhibit features of the human disease, as previously described [14, 16, 21, 22, 28]. For each set of experiments, we used five to eight different mice. Mice were anaesthetized with 5% isoflurane (1.5 L/min O_2). Blood was collected via cardiac puncture, which is a terminal procedure, and tissues were collected for analysis. For each set of experiments, we used five to eight different mice. Blood and tissues were collected for experiments. Small mesenteric arteries were used to assess vascular function and molecular studies. In previous study we demonstrated that this is a gain-of-function mutation, where increased Notch3 signalling is observed in VSMCs and arteries. Body weight and systolic blood pressure of TgNotch3^{R169C} mice were similar to wild-type (TgNotch3^{WT}) controls. Cardiac function and structure were similar between groups. Clinical features of subjects and mice phenotype were also shown previously [12].

Transmission Electron Microscopy (TEM)

GOM deposition in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice was examined by transmission electron microscopy. Arteries (2 mm thick slices) were fixed in 2% Glutaraldehyde / 2% Paraformaldehyde / 0.1M Sodium Cacodylate buffer overnight at 4°C and rinsed with 0.1M Sodium Cacodylate buffer three times of 5 minutes before post fixation in 1% Osmium Tetroxide for 1 hour. Osmium Tetroxide was removed with changes of distilled H_2O (dH_2O) three times of 10 minutes each. Samples were then stained with 0.5% Uranyl Acetate / dH_2O for 1 hour in the dark prior dehydration through an ethanol series of 30, 50, 70, 90% (15 minutes each), 100% ethanol (4 times of 5 minutes), dried 100% ethanol (plus 3A Molecular sieve) (4 times of 5 minutes), followed by three times changes of Propylene Oxide for 5 minutes

each. Samples were then placed into a mix of Propylene Oxide: EPON 812 resin 1:1 pure resin overnight, fresh resin embedded next day in moulds and polymerised at 60°C for 48 hours. Ultrathin sections (50-70 nm) were cut using a DRUKKER diamond ultratome Knife and a LEICA Ultracut UTC. Sections were collected on Formvar coated 100 mesh copper grids and contrast was stained with 2% Uranyl Acetate for 5 minutes and Reynolds Lead Citrate also for 5 minutes. Tissue samples were viewed on a JEOL 1200EX TEM running at 80kv and digital images captured using a Cantega 2K X 2K camera and Olympus ITEM software.

Vascular functional studies

Mouse mesenteric resistance arteries (first and second order; ~300-350 µm) were isolated from TgNotch3^{WT} and TgNotch3^{R169C} mice. Briefly, arterial segments were mounted on isometric wire myographs (Danish Myo Technology, Denmark) filled with 5ml of physiological saline solution [(in mmol/L: 130 NaCl, 14.9 $NaHCO_3$, 4.7 KCl, 1.18KH_2PO_4, 1.17 $MgSO_4.7H_2O$, 5.5 glucose, 1.56 $CaCl_2.2H_2O$, and 0.026 EDTA] and continuously gassed with a mixture of 95% O_2 and 5% CO_2 while being maintained at a constant temperature of 37±0.5°C. Following 30 minutes of equilibration, the contractile responses of arterial segments were assessed by the addition of KCl (62.5mmol/L). The integrity of the endothelium was verified by relaxation induced by acetylcholine (ACh) ($3x10^{-6}$ mol/L) in arteries pre-contracted with thromboxane A2 agonist (U46619) ($3x10^{-8}$ mol/L). Endothelium-dependent relaxation was assessed as a dose-response to acetylcholine (ACh, 10^{-9}-10^{-5} mol/L). Endothelium-independent vasorelaxation was assessed by a dose-response to sodium nitroprusside (SNP) (10^{-10} – 10^{-5} mol/L), BAY 58-2667 (10^{-12} – 10^{-5} mol/L) and 8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP) (10^{-8} – 10^{-4} mol/L). Concentration-response curves to phenylephrine (Phe) (10^{-9} – $3x10^{-5}$ mol/L), U46619 (10^{-10} – 10^{-6} mol/L) and

angiotensin II (Ang II) (10^{-10} – $3x10^{-5}$ mol/L) were performed to evaluate vasoconstriction. Vascular functional responses were also assessed in the absence and presence of 4-Phenylbutyric acid (4-PBA) (ER stress inhibitor; 1 mmol/L, 30 minutes) or fasudil (Rho Kinase inhibitor; 1 µmol/L, 30 minutes).

Vascular smooth muscle cell isolation

Methods for the isolation and culture of human VSMCs (from isolated small arteries from gluteal biopsies) and mice (from mesenteric arteries) have been previously described [24]. Briefly, cleaned arteries were placed in Ham's F-12 culture medium containing 1% gentamicin, collagenase (type 1), elastase, soybean trypsin inhibitor and BSA, and were incubated for 30 to 60 minutes at 37°C under constant agitation. The digested tissue was further dissociated by repeated aspiration through a syringe with 20G needle. The cell suspension was centrifuged (2000 rpm, 4 minutes) and the cell pellet was resuspended in Ham's F-12 culture medium containing 10% FBS. Cells were seeded onto 25mm flask. VSMCs were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin (50 µg/ml). Before experimentation, cells were rendered quiescent by maintenance in a reduced growth supplement medium (0.5% FBS) overnight. Only primary, low passage cells (passages 4 to 8) were studied. In some protocols, the role of ER stress, Rho kinase and Notch signalling was assessed using pharmacological inhibitors: ER stress inhibitor, 4-phenylbutyrate (4-PBA, 1 mmol/L, Sigma-Aldrich, UK) and Rho kinase inhibitor, fasudil (10 µmol/L, Tocris, UK). Cells were pre-exposed to 4-PBA and fasudil for 24 hours.

Quantitative real-time Polymerase Chain Reaction

Quantitative real-time Polymerase Chain Reaction (qPCR) (Qiagen, UK) was used to assess mRNA expression in TgNotch3^{WT} and TgNotch3^{R169C} mice VSMCs and

mesenteric arteries. For some experiments, wildtype FVB mice were used as a control for Notch3 and Notch3 target gene expression since TgNotch3^{WT} and TgNotch3^{R169C} mice were on a FVB background. Briefly, total RNA was extracted from tissues using TRIzol (Qiagen, Manchester, UK), treated with RNase-free DNAse I, and 2 µg of RNA was reverse transcribed in a reaction containing 100 µg/mL oligo-dT, 10 mmol/L of 2′-deoxynucleoside 5′-triphosphate, 5×first-Strand buffer, and 2 µL of 200-U reverse transcriptase. For real-time PCR amplification, 3 µL of each reverse transcription product were diluted in a reaction buffer containing 5 µL of SYBR Green PCR master mix and 300 nmol/L of primers in a final volume of 10 µL per sample. The reaction conditions consisted of 2 steps at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 3 steps, 15-second denaturation at 95°C, 60-second annealing at 60°C, and 15 seconds at 72°C. Mouse primers used are detailed in supplementary table S2. Data are expressed as target gene/GAPDH housekeeping gene. Relative gene expression was calculated using the $2^{\Delta\Delta Ct}$ method.

Measurement of intracellular Ca^{2+} transients in VSMCs

VSMC Ca^{2+} signalling was assessed using the fluorescent Ca^{2+} indicator, Cal-520 acetoxymethyl ester (Cal-520/AM; Abcam; 10 µmol/L). Cells were grown in 12-well plates and following removal of culture media were incubated with Cal-520 AM in 0.5% FBS at 37°C for 75 minutes followed by 30 minutes at room temperature. Following incubation, the dye solution was replaced with HEPES physiological saline solution ($1.3x10^{-1}$ mol/L NaCl, $5x10^{-3}$ mol/L KCl, 10^{-3} mol/L CaCl, 10^{-3} mol/L MgCl, $2x10^{-2}$ mol/L HEPES, and 10^{-2} mol/L D-glucose, pH 7.4) for 30 minutes prior to imaging. Fluorescence intensity as a measure of $[Ca^{2+}]_i$, was monitored for 30 seconds in basal condition and 180 minutes under U46619 (1 µmol/L) stimulation. In some experiments, VSMCs were pre-treated for 24 hours with 4-PBA. Fluorescence-based

measurements of Ca^{2+} signals were performed using an inverted epifluorescence microscope (Axio Observer Z1 Live-Cell imaging system; Zeiss, Cambridge, UK) with excitation/emission wavelengths 490/535nm, respectively. Images were acquired and analysed using Zen Blue Program (Zeiss, Cambridge, UK).

Immunoblotting

Protein was extracted from mesenteric arteries isolated from TgNotch3^{WT} and TgNotch3^{R169C} mice. Protein (30µg) was separated by electrophoresis on a polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) solution. Membranes were then incubated with specific antibodies overnight at 4°C. Membranes were washed 3 times with TBS-Tween20 and incubated with infrared dye-labelled secondary antibodies for 1 hour at room temperature. Membranes were visualized using an Odyssey CLx infrared imaging system (LiCor Biosciences UK Ltd, UK) and results were normalized to β-actin protein and are expressed in arbitrary units compared to wildtype group, which was taken as 100. Antibodies used were as follows: anti-β-actin (1:5000; Sigma-Aldrich, UK); anti-phospho-eNOS^{Thr495} (1:500; Santa Cruz, UK); anti-phospho-eNOS^{Ser1177} (1:1000; Cell signalling, UK); anti-total-eNOS (1:1000; Cell signalling, UK), anti-ERO1 (1:1000; Santa Cruz, UK).

Affinity capture of sulfenylated proteins

In order to investigate sGCβ1 oxidation, sulfenylated proteins were captured using a biotin-tagged dimedone-based probe (DCP-Bio, Merck NS1226-5MG) that specifically binds sulfenic acid groups (SOH) in proteins [29]. Mesenteric arteries from FVB, TgNotch3^{WT} and TgNotch3^{R169C} mice and VSMC isolated from control and CADASIL patients, after homogenization in lysis buffer were supplemented with DCP-Bio1 (1mM), N-methylmalemide (10mM), catalase (200U) and protease inhibitors (1mM

PMSF and 1 µg/mL of aprotinin, leupeptin and pepstatin). Samples were kept on ice for 30 minutes and centrifuged at 12,000g for 4 minutes at 4˚C. Supernatants were collected and DCP-Bio1 excess was removed by acetone precipitation. The pellet was washed in 70% acetone and suspended in non-supplemented lysis buffer. Protein levels were determined, and 300 µg of total protein was added to a 50 µl slurry of non-liganded support beads (sepharose CL-4B beads, Sigma-Aldrich, Seelze, Germany) to remove proteins with a tendency to bind non-specifically and incubated for 2 hours at 4˚C with constant rotation. Beads were centrifuged at 1,000 x g for 2 minutes. The supernatant was collected and incubated with streptavidin beads (High Capacity Streptavidin–Agarose Resin, Thermo Scientific, Illinois, USA) overnight at 4˚C with constant rotation. After the incubation steps beads were centrifuged at 1,000 x g for 2 minutes and washed with PBS three times. Proteins were then eluted in 50 µl of 2x sample buffer for western blotting and boiled at 95˚C for 5 min. As a procedural control for the affinity capture, it was used Biotinylated-Trx Loading Control Protein (Kerafast EE0035). In order to concentrate the proteins for the protocol, a pool of five samples from different mice or patients was made. Sulfenylated protein-enriched lysates were used for immunoblotting and membranes were probed for sGCβ1 (anti-sGCβ1; 1:500; Cayman Chemical, UK).

Immunofluorescence

Immune staining was performed for an endoplasmic reticulum (ER) stress regulator, BiP, and 8-Hydroxyguanosine (8-OHG), an indirect oxidative stress marker. 8-OHG is a modified guanosine that occurs in DNA/RNA due to attack by hydroxyl radicals that are formed as by products and intermediates of aerobic metabolism and during oxidative stress. 8-OHG immunohistochemistry has been widely used as a sensitive, stable and integral biomarker of oxidative stress-induced DNA and RNA damage [30].

GRP78/BiP is a major ER chaperone protein critical for protein quality control of the ER, regulating ER stress-signalling pathways leading to unfolded protein responses (UPR) survival and apoptosis responses [31]. Paraffin sections (5 µm) of brain from control and CADASIL patients and mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice were deparaffinized in xylene, rehydrated through graded ethanol, and washed in water. All sections were incubated in EDTA (pH 8.0) and boiled for 15 minutes at 95°C for antigen unmasking. Slides were cooled to room temperature, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes, and blocked with 10% donkey serum, 1% bovine serum albumin (BSA) in 1x Tris-buffered saline and Tween 20 (TBS-T) for 1 hour at room temperature in a humidified chamber. For 8-OHG immunostaining, slides were incubated overnight with anti- 8-OHG goat polyclonal antibody (Abcam ab10802, 1:200 diluted in 5% donkey serum, 0.02% BSA, 0.0025% Tween-20 in 1x TBS solution) in a humidified chamber; and Alexa-fluor-488-conjugated donkey anti-goat antibody (Molecular probes, A-11055, 1:300 dilution in 5% donkey, 0.02% BSA, 0.0025% Tween-20 in 1xTBS solution) was used as secondary antibody. For BiP immunostaining, slides were incubated overnight with anti-BiP rabbit antibody (Cell 3177, 1:200 diluted in 5% donkey serum, 0.02% BSA, 0.0025% Tween-20 in 1x TBS solution) in a humidified chamber, and Alexa-fluor-488-conjugated donkey anti-rabbit (Molecular probes, A-11034, 1:300 dilution in 5% donkey, 0.02% BSA, 0.0025% Tween-20 in 1xTBS solution) secondary antibody was used. For both staining, after primary antibody incubation, secondary antibodies were incubated for 1 hour at room temperature in the dark. Slides were treated with 0.1% Sudan Black B (Sigma Aldrich, 199664) in methanol for 10 minutes to minimise autofluorescence. Sections were mounted with a coverslip using ProLong Gold anti-fade mounting media containing DAPI (Molecular probes, P-36931) at room

temperature and then stored at 4°C in the dark. Fluorescence images were captured at 20X (brain vessels from patients) or 63X (mesenteric arteries from mice) magnification using an inverted epifluorescence microscope (Axio Observer Z1, Zeiss) and a dedicated software (Zen Blue Program, Zeiss). Laser excitation and acquisition settings were maintained constant across all slides. Image analyses were performed using the software ImageJ®, where a mean of the green fluorescence intensity from at least 3 vessels from each mice or patient was taken.

cGMP ELISA

Cyclic guanosine 3',5'-monophosphate (cGMP) levels were detected in this study in VSMC isolated from TgNotch3^{WT} and TgNotch3^{R169C} mice and control and CADASIL patients by ELISA assay according the manufacturer's protocol (Cyclic GMP Assay kit, Cell Signaling Technology). Results were normalized by concentration of protein.

Lucigenin-enhanced chemiluminescence

Lucigenin-derived chemiluminescence assay was used to determine NADPH-dependent ROS production in mesenteric arteries homogenates from TgNotch3^{WT} and TgNotch3^{R169C} mice as we previously described [32]. Briefly, tissues were homogenized in lysis buffer (20 mmol/L of KH_2PO_4, 1 mmol/L of EGTA, 1 µg/mL of aprotinin, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin, and 1 mmol/L of PMSF). 50 µl of the sample were added to a suspension containing 175 µl of assay buffer (50 mmol/L of KH_2PO_4, 1 mmol/L of EGTA, and 150 mmol/L of sucrose) and lucigenin (5 µmol/L). Luminescence was measured with a luminometer (AutoLumat LB 953, Berthold) before and after stimulation with nicotinamide adenine dinucleotide phosphate (NADPH, 100 µmol/l). A buffer blank was subtracted from each reading. Results were normalized by concentration of protein, as measured by the BCA assay.

Amplex Red assay

Hydrogen peroxide (H_2O_2) levels was assessed by Amplex red® assay in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice. Protocols were made according to the manufacture's instruction using the horseradish peroxidase-linked Amplex Red fluorescence assay (A22188; Life Technologies). Fluorescence readings were made in a 96-well plate at Ex/Em = 530/590 nm. H_2O_2 production was normalized to protein concentration.

Plasma TBARS measurement

Blood was collected under isoflurane anesthesia (3% induction; 1.5% maintenance) by cardiac puncture immediately prior to sacrifice. Blood was collected in heparinized tubes (TekLab, County Durham, UK). Plasma was separated by centrifugation (2,000 rpm, 10 min) (Heraeus Megafuge 16R; ThermoScientific). Thiobarbituric acid reactive substances (TBARS) are a well-established indicator of oxidative stress in cells, plasma and tissues. Its products were detected in this study in plasma from TgNotch3^{WT} and TgNotch3^{R169C} mice by colorimetric (532-535nm) assay according the manufacturer's protocol (Cayman's TBARS Assay Kit, Cayman Chemical - CAY700870).

Urine biochemistry

Spot urine was collected from the bladder during sacrifice. Urine were aliquoted, snap frozen and stored at -80 ^{o}C. Albumin and creatinine were determined by an automated analyzer (Roche/Hitachi cobas c systems - cobas c 311 Autoanalyser).

Measurement of $O_2^{\bullet -}$ by electron paramagnetic resonance

Superoxide anion ($O_2^{\bullet -}$) was measured by electron paramagnetic resonance [33]. Production of $O_2^{\bullet -}$ was measured in in Krebs–Hepes buffer (99 mM NaCl, 4.69 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4,

5.6 mM D (+) glucose, 20 mM HEPES, pH 7.4) containing chelating agents, deferoxamine (25 µM, Sigma) and sodium diethyldithiocarbamate trihydrate (5 µM, Sigma). Confluent cells were incubated with hydroxylamine spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 1 mM; Enzo Life Sciences) for 10 min at 37°C, and then washed with PBS. Then cells were collected and placed into 50µl glass capillary tubes (Hirschmann Laborgeräte, Eberstadt, Germany) and subsequently assessed in an EPR spectrometer (e-scan Research Bruker® Biospin Corporation) equipped with a super-high Q microwave cavity at room temperature. Oxidation of CMH by $O_2^{\bullet -}$ results in formation of the stable nitroxide radical, 3-methoxy-carbonyl (CM). Therefore, the amount of CM formed equals the concentration of the reacting oxidant species. The concentration of CM was determined from the amplitude of the low field component of EPR spectra according to 1 mM stock solution of CMH dissolved in Krebs-HEPES buffer. Counts were recorded once a minute for 10 minutes and $O_2^{\bullet -}$ formation recorded as µmol/minute. EPR spectra and kinetics were recorded from cell suspensions in 50 µl. Instrument settings were: 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.

Statistical analysis

For vascular functional studies, concentration-response curves were generated and the maximal effect (E_{max}) and the agonist concentration that produced 50% of the maximal response (log EC50) were calculated using nonlinear regression analysis. pD_2 (defined as the negative logarithm of the EC_{50} values) and E_{max} were compared by Student's *t*-test or two-way analysis of variance (ANOVA) with Bonferroni post-test,

as appropriate. For the other experiments, statistical comparisons between groups were performed using two-tailed student's *t*-test and or one-way ANOVA. Bonferroni or Dunnett post-test were used as appropriate. $p<0.05$ was considered statistically significant. Data analysis was conducted using GraphPad Prism® 6.0 (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM.

Results

GOM deposits in small peripheral arteries

A characteristic feature of CADASIL is cerebrovascular GOM deposition [8]. GOM deposits have been identified in VSMC in skin biopsies from CADASIL patients [34], however whether GOM accumulates around VSMCs in peripheral small arteries is unclear. As shown in figures 1A and 1B, electron microscopic analysis revealed GOM deposits in mesenteric resistance arteries from TgNotch3^{R169C} mice but not in TgNotch3^{WT} mice. GOM deposits were located close to the smooth muscle cells (SMCs), often within an infolding of the cell membrane (Figure 1B, arrows). The intercellular space between SMCs from TgNotch3^{R169C} mice is also enlarged when compared to wildtype mice, which might be associated with increased deposition of ECM components such as MMP2 and MMP9. Previous studies in cerebral vessels from TgNotch3^{R169C} mice showed increased MMP expression [14, 21]. Here we corroborate this in peripheral vessels, since mRNA expression of MMP2 and MMP9 was increased in CADASIL mice (Supplementary figure 2A, 2B).

Altered vascular function of peripheral small arteries in CADASIL

Small resistance arteries were studied by wire myography to assess vascular contraction and relaxation. In small arteries from TgNotch3^{R169C} mice, contractile responses to multiple vasoconstrictors including Phe, U46619 and Ang II were

increased compared with vessels from TgNotch3^{WT} mice (Supplementary figure 1A, 1B, 1C). These responses were restored when arteries were exposed to inhibitors of Rho kinase (fasudil; Figures 2A, 2C, 2E) or ER stress (4-PBA; Figures 2B, 2D, 2F). No changes were observed in vessels from TgNotch3^{WT} mice incubated with fasudil or 4-PBA.

In addition to hypercontractile responses, CADASIL mice exhibited significantly reduced endothelium-dependent (ACh-induced) and endothelium-independent vasorelaxation (SNP-induced) compared with wildtype vessels (Figures 2G, 2H). Maximum response for ACh-induced relaxation in TgNotch3^{R169C} was 73.7±3.9 versus 103.1±7.5 in TgNotch3^{WT} mice ($p<0.05$). EC_{50} for SNP-induced vasorelaxation was 7.0±0.2 in TgNotch3^{R169C} versus 7.8±0.1 in wildtype mice ($p<0.05$). Pre-treatment of vessels with 4-PBA normalized ACh-induced vasorelaxation in CADASIL mice (Figure 2G).

Molecular mechanisms underlying vascular dysfunction in peripheral small arteries in CADASIL

To explore putative mechanisms underlying augmented vasoconstriction in CADASIL vessels, we assessed some of the molecular machinery and signalling pathways that control VSMC contraction. In particular we measured changes in agonist-stimulated Ca^{2+} responses and Ca^{2+} channels and activation of the Rho kinase and ER stress pathways in mesenteric arteries and VSMCs. As shown in figure 3, Ca^{2+} transients and expression of various Ca^{2+} channels were altered in TgNotch3^{R169C}-derived VSMCs, compared with wildtype VSMCs. Agonist-stimulated Ca^{2+} responses (Figure 3A) and the calculated area under the curve (Figure 3B) were significantly increased in the TgNotch3^{R169C} group compared with control mice. Pre-treatment with 4-PBA attenuated Ca^{2+} responses in CADASIL VSMCs. Gene expression of the Ca^{2+}

channels voltage-dependent L-type calcium channel, subunit α_{1S} (*Cav1.1*), transient receptor potential cation channel, subfamily M, member 2 (*TRPM2*) and ryanodine receptor 1 (*RyR1*) was augmented in TgNotch3^{R169C}-derived VSMCs, whereas no changes were observed for inositol 1,4,5-trisphosphate (IP_3) receptor *(IP3R)*, ryanodine receptor 2 *(RyR2),* ryanodine receptor 3 *(RyR3)* and sarcoplasmic reticulum Ca^{2+}-ATPase *(SERCA)* (Figure 3C).

Having demonstrated that inhibitors of Rho kinase and ER stress ameliorate hypercontractile responses in CADASIL vessels, some of the elements of these systems were assessed. Vascular expression of the Rho guanine nucleotide-exchange factors (GEFs), *Pdz* and *Larg*, was increased in TgNotch3^{R169C} mice compared to TgNotch3^{WT} (Figure 4A). Gene expression of *p115* was unchanged in TgNotch3^{R169C} mice. Arteries from TgNotch3^{R169C} mice also exhibited increased transcription of ER stress genes: X-box binding protein 1 (*XBP1*), activating transcription factor 4 (*ATF4*), binding immunoglobulin protein (*BiP*), and C/EBP homologous protein (*CHOP*) (Figure 4B). Increased ER stress during Notch3 gain-of-function mutation was confirmed in mesenteric arteries stained with BiP, a major ER chaperone and a central regulator of ER stress, which was significantly increased in TgNotch3^{R169C} vessels (Figure 4C). ERO1, a natural UPR target promoter triggered by ER stress, was also increased in TgNotch3^{R169C} mesenteric arteries (Figure 4D). Together these data indicate that a Notch3 gain-of-function mutation during CADASIL is associated with upregulation of Rho kinase, ER stress responses and changes in Ca^{2+} homeostasis in small peripheral arteries.

Notch3 gain-of-function impairs NO signalling in peripheral small arteries

Impaired vasorelaxation in TgNotch3^{R169C} mesenteric arteries was associated with alterations in signalling pathways that control endothelial and vascular

contraction/dilation. As shown in figure 5A, phosphorylation of the inhibitory site of endothelial nitric oxide synthase (eNOS; Thr^{495}) was significantly increased in TgNotch3^{R169C} mice whereas phosphorylation of the activator site of eNOS (Ser^{1177}) was unchanged (Supplementary figure 3). These responses were associated with decreased cGMP levels in CADASIL VSMCs (Figure 5B). cGMP levels were not altered by fasudil or 4-PBA in CADASIL and control VSMCs.

Impaired vasorelaxation involves redox-sensitive PKG-dependent pathways in TgNotch3^{R169C} mice

To further dissect possible mechanisms underlying reduced vasorelaxation and perturbed vascular NO/cGMP signalling in TgNotch3^{R169C} mice, we interrogated vasodilator pathways mediated by redox-sensitive protein kinase G (PKG), which has been shown to regulate vasorelaxation through H_2O_2-dependent pathways. Vasorelaxation concentration-response curves to BAY 58-2667, a potent soluble guanylyl cyclase (sGC) activator, and 8-Br-cGMP, which activates cGMP-dependent PKG, were performed in mesenteric arteries from TgNotch3^{R169C} mice. Vasodilation induced by both BAY 58-2667 (Figure 6A) and 8-Br-cGMP, a cGMP analogue (Figure 6B), was reduced in vessels from TgNotch3^{R169C} mice, suggesting an impairment in sGC and PKG activity in these mice. At the molecular level, this was associated with augmented generation of vascular ROS (Figure 6C, Supplementary figure 4B), systemic oxidative stress (Supplementary figure 4A), increased oxidation of sGCβ1 (Figure 6D) and increased expression of Nox1 in TgNotch3^{R169C} arteries (Figure 6E). No changes in Nox 2 and 4 were observed between groups (Supplementary figure 5). Associated with increased NADPH-derived $O_2^{\bullet -}$ production was reduced bioavailability of H_2O_2, a putative endothelium-derived relaxing factor (EDRF) [35, 36], in TgNotch3^{R169C} arteries (Figure 6F). Vascular expression of the antioxidant enzymes

catalase and glutathione peroxidase 1 (GPX1), which catalyse H_2O_2 to O_2 and H_2O, was increased in CADASIL mice (Figures 6G, 6H).

Renal dysfunction in CADASIL mice

To determine whether vascular abnormalities in CADASIL mice are associated with altered organ function, we assessed renal function in CADASIL mice by measuring urine albumin:creatinie ratio. TgNotch3^{R169C} mice also showed a significant increase in albumin:creatinine ratio in urine, suggesting an increase in vascular permeability and endothelial dysfunction (Figure 7).

Impaired sGC/cGMP and oxidative status in VSMCs and cerebral vessels from patients with CADASIL

To determine whether the vascular alterations identified in experimental models of CADASIL are also present in human vessels, we explored some of the molecular processes in VSMCs from patients with CADASIL. We also studied post-mortem brain sections from patients who had CADASIL. As shown in figure 7A, levels of cGMP, an important regulator of VSMC relaxation, were significantly reduced in VSMCs from patients with CADASIL. Pre-treatment of VSMCs with fasudil or 4-PBA normalised cGMP levels (Figure 8A). Oxidation of sGCβ1 was higher in CADASIL VSMCs compared with control VSMCs (Figure 8B). We previously showed that VSMCs from CADASIL patients have increased oxidative stress [12]. Levels of DNA oxidation, assessed as the 8-OHG content, were significantly increased in brain vessels in CADASIL patients compared with controls (Figures 8C, 8D).

Discussion

CADASIL is typically associated with small vessel disease of the brain causing migraine with aura, ischaemic structural changes in white and deep grey matter

structures, cognitive impairment, and recurrent small vessel ischemic strokes leading to vascular dementia [7, 9]. However, growing pre-clinical and clinical evidence indicates that peripheral small arteries are also dysfunctional in CADASIL [11, 37-39]. We recently reported that patients with CADASIL have impaired endothelial function and altered vascular contractile responses, processes associated with increased Rho kinase activation and ER stress [12]. To advance these findings and to further dissect underlying molecular mechanisms we studied TgNotch3^{R169C} mice that express the CADASIL-causing Notch3(R169C) mutant protein [22]. We also probed some molecular processes in cerebral vessels in brain tissue obtained post-mortem from patients with CADASIL. Major findings from our study show that peripheral small arteries from TgNotch3^{R169C} mice exhibit GOM deposits, typically observed in the cerebrovascular bed in CADASIL. Functional alterations were defined by hypercontractility and impaired endothelium-dependent and -independent vasorelaxation. At the molecular level, vascular abnormalities in TgNotch3^{R169C} mice were linked to increased Ca^{2+} transients and upregulation of Ca^{2+} channels, processes associated with Rho kinase activation and ER and oxidative stress. In addition, we identified blunting of eNOS/NO/cGMP-induced vasorelaxation through processes involving increased oxidation of sGC. These phenomena in mice were recapitulated in human studies, where we observed increased oxidative stress-induced DNA and RNA damage and sGC oxidation in VSMCs and cerebral arteries from CADASIL patients. Exact processes linking Notch3 to perturbed vascular signaling in CADASIL remain unclear, but oxidative stress may be a common driver by promoting oxidation of downstream proteins.

Pathological hallmarks of the vasculopathy in CADASIL include accumulation of the extracellular domain of Notch3 (Notch3^{ECD}) and the presence of GOM deposits

on SMCs from small arteries [40, 41]. In aging mice GOM deposits progress in size over time and new GOM deposits are continuously being formed [41]. Here we demonstrated by electron microscopy that peripheral arteries from TgNotch3^{R169C} mice have GOM deposits, processes associated with altered ECM protein expression and enlargement of SMC intercellular space in arteries. These findings recapitulate features in cerebral vessels [40], confirming that manifestations of the Notch3 mutation in CADASIL are not restricted to the cerebrovascular system but are likely present in small arteries in multiple vascular beds [40].

We provide evidence that CADASIL-causing Notch3 mutations cause functional changes in peripheral arteries. These process seem to be linked to the pathophysiological manifestations of CADASIL because vascular changes only become evident at 6 months when mice exhibit features of CADASIL, as previously reported [22, 42]. In addition CADASIL mice exhibited some functional changes at the kidney level because albumin:creatinine ratio was increased in TgNotch3^{R169C} mice. Albuminuria represents increased endothelial permeability and dysfunction and is an accepted cardiovascular risk factor clinically [43]. Proteinuria has also been described in patients with CADASIL and may represent systemic vascular dysfunction in these patients [44, 45].

Vasoconstriction to three different agonists (phenylephrine, Ang II and U44619), was increased in TgNotch3^{R169C} mice, indicating a generalized phenomenon rather than an agonist-specific effect. These findings are in contrast to what was demonstrated in CADASIL patients where peripheral arteries showed reduced vasoreactivity [11, 12, 46]. Reasons for these differences are unclear but may relate to relative chronicity of the disease, since human studies were carried out in patients later in life, whereas our experimental studies here were performed in mice at a

relatively young age (6 months). It may be possible that with aging and progression of disease in TgNotch3^{R169C} mice, vasocontractile responses may change. However this awaits confirmation. Vascular smooth muscle cell contraction is regulated primarily by dynamic changes in Ca^{2+} homeostasis and Ca^{2+} channel activity/expression [47]. Our findings showed hypercontractility of TgNotch3^{R169C} arteries, in addition to augmented agonist-stimulated Ca^{2+} transients and increased expression of Ca^{2+} channels. These processes are highly regulated since Cav1.1, IP3R and RyR1 were upregulated in CADASIL mice, whereas TRPM2, SERCA, RyR2 and RyR3 were not altered compared with control mice. Previous studies showed an important role for Notch in Ca^{2+} regulation, since Notch increases expression/activity of store-operated Ca^{2+} entry (SOCE) and canonical transient receptor potential (TRPC6) channels in VSMCs [48]. Moreover Ca^{2+} channel blockers seem to improve cognitive decline and cerebral hypoperfusion in CADASIL patients [49], although this aspect warrants further investigation.

Vascular smooth muscle cells are highly plastic and in disease states undergo phenotypic switching from a contractile to a proliferative and pro-inflammatory state. While VSMC contraction is triggered by an increase in $[Ca^{2+}]_i$ which promotes actin-myosin interaction, it is also regulated by Ca^{2+}-independent processes involving RhoA-Rho kinase and MAP kinases, ROS amongst other systems [47]. These phenomena are especially important in pathological conditions as we demonstrate here, where vascular dysfunction was associated not only with amplification of Ca^{2+} transients, but also with systems involving Rho kinase, oxidative and ER stress. Pharmacological inhibitors of Rho kinase and ER stress normalised hypercontractile responses in CADASIL mice, indicating involvement of these systems in Notch3-regulated contraction. Supporting this notion, at the molecular level, elements of RhoA/Rho

kinase signalling, and the ER stress response were perturbed in TgNotch3^{R169C} VSMCs. RhoA, a member of the Rho GTPase family and regulated by Rho GEFs, is a master regulator of cytoskeletal dynamics and VSMC function [50, 51]. Expression of Rho GEFs was altered in CADASIL mice. In particular, mRNA expression of PDZ and LARG, but not p115, was increased in TgNotch3^{R169C} vessels. This is not surprising since PDZ and LARG are crucial elements involved in VSMC contractile signalling, whereas p115 influences destabilization of endothelial cell-cell junctions [51]. Our findings are in line with those observed in VSMCs from CADASIL patients, where the RhoA/Rho kinase pathway is upregulated [12].

Among the many systems implicated in abnormal VSMC function in CADASIL, is abnormal handling and folding of mutant Notch3 protein, processes that involve the ER [52]. Under stress conditions, proteins become misfolded and accumulate in the ER provoking the unfolded ER protein response. Prolonged retention of ER mutant Notch3 aggregates and ER stress influence VSMC function and GOM deposition and may be important pathogenic mechanisms contributing to the vasculopathy in CADASIL. Supporting this, expression of ER stress markers XBP1, ATF4, Bip, CHOP and ERO1 was increased in CADASIL vessels, processes that involve Rho kinase activation as we previously demonstrated [12]. ER stress is downstream of Rho kinase because fasudil inhibits ER stress-induced responses by modulating the unfolded protein response in vascular cells [53]. Functionally, ER stress influences vascular function since 4-PBA attenuated hypercontractile responses in TgNotch3^{R169C} mice. Corroborating these findings, previous studies showed that aberrant ER stress in VSMCs causes increased vascular contraction [54].

Similar to what we found in peripheral and cerebral vessels in CADASIL patients [12, 55], agonist-stimulated relaxation was impaired in mesenteric arteries in

TgNotch3^{R169C} mice. Both endothelium-dependent and endothelium-independent vasorelaxation were reduced in CADASIL mice, analogous to what was reported in the cerebrovascular system of these mice [42]. Endothelial NOS is the primary source of NO in endothelial cells and is the key regulator of endothelial function [56]. Impaired endothelium-dependent relaxation in TgNotch3^{R169C} arteries was associated with decreased eNOS phosphorylation and oxidative stress, which lead to reduced eNOS activation and decreased bioavailability of the vasodilator NO, which is vasoinjurious [56]. Notch signalling plays an important role in cell-cell communication between endothelial cells and VSMCs, but exactly how VSMC Notch3 influences endothelial cell function remains unclear. It may be possible that endothelial injury is secondary to VSMC dysfunction, vascular remodelling and GOM accumulation.

The importance of perturbed VSMC function in CADASIL is further evidenced by our findings that endothelium-independent vasorelaxation (SNP-induced responses) was impaired in TgNotch3^{R169C} mice. VSMCs constitute the bulk of the vascular media and are largely responsible for maintaining vascular contraction/dilation and arterial tone. The major molecular system controlling VSMC dilation is the sGC/cGMP pathway**.** Activation of sGC increases production of the second messenger cGMP, which influences downstream signalling through cGMP-dependent protein kinase (PKG) [57, 58]. PKG is a potent vasodilator and mediates effects in part through H_2O_2 [36]. PKG is also regulated by oxidant-induced interprotein disulphide formation. This oxidation-induced activation of PKG represents an alternate cGMP-independent mechanism regulating vascular function [59, 60]. The potential role of sGC/cGMP/PKG in endothelium-independent vasorelaxation in CADASIL was probed in VSMCs and vessels from TgNotch3^{R169C} mice and patients with CADASIL. Vascular sensitivity to BAY 58-2667, a sGC activator that bypasses the impaired

NO/sGC/cGMP pathway by activating the oxidized form of the enzyme [61], was reduced in TgNotch3^{R169C} mice, suggesting less activity of sGC due to its higher oxidation. Supporting this, vascular oxidative stress, driven by Nox1-mediated ROS production, and associated oxidation of sGCβ1 were increased in tissue from TgNotch3^{R169C} mice. Decreased activation of sGC culminates in reduced PKG activity and decreased vasodilation, which might be aggravated by decreased H_2O_2 levels, since H_2O_2 is an important vasodilator [59, 60, 62]. This may be important in TgNotch3^{R169C} mice, where downregulation of the sGC/cGMP system was associated with decreased vascular H_2O_2 production compared with control mice. While these observations were made in peripheral vessels, they are especially pertinent in the cerebral circulation where H_2O_2 rather than NO seems to be the major vasodilator [36, 63]. Hence, cerebrovascular dysfunction in CADASIL may be linked, at least in part, to defective H_2O_2-mediated vasorelaxation. Vascular H_2O_2 downregulation seems to be associated with increased catalase and GPX1 whereas increased superoxide levels may be linked to Nox1, an important source of vascular ROS [32].

Translating our pre-clinical studies to humans, we studied VSMCs and brain sections from CADASIL patients and found increased vascular oxidative stress and sGCβ1 oxidation and extensive DNA damage. These vascular abnormalities may play a role in cerebrovascular pathology underlying CADASIL. Moreover, since many of the vascular signaling pathways we studied are similarly dysregulated in patients with CADASIL [12] and in our mouse model, we suggest that TgNotch3^{R169C} mice may be a suitable experimental model of human disease.

In conclusion, we demonstrate that peripheral small arteries from TgNotch3^{R169C} mice exhibit hypercontractility, impaired endothelium-dependent and -independent vasorelaxation, processes associated with altered Ca^{2+} homeostasis,

upregulation of Rho kinase and ER stress. Moreover, we define novel pathways of impaired eNOS/sGC/cGMP signalling in CADASIL through hyperoxidation, likely driven by Nox1-mediated oxidative stress (Figure 9). Our data identify novel molecular mechanisms whereby Notch3 gain-of-function mutation causes vascular dysfunction and highlight the concept that, although the arteriopathy of CADASIL is primarily cerebral, peripheral vessels are also affected. This has important clinical implications, because systemic vasculopathy and dysfunctional vasoreactivity may be associated with peripheral vascular disease in CADASIL patients [37]. Additionally, given the current elusive knowledge of the downstream Notch3-mediated mechanisms in the vasculature and the ubiquitous expression in all VSMCs, our data are not just useful for examining CADASIL, but are translatable to vascular Notch3 signalling in general and in other vascular diseases.

Data availability Statement

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

Clinical Perspectives

- **Background as to why the study was undertaken**

CADASIL is typically defined as a small vessel disease of the brain. However growing evidence indicates that peripheral small vessels are also dysfunctional, which may be a marker of cerebral vessel dysfunction and/or contribute to peripheral vascular disease. Molecular mechanisms underlying the peripheral vasculopathy in CADASIL remain unclear, although Rho kinase and ER- and oxidative stress may be important as we showed. Here we advance this notion and define novel signaling casades in the

vasculopathy of CADASIL, particularly related to interplay between oxidatie stress and the eNOS/NO/sGC/cGMP pathway.

A brief summary of the results

CADASIL mice with Notch3 gain of function mutation exhibit peripheral vascular dysfunction characterised by impaired vasorelaxation and hypercontractility. These process involve altered vascular Ca^{2+} homeostasis, upregulation of Rho kinase, and ER-and Nox1-mediated oxidative stress that cause hyperoxidation of vascular signaling molecules and blunting of the eNOS/sGC/cGMP pathway. Findings in CADASIL mice were recapitulated in post-mortem cerebral vessels from patients with CADASIL

The potential significance of the results to human health and disease

We identify novel pathways whereby Notch3 gain-of-function mutation causes vascular dysfunction and highlight the concept that, although the arteriopathy of CADASIL is primarily cerebral, peripheral vessels are also affected. This has important clinical implications, because systemic vasculopathy may predispose to peripheral vascular disease in CADASIL patients. Additionally, given the gap in knowledge of Notch3-mediated downstream pathways and the ubiquitous expression in VSMCs, our data provide insights into vascular Notch3 signalling in general, beyond CADASIL.

Competing Interests

None.

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Abbreviations

CADASIL **–** Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoenephalopathy

Notch3^{ECD} - extracellular domain of Notch3

GOM **-** granular osmiophilic material

VSMC - vascular smooth muscle cells

ER - endoplasmic reticulum

sGC – soluble guanylate cyclase

NO – nitric oxide

eNOS – endothelial nitric oxide synthase

cGMP – cyclic guanosine monophosphate

Kv1 - voltage-dependent potassium (Kv1) channels

ROS - reactive oxygen species

FBS **-** fetal bovine serum

4-PBA - 4-phenylbutyrate

SOH - sulfenic acid groups

8-OHG - 8-Hydroxyguanosine

UPR - unfolded protein responses

H_2O_2 - Hydrogen peroxide

TBARS - Thiobarbituric acid reactive substances

ACh – acetylcholine

SNP – sodium nitroprusside

Phe - phenylephrine

Cav1.1 - Ca^{2+} channels voltage-dependent L-type calcium channel, subunit α_{1S}

TRPM2 - transient receptor potential cation channel, subfamily M, member 2

RyR - ryanodine receptor

IP_3R - inositol 1,4,5-trisphosphate

SERCA - sarcoplasmic reticulum Ca^{2+}-ATPase

GEFs - guanine nucleotide-exchange factors

XBP1 - X-box binding protein 1

ATF4 - activating transcription factor 4

BiP - binding immunoglobulin protein

CHOP - C/EBP homologous protein

GPX1 - glutathione peroxidase 1

SOCE - store-operated Ca^{2+} entry (SOCE)

TRPC6 - canonical transient receptor potential

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. *Science*. 1999;**284**:770-776
2. Fleming RJ. Structural conservation of notch receptors and ligands. *Semin Cell Dev Biol*. 1998;**9**:599-607
3. Kopan R, Ilagan MX. The canonical notch signaling pathway: Unfolding the activation mechanism. *Cell*. 2009;**137**:216-233
4. Morris HE, Neves KB, Montezano AC, MacLean MR, Touyz RM. Notch3 signalling and vascular remodelling in pulmonary arterial hypertension. *Clin Sci (Lond)*. 2019;**133**:2481-2498
5. Baeten JT, Lilly B. Notch signaling in vascular smooth muscle cells. *Adv Pharmacol*. 2017;**78**:351-382
6. Wang T, Baron M, Trump D. An overview of notch3 function in vascular smooth muscle cells. *Prog Biophys Mol Biol*. 2008;**96**:499-509
7. Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, Alamowitch S, Domenga V, Cecillion M, Marechal E, Maciazek J, Vayssiere C, Cruaud C, Cabanis EA, Ruchoux MM, Weissenbach J, Bach JF, Bousser MG, Tournier-Lasserve E. Notch3 mutations in cadasil, a hereditary adult-onset condition causing stroke and dementia. *Nature*. 1996;**383**:707-710
8. Ishiko A, Shimizu A, Nagata E, Takahashi K, Tabira T, Suzuki N. Notch3 ectodomain is a major component of granular osmiophilic material (gom) in cadasil. *Acta Neuropathol*. 2006;**112**:333-339
9. Moreton FC, Razvi SS, Davidson R, Muir KW. Changing clinical patterns and increasing prevalence in cadasil. *Acta Neurol Scand*. 2014;**130**:197-203
10. Di Donato I, Bianchi S, De Stefano N, Dichgans M, Dotti MT, Duering M, Jouvent E, Korczyn AD, Lesnik-Oberstein SA, Malandrini A, Markus HS, Pantoni L, Penco S, Rufa A, Sinanovic O, Stojanov D, Federico A. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (cadasil) as a model of small vessel disease: Update on clinical, diagnostic, and management aspects. *BMC Med*. 2017;**15**:41
11. Moreton FC, Cullen B, Delles C, Santosh C, Gonzalez RL, Dani K, Muir KW. Vasoreactivity in cadasil: Comparison to structural mri and neuropsychology. *J Cereb Blood Flow Metab*. 2018;**38**:1085-1095
12. Neves KB, Harvey AP, Moreton F, Montezano AC, Rios FJ, Alves-Lopes R, Nguyen Dinh Cat A, Rocchicciolli P, Delles C, Joutel A, Muir K, Touyz RM. Er stress and rho kinase activation underlie the vasculopathy of cadasil. *JCI Insight*. 2019;**4**
13. Ling C, Liu Z, Song M, Zhang W, Wang S, Liu X, Ma S, Sun S, Fu L, Chu Q, Belmonte JCI, Wang Z, Qu J, Yuan Y, Liu GH. Modeling cadasil vascular

pathologies with patient-derived induced pluripotent stem cells. *Protein Cell*. 2019;**10**:249-271
14. Capone C, Dabertrand F, Baron-Menguy C, Chalaris A, Ghezali L, Domenga-Denier V, Schmidt S, Huneau C, Rose-John S, Nelson MT, Joutel A. Mechanistic insights into a timp3-sensitive pathway constitutively engaged in the regulation of cerebral hemodynamics. *Elife*. 2016;**5**
15. Ihalainen S, Soliymani R, Iivanainen E, Mykkanen K, Sainio A, Poyhonen M, Elenius K, Jarvelainen H, Viitanen M, Kalimo H, Baumann M. Proteome analysis of cultivated vascular smooth muscle cells from a cadasil patient. *Mol Med*. 2007;**13**:305-314
16. Monet-Lepretre M, Haddad I, Baron-Menguy C, Fouillot-Panchal M, Riani M, Domenga-Denier V, Dussaule C, Cognat E, Vinh J, Joutel A. Abnormal recruitment of extracellular matrix proteins by excess notch3 ecd: A new pathomechanism in cadasil. *Brain*. 2013;**136**:1830-1845
17. Dong H, Blaivas M, Wang MM. Bidirectional encroachment of collagen into the tunica media in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Brain Res*. 2012;**1456**:64-71
18. Panahi M, Yousefi Mesri N, Samuelsson EB, Coupland KG, Forsell C, Graff C, Tikka S, Winblad B, Viitanen M, Karlstrom H, Sundstrom E, Behbahani H. Differences in proliferation rate between cadasil and control vascular smooth muscle cells are related to increased tgfbeta expression. *J Cell Mol Med*. 2018;**22**:3016-3024
19. Tikka S, Ng YP, Di Maio G, Mykkanen K, Siitonen M, Lepikhova T, Poyhonen M, Viitanen M, Virtanen I, Kalimo H, Baumann M. Cadasil mutations and shrna silencing of notch3 affect actin organization in cultured vascular smooth muscle cells. *J Cereb Blood Flow Metab*. 2012;**32**:2171-2180
20. Viitanen M, Sundstrom E, Baumann M, Poyhonen M, Tikka S, Behbahani H. Experimental studies of mitochondrial function in cadasil vascular smooth muscle cells. *Exp Cell Res*. 2013;**319**:134-143
21. Dabertrand F, Kroigaard C, Bonev AD, Cognat E, Dalsgaard T, Domenga-Denier V, Hill-Eubanks DC, Brayden JE, Joutel A, Nelson MT. Potassium channelopathy-like defect underlies early-stage cerebrovascular dysfunction in a genetic model of small vessel disease. *Proc Natl Acad Sci U S A*. 2015;**112**:E796-805
22. Baron-Menguy C, Domenga-Denier V, Ghezali L, Faraci FM, Joutel A. Increased notch3 activity mediates pathological changes in structure of cerebral arteries. *Hypertension*. 2017;**69**:60-70
23. Schiffrin EL, Park JB, Intengan HD, Touyz RM. Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation*. 2000;**101**:1653-1659
24. Montezano AC, Lopes RA, Neves KB, Rios F, Touyz RM. Isolation and culture of vascular smooth muscle cells from small and large vessels. *Methods Mol Biol*. 2017;**1527**:349-354
25. Graham DI, Gentleman SM, Lynch A, Roberts GW. Distribution of beta-amyloid protein in the brain following severe head injury. *Neuropathol Appl Neurobiol*. 1995;**21**:27-34
26. Paraskevas GP, Bougea A, Synetou M, Vassilopoulou S, Anagnostou E, Voumvourakis K, Iliopoulos A, Spengos K. Cadasil and autoimmunity: Coexistence in a family with the r169c mutation at exon 4 of the notch3 gene. *Cerebrovasc Dis*. 2014;**38**:302-307

27. Rutten JW, Hack RJ, Duering M, Gravesteijn G, Dauwerse JG, Overzier M, van den Akker EB, Slagboom E, Holstege H, Nho K, Saykin A, Dichgans M, Malik R, Lesnik Oberstein SAJ. Broad phenotype of cysteine-altering notch3 variants in uk biobank: Cadasil to nonpenetrance. *Neurology*. 2020;**95**:e1835-e1843
28. Lacombe P, Oligo C, Domenga V, Tournier-Lasserve E, Joutel A. Impaired cerebral vasoreactivity in a transgenic mouse model of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy arteriopathy. *Stroke*. 2005;**36**:1053-1058
29. Nelson KJ, Klomsiri C, Codreanu SG, Soito L, Liebler DC, Rogers LC, Daniel LW, Poole LB. Use of dimedone-based chemical probes for sulfenic acid detection methods to visualize and identify labeled proteins. *Methods Enzymol*. 2010;**473**:95-115
30. Kasai H, Crain PF, Kuchino Y, Nishimura S, Ootsuyama A, Tanooka H. Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis*. 1986;**7**:1849-1851
31. Wang M, Wey S, Zhang Y, Ye R, Lee AS. Role of the unfolded protein response regulator grp78/bip in development, cancer, and neurological disorders. *Antioxid Redox Signal*. 2009;**11**:2307-2316
32. Lopes RA, Neves KB, Tostes RC, Montezano AC, Touyz RM. Downregulation of nuclear factor erythroid 2-related factor and associated antioxidant genes contributes to redox-sensitive vascular dysfunction in hypertension. *Hypertension*. 2015;**66**:1240-1250
33. Dikalov SI, Kirilyuk IA, Voinov M, Grigor'ev IA. Epr detection of cellular and mitochondrial superoxide using cyclic hydroxylamines. *Free Radic Res*. 2011;**45**:417-430
34. Lorenzi T, Ragno M, Paolinelli F, Castellucci C, Scarpelli M, Morroni M. Cadasil: Ultrastructural insights into the morphology of granular osmiophilic material. *Brain Behav*. 2017;**7**:e00624
35. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;**106**:1521-1530
36. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pflugers Arch*. 2010;**459**:915-922
37. Briceno DF, Bhattacharjee MB, Supsupin E, Jr., Navarro P, Bhattacharjee M. Peripheral artery disease as a manifestation of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (cadasil) and practical implications. *Circulation*. 2013;**127**:e568-570
38. Fujiwara Y, Mizuno T, Okuyama C, Nagakane Y, Watanabe-Hosomi A, Kondo M, Kuriyama N, Tokuda T, Matsushima S, Nishimura T, Nakagawa M. Simultaneous impairment of intracranial and peripheral artery vasoreactivity in cadasil patients. *Cerebrovasc Dis*. 2012;**33**:128-134
39. Stenborg A, Kalimo H, Viitanen M, Terent A, Lind L. Impaired endothelial function of forearm resistance arteries in cadasil patients. *Stroke*. 2007;**38**:2692-2697
40. Ruchoux MM, Domenga V, Brulin P, Maciazek J, Limol S, Tournier-Lasserve E, Joutel A. Transgenic mice expressing mutant notch3 develop vascular alterations characteristic of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Am J Pathol*. 2003;**162**:329-342

41. Gravesteijn G, Munting LP, Overzier M, Mulder AA, Hegeman I, Derieppe M, Koster AJ, van Duinen SG, Meijer OC, Aartsma-Rus A, van der Weerd L, Jost CR, van den Maagdenberg A, Rutten JW, Lesnik Oberstein SAJ. Progression and classification of granular osmiophilic material (gom) deposits in functionally characterized human notch3 transgenic mice. *Transl Stroke Res*. 2019
42. Capone C, Cognat E, Ghezali L, Baron-Menguy C, Aubin D, Mesnard L, Stohr H, Domenga-Denier V, Nelson MT, Joutel A. Reducing timp3 or vitronectin ameliorates disease manifestations in cadasil mice. *Ann Neurol*. 2016;**79**:387-403
43. Paisley KE, Beaman M, Tooke JE, Mohamed-Ali V, Lowe GD, Shore AC. Endothelial dysfunction and inflammation in asymptomatic proteinuria. *Kidney Int*. 2003;**63**:624-633
44. Ragno M, Trojano L, Pianese L, Boni MV, Silvestri S, Mambelli V, Lorenzi T, Scarpelli M, Morroni M. Renal involvement in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (cadasil): Report of a case with a six-year follow-up. *Histol Histopathol*. 2012;**27**:1307-1314
45. Kusaba T, Hatta T, Kimura T, Sonomura K, Tanda S, Kishimoto N, Kameyama H, Okigaki M, Mori Y, Ishigami N, Mizuno T, Nakagawa M, Matsubara H. Renal involvement in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (cadasil). *Clin Nephrol*. 2007;**67**:182-187
46. Hussain MB, Singhal S, Markus HS, Singer DR. Abnormal vasoconstrictor responses to angiotensin ii and noradrenaline in isolated small arteries from patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (cadasil). *Stroke*. 2004;**35**:853-858
47. Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, Montezano AC. Vascular smooth muscle contraction in hypertension. *Cardiovasc Res*. 2018;**114**:529-539
48. Smith KA, Voiriot G, Tang H, Fraidenburg DR, Song S, Yamamura H, Yamamura A, Guo Q, Wan J, Pohl NM, Tauseef M, Bodmer R, Ocorr K, Thistlethwaite PA, Haddad GG, Powell FL, Makino A, Mehta D, Yuan JX. Notch activation of ca(2+) signaling in the development of hypoxic pulmonary vasoconstriction and pulmonary hypertension. *Am J Respir Cell Mol Biol*. 2015;**53**:355-367
49. Mizuno T, Kondo M, Ishigami N, Tamura A, Itsukage M, Koizumi H, Isayama R, Hosomi A, Nagakane Y, Tokuda T, Sugimoto E, Ushijima Y, Nakagawa M. Cognitive impairment and cerebral hypoperfusion in a cadasil patient improved during administration of lomerizine. *Clin Neuropharmacol*. 2009;**32**:113-116
50. van Buul JD, Geerts D, Huveneers S. Rho gaps and gefs: Controling switches in endothelial cell adhesion. *Cell Adh Migr*. 2014;**8**:108-124
51. Hodge RG, Ridley AJ. Regulating rho gtpases and their regulators. *Nat Rev Mol Cell Biol*. 2016;**17**:496-510
52. Takahashi K, Adachi K, Yoshizaki K, Kunimoto S, Kalaria RN, Watanabe A. Mutations in notch3 cause the formation and retention of aggregates in the endoplasmic reticulum, leading to impaired cell proliferation. *Hum Mol Genet*. 2010;**19**:79-89

53. Kawanami D, Matoba K, Okada R, Tsukamoto M, Kinoshita J, Ishizawa S, Kanazawa Y, Yokota T, Utsunomiya K. Fasudil inhibits er stress-induced vcam-1 expression by modulating unfolded protein response in endothelial cells. *Biochem Biophys Res Commun*. 2013;**435**:171-175
54. Liang B, Wang S, Wang Q, Zhang W, Viollet B, Zhu Y, Zou MH. Aberrant endoplasmic reticulum stress in vascular smooth muscle increases vascular contractility and blood pressure in mice deficient of amp-activated protein kinase-alpha2 in vivo. *Arterioscler Thromb Vasc Biol*. 2013;**33**:595-604
55. Chabriat H, Pappata S, Ostergaard L, Clark CA, Pachot-Clouard M, Vahedi K, Jobert A, Le Bihan D, Bousser MG. Cerebral hemodynamics in cadasil before and after acetazolamide challenge assessed with mri bolus tracking. *Stroke*. 2000;**31**:1904-1912
56. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation*. 2006;**113**:1708-1714
57. Park M, Sandner P, Krieg T. Cgmp at the centre of attention: Emerging strategies for activating the cardioprotective pkg pathway. *Basic Res Cardiol*. 2018;**113**:24
58. Cuello F, Eaton P. Cysteine-based redox sensing and its role in signaling by cyclic nucleotide-dependent kinases in the cardiovascular system. *Annu Rev Physiol*. 2019;**81**:63-87
59. Dou D, Zheng X, Liu J, Xu X, Ye L, Gao Y. Hydrogen peroxide enhances vasodilatation by increasing dimerization of cgmp-dependent protein kinase type ialpha. *Circ J*. 2012;**76**:1792-1798
60. Sheehe JL, Bonev AD, Schmoker AM, Ballif BA, Nelson MT, Moon TM, Dostmann WR. Oxidation of cysteine 117 stimulates constitutive activation of the type ialpha cgmp-dependent protein kinase. *J Biol Chem*. 2018;**293**:16791-16802
61. Shah RC, Sanker S, Wood KC, Durgin BG, Straub AC. Redox regulation of soluble guanylyl cyclase. *Nitric Oxide*. 2018;**76**:97-104
62. Stubbert D, Prysyazhna O, Rudyk O, Scotcher J, Burgoyne JR, Eaton P. Protein kinase g ialpha oxidation paradoxically underlies blood pressure lowering by the reductant hydrogen sulfide. *Hypertension*. 2014;**64**:1344-1351
63. Wei EP, Kontos HA, Beckman JS. Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *Am J Physiol*. 1996;**271**:H1262-1266

Figures and Figure Legends

Figure 1. GOM deposition in CADASIL peripheral small arteries. GOM deposits were identified in mesenteric arteries from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} mice via electron microscopy. Ultrathin vessel sections from TgNotch3^{WT} (A) and TgNotch3^{R169C} (B) mice were examined by electron microscopy (n=2). Vessels from TgNotch3^{R169C} exhibited electron-dense granular deposits corresponding to GOM (arrows) within the basement membrane. SMC, smooth muscle cell; EC, endothelial cell; BM, basal membrane. Magnification 600x; Scale bar 5 µm.

Figure 2. Role of Rho kinase signalling pathway and ER stress in the vascular dysfunction observed in TgNotch3^{R169C} peripheral small arteries. Vascular functional responses in mesenteric arteries obtained from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} in response to phenylephrine (Phe), U46619, Angiotensin II (Ang II), acetylcholine (ACh) and sodium nitroprusside (SNP) was assessed by wire myography. The increase in contraction observed in TgNotch3^{R169C} mice was ameliorated in vessels pre-treated with the Rho kinase inhibitor fasudil (A, C, E) and the ER stress inhibitor 4-PBA (B, D, F) (n=5-6; Two-way ANOVA with Bonferroni post-test). Curves represent the mean±SEM. (G) Endothelium-dependent vasorelaxation in response to acetylcholine (ACh) was decreased in TgNotch3^{R169C} vessels, which was improved by the ER stress inhibitor 4-PBA (n=4-5). (H) Endothelium-independent vasorelaxation in response to sodium nitroprusside (SNP) was decreased in TgNotch3^{R169C} vessels (n=9-12). Responses were expressed as percentage of U46619-induced pre-constriction. Curves represent the mean±SEM. Two-way ANOVA with Bonferroni post-test. # $p<0.05$ *vs.* TgN3^{WT}, † *vs.* TgN3^{R169C}.

Figure 3. Increased $[Ca^{2+}]_i$ transients and Ca^{2+} channels gene expression in TgNotch3^{R169C} VSMCs. (A) Calcium transients were measured by live cell fluorescence imaging using the fluoroprobe Cal-520 AM. Representative tracings of VSMCs $[Ca^{2+}]_i$ responses to U46619 (1 µmol/L) in TgNotch3^{WT} and TgNotch3^{R169C} VSMCs in presence or absence of 4-PBA. Experiments were repeated 6 times/group with >30 cells studied/field. (B) $[Ca^{2+}]_i$ calculated as the area under the curve (n=6; One-way ANOVA with Dunnett post-test). (C) *Cav1.1*, *TRPM2*, *IP3R*, *SERCA*, *RyR1*, *RyR2* and *RyR3* gene expression in VSMCs isolated from TgNotch3^{WT} and TgNotch3^{R169C} mice. Analysis was by qPCR and gene expression was normalised to GAPDH (n=5-11; One-way ANOVA with Dunnett post-test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}, † *vs.* TgN3^{R169C}.

Figure 4. Rho kinase and ER stress markers are increased in TgNotch3^{R169C} arteries. (A) *PDZ*, *LARG* and *p115* gene expression in mesenteric arteries from FVB, TgNotch3^{WT} and TgNotch3^{R169C} mice (n=6-8). (B) *XBP1*, *ATF4, BiP* and *CHOP* gene expression in FVB, TgNotch3^{WT} and TgNotch3^{R169C} mice (n=5-8). Analysis was performed by qPCR and gene expression was normalised to GAPDH (One-way ANOVA with Dunnett post-test). (C) Representative images and fluorescence quantification of BiP (ER stress marker) in mesenteric arteries from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} mice. Nuclei are in blue (DAPI) and BiP in green. Scale bars = 20 µm; 63x (n=4; Student's *t* test). (D) Upper panel: representative immunoblot for ERO1 in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice; Lower panels: quantification of ERO1. Protein expression was normalised to β-actin. Results are expressed as mean±SEM *p<0.05 vs FVB, # *vs.* TgN3^{WT}.

Figure 5. Vasodilation impairment in TgNotch3^{R169C} arteries involves downregulation of eNOS activity and cGMP levels. (A) Upper panel: representative immunoblot for the phosphorylation of the inhibitory site of eNOS (Thr^{495}) in mesenteric arteries from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} mice; Lower panels: quantification of p-eNOS. Protein expression was normalised to t-eNOS (n=7; Student's *t* test). (B) Levels of cGMP in VSMCs isolated from TgNotch3^{WT} and TgNotch3^{R169C} mice in presence or absence of fasudil and 4-PBA (n=5-9; One-way ANOVA with Dunnett post-test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}.

Figure 6. Downregulation of NO/sGC/cGMP signalling pathway in VSMCs during Notch3 gain-of-function mutation is associated with redox-sensitive processes. Concentration-response curves to (A) BAY 58-2667 (sGC activator) and (B) 8-Br-cGMP (PKG activator) in TgNotch3^{WT} and TgNotch3^{R169C} mesenteric arteries. Responses were expressed as percentage of U46619-induced pre-constriction (n=4-7; Two-way ANOVA with Bonferroni post-test). (C) Reactive oxygen species (ROS) production measured by EPR in mesenteric arteries from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} mice (n=7; Student's *t* test). Results are normalized by protein content. (D) sGCβ1oxidation was also assessed in arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice (pool of five different samples) by using a biotin-tagged dimedone-based probe that captures specifically sulfenylated proteins. (E) *Nox1* gene expression in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice. Analysis was by qPCR and gene expression was normalised to GAPDH (n=6-7; Student's *t* test). (F) H_2O_2 levels in TgNotch3^{WT} and TgNotch3^{R169C} mesenteric arteries were measured by

Amplex Red (n=6-7; Student's *t* test). (G) *Catalase* and (H) *GPX1* gene expression in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice. Analysis was by qPCR and gene expression was normalised to GAPDH (n=6-8; Student's *t* test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}.

Figure 7. Renal dysfunction in CADASIL mice. Renal function in in urine from TgNotch3^{WT} and TgNotch3^{R169C} mice mice was assessed by measuring urine albumin:creatinie ratio (n=9; Student's *t* test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}.

Figure 8. Human CADASIL vessels exhibit increased oxidative stress and sGCβ1 oxidation. (A) Levels of cGMP in VSMCs from control and CADASIL patients in the presence and absence of fasudil or 4-PBA (n=5; One-way ANOVA with Dunnett post-test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}. (B) sGCβ1 oxidation assessed in control and CADASIL VSMCs (pool of five different samples) using a biotin-tagged dimedone-based probe that captures specifically sulfenylated proteins. (C) Representative images from two different control and CADASIL patients and (D) analysis of 8-hydroxyguanosine (8-OHG) in brain vessels. Nuclei are in blue (DAPI) and 8-OHG in green (arrows). Scale bars = 50 μm; 20x (n=3-4; Student's *t* test). Results are expressed as mean±SEM. *** p<0.001 *vs.* Control.

Figure 9. Schematic demonstrating putative molecular mechanisms underlying the peripheral vasculopathy associated with CADASIL. In vascular smooth muscle cells, gain of function of Notch3 leads to oxidative stress and decreased activation of the eNOS/NO/cGMP pathway, processes associated with Rho kinase activation and

ER stress. These signaling events promote increased vasoconstriction and reduced vasodilation, leading to impaired vascular function in CADASIL. eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, cyclic GMP.