Peripheral arteriopathy caused by Notch3 gain-of-function mutation is associated with increased Ca^{2+} and redox signaling 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 have taken place 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). Therefore, only male mice were used in this study. 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, 26]. For each set of experiments, we used five to eight different mice. Mice were anaesthetized with 5% isoflurane (1.5 L/min O_2) for blood collection via cardiac puncture, which is a terminal procedure, and

tissues were collected for future experiments. 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-sGCβ1 (1:500; Cayman Chemical, UK), anti-ERO1 (1:1000; Santa Cruz, UK).

Affinity capture of sulfenylated proteins

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 [27]. 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.

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 [28]. 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 [29]. 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 [30]. 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

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).

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. *p*D_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 [31], 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 5C). 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), systemic oxidative stress (Supplementary figure 4), increased oxidation of sGCβ1 (Figure 6D) and increased expression of Nox1 in TgNotch3^{R169C} arteries (Figure 6E). Associated with increased NADPH-derived O_2^- production was reduced bioavailability of H_2O_2, a putative endothelium-derived relaxing factor (EDRF) [32, 33], 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 reduced in CADASIL mice (Figures 6G, 6H).

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 7A). Oxidation of sGCβ1 was higher in CADASIL VSMCs compared with control VSMCs (Figure 7B). 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 7C, 7D).

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, 34-36]. 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 dysregulation of Ca^{2+} homeostasis, Rho kinase activation, ER stress response, and blunting of the eNOS/NO/sGC/cGMP pathway. Perturbed VSMC signalling and vascular dysfunction in CADASIL mice were associated with an increase in ROS production, highlighting a role for redox-dependent processes in the

vasculopathy of CADASIL. 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. Our novel findings indicate that a Notch3 gain-of-function mutation is associated with upregulation of Rho kinase, ER stress responses and redox-sensitive processes affecting the eNOS/sGC/cGMP axis leading to vascular dysfunction.

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 [37, 38]. In aging mice GOM deposits progress in size over time and new GOM deposits are continuously being formed [38]. 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 [37], 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 [37].

We provide evidence that CADASIL-causing Notch3 mutations cause functional changes in peripheral arteries. 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, 39]. 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). Vascular smooth muscle cell contraction is

525 regulated primarily by dynamic changes in Ca^{2+} homeostasis and Ca^{2+} channel
526 activity/expression [40]. Our findings showed hypercontractility of TgNotch3^{R169C}
527 arteries, in addition to augmented agonist-stimulated Ca^{2+} transients and increased
528 expression of Ca^{2+} channels. These processes are highly regulated since Cav1.1,
529 IP3R and RyR1 were upregulated in CADASIL mice, whereas TRPM2, SERCA, RyR2
530 and RyR3 were not altered compared with control mice. Previous studies showed an
531 important role for Notch in Ca^{2+} regulation, since Notch increases expression/activity
532 of store-operated Ca^{2+} entry (SOCE) and canonical transient receptor potential
533 (TRPC6) channels in VSMCs [41]. Moreover Ca^{2+} channel blockers seem to improve
534 cognitive decline and cerebral hypoperfusion in CADASIL patients [42], although this
535 aspect warrants further investigation.

536 Vascular smooth muscle cells are highly plastic and in disease states undergo
537 phenotypic switching from a contractile to a proliferative and pro-inflammatory state.
538 While VSMC contraction is triggered by an increase in $[Ca^{2+}]_i$ which promotes actin-
539 myosin interaction, it is also regulated by Ca^{2+}-independent processes involving RhoA-
540 Rho kinase and MAP kinases, ROS amongst other systems [40]. These phenomena
541 are especially important in pathological conditions as we demonstrate here, where
542 vascular dysfunction was associated not only with amplification of Ca^{2+} transients, but
543 also with systems involving Rho kinase, oxidative and ER stress. Pharmacological
544 inhibitors of Rho kinase and ER stress normalised hypercontractile responses in
545 CADASIL mice, indicating involvement of these systems in Notch3-regulated
546 contraction. Supporting this notion, at the molecular level, elements of RhoA/Rho
547 kinase signalling, and the ER stress response were perturbed in TgNotch3^{R169C}
548 VSMCs. RhoA, a member of the Rho GTPase family and regulated by Rho GEFs, is
549 a master regulator of cytoskeletal dynamics and VSMC function [43, 44]. 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 [44]. 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 [45]. 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 [46]. 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 [47].

Similar to what we found in peripheral and cerebral vessels in CADASIL patients [12, 48], 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 [49]. Endothelial NOS is the primary source

of NO in endothelial cells and is the key regulator of endothelial function [50]. 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 [50]. 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) [51, 52]. PKG is a potent vasodilator and mediates effects in part through H_2O_2 [33]. 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 [53, 54]. 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 [55], was reduced in TgNotch3^{R169C} mice, suggesting less activity of sGC due to its higher oxidation. Supporting this, vascular oxidative stress 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 [53, 54, 56]. 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 [33, 57]. 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 [30]. 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.

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, activation of ER stress and impaired eNOS/sGC/cGMP signalling. 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 and dysfunctional vasoreactivity may be associated with peripheral vascular disease in CADASIL

patients [34]. 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 pathways in the vasculopathy of CADASIL

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 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

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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}.

945

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

957

958 **Figure 4. Rho kinase and ER stress markers are increased in TgNotch3^{R169C}**
959 **arteries.** (A) *PDZ*, *LARG* and *p115* gene expression in mesenteric arteries from FVB,
960 TgNotch3^{WT} and TgNotch3^{R169C} mice (n=6-8). (B) *XBP1*, *ATF4, BiP* and *CHOP* gene
961 expression in FVB, TgNotch3^{WT} and TgNotch3^{R169C} mice (n=5-8). Analysis was
962 performed by qPCR and gene expression was normalised to GAPDH (One-way
963 ANOVA with Dunnett post-test). (C) Representative images and fluorescence
964 quantification of BiP (ER stress marker) in mesenteric arteries from 24-week-old
965 TgNotch3^{WT} and TgNotch3^{R169C} mice. Nuclei are in blue (DAPI) and BiP in green.
966 Scale bars = 20 µm; 63x (n=4; Student's *t* test). (D) Upper panel: representative
967 immunoblot for ERO1 in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C}
968 mice; Lower panels: quantification of ERO1. Protein expression was normalised to β-
969 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 lucigenin in mesenteric arteries from 24-week-old TgNotch3^{WT} and TgNotch3^{R169C} mice (n=6-8; 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). (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

995 expression in mesenteric arteries from TgNotch3^{WT} and TgNotch3^{R169C} mice. Analysis
996 was by qPCR and gene expression was normalised to GAPDH (n=6-8; Student's *t*
997 test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}.
998
999 **Figure 7. Human CADASIL vessels exhibit increased oxidative stress and sGCβ1**
1000 **oxidation.** (A) Levels of cGMP in VSMCs from control and CADASIL patients in the
1001 presence and absence of fasudil or 4-PBA (n=5; One-way ANOVA with Dunnett post-
1002 test). Results are expressed as mean±SEM. # p<0.05 *vs.* TgN3^{WT}. (B) sGCβ1
1003 oxidation assessed in control and CADASIL VSMCs (pool of five different samples).
1004 (C) Representative images from two different control and CADASIL patients and (D)
1005 analysis of 8-hydroxyguanosine (8-OHG) in brain vessels. Nuclei are in blue (DAPI)
1006 and 8-OHG in green (arrows). Scale bars = 50 μm; 20x (n=3-4; Student's *t* test).
1007 Results are expressed as mean±SEM. *** p<0.001 *vs.* Control.
1008
1009 **Figure 8. Schematic with mechanisms underlying the peripheral vasculopathy**
1010 **associated with CADASIL.** The vasculopathy associated with CADASIL is also
1011 present in peripheral small vessels where Rho kinase, ER stress and impaired eNOS
1012 activation and redox-regulated sGC/cGMP signalling may be important. We define new
1013 molecular mechanisms underlying the vasculopathy of CADASIL and bring new
1014 insights for novel and attractive therapeutic targets in CADASIL.