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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Notch3 mutations cause Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), which predisposes to stroke and dementia. CADASIL is characterised 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 (VSMCs) from TgNotch3R169C mice (CADASIL model), (ii) VSMCs from peripheral arteries from CADASIL patients, and (iii) post-mortem brains from CADASIL individuals. TgNotch3R169C vessels exhibited GOM deposits, increased vasoreactivity and impaired vasorelaxation. Hypercontractile responses were normalised by fasudil (Rho kinase inhibitor) and 4-phenylbutyrate (4-PBA; endoplasmic-reticulum (ER) stress inhibitor). 

Ca2+ transients and Ca2+ channel expression were increased in CADASIL VSMCs, with increased expression of Rho guanine nucleotide-exchange factors (GEFs) and ER stress proteins. Vasorelaxation mechanisms were impaired in CADASIL, evidenced by decreased endothelial nitric oxide synthase (eNOS) phosphorylation and reduced cyclic guanosine 3’,5’-monophosphate (cGMP) levels, with associated increased soluble guanylate cyclase (sGC) oxidation, decreased sGC activity and reduced levels of the vasodilator hydrogen peroxide (H2O2). In VSMCs from CADASIL patients, sGC oxidation was increased and cGMP levels decreased, effects normalised 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 Ca2+ 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.

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 remodelling 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 Infarcts 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 remodelling 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, TgNotch3R169C mice, demonstrated cerebrovascular dysfunction and thickening, with associated increased deposition of ECM proteins, up-regulation of voltage-dependent potassium (Kv1) channels, blunted membrane depolarisation 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, U.K.

**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 anaesthesia, 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 summarised 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, standardised 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, TgNotch3WT and TgNotch3R169C, have been previously characterised and described. Briefly, TgNotch3WT and TgNotch3R169C mice (on an FVB background) express rat wildtype 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 one X chromosome in females results in mosaic expression of the mutant protein in TgNotch3R169C 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 (22–24°C), and in light/dark cycles of 12 h with free access to food and tap water. They were studied at 24 weeks of age, at which stage features consistent with CADASIL were well established and they exhibited 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 anaesthetised with 5% isoflurane (1.5 l/min O2). 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 TgNotch3R169C mice were similar to wildtype (TgNotch3WT) controls. Cardiac function and structure were similar between groups. Clinical features of subjects and mice phenotype were also shown previously [12].

**Transmission electron microscopy**

GOM deposition in mesenteric arteries from TgNotch3WT and TgNotch3R169C mice was examined by transmission electron microscopy (TEM). Arteries (2 mm thick slices) were fixed in 2% Glutaraldehyde/2% Paraformaldehyde/0.1 M Sodium Cacodylate buffer overnight at 4°C and rinsed with 0.1 M Sodium Cacodylate buffer three times of 5 min before post-fixation in 1% Osmium Tetroxide for 1 h. Osmium tetroxide was removed with changes of distilled H2O (dH2O) three times of 10 min each. Samples were then stained with 0.5% Uranyl Acetate/dH2O for 1 h in the dark prior dehydration through an ethanol series of 30, 50, 70, 90% (15 min each), 100% ethanol (four times of 5 min), dried 100% ethanol (plus 3A molecular sieve) (four times of 5 min), followed by three-times changes of Propylene Oxide for 5 min 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 h. 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 min and Reynolds Lead Citrate also for 5 min. Tissue samples were viewed on a JEOL 1200EX TEM running at 80 kV 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 TgNotch3WT and TgNotch3R169C mice. Briefly, arterial segments were mounted on isometric wire myographs (Danish Myo Technology, Denmark) filled with 5 ml of physiological saline solution [(in mmol/l): 130 NaCl, 14.9 NaHCO3, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4, 7H2O, 5.5 glucose, 1.56 CaCl2, 2H2O and 0.026 EDTA] and continuously gassed with a mixture of 95% O2 and 5% CO2 while being maintained at a constant temperature of 37 ± 0.5°C. Following 30 min of equilibration, the contractile responses of arterial segments were assessed by the addition of KCl (62.5 mmol/l). Endothelium-dependent relaxation was assessed as a dose-response to ACh (10−8–10−5 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 to 3 × 10−5 mol/l), U46619 (10−10–10−6 mol/l) and angiotensin II (Ang II) (10−10 to 3 × 10−5 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 min) or fasudil (Rho kinase inhibitor; 1 μmol/l, 30 min).

**VSMC 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 bovine serum albumin (BSA), and were incubated for 30–60 min 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 min) and the cell pellet was resuspended in Ham’s F-12 culture medium containing 10% foetal bovine serum (FBS). Cells were seeded on to 25 mm flask. VSMCs were maintained in DMEM media supplemented with 10% 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 4–8) were studied. In some protocols, the role of ER stress, Rho kinase and Notch signalling was assessed using pharmacological inhibitors: ER stress inhibitor, 4-PBA (1 mmol/l, Sigma–Aldrich, U.K.) and Rho kinase inhibitor, fasudil (10 μmol/l, Tocris, U.K.). Cells were pre-exposed to 4-PBA and fasudil for 24 h.

**Quantitative real-time polymerase chain reaction**

Quantitative real-time polymerase chain reaction (qPCR) (Qiagen, U.K.) was used to assess mRNA expression in TgNotch3WT and TgNotch3R169C 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 TgNotch3R169C mice were on an FVB background. Briefly, total RNA was extracted from tissues using TRIZol (Qiagen, Manchester, U.K.), 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 two steps at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of three steps, 15-s denaturation at 95°C, 60-s annealing at 60°C and 15 s 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^ΔΔCt method.

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

VSMC Ca^{2+} signalling was assessed using the fluorescent Ca^{2+} indicator, Cal-520 acetoxymethylester (Cal-520/AM; CellSignaling, U.K.). Cellswerepre-exposedto4-PBAandfasudilfor24h.

**Immunoblotting**

Protein was extracted from mesenteric arteries isolated from TgNotch3WT and TgNotch3R169C mice. Protein (30 μg) was separated by electrophoresis on a polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 3% BSA in Tris-buffered saline (TBS) solution. Membranes were then incubated with specific antibodies overnight at 4°C. Membranes were washed three times with TBS-Tween 20 and incubated with infrared dye-labelled secondary antibodies for 1 h at room temperature. Membranes were visualised using an Odyssey CLx infrared imaging system (LI-COR Biosciences UK Ltd, U.K.) and results were normalised to β-actin protein and are expressed in arbitrary units compared with wildtype group, which was taken as 100. Antibodies used were as follows: anti-β-actin (1:5000; Sigma–Aldrich, U.K.); anti-phospho-eNOSThr495 (1:500; Santa Cruz, U.K.); anti-phospho-eNOSSer1177 (1:1000; Cell Signaling, U.K.); anti-total-endothelial nitric oxide synthase (eNOS) (1:1000; Cell Signaling, U.K.); anti-EROD1 (1:1000; Santa Cruz, U.K.).
Affinity capture of sulphenylated proteins

In order to investigate sGCβ1 oxidation, sulphenylated proteins were captured using a biotin-tagged dimedone-based probe (DCP-Bio, Merck NS1226-5MG) that specifically binds sulphenic acid groups (SOH) in proteins [29]. Mesenteric arteries from FVB, TgNotch3WT and TgNotch3R169C mice and VSMCs isolated from control and CADASIL patients, after homogenisation in lysis buffer were supplemented with DCP-Bio1 (1 mM), N-methylmaleimide (10 mM), catalase (200 U) and protease inhibitors (1 mM PMSF and 1 μg/ml of aprotinin, leupeptin and pepstatin). Samples were kept on ice for 30 min and centrifuged at 12000 × g for 4 min 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-ligand support beads (sepharose CL-4B beads, Sigma–Aldrich, Seelze, Germany) to remove proteins with a tendency to bind non-specifically and incubated for 2 h at 4°C with constant rotation. Beads were centrifuged at 1000 × g for 2 min. The supernatant was collected and incubated with streptavidin beads (High Capacity Streptavidin–Agarose Resin, Thermo Scientific, Illinois, U.S.A.) overnight at 4°C with constant rotation. After the incubation steps beads were centrifuged at 1000 × g for 2 min and washed with PBS three times. Proteins were then eluted in 50 μl of 2× 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. Sulphenylated protein-enriched lysates were used for immunoblotting and membranes were probed for sGCβ1 (anti-sGCβ1; 1:500; Cayman Chemical, U.K.).

Immunofluorescence

Immune staining was performed for an ER stress regulator, binding immunoglobulin protein (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 (UPRs) survival and apoptosis responses [31]. Paraffin sections (5 μm) of brain from control and CADASIL patients and mesenteric arteries from TgNotch3WT and TgNotch3R169C mice were deparaffinised in xylene, hydrated through graded ethanol and washed in water. All sections were incubated in EDTA (pH 8.0) and boiled for 15 min at 95°C for antigen unmasking. Slides were cooled to room temperature, permeabilised in 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, and blocked with 10% donkey serum, 1% BSA in 1× TBS and Tween 20 (TBS-T) for 1 h 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 1× 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 1× TBS 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 1× 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 1× TBS solution) secondary antibody was used. For both staining, after primary antibody incubation, secondary antibodies were incubated for 1 h at room temperature in the dark. Slides were treated with 0.1% Sudan Black B (Sigma–Aldrich, 199664) in methanol for 10 min 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 20× (brain vessels from patients) or 63× (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®

Cyclic guanosine 3′,5′-monophosphate ELISA

Cyclic guanosine 3′,5′-monophosphate (cGMP) levels were detected in the present study in VSMCs isolated from TgNotch3WT and TgNotch3R169C mice and control and CADASIL patients by ELISA according to the manufacturer’s protocol (Cyclic GMP Assay kit, Cell Signaling Technology). Results were normalised by concentration of protein.
Lucigenin-enhanced chemiluminescence

Lucigenin-derived chemiluminescence assay was used to determine NADPH-dependent ROS production in mesenteric arteries homogenates from TgNotch3WT and TgNotch3R169C mice as we previously described [32]. Briefly, tissues were homogenised in lysis buffer (20 mmol/l of KH2PO4, 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). Fifty microlitres of the sample was added to a suspension containing 175 μl of assay buffer (50 mmol/l of KH2PO4, 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 normalised by concentration of protein, as measured by the BCA assay.

Amplex red assay

Hydrogen peroxide (H2O2) levels was assessed by Amplex red® assay in mesenteric arteries from TgNotch3WT and TgNotch3R169C mice. Protocols were made according to the manufacturer’s instructions using the horseradish peroxidase-linked Amplex Red fluorescence assay (A22188; LifeTechnologies). Fluorescence readings were made in a 96-well plate at Ex/Em = 530/590 nm. H2O2 production was normalised to protein concentration.

Plasma thiobarbituric acid reactive substances measurement

Blood was collected under isoflurane anaesthesia (3% induction; 1.5% maintenance) by cardiac puncture immediately prior to killing. Blood was collected in heparinised tubes (TekLab, County Durham, U.K.). Plasma was separated by centrifugation (2000 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 the present study in plasma from TgNotch3WT and TgNotch3R169C mice by colorimetric (532–535 nm) assay according the manufacturer’s protocol (Cayman’s TBARS Assay Kit, Cayman Chemical - CAY700870).

Urine biochemistry

Spot urine was collected from the bladder during killing. Urine was aliquoted, snap frozen and stored at −80°C. Albumin and creatinine were determined by an automated analyser (Roche/Hitachi cobas c systems - cobas c 311 Autoanalyser).

Measurement of O2•− by electron paramagnetic resonance

Superoxide anion (O2•−) was measured by electron paramagnetic resonance [33]. Production of O2•− was measured in in Krebs–Hepes buffer (99 mM NaCl, 4.69 mM KCl, 2.5 mM CaCl2,2H2O, 1.2 mM MgSO4.7H2O, 25 mM NaHCO3, 1.03 mM KH2PO4, 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 50 μl 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 O2•− 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 min and O2•− 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 × 105. Results were normalised by protein content.

Statistical analysis

For vascular functional studies, concentration–response curves were generated and the maximal effect (Emax) and the agonist concentration that produced 50% of the maximal response (log EC50) were calculated using non-linear regression analysis. pD2 (defined as the negative logarithm of the EC50 values) and Emax were compared by Student’s t test or two-way analysis of variance (ANOVA) with Bonferroni’s 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.
Results

GOM deposits in small peripheral arteries

A characteristic feature of CADASIL is cerebrovascular GOM deposition [8]. GOM deposits have been identified in VSMCs in skin biopsies from CADASIL patients [34], however whether GOM accumulates around VSMCs in peripheral small arteries is unclear. As shown in Figure 1A,B, electron microscopic analysis revealed GOM deposits in mesenteric resistance arteries from TgNotch3R169C mice but not in TgNotch3WT 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 TgNotch3R169C mice is also enlarged when compared with wildtype mice, which might be associated with increased deposition of ECM components such as MMP2 and MMP9. Previous studies in cerebral vessels from TgNotch3R169C 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 S2A,B).

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 TgNotch3R169C mice, contractile responses to multiple vasoconstrictors including Phe, U46619 and Ang II were increased compared with vessels from TgNotch3WT mice (Supplementary Figure S1A–C). These responses were restored when arteries were exposed to inhibitors of Rho kinase (fasudil; Figure 2A,C,E) or ER stress (4-PBA; Figure 2B,D,F). No changes were observed in vessels from TgNotch3WT mice incubated with fasudil or 4-PBA.

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.
Figure 2. Role of Rho kinase signalling pathway and ER stress in the vascular dysfunction observed in TgNotch3R169C peripheral small arteries.

Vascular functional responses in mesenteric arteries obtained from 24-week-old TgNotch3WT and TgNotch3R169C in response to Phe, U46619, Ang II, ACh and SNP was assessed by wire myography. The increase in contraction observed in TgNotch3R169C 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's post-test). Curves represent the mean ± SEM. (G) Endothelium-dependent vasorelaxation in response to ACh was decreased in TgNotch3R169C vessels, which was improved by the ER stress inhibitor 4-PBA (n=4–5). (H) Endothelium-independent vasorelaxation in response to SNP was decreased in TgNotch3R169C vessels (n=9–12). Responses were expressed as percentage of U46619-induced pre-constriction. Curves represent the mean ± SEM. Two-way ANOVA with Bonferroni's post-test. # P<0.05 vs. TgN3WT, † vs. TgN3R169C.
In addition to hypercontractile responses, CADASIL mice exhibited significantly reduced endothelium-dependent (ACh-induced) and endothelium-independent vasorelaxation (SNP-induced) compared with wildtype vessels (Figure 2G,H). Maximum response for ACh-induced relaxation in TgNotch3R169C was 73.7 ± 3.9 vs. 103.1 ± 7.5 in TgNotch3WT mice (P < 0.05). EC50 for SNP-induced vasorelaxation was 7.0 ± 0.2 in TgNotch3R169C vs. 7.8 ± 0.1 in wildtype mice (P < 0.05). Pre-treatment of vessels with 4-PBA normalised 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 VSMCs contraction. In particular we measured changes in agonist-stimulated Ca2+ responses and Ca2+ channels and activation of the Rho kinase and ER stress pathways in mesenteric arteries and VSMCs. As shown in Figure 3, Ca2+ transients and expression of various Ca2+ channels were altered in TgNotch3R169C-derived VSMCs, compared with wildtype VSMCs. Agonist-stimulated Ca2+ responses (Figure 3A) and the calculated area under the curve (Figure 3B) were significantly increased in the TgNotch3R169C group compared with control mice. Pre-treatment with 4-PBA attenuated Ca2+ responses in CADASIL VSMCs. Gene expression of the Ca2+ channels voltage-dependent L-type calcium channel, subunit α1S (Cav1.1), transient receptor potential cation channel, subfamily M, member 2 (TRPM2) and ryanodine receptor 1 (RyR1) was augmented in TgNotch3R169C-derived VSMCs, whereas no changes were observed for inositol 1,4,5-trisphosphate (IP3) receptor (IP3R), ryanodine receptor 2 (RyR2), ryanodine receptor 3 (RyR3) and sarcoplasmic reticulum Ca2+-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 TgNotch3R169C mice compared with TgNotch3WT (Figure 4A). Gene expression of p115 was unchanged in TgNotch3R169C mice. Arteries from TgNotch3R169C mice also exhibited increased transcription of ER stress genes: X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4), 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 TgNotch3R169C vessels (Figure 4C). ERO1, a natural UPR target promoter triggered by ER stress, was also increased in TgNotch3R169C mesenteric arteries (Figure 4D). Together these data indicate that a Notch3 gain-of-function mutation during CADASIL is associated with up-regulation of Rho kinase, ER stress responses and changes in Ca2+ homeostasis in small peripheral arteries.

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

Impaired vasorelaxation in TgNotch3R169C 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 eNOS (Thr495) was significantly increased in TgNotch3R169C mice whereas phosphorylation of the activator site of eNOS (Ser1177) was unchanged (Supplementary Figure S3). 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 TgNotch3R169C mice

To further dissect possible mechanisms underlying reduced vasorelaxation and perturbed vascular NO/cGMP signalling in TgNotch3R169C mice, we interrogated vasodilator pathways mediated by redox-sensitive protein kinase G (PKG), which has been shown to regulate vasorelaxation through H2O2-dependent pathways. Vasorelaxation concentration-response curves to BAY 58-2667, a potent soluble guanylate cyclase (sGC) activator, and 8-Br-cGMP, which activates cGMP-dependent PKG, were performed in mesenteric arteries from TgNotch3R169C mice. Vasodilation induced by both BAY 58-2667 (Figure 6A) and 8-Br-cGMP, a cGMP analogue (Figure 6B), was reduced in vessels from TgNotch3R169C 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 S4B), systemic oxidative stress (Supplementary Figure S4A), increased oxidation of sGCβ1 (Figure 6D) and increased expression of Nox1 in TgNotch3R169C arteries (Figure 6E). No changes in Nox 2 and 4 were observed between groups (Supplementary Figure S5). Associated with increased NADPH-derived O2•− production was reduced bioavailability of H2O2,
Figure 3. Increased [Ca\textsuperscript{2+}]\textsubscript{i} transients and Ca\textsuperscript{2+} channels gene expression in TgNotch\textsuperscript{3R169C} VSMCs

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

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:creatinine ratio. TgNotch\textsuperscript{3R169C} mice also showed a significant increase in albumin:creatinine ratio in urine, suggesting an increase in vascular permeability and endothelial dysfunction (Figure 7).
Figure 4. Rho kinase and ER stress markers are increased in TgNotch3R169C arteries

(A) PDZ, LARG and p115 gene expression in mesenteric arteries from FVB, TgNotch3WT and TgNotch3R169C mice (n=6–8). (B) XBP1, ATF4, BiP and CHOP gene expression in FVB, TgNotch3WT and TgNotch3R169C mice (n=5–8). Analysis was performed by qPCR and gene expression was normalised to GAPDH (one-way ANOVA with Dunnett’s post-test). (C) Representative images and fluorescence quantification of BiP (ER stress marker) in mesenteric arteries from 24-week-old TgNotch3WT and TgNotch3R169C mice. Nuclei are in blue (DAPI) and BiP in green. Scale bars = 20 μm; 63× (n=4; Student’s t test). (D) Upper panel: representative immunoblot for ERO1 in mesenteric arteries from TgNotch3WT and TgNotch3R169C 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. TgN3WT.
Figure 5. Vasodilation impairment in TgNotch3R169C arteries involves down-regulation of eNOS activity and cGMP levels

(A) Upper panel: representative immunoblot for the phosphorylation of the inhibitory site of eNOS (Thr495) in mesenteric arteries from 24-week-old TgNotch3WT and TgNotch3R169C 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 TgNotch3WT and TgNotch3R169C mice in presence or absence of fasudil and 4-PBA (n=5–9; one-way ANOVA with Dunnett’s post-test). Results are expressed as mean ± SEM. *P<0.05 vs. TgN3WT.

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 (Figure 8C,D).

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 ischaemic 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 TgNotch3R169C mice that express the CADASIL-causing Notch3(R169C) mutant protein [22]. We also proved 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 TgNotch3R169C 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 TgNotch3R169C mice were linked to increased Ca²⁺ transients and up-regulation of Ca²⁺ 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
Figure 6. Down-regulation 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 TgNotch3WT and TgNotch3R169C mesenteric arteries. Responses were expressed as percentage of U46619-induced pre-constriction (n=4–7; two-way ANOVA with Bonferroni’s post-test). (C) ROS production measured by EPR in mesenteric arteries from 24-week-old TgNotch3WT and TgNotch3R169C mice (n=7; Student’s t test). Results are normalised by protein content. (D) sGCβ1 oxidation was also assessed in arteries from TgNotch3WT and TgNotch3R169C mice (pool of five different samples) by using a biotin-tagged dimedone-based probe that captures specifically sulphenylated proteins. (E) Nox1 gene expression in mesenteric arteries from TgNotch3WT and TgNotch3R169C mice. Analysis was by qPCR and gene expression was normalised to GAPDH (n=6–7; Student’s t test). (F) H2O2 levels in TgNotch3WT and TgNotch3R169C 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 TgNotch3WT and TgNotch3R169C 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. TgN3WT.
Figure 7. Renal dysfunction in CADASIL mice

Renal function in urine from TgNotch3WT and TgNotch3R169C mice was assessed by measuring urine albumin:creatinine ratio (n=9; Student’s t test). Results are expressed as mean ± SEM. #P<0.05 vs. TgN3WT.

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 (Notch3ECD) and the presence of GOM deposits on SMCs from small arteries [40,41]. In ageing 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 TgNotch3R169C 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 TgNotch3R169C 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 (Phe, Ang II and U44619), was increased in TgNotch3R169C mice, indicating a generalised phenomenon rather than an agonist-specific effect. These findings are in contrast with 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 ageing and progression of disease in TgNotch3R169C mice, vasocontractile responses may change. However this awaits confirmation. VSMC contraction is regulated primarily by dynamic changes in Ca2+ homoeostasis and Ca2+ channel activity/expression [47]. Our findings showed hypercontractility of TgNotch3R169C arteries, in addition to augmented antagonist-stimulated Ca2+ transients and increased expression of Ca2+ channels. These processes are highly regulated since Cav1.1, IP3R and RyR1 were up-regulated 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 Ca2+ regulation, since Notch increases expression/activity of store-operated Ca2+ entry (SOCE) and canonical transient receptor potential (TRPC6) channels in VSMCs [48]. Moreover Ca2+ channel blockers seem to improve cognitive decline and cerebral hypoperfusion in CADASIL patients [49], although this aspect warrants further investigation.

VSMCs 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 [Ca2+], which promotes actin–myosin interaction, it is also regulated by Ca2+-independent processes involving RhoA-Rho kinase and MAP kinases, ROS among other systems [47]. These phenomena are especially important in pathological conditions as
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’s post-test). Results are expressed as mean ± SEM. *P<0.05 vs. TgN3WT. (B) sGCβ1 oxidation assessed in control and CADASIL VSMCs (pool of five different samples) using a biotin-tagged dimedone-based probe that captures specifically sulphenylated proteins. (C) Representative images from two different control and CADASIL patients and (D) analysis of 8-OHG in brain vessels. Nuclei are in blue (DAPI) and 8-OHG in green (arrows). Scale bars = 50 μm; 20× (n=3–4; Student’s t test). Results are expressed as mean ± SEM. ***P<0.001 vs. Control.
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 VSMCs 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 destabilisation 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 up-regulated [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 VSMCs function and GOM deposition and may be important pathogenic mechanisms contributing to the vasculopathy in CADASIL. Supporting this, expression of ER stress markers XBPI, 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 UPR 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 VSMCs 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\(_2\)O\(_2\) [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 BAY58-2667, a sGC activator and ER 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\(_2\)O\(_2\) levels, since H\(_2\)O\(_2\) is an important vasodilator [59,60,62]. This may be important in TgNotch3\(^{R169C}\) mice, where down-regulation of the sGC/cGMP system was associated with decreased vascular H\(_2\)O\(_2\) production compared with control mice. While these observations were made in peripheral vessels, they are especially pertinent in the cerebral circulation where H\(_2\)O\(_2\) 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\(_2\)O\(_2\)-mediated vasorelaxation. Vascular H\(_2\)O\(_2\) down-regulation 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 Ca²⁺ homeostasis, up-regulation 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.

**Clinical perspectives**

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

- 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+} homoeostasis, up-regulation of Rho kinase, and ER- and Nox1-mediated oxidative stress that cause hyperoxidation of vascular signalling molecules and blunting of the eNOS/sGC/cGMP pathway. Findings in CADASIL mice were recapitulated in post-mortem cerebral vessels from patients with CADASIL.

- 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.

**Data Availability**

The data underlying the present study will be shared on request to the corresponding authors.

**Competing Interests**

The authors declare that there are no competing interests associated with the manuscript.

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**CRediT Author Contribution**

Karla B. Neves: Conceptualisation, Formal analysis, Investigation, Methodology, Writing—original draft. Hannah Morris: Conceptualisation, Investigation, Methodology, Writing—review and editing. Rhéure Alves-Lopes: Investigation, Methodology, Writing—review and editing. Keith W. Muir: Conceptualisation, Funding acquisition, Writing—review and editing. Fiona Moreton: Conceptualisation, Writing—review and editing. Christian Delles: Writing—review and editing. Augusto C. Montezano: Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing. Rhian M. Touyz: Resources, Supervision, Funding acquisition, Project administration, Writing—review and editing.
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Abbreviations

ACh, acetylcholine; ATF4, activating transcription factor 4; BiP, binding immunoglobulin protein; BSA, bovine serum albumin; CADASIL, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy; Cav1.1, Ca2+ channels voltage-dependent L-type calcium channel, subunit α1S; cGMP, cyclic guanosine 3′,5′-monophosphate; CHOP, C/EBP homologous protein; dH2O2, distilled H2O; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; FBS, foetal bovine serum; GEF, guanine nucleotide-exchange factor; GOM, granular osmiophilic material; GPX1, glutathione peroxidase 1; H2O2, hydrogen peroxide; IP3R, inositol 1,4,5-trisphosphate; Phe, phenylephrine; ROS, reactive oxygen species; RyR1/2/3, ryanodine receptor 1/2/3; SERCA, sarcoplasmic reticulum Ca2+-ATPase; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; TBARS, thiobarbituric acid reactive substance; TBS, Tris-buffered saline; TRPM2, transient receptor potential cation channel, subfamily M, member 2; UPR, unfolded protein response; VSMC, vascular smooth muscle cell; XBP1, X-box binding protein 1; 4-PBA, 4-phenylbutyrate; 8-OHG, 8-Hydroxyguanosine.

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