Abnormal Vasoconstrictor Responses to Angiotensin II and Noradrenaline in Isolated Small Arteries From Patients With Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy (CADASIL)

Monira B. Hussain, PhD; Sumeet Singhal, BM, BCh, BSc; Hugh S. Markus, BMBCh, MD, FRCP; Donald R.J. Singer, BMedBiol, MD, FRCP

Background and Purpose—Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is characterized by ultrastructural abnormalities in small cerebral and systemic vessels. We assessed vasomotor function in systemic small arteries in CADASIL.

Methods—We studied 10 CADASIL patients and 10 control subjects. Resistance arteries isolated from gluteal biopsies were mounted on small-vessel myographs, and concentration responses were determined for vasoconstrictors (noradrenaline, angiotensin II, and endothelin-I) and vasodilators (acetylcholine, bradykinin, spermine-NONOate, and nifedipine). Maximum data are shown as percent potassium contraction.

Results—There was reduced potency for noradrenaline in CADASIL (CADASIL [38 arteries]: EC50, 240 nmol/L; control subjects [27 arteries]: EC50, 100 nmol/L; 2-way analysis of variance, F = 9.76, P = 0.002). Maximum response to angiotensin II was greater in CADASIL (120% versus 97% in control subjects; F = 4.28, P = 0.043). Tachyphylaxis to angiotensin II occurred in all control subjects studied but in only 3 of 9 CADASIL subjects (P = 0.011, Fisher’s exact test). Vasodilation was similar in CADASIL patients compared with control subjects for endothelium-dependent dilators (acetylcholine and bradykinin) and endothelium-independent dilators (spermine-NONOate and nifedipine).

Conclusions—These results suggest a selective systemic microvascular vasoconstrictor abnormality in CADASIL in noradrenaline and angiotensin II pathways that is not explained by vasodilator impairment in endothelium or vascular smooth muscle. This could have important implications for prophylaxis and treatment of CADASIL. (Stroke. 2004;35:853-858.)

Key Words: angiotensins ■ arteries ■ CADASIL ■ muscle, smooth ■ noradrenaline

Cerebral autosomal dominant arteriopathy subcortical infarcts and leukoencephalopathy (CADASIL) is an as-yet-untreatable inherited neurodegenerative disorder typified by early-onset lacunar strokes, subcortical dementia, psychiatric disturbances, and migraine.1 The pathology is a nonarteriosclerotic nonamyloid angiopathy affecting capillaries and small arteries, with brain arterioles most severely affected and skin, nerve, and muscle also involved. Characteristic features are degeneration of arterial vascular smooth muscle cells (VSMCs), with accumulation of granular osmiophilic material (GOM) related to Notch3 extracellular domain (ECD) immunoreactivity,2 endothelial cell thinning, and basal lamina irregularities.1 Notch3 gene mutations are responsible and may occur at multiple sites.3

Signaling by Notch3 family receptors influences vascular, neural, and hemopoietic cell differentiation, growth, and apoptosis.4,5 The Notch3 ECD contains multiple epidermal growth factor–like repeats, each with 3 pairs of cysteine residues. Notch3 is expressed largely in membranes of VSMCs in adult mammalian arteries, capillaries, and veins within cerebral and systemic vasculature.6 DSL (Delta, Serrate, LAG2) ligand binding physiologically activates Notch3 receptors, leading to proteolytic cleavage of the Notch3 internal component, a nuclear activation domain, which binds to the transcription factor RBP-Jk/Cbf. Factors regulating Notch3 pathways include angiotensin II.4

CADASIL-causing Notch3 mutations described so far result in an unpaired cysteine residue. Effects of these mutations include abnormal Notch3 receptor trafficking7 or accumulation8 or shedding of mutant Notch3 receptor ECDs and disrupted Notch3 signaling.2 Increased ligand clearance by mutant Notch3 ECDs could reduce availability of these
ligands to other normal receptors, reducing normal pathway activation. \(^8\)

Human Notch3 carrying an archetypal Arg90Cys mutation has been expressed in VSMCs in transgenic mice, \(^9\) which develop 2 hallmarks of the CADASIL angiopathy, GOM deposits and Notch3 accumulation, within cerebral and peripheral arteries. They show disrupted anchorage of VSMCs to adjacent extracellular matrix and cells and early signs of VSMC degeneration, which precedes accumulation of Notch3 and GOM. \(^9\)

Notch3 is constitutively expressed in smooth muscle in peripheral arteries, \(^4\) and nocturnal dipping of blood pressure is blunted in subjects with CADASIL. \(^10\) We therefore assessed vasoconstrictor function in systemic small arteries from CADASIL patients compared with control subjects well matched for cardiovascular risk factors.

**Subjects and Methods**

Subjects were recruited from a national CADASIL clinical service and were excluded if they had had stroke-like events in the 3 previous months. Control subjects were healthy volunteers from the community or hospital matched for age, sex, and blood pressure. Exclusion criteria for control subjects included previous cerebrovascular or cardiovascular events. Twelve CADASIL patients and 12 healthy volunteers were recruited. In 2 patients and 2 control subjects, insufficient material was obtained for vascular studies. Therefore, 10 CADASIL patients and 10 control subjects were studied. This study conformed to the principles outlined in Declaration of Helsinki \(^11\) and was approved by the local Hospital Research Ethics Committee. Written informed consent was obtained.

**Diagnosis and Assessment of CADASIL**

The Expanded Disability Status Scale (EDSS) and lesion load on MRI scan were used to assess disease severity of CADASIL. The EDSS is used routinely to assess disability from multiple sclerosis, another neurological disease in which disability results primarily from damage to white matter structures. On this scale, a score of 0 means the absence of symptoms or signs of disease and 10 is death as a result of disease. \(^12\) Lesion load was determined quantitatively from digitized axial T2-weighted MRI scans with in-house software (Dispunc, D.L. Plummer, University College London). Hyperintense lesions, brain circumference, and lateral ventricles for all supra- and infratentorial slices were identified and circumscribed with image analysis software (Dispunc, D.L. Plummer, University College London). Brain parenchymal area was calculated from the difference between brain circumferential and lateral ventricle areas; total lesion load was expressed as percentage of brain parenchyma.

**Clinical Assessment**

Smoking history, height, and weight were recorded. Sitting blood pressure and pulse were measured with an Omron automated sphygmomanometer. \(^13\) Subjects were classified as hypertensive if they had a history of hypertension, were on treatment for high blood pressure, or had a blood pressure \(140/85 \text{ mm Hg}\) on the study day. Random serum cholesterol, homocysteine, and blood glucose were measured with standard methods. Fasting glucose was checked. Diabetes was defined as fasting glucose \(>6.0 \text{ mmol/L}\) or history of diabetes. Hypercholesterolemia was defined as random serum level \(>5.6 \text{ mmol/L}\) based on the reference range of our biochemistry laboratory.

**Myograph Studies**

Subjects were asked to discontinue any antiplatelet agents or cholesterol-lowering drugs for 72 hours before biopsy and blood pressure-lowering drugs 12 hours before biopsy. Smokers were asked not to smoke on the day of biopsy. Resistance arteries were dissected from subcutaneous fat obtained from gluteal biopsies of skin and adipose tissue obtained under local anesthesia (2% lidocaine). Each biopsy was collected in HEPES, stored overnight at 4°C, and studied the next day. \(^14\)

Multiple arteries were obtained from different biopsies for construction of concentration-response curves (CRCs) to the reagents noted below (Figure 1). Arterial rings were mounted on a small-vessel myograph (Danish Myotech) in Krebs’ solution at 37°C and bubbled with 95% O\(_2\)/5% CO\(_2\). Vessels were equilibrated for 1 hour and normalized to 90% of the diameter achieved at a transmural pressure of 100 mm Hg. \(^14\) Arterial segments were repeatedly exposed to high potassium solution (K\(^+\): 124 mmol/L, potassium chloride solution) until reproducible responses were obtained (within 10%). Tissue was discarded if K\(^+\) contractions were less than during normalization.

Half-log molar incremental CRCs to noradrenaline were obtained. After 30 minutes of washing, tissues were preconstricted to noradrenaline to give a 70% to 80% response. CRCs were then obtained with endothelium-dependent (acetylcholine or bradykinin) or endothelium-independent (spermine-NONOate) vasorelaxants. Each concentration of drug was set in contact with tissue until a plateau response was seen before the next concentration was added.

Tissues were again washed for 30 minutes before further CRCs to endothelin-1, angiotensin II, or nifedipine were obtained. In view of ethics regarding biopsy size, there was not always a sufficient number of arteries obtained to allow testing of all drugs in all subjects. Tachyphylaxis to angiotensin II was tested in 9 patients and 6 control subjects in whom arteries were available for these studies. Nifedipine responses were obtained after preconstriction of arteries with noradrenaline, with each concentration of nifedipine in contact with tissue for 4 minutes.

**Data and Statistical Analyses**

Responses to noradrenaline, angiotensin II, and endothelin-1 are represented as percentage maximum K\(^+\) contraction. Responses for vasodilators are given as percentage reversal of preconstricted noradrenaline tone. The antilogarithm of half-maximal response concentrations (pEC\(_{50}\)) of response) for vasoactive agents was calculated from nonlinear regression curve fitting with GraphPad Prism. CRCs between CADASIL patients and control subjects were compared by 2-way analysis of variance for repeated measures, with Bonferroni’s post-hoc testing when appropriate. Pearson’s correlation coefficient (\(r\)) was used for parametrically distributed variables. For potency and EDSS score, we used Spearman’s correlation coefficient (\(p\)). We compared differences in tachyphylaxis between patients and control subjects using Fisher’s exact test.

![Figure 1. Pathways studied. a1 indicates a1 adrenoceptor; Ach, acetylcholine; Ang II, angiotensin II; AT1, angiotensin II type 1 receptor;BK, bradykinin; B2, bradykinin type 2 receptor; [Ca\(^{2+}\)], intracellular free calcium ion concentration; CC, L-type calcium channel; ET-1, endothelin-1; ET A/B, endothelin type A or B receptor; M3, muscarinic-3 receptor; NIF, nifedipine; and SPER-NO, spermine-NONOate.](image-url)
Table 1. Demographic and Blood Results in CADASIL (n=10) and Controls (n=10)

<table>
<thead>
<tr>
<th></th>
<th>CADASIL Patients</th>
<th>Control Subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>47±4</td>
<td>50±4</td>
<td>0.70</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>123±6/76±4</td>
<td>118±3/80±2</td>
<td>0.55/0.45</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.2±5.2</td>
<td>28.1±4.8</td>
<td>0.47</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.2±0.3</td>
<td>5.6±0.3</td>
<td>0.71</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>13.7±2.2</td>
<td>12.1±1.5</td>
<td>0.57</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.0±0.2</td>
<td>5.0±0.3</td>
<td>0.62</td>
</tr>
<tr>
<td>Current/ex-smoker, n</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Hypertensive, n</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive therapy, n</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia, n</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Statin therapy, n</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM when appropriate.

* Lisinopril 10 mg OD in both cases.
† Simvastatin 10 mg, fluvastatin 40 mg, atorvastatin 10 mg, or pravastatin 20 mg, all OD.

Results

The diagnosis of CADASIL was made from a known Notch3 gene mutation in all cases. All CADASIL subjects were symptomatic; 3 had experienced only migraine with aura. Patients and control subjects were well matched for age, sex, and cardiovascular risk factors (Table 1). Treatments for blood pressure and raised cholesterol are shown in Table 1. No patients were taking vasoactive drugs. Three were taking folic acid. No control subjects were taking vasoactive drugs.

Responses to Vasoconstrictors

Contractions to 124 mmol/L potassium chloride reached a plateau within 2 minutes, with similar maximum contraction for CADASIL subjects (7.9±0.7 mN; 38 vessels from 10 subjects) and control subjects (8.1±0.8 mN; 27 vessels from 10 subjects). Potency for noradrenaline contraction was reduced: EC50 for noradrenaline was greater in CADASIL patients (120±8%) compared with control subjects (97±5%, F=4.28; P=0.043; Figure 2b and Table 2). Tachyphylaxis to angiotensin II was observed in all 6 control subjects (P=0.011, Fisher’s exact test).

Responses to Vasodilators

Within treatments, for the endothelium-dependent vasodilators acetylcholine and bradykinin (Figure 3a and Table 2) and the endothelium-independent vasodilators spermine-NONOate and the L-type calcium channel antagonist nifedipine (Figure 4a and Table 2), there was similar reversal of noradrenaline precontracted tone in CADASIL patients compared with control subjects.

Relationships Between Vasoactive Responses and Cardiovascular Risk Factors

There were no age-related changes in vasoactive responses. Subjects with higher body mass index had reduced maximum relaxant response to angiotensin II (r=−0.564, P=0.023). Serum cholesterol was correlated with potencies for noradrenaline (r=0.683, P=0.002), angiotensin II (r=0.541, P=0.031), and endothelin-1 (r=0.520, P=0.027).

Relationships Within Vasoactive Responses

There was a significant direct correlation between efficacy (maximum vasoconstrictor response) to angiotensin II and to endothelin-1 (r=0.83, P=0.019). The pEC50 for relaxation to the vasodilator bradykinin was positively related to that for spermine-NONOate (r=0.94, P<0.0001). However, efficacy (maximum vasodilation) for acetylcholine was inversely related to that for nifedipine (r=−0.76, P=0.028).

Relationships Between Vasoactive Responses and Severity of CADASIL

The severity of CADASIL involvement as assessed by the EDSS was positively correlated to pEC50 for endothelin-1 (ρ=0.64, P=0.048) and noradrenaline (ρ=0.82, P=0.004). The EDSS was inversely associated with the maximum contractile response to angiotensin II (ρ=−0.71, P=0.032). Lesion load was positively correlated with noradrenaline...
Discussion
This study demonstrated selective abnormalities in vasoconstrictor reactivity in systemic small arteries from subjects with CADASIL, an important inherited cause of premature stroke and dementia. We found both increased maximal responsiveness to the constrictor effects of angiotensin II and reduced sensitivity to the constrictor effects of the catecholamine noradrenaline, but we saw no difference in endothelin-1 responsiveness. Because there were no major differences in response to a wide range of endothelium-dependent and -independent vasodilators, our findings point to functional abnormalities in CADASIL within vasoconstrictor pathways in systemic small artery smooth muscle rather than indirect effects resulting from altered vasorelaxant pathways. These results support our hypothesis regarding impaired vasoreactivity of small arteries in peripheral tissue in CADASIL.

Our study is the first to provide direct evidence for abnormal vasoconstrictor reactivity in the systemic arterial circulation in CADASIL. Previous studies had reported impaired cerebral vasoreactivity in CADASIL as determined by blunted vasodilation to inhaled carbon dioxide\(^1\) or the carbonic anhydrase inhibitor acetazolamide.\(^2\) There has been possible indirect evidence for impaired vasoreactivity in the systemic circulation from a study using ambulatory blood pressure monitoring\(^3\) that reported blunting of the normal nocturnal blood pressure dip in subjects with CADASIL compared with matched control subjects. However, abnormal central sympathetic outflow could have been responsible for the relatively raised nighttime blood pressure reported. Our results provide a further systemic mechanism for impaired circadian blood pressure regulation in CADASIL: abnormal vasoconstrictor responses in systemic small arteries, a major contributory site for regulation of systemic vascular resistance. The resulting increase in cumulative daily blood pressure load for a given office blood pressure could contribute to the pathogenesis of arterial disease in CADASIL.

We identified 2 major abnormalities in angiotensin II responsiveness in CADASIL. First, there was an increase in the maximum vasoconstrictor response in CADASIL patients compared with control subjects. This suggests greater bioactivity of the angiotensin II signal transduction pathway. We excluded impaired vasodilator responsiveness as a mechanism for this because relaxation to 2 different endothelium-dependent dilators, acetylcholine and bradykinin, was similar to control subjects.

### Table 2: Maximum (Percent of K\(^+\)-Induced Constriction) and pEC\(_{50}\) for Constrictor and Dilator Responses in CADASIL Patients Versus Control Subjects

<table>
<thead>
<tr>
<th>Drugs</th>
<th>CADASIL Patients pEC(<em>{50}) (EC(</em>{50}), nmol/L)</th>
<th>Control Subjects pEC(<em>{50}) (EC(</em>{50}), nmol/L)</th>
<th>CADASIL Patients Maximum</th>
<th>Control Subjects Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenaline</td>
<td>6.6±0.1 (240)</td>
<td>7.0±0.2 (100)</td>
<td>153±6</td>
<td>151±29</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>8.3±0.1 (5.4)</td>
<td>8.2±0.1 (6.7)</td>
<td>138±11</td>
<td>154±18</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>8.7±0.2 (2.0)</td>
<td>8.7±0.1 (2.0)</td>
<td>97±15</td>
<td>120±8</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>7.8±0.1 (16.0)</td>
<td>7.9±0.1 (13.0)</td>
<td>88±3</td>
<td>96±4</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>8.3±0.1 (5.6)</td>
<td>8.2±0.1 (6.8)</td>
<td>86±4</td>
<td>94±5</td>
</tr>
<tr>
<td>Spermine-NONOate</td>
<td>6.6±0.1 (230)</td>
<td>6.5±0.1 (280)</td>
<td>94±7</td>
<td>104±10</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>8.4±0.3 (4.0)</td>
<td>8.2±0.3 (5.9)</td>
<td>75±8</td>
<td>73±7</td>
</tr>
</tbody>
</table>

pEC\(_{50}\) (\(\rho=0.806, P=0.005\)) and endothelin-1 pEC\(_{50}\) (\(\rho=0.648, P=0.043\)).

**Figure 3.** CRCs to acetylcholine (a; Ach) and bradykinin (b; BK) in CADASIL and control subjects.

**Figure 4.** CRCs to spermine-NONOate (a; SPER-NO) and nifedipine (b) in CADASIL and control subjects.
in CADASIL, compared with control subjects, was a relaxation to the endothelium-independent dilator nitric oxide donor spermine-NONOate and the calcium channel blocker nifedipine. Mechanisms for increased vasoconstriction to angiotensin II may include increased activity of classic angiotensin II type 1 receptor–mediated signal transduction pathways such as activation of phospholipase C with generation of inositol phosphate, a rise in intracellular Ca2++, protein kinase C activation, and alternative pathway activation such as oxidant stress-mediated contraction through stimulation of NAD(P)H oxidase and other oxidant enzymes.17

Second, we found marked blunting of tachyphylaxis to the vasoconstrictor effects of angiotensin II in CADASIL. It is well established that the normal vasoactive small artery response to angiotensin II is initial contraction, followed by desensitization or tachyphylaxis.18 In our study, tachyphylaxis to angiotensin II–mediated contraction occurred as expected in all control subjects studied but in only 30% of the CADASIL group. The cause is unclear, but loss of tachyphylaxis may have contributed to the enhanced contractile response to angiotensin II found in the CADASIL arteries. Tachyphylaxis has been reported to be blunted when endothelial bioactivity is impaired. Postulated mechanisms for this endothelium-dependent desensitization include mediation by a cyclooxygenase cytochrome P450 product, which could act by increasing K(Ca2+) channel activity.19 However, impaired endothelial function does not appear to be a major explanation for blunted tachyphylaxis in CADASIL because in the present study endothelium-dependent relaxant responses to both acetylcholine and bradykinin appeared similarly preserved in CADASIL subjects compared with matched control subjects.

Angiotensin II downregulates Notch3 ligand (Jagged 1) and Notch3 receptor expression in rat aortic smooth muscle cells and inactivates endothelial cell–derived Jagged 1 by glycosylation.4 In CADASIL, mutant Notch3 receptors are not effectively downregulated. An intriguing possibility is that increased angiotensin II vasoconstriction may reflect general upregulation of angiotensin II pathways in response to Notch3 accumulation in CADASIL. If so, this would appear to be an early feature of the natural history of CADASIL because angiotensin II maximal contraction was inversely related to disease severity as assessed by EDSS.

Noradrenaline potency was 2.4-fold lower in CADASIL compared with control subjects. This decreased sensitivity to noradrenaline may reflect overactivity of sympatheic outflow in CADASIL. Altered α-1 adrenoceptor–mediated tone may affect cerebral perfusion by restricting the ability of affected vessels in CADASIL to autoregulate during periods of low perfusion pressure.

There are structural abnormalities in small penetrating arteries supplying subcortical structures in CADASIL, with accumulation of GOM in the basement membrane of VSMCs of cerebral arterioles.20 Our study provides a mechanism for functionally impaired vasoreactivity in the form of increased vasoconstriction to angiotensin II in CADASIL, compounded by reduced tachyphylaxis. The resulting failure of appropriate systemic vasoregulation, accompanied by direct or indirect impairment of regulation of the cerebral blood supply, could contribute to recurrent brain hypoperfusion injury.

Although noradrenaline potency was reduced in CADASIL, subjects with higher noradrenaline potency had greatest disease severity for CADASIL on the basis of both EDSS and lesion load assessed by brain imaging. One possible explanation is that reduced noradrenaline potency may be a secondary, perhaps compensatory, response to the primary pathogenetic insult in CADASIL. If patients with more severe disease were less able to suppress noradrenaline potency, a spurious relationship between potency and disease severity may have arisen.

Further studies are needed to explore mechanisms for the contrast between reactivity to endothelin and other vasoconstrictors. However, there are clear precedents for dissociation between response to endothelin and to other vasoconstrictors in pathophysiology.21

Aspirin was stopped for only 3 days in view of the stroke risk associated with CADASIL. Prolonged posttreatment actions of aspirin result largely from residual effects on platelets. In contrast, vascular cells recover rapidly from the effects of previous aspirin treatment because unlike platelets they can synthesize new COX enzyme.22 Confounding by recent aspirin treatment is therefore less a concern for these ex vivo studies than would be the case for in vivo studies. Statins may improve endothelium-dependent relaxation23; however, we found no evidence that altered endothelium-dependent relaxation accounted for differences in vasoconstrictor responses between CADASIL and control arteries. Because only 2 of our CADASIL patients were on prior angiotensin-converting enzyme inhibitor treatment and this treatment was stopped at least 12 hours before biopsy, we think it unlikely that this treatment would have greatly influenced our findings.

Our study suggests that vasoactive consequences of the initial pathogenetic defect in CADASIL are highly selective, with preservation of a wide range of relaxant pathways and selective changes in α-adrenergic and angiotensin II pathways. These are ex vivo studies; further in vivo studies are needed to establish the clinical relevance of our findings. The above observations have 2 major therapeutic implications. They provide a focus for exploring signal transduction for selected vasoconstrictor targets in the future treatment of CADASIL. Second, preservation of relaxant pathways indicates that there may be a wide range of endothelium-dependent and independent dilator agents that could be used in trials of treatment of CADASIL.

Acknowledgments

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References


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