Alterations in the modulation of cerebrovascular tone and blood flow by nitric oxide synthases in SHRsp with stroke

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Received 8 July 2009; revised 3 December 2009; accepted 7 December 2009; online publish-ahead-of-print 15 December 2009

Time for primary review: 25 days

Aims
The modulation of myogenic function and cerebral blood flow (CBF) by nitric oxide (NO) synthases (NOS) was assessed in the middle cerebral arteries (MCAs) of Kyoto Wistar stroke prone hypertensive rats (SHRsp) in relation to haemorrhagic stroke development.

Methods and results
MCAs were studied with a pressure myograph. CBF in MCA perfusion domain was measured using laser Doppler techniques. NOS isozymes were identified using immunohistochemistry. MCAs expressed endothelial, neuronal, and inducible NOS (eNOS, nNOS, and iNOS, respectively) in the endothelium, nNOS and traces of iNOS in smooth muscle and adventitial cells. Before stroke, MCA pressure-dependent constriction (PDC) was superimposed over basal non-pressure-dependent tone (BNPDT). Endothelial NO generation and non-endothelial nNOS but not iNOS reduced BNPDT and increased the lumen diameter at which PDC initiated without altering the amplitude of PDC. NOS inhibition decreased CBF and increased the upper blood pressure limit of autoregulation. PDC, CBF autoregulation, and NOS dilatory influence were lost, and BNPDT was increased in MCAs from SHRsp with stroke. The expression of NOS isozymes and MCA reactivity to NO donors was not altered. NOS activity was not recovered by in vitro L-arginine or tetrahydrobiopterin supplementation, L-arginase inhibition or superoxide scavengers.

Conclusion
The loss of PDC and CBF autoregulation during hypertension may facilitate over-perfusion and cerebral haemorrhage formation in SHRsp. NOS dysfunction in MCAs preceded stroke and involved the inactivation of eNOS and nNOS in areas not subjected to hyper-distension. The elevation in BNPDT due to NOS inactivation may oppose over-perfusion in the absence of CBF autoregulation.

Keywords
Haemorrhagic stroke • Middle cerebral artery • Myogenic response • Nitric oxide synthase • CBF autoregulation

1. Introduction
Constant cerebral blood flow (CBF) regulation over varying blood pressures (BP) protects the brain from ischaemia when BP drops and limits over-perfusion when BP is elevated. Elevations in BP initiate cerebrovascular pressure-dependent constriction (PDC) which opposes the increase in CBF allowing flow to remain constant. The mechanisms promoting PDC are endothelial independent. Despite this, the pharmacological influence of the endothelium can modify the dynamics of PDC.

Haemorrhagic stroke development in Kyoto Wistar stroke prone spontaneously hypertensive rats (SHRsp) is preceded by the loss of CBF autoregulation in the middle cerebral artery (MCA) perfusion domain and PDC in isolated MCAs. Before stroke, MCAs that elicit PDC also constrict to nitric oxide (NO) synthase (NOS) inhibition with Nω-nitro-L-arginine methyl-ester (L-Name). This suggests that the spontaneous generation of NO governs tone. This function is also lost prior to cerebral haemorrhage in SHRsp that develop hypertensive encephalopathy. The impact of NOS dysfunctions on PDC and CBF autoregulation have not been studied.

The influence of the endothelial NOS (eNOS) and non-eNOS activity on basal non-pressure-dependent tone (BNPDT), PDC, and CBF were measured in relation to stroke in SHRsp. NOS isozyme expression and MCA reactivity to NO were studied and the ability to...
restore NOS function by manipulating L-arginine, NOS cofactors, and superoxide was assessed. We determined whether the in vitro dysfunctions observed in isolated MCAs influenced in vivo CBF regulation.

2. Methods

Detailed methods are presented in Supplementary material online. Experiments were performed with institutional approval (Memorial University of Newfoundland, Protocol-08/09-25-JS) and followed the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23).

Male SHRsp were fed a 4% NaCl Japanese-style stroke-prone (stroke-genic diet) or a Purina diet (0.7% NaCl) that delays the onset of stroke. SHRsp fed the stroke-genic diet were sampled before and after stroke. The systolic BP (sBP) was measured using a tail cuff compression method. PDC to a 100 mmHg pressure step and BNPDT were measured in isolated MCAs using a pressure myograph. The methodology used to measure PDC and BNPDT is outlined in detail (see Supplementary material online) and is summarized in Figure 1. MCAs were pressurized to 100 mmHg for 30 min allowing constriction to develop. Subsequently, PDC was inactivated by reducing pressure to 0 mmHg for 6 min. The MCAs were then repressurized to 100 mmHg. The change in lumen diameter (LD) between 1 s and 4 min after repressurization was used as a measure of PDC. The LD of MCAs never expanded to maximal dilation 1 s after repressurization to 100 mmHg. We have called the residual tone maintaining constriction at 100 mmHg in the absence of PDC BNPDT. BNPDT and the operating range PDC were measured in MCAs by repeating the above pressurization paradigm in the absence and presence of NO synthase inhibitors and/or endothelial removal.

The expression and distribution of eNOS, nNOS, and iNOS in the MCAs of SHRsp was analysed. Frozen MCA sections were fixed. Monoclonal mouse primary antibodies capable of detecting rat eNOS, nNOS, or iNOS were applied, conjugated with fluorescent tags and analysed with a laser confocal microscope.

CBF was measured in the MCA perfusion domain in anaesthetized SHRsp using laser Doppler techniques. The increase in relative CBF to elevations in mean BP produced following abdominal compression and subsequent intravenous phenylephrine infusion were measured in the absence and presence of NOS inhibition produced by the intravenous infusion of L-Name (40 mg/kg).

2.1 Data analysis and statistical procedures

An analysis of variance (ANOVA) was used to determine ‘between group’ differences and a Fisher’s post hoc test assessed subgroups differences (ANOVA + post hoc). A general linear model of multivariate analysis determined differences between dose–response curves, alterations in CBF with time or BP. Relationships were assessed using linear regression and Pearson product correlations (r-values). Values are expressed as the mean ± SEM for the SHRsp used in the experiment and considered significant at P < 0.05.

3. Results

3.1 Stroke development in SHRsp

Stroke development in SHRsp fed stroke-genic diet was associated with seizures followed by immobility. SHRsp exhibited intracerebral
haemorrhage, oedema, the extravasation of albumin and herniation.3,4 Lesions occurred in the cerebral hemispheres predominantly in the MCA perfusion domain.1 A video demonstrating seizures and photographs showing cerebral haemorrhage, oedema, and herniation in SHRsp is present online (see Supplementary material online, Video S1 and Figure S1).

The mean onset of stroke occurred at 14.5 ± 0.2 weeks (wks) of age (n = 62, sBP of 257 ± 4 mmHg). The first SHRsp developed stroke at 11.7 wks (see Supplementary material online, Figure S2). Post-stroke SHRsp were sampled at the first signs of stroke and compared with 9 and 10-wk-old prestroke SHRsp (n = 59, age = 10.3 ± 0.1 wks, sBP = 225 ± 4 mmHg), which exhibited normal behaviour and no brain lesions.

3.2 PDC and BNPDT in MCAs

PDC was studied in MCAs mounted in a pressure myograph as outlined in Figure 1 and described in Supplementary material online. After 30 min equilibration at 100 mmHg, pressure was lowered to 0 mmHg for 6 min. This inactivated the PDC. Pressure was then instantaneously increased to 100 mmHg for 4 min. The decrease in LD between 1 s and 4 min after the reaplication of pressure was used as a measure of PDC. Nifedipine (3 μmol/L) produced maximal dilation which was not enhanced with EGTA (LD before vs. after 7.8 mmol/L EGTA in the presence of nifedipine, 193 ± 4 vs. 194 ± 4 μm, n = 14 MCA). We have previously shown that MCA fusion with Ca2+ free Krebs containing 1 mmol/L EGTA or 10 μmol/L verapamil can not further dilate the MCAs after 3 μmol/L nifedipine.2 The LD presents 1 s after the application of 100 mmHg never reached maximal vasodilation (170 vs. 192 μm, Figure 1). The difference in tone between the latter two conditions represents the level of BNPDT present at 100 mmHg.

3.3 Alterations in MCA NOS function

MCAs from post-stroke SHRsp exhibited large levels of BNPDT and smaller LDs 1 s after the reaplication of 100 mmHg pressure (Table 1 and Figure 2A) but did not constrict to pressure (Figure 2A). MCAs from prestroke SHRsp exhibited lower levels of BNPDT. However, when pressurized, their ability to elicit PDC allowed them to achieve greater total constriction and smaller LDs (Table 1, 4 min after 100 mmHg) than MCAs from poststroke SHRsp.

SHRsp fed a Purina diet exhibit a delayed onset of stroke (50% mortality at 26 vs. 14 wks of age).4 Stroke-free (age = 16–17 wks) Purina fed SHRsp had comparable sBP and were older than post-stroke SHRsp (Table 1). MCAs from Purina and prestroke strokegenic diet fed SHRsp exhibited comparable PDC and BNPDT (Table 1 and Figure 2A).

After measuring PDC, NOS was inhibited with L-Name (100 μmol/L, at 100 mmHg for 8 min as shown in Figure 1). NOS inhibition constricted MCAs from prestroke and Purina fed SHRsp (Figure 2A) suggesting that spontaneous NO generation opposed tone development, whereas those from poststroke SHRsp could not constrict to L-Name (Figure 2A). The application of hydroxocobalamin (100 μmol/L, an NO scavenger) after L-Name did not enhance constriction.2 Reactivity to sodium nitroprusside, (an NO donor tested in the presence of 100 μmol/L L-NAME) was not altered in MCAs from poststroke SHRsp (Figure 2B). An age-related decline in MCA constriction to NOS inhibition with L-NAME was observed prior to stroke in SHRsp between the ages of 10 and 13.5 wks indicating that the development of this dysfunction preceded stroke in SHRsp (see Supplementary material online, Figure S3).

In an attempt to restore NOS function, MCAs from poststroke SHRsp were equilibrated (20 min at 100 mmHg) with a combinations of (i) superoxide scavenger (Tiron, 1 mmol/L), sepiapterin, (5 μmol/L, a precursor enhancing tetrahydrobiopterin production), and L-arginine (100 μmol/L, NOS substrate) or by (ii) 1 mmol/L Tiron, L-arginase inhibitor Nα-hydroxy-nor-L-arginine (135 μmol/L), and (6R)-5,6,7,8-tetrahydrobiopterin (BH4, 64 μmol/L). These treatments did not enhance constriction to NOS inhibition (Figure 2C) or PDC [% PDC to a 100 mmHg pressure step—poststroke (no treatment) = 46.8 ± 4.7, n = 6/6; poststroke: no treatment = 5.7 ± 3.5, n = 6/6; treatment (i) = 1.2 ± 5.4, n = 5/5; treatment (ii) = 6.9 ± 5.1, n = 5/5; statistics: ANOVA + post hoc, pretreatment vs. other groups P < 0.05, other comparisons NS] Following treatments (i) or (ii), vasopressin (0.12 μmol/L) readily constricted the MCAs [34.7 ± 3.7%, n = 10 MCA/10 SHRsp, experiments (i) and (ii) combined] discounting the possibility that the MCAs were not capable of constriction.

3.4 Relation between basal NOS activity and non-pressure-dependent tone in MCAs

MCAs exhibited an inverse relationship between BNPDT and their subsequent ability to constrict to L-NAME (Figure 3A). NO generation by the MCAs, indicated by the level of constriction to L-NAME, reduced the level of BNPDT, causing the initiation of PDC [1 s after

**Table 1 Physical characteristics of SHRsp and PDC in the MCAs**

<table>
<thead>
<tr>
<th>SHRsp groups</th>
<th>n (MCA/SHRsp)</th>
<th>Diet</th>
<th>Age (wks)</th>
<th>Systolic BP (mmHg)</th>
<th>MCA lumen diameter (μm) at 1 s and 4 min after 100 mmHg pressure step and at MD*</th>
<th>ANOVA (P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Prestroke</td>
<td>21/21</td>
<td>JSD</td>
<td>10.6 ± 0.2</td>
<td>219 ± 7</td>
<td>172 ± 4 104 ± 5 200 ± 2 (a) vs. (b) and (c) (b) vs. (a) and (c) (b) vs. (a) and (c) (a) vs. (b)</td>
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</tr>
<tr>
<td>(b) Poststroke</td>
<td>39/39</td>
<td>JSD</td>
<td>14.4 ± 0.2</td>
<td>256 ± 5</td>
<td>136 ± 4 125 ± 3 182 ± 2 (a) vs. (b) and (c) (b) vs. (a) and (c) (a) vs. (b)</td>
<td></td>
</tr>
<tr>
<td>(c) Purina fed (no stroke)</td>
<td>5/5</td>
<td>Purina</td>
<td>16.7 ± 1.3</td>
<td>250 ± 11</td>
<td>163 ± 3 99.2 ± 8 191 ± 7 (a) vs. (b) and (c) (b) vs. (a) and (c) (a) vs. (b)</td>
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MD, maximal dilation; JSD, Japanese-style diet; Purina—Ralston Purina Rat Chow. *See Figure 1 for the experimental details.
pressurization (Figure 1)] to be shifted to larger LDs (Figure 3B). This modified the operating range of PDC in MCAs from poststroke SHRsp towards more dilated LDs. The low level of spontaneous NO generation in MCAs from poststroke SHRsp (indicated by unresponsiveness towards more dilated LDs) permitted the development of large levels of BNPDT (Figure 2A).

3.5 NOS function in the MCAs of stroke resistant SHR

We also assessed the effects of NOS inhibition with \( N_\text{omega}-\text{nitro-L-arginine (NLA, 100 \mu mol/L)} \) on myogenic function in MCAs from poststroke SHRsp \((n = 16, \text{age} = 14.7 \pm 0.5 \text{ wks})\) and age-matched regular SHR \((n = 19, \text{age} = 15.6 \pm 0.6 \text{ wks})\) fed the stroke-genic diet (see Supplementary material online, Figure S5A–D). Regular SHR (subsequently called srSHR) fed the latter diet develop comparable sBP to SHRsp but live to a median age of 65 wks without developing stroke. MCAs from srSHR (12 MCAs/12 srSHR) exhibited similar constriction to a 100 mmHg pressure step (36.9 ± 5.1%) and NOS inhibition (with NLA, 28.6 ± 2.8% at 100 mmHg) and smaller levels of BNPDT (3.26 ± 1.56%) when compared with the prestroke SHRsp described in Figure 2A. The MCAs of poststroke SHRsp (7 MCAs/7 SHRsp, co-studied with srSHR) did not constrict to pressure (5.1 ± 6.7% dilatation) or NOS inhibition with LNA (2.4 ± 4.0%) and exhibited large levels of BNPDT (24.7 ± 4.8%). MCAs from srSHR and SHRsp subjected to NOS inhibition with LNA exhibited significant correlations (19 MCAs/19 SHR, \( r > 0.770, P < 0.0001 \)) between BNPDT, LD, and constriction to NOS inhibition. The correlations were nearly identical to those outlined in Figure 3A and B involving the MCAs of the SHRsp subjected to NOS inhibition with L-Name. The MCAs of srSHR (7 MCAs/7 srSHR) and poststroke SHRsp (9 MCAs/9 SHRsp) also responded equally to the Na-nitroprusside in the presence of NLA. This indicated that the loss of constriction to pressure and NOS inhibition in the MCAs from poststroke SHRsp was not the consequence of feeding a high-salt diet to a hypertensive rat or a unique effect of L-Name.

3.6 Modulation of PDC and BNPDT by NOS isozymes

MCAs were sampled from prestroke \((n = 19, \text{age} = 10.4 \pm 0.1 \text{ wks})\), sBP = 229 ± 7 mmHg) and poststroke \((n = 10, \text{age} = 13.7 \pm 0.3 \text{ wks})\), sBP = 264 ± 8 mmHg) SHRsp. BNPDT and PDC were measured during sequential pressure steps in the same MCAs under conditions of endothelial removal, NOS inhibition, and an NO scavenger. Endothelial removal was judged by the absence of endothelial-dependent vasodilation to bradykinin and PAR2 agonist, 2-furoyl-leucine-isoleucine-glycine-arginine-leucine-ornithine-amide (see Supplementary material online, Methods). Figure 4A–E represents MCA data from prestroke SHRsp. Endothelial removal and NOS inhibition in de-endothelialized MCAs sequentially increased BNPDT, indicating that endothelial and non-endothelial sources of NOS influenced BNPDT (Figure 4A). The LD alterations corresponding to the sequential PDCs in Figure 4A.
are presented in Figure 4B. PDC (% decrease in LD from 1 s to 4 min after a 100 mmHg pressure step) was not altered. Elevations in BNPDT to the above manipulations shifted the starting point of PDC (1 s after 100 mmHg pressure) to smaller LDs, moving the operating range of PDC to more constricted LDs.

The ability of L-Name to increase BNPDT in de-endothelialized MCAs was duplicated by AAAN (30 μmol/L, N-[2-(aminoethyl)amino]pentyl-N-nitroguanidine), a specific nNOS inhibitor, and hydroxocobalamin (250 μmol/L), an NO scavenger (Figure 4C and D). The co-addition of L-Name to either AAAN or hydroxocobalamin did not further enhance BNPDT. In contrast, 1400 W (1.13 μmol/L), a specific iNOS inhibitor, did not alter BNPDT, whereas the subsequent addition of L-Name to 1400 W raised BNPDT in endothelial-intact MCAs (Figure 4E). AAAN is a selective inhibitor of nNOS over eNOS (respectively 97 vs. 1% inhibition, at 30 μmol/L), whereas 1400 W selectively inhibits iNOS over nNOS or eNOS (respectively 99% vs. 6 and 0.3% inhibition at 1.13 μmol/L) (see Supplementary material online, Table S1). The results suggested that NO generation by non-endothelial nNOS rather than arterial iNOS modified BNPDT in the MCAs of prestroke SHRsp. In each experiment (Figure 4C–E), PDC was not altered. Increases in BNPDT shifted the starting point and operating range of PDC to more constricted LDs (see Supplementary material online, Table S2).

Endothelial removal from the MCAs of prestroke SHRsp only elevated BNPDT in the absence but not the presence of L-Name (see Supplementary material online, Figure S6A and Table S2), indicating that endothelial removal raised BNPDT by eliminating NO activity as opposed to removing a non-NO endothelial vasodilator. L-Name (a competitive NOS inhibitor) did not alter BNPDT in de-endothelialized or endothelial-intact MCAs from prestroke SHRsp in the presence of 2 mmol/L L-arginine (Figure 56B, see Supplementary material online). This further supported the conclusion that BNPDT was altered by NOS generated NO.

L-Name did not alter BNPDT in endothelial-intact or de-endothelialized MCAs from poststroke SHRsp (Figure 4F). These MCAs exhibited large levels of BNPDT and could not elicit PDC (Figure 4G).

3.7 Localization of NOS isozymes in the MCAs of prestroke and poststroke SHRsp

The levels and patterns of fluorescent eNOS, nNOS, and iNOS labelling were comparable in MCAs from prestroke and poststroke SHRsp (Figure 5). All three isozymes were present in the endothelium. eNOS was only found in the endothelium. nNOS partially co-localized with eNOS in the endothelium, was present at moderate levels in the medial smooth muscle cells, and high levels in the adventitial cells. The requirement of a differing fixation prevented the assessment of iNOS co-localization with eNOS or nNOS in the same MCA sections (see Supplementary material online, Methods). iNOS was localized in the endothelium with trace levels observed in adventitial cells.

3.8 Effects of NOS inhibition on CBF regulation in prestroke and poststroke SHRsp

Prestroke SHRsp (n = 13, age = 9.9 ± 0.5 wks, sBP = 222 ± 6 mmHg) and poststroke SHRsp (n = 11, age = 16.6 ± 0.8 wks,
sBP = 258 ± 7 mmHg) were sampled. CBF in the MCA perfusion domain was measured using laser Doppler techniques (see Supplementary material online, Methods). L-Name infusion (40 mg/kg, i.v.) into anaesthetized SHRsp equally raised mean BP by 30 mmHg in both groups. The final mean BP at 350–521 s after L-Name infusion was higher in poststroke vs. prestroke SHRsp (Figure 6A). L-Name produced a greater proportional decrease in CBF in prestroke vs. poststroke SHRsp (Figure 6A). Elevations in mean BP produced smaller elevations in CBF in prestroke vs. poststroke SHRsp (Figure 6B). L-Name infusion attenuated the ability of BP to increase CBF to a greater degree in prestroke (Figure 6C) vs. poststroke (Figure 6D) SHRsp.

In other experiments, BP was slowly raised from a mean BP of 48 mmHg to rest following abdominal compression then further elevated to maximum possible levels with phenylephrine infusion (Figure 6E and F). The CBF present at a BP of 80 mmHg was given a value of 1 and the relative increase in CBF was measured. In the absence of L-Name, prestroke SHRsp moderately increased in CBF up to a mean upper BP limit of 205 mmHg, after which cerebrovascular forced dilation massively elevated CBF (Figure 6E). Following

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were sampled. CBF in the MCA perfusion domain was measured using laser Doppler techniques (see Supplementary material online, Methods). L-Name infusion (40 mg/kg, i.v.) into anaesthetized SHRsp equally raised mean BP by ~30 mmHg in both groups. The final mean BP at 350–521 s after L-Name infusion was higher in poststroke vs. prestroke SHRsp (Figure 6A). L-Name produced a greater proportional decrease in CBF in prestroke vs. poststroke SHRsp (Figure 6A). Elevations in mean BP produced smaller elevations in CBF in prestroke vs. poststroke SHRsp (Figure 6B). L-Name infusion attenuated the ability of BP to increase CBF to a greater degree in prestroke (Figure 6C) vs. poststroke (Figure 6D) SHRsp.

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15 min of L-Name infusion prestroke SHRsp exhibited reduced elevations in relative CBF when BP was elevated and we could not raise the BP (via phenylephrine infusion) to levels that exceeded the upper BP limit of CBF autoregulation. Poststroke SHRsp exhibited near linear increases in CBF with elevated BP and did not autoregulate the upper BP limit of CBF autoregulation. Poststroke SHRsp exhibited a raise in BP (via phenylephrine infusion) to levels that exceeded the elevations in relative CBF when BP was elevated and we could not observe a difference in constitutive eNOS + nNOS and iNOS activity or constriction to NOS inhibition. We observed that age-matched stroke resistant SHR fed a stroke-genic diet with comparable sBP to poststroke SHRsp did not develop the defects in myogenic or NOS function. In the previous studies, we found that PDC declined in MCAs after 10 wks of age and that PDC and NOS function was lost prior to intracerebral haemorrhage formation in SHRsp experiencing hypertensive encephalopathy. In the current study, the ability of the MCAs to constric to NOS inhibition declined in SHRsp prior to stroke between 10 and 13.5 wks of age. This suggests that these defects precede stroke in SHRsp and are not a general feature of hypertension, high-salt loading, or a consequence of cerebral haemorrhage.

Superoxide generation is increased and antioxidant capacity decreased in arterial tissues and the brain of SHRsp in relation to normotensive rats or when stroke development is enhanced with a high sodium intake. NO reacts with superoxide to form peroxynitrite (OONO−), Peroxynitrite formation has been observed in the cerebral arterial and neuronal cells near brain lesions in post-stroke SHRsp. Peroxynitrite also inhibits superoxide dismutase, reducing tissue antioxidant capacity. When tetrahydrobiopterin (BH4, NOS co-factor) and/or L-arginine (NOS substrate) are limited, NOS generates superoxide rather than NO. Superoxide and peroxynitrite both oxidize BH4, which further shifts NOS function towards the formation of peroxynitrite. Peroxynitrite also induces the nitrosylation and depolymerization of smooth muscle F-actin, causing the loss of myogenic tone in rat cerebral arteries. Vascular NOS dysfunction associated with a variety of pathological conditions (ageing, diabetes, atherosclerosis, and hypertension) has been reversed by the in vitro addition of L-arginase inhibitors, O2 scavengers, or supplementation with L-arginine substrate. BH4 or sepiapterin similarly manipulations did not restore NOS or myogenic function in the MCAs of poststroke SHRsp. In other studies, we found treatment

4. Discussion

Brain over-perfusion under conditions of hypertension is counteracted by the development of appropriate cerebrovascular constriction. Two categories of tone maintained constriction in pressurized MCAs. PDC was superimposed over a static component of BNPDT. Spontaneous NO release by the MCA’s reduced BNPDT. The presence of nNOS, eNOS, and iNOS has been demonstrated in the Circle of Willis of Wistar Kyoto normotensive (WKY), SHR, and basilar arteries from SHRsp. Our study is unique in demonstrating that NO generation by non-endothelial nNOS in addition to eNOS activity profoundly influences myogenic function by altering BNPDT. The presence of a non-functional iNOS in the MCAs of SHRsp is consistent with similar observations in the MCAs of SHR. The lack of iNOS influence could reside in the low-activity levels of this isozyme (25% of the activity of constitutive NOS in SHR cerebral arteries) and/or the possibility that iNOS may be maintained in the inactivated state.

Studies comparing the MCAs of SHR and WKY have failed to observe a difference in constitutive eNOS + nNOS and iNOS activity or constriction to NOS inhibition. We observed that age-matched stroke resistant SHR fed a stroke-genic diet with comparable sBP to poststroke SHRsp did not develop the defects in myogenic or NOS function. In the previous studies, we found that PDC declined in MCAs after 10 wks of age and that PDC and NOS function was lost prior to intracerebral haemorrhage formation in SHRsp experiencing hypertensive encephalopathy. In the current study, the ability of the MCAs to constrain to NOS inhibition declined in SHRsp prior to stroke between 10 and 13.5 wks of age. This suggests that these defects precede stroke in SHRsp and are not a general feature of hypertension, high-salt loading, or a consequence of cerebral haemorrhage.

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Figure 5 Fluorescent labelling of NO synthase (NOS) isozymes in the middle cerebral arteries (MCAs) of SHRsp. eNOS was only found in the endothelium (A and E—cy2; green), whereas nNOS was prevalent in the endothelium and adventitia with a weak presence in the medial smooth muscle (B and F—cy5; red). Co-localization studies indicated areas of eNOS and nNOS overlap (yellow in C and G) in the endothelium. iNOS was non-uniformly distributed within the endothelium (D and H—cy2; green). The NOS isozyme labelling densities and patterns were similar in MCAs from prestroke vs. poststroke SHRsp. (bars = 50 μm).
of SHRsp with the antioxidant tempol (oral, 1 mmol/L) prior to (10 or 12 wks of age) or after stroke did not alter the onset of stroke and death in SHRsp or the PDC and NOS dysfunctions in the MCAs described in the current study (unpublished results).

We have shown that SHRsp develop renal damage which produces a reduction in glomerular filtration. This results in the activation of the renin–angiotensin system. High-salt diets accelerate renal dysfunction and stroke in SHRsp, hence the onset of stroke is delayed in SHRsp fed a normal salt Purina diet. We found that the treatment of SHRsp with captopril (angiotensin-converting enzyme inhibitor) prior to stroke retards the onset of stroke and similar treatment with captopril or losartan (AT-1 receptor blocker) initiated after stroke retards the onset of death (from 12 days to >2 months). Losartan treatment of poststroke SHRsp restored PDC and constriction to NOS inhibition in isolated MCAs as well as CBF autoregulation in the MCAs perfusion domain. The above beneficial effects of captopril or losartan treatment occur in the absence of an antihypertensive effect. The ability of eNOS to synthesize NO is inhibited when it is associated with caveolin-1. Ca²⁺ influx into the cells promotes NOS–caveolin-1 dissociation leading to the activation of eNOS.
over-perfusion and bleeding and thus potentially oppose the onset where PDC is lost, an elevation in cerebrovascular tone mediated dysfunction are well documented. However, under conditions of high All, alterations in the phosphorylation of NOS or modifications in the presence or function of chaperone proteins (HSP90) may contribute to NOS inactivation.11,24 AT-1 receptor blockade via losartan treatment could interrupt the above pathological processes and thus enable the restoration of NOS function in the MCAs of SHRsp after stroke.3

We believe that the loss of PDC in the MCAs prior to stroke4 contributes to the loss of CBF autoregulation in the MCA perfusion domain in SHRsp.1 This could elevate cerebrovascular pressures and promote over-perfusion under hypertensive conditions, increasing the probability of intracerebral haemorrhage formation. The continued presence of these defects after stroke would enhance the secondary haemorrhage development and amplify cerebral damage. Although the loss of PDC and NOS function co-incidently occurs within the MCAs in SHRsp that develop stroke, the mechanisms promoting these dysfunctions are likely distinct. The loss of PDC is associated with the development of defects in electromechanical coupling2 and protein kinase C function25 in the smooth muscle. As the primary location of the NOS isoforms is within the endothelium and adventitial cells, unrelated cellular defects are likely responsible for the loss of NOS function in the MCAs.

Vascular constrictor in response to NOS inhibition under constant blood flow conditions and flow-induced NO vasodilation have been observed in vivo within the same blood vessels.26 Likely, basal- and flow-related NO dilatory influences also affected cerebrovascular tone in vivo within our study. The limited ability of NOS inhibition to alter CBF in the MCA perfusion domain after stroke despite the predicted presence of over-perfusion would suggest that both basal- and flow-induced NO dilation were attenuated. The observations of similar elevations in BP and an attenuated depression in CBF in response to NOS inhibition within poststroke vs. prestroke SHRsp further suggests that NOS function may have been compromised to a greater degree in the cerebrovasculature relative to other systemic beds.

The detrimental pathological consequences associated with NOS dysfunction are well documented. However, under conditions where PDC is lost, an elevation in cerebrovascular tone mediated by a decrease in basal NOS function could reduce cerebrovascular over-perfusion and bleeding and thus potentially oppose the onset of stroke or reduce the degree of further tissue damage after stroke.

Supplementary material
Supplementary Material is available at Cardiovascular Research online.

Conflict of interest: none declared.

Funding
Supported by a grant from Canadian Institutes of Health Research to J.S.S.

References