Mechanism of Enlargement of Major Cerebral Collateral Arteries in Rabbits

R. Michael Lehman, BA; Gary K. Owens, PhD; Neal F. Kassell, MD; and Kazuhiro Hongo, MD, DMSc

Major cerebral collateral arteries enlarge following bilateral ligation of the common and internal carotid arteries. The purpose of this investigation was to determine the relative contribution of cellular hypertrophy versus cellular hyperplasia to this vessel change in a morphometric analysis as well as the functional properties of remodeled vessels in an in vitro study. We assessed cell number and vessel dimensions by morphometric analysis of 16 perfusion-fixed rabbit basilar arteries. Results demonstrated significant increases in luminal diameter from 761 to 946 μm (p<0.01), medial cross-sectional area from 5.1x10^4 to 7.6x10^4 μm^2 (p<0.005), smooth muscle cell volume from 9.19x10^5 to 1.44x10^6 μm^3 (p<0.0005), and overall arterial length from 17.41 to 20.36 mm (p<0.005) in basilar arteries from the eight ligated rabbits compared with the eight sham-operated controls. Smooth muscle cell volume fraction and cell numerical density were unchanged whereas the number of cells per unit length of artery was increased significantly from 21.5 to 31.0 cells/μm (p<0.05). These data indicate that smooth muscle cell hyperplasia rather than hypertrophy contributes to increases in vessel mass. Functional properties of the basilar arteries from 10 ligated and 10 normal control rabbits were analyzed in vitro. Results showed increased contraction to potassium chloride (approximately 74%) (p<0.01) and increased sensitivity of smooth muscle to acetylcholine (p<0.05) while maximal relaxation was the same as control in the ligated animals. In our study, basilar arteries subjected to increased load became remodeled into longer, thicker, wider arteries by the process of hyperplasia and exhibited greater contractility and capacity for vasodilation. (Stroke 1991;22:499-504)

The capability of the collateral circulation to adapt to increases in blood flow when the patient suffers from arterial occlusive disease of the primary pathways is of particular interest in those patients considered at risk for stroke. Blood flow increases in collateral arteries following occlusion of the preferred flow path, with the pressure gradient between the two pathways constituting the driving force. Flow-induced changes, acute and chronic, in arterial diameter are generally acknowledged. Rodbard2 provides a thorough review of this phenomenon, suggesting that chronic changes in blood flow induce some "anatomic reorganization of the vessel" to accommodate the enlarged lumen. It has been reported that chronically increasing the blood flow in the collateral circulation results in an increase in arteriolar diameter and length in those animals that survive the experimental occlusion of the primary pathways.3 The possible mechanisms responsible for this arterial enlargement are simple dilatation or reconstruction of the artery, including smooth muscle cell proliferation, enlargement of preexisting smooth muscle cells or cellular hypertrophy, enlargement of the extracellular medial matrix, intimal proliferation, or a combination of these factors. It is suspected that these changes are induced via local mechanisms.4-6

We sought to determine the contribution of cellular hypertrophy versus hyperplasia to arterial enlargement in a morphometric analysis and to characterize the reactivity of the enlarged vessels in an in vitro muscle bath system by developing a rabbit model that would simulate the condition of increased blood flow in the collateral arteries due to occlusion of the primary pathways.

Materials and Methods

The rabbit is a suitable model for this investigation because 1) the circle of Willis is well-developed7 and enables animals to survive the procedure and 2) the posterior circulation receives nearly the full load when blood flow is halted in the internal carotid

From the Department of Neurological Surgery, University of Virginia Health Sciences Center, Charlottesville, Va.
Address for correspondence: Neal F. Kassell, MD, Department of Neurosurgery, Box 212, University of Virginia Health Sciences Center, Charlottesville, VA 22908.
Received May 15, 1990; accepted December 17, 1990.
arteries since rabbits possess little flow in the ventral spinal artery under normotensive conditions and the anastomoses in the neck are extremely small and negligible. We used 55 male New Zealand White rabbits weighing 3–3.5 kg. Ten normal rabbits served as controls in the in vitro study, and the remaining 45 animals were anesthetized with 30 mg/kg i.m. ketamine and 6 mg/kg i.m. xylazine and orally intubated but mechanically ventilated (model 683 rodent respirator, Harvard Apparatus, South Natick, Mass.) only if spontaneous respiration ceased during the procedure. All 45 rabbits received 0.028 mg i.m. atropine and 50,000 units i.m. penicillin to mitigate inflammation and infection, respectively. Mean systemic arterial blood pressure was continuously monitored via a 24-gauge Teflon catheter inserted percutaneously into the marginal ear artery and connected to a pressure transducer and chart recorder (pressure transducer 1290A, option 6; monitor 78172A; Hewlett-Packard Co., Andover, Mass.). The ventral cervical region was shaved and prepared with ethanol and providone-iodine. The remainder of the animal was covered with a sterile drape and, using sterile technique, a midline cervical incision approximately 1 in. long was made. The carotid arteries were exposed bilaterally at the level of the internal/external bifurcation. Thirty-seven rabbits underwent bilateral ligation of the common carotid artery just proximal to the bifurcation and of the external carotid artery just distal to the bifurcation, which prevented retrograde blood flow in the internal carotid artery. Ligation was accomplished with 2/0 sterile nylon suture. The wound was closed with 3/0 sterile Vicryl suture (Ethicon, Inc., Summerville, N.J.), and the rabbit was allowed to recover. The remaining eight rabbits were sham-operated to serve as controls for the morphometric analysis and received the same treatment as the ligated animals, excepting arterial ligation.

Of the 37 rabbits that received bilateral ligation, 19 died or were euthanized before surviving 4 weeks, representing a procedural mortality of 51%. The rabbits that died usually did so during the several days immediately following the procedure, often with evidence of seizure. Postmortem examination revealed gross infarction and general cerebral ischemia as evidenced by the presence of pyknotic neurons. The surviving 18 ligated rabbits were observed twice daily for nutritional status and neurological deficiencies. The ligated and sham-operated control rabbits were fed standard chow with water, excepting animals not feeding postoperatively, which received subcutaneous Nutri-cal supplements (EVSCO Pharmaceuticals, Immunogenetics, Inc., Buena, N.J.), lettuce, and hay. Both groups of rabbits averaged a slight gain in weight, about 0.2 kg, over the 4-week experimental period.

After 4 weeks, 16 rabbits (eight ligated and eight sham-operated controls) were used for morphometric analysis were anesthetized, intubated, paralyzed with 0.1 mg/kg pancuronium bromide, and ventilated. Arterial blood pressure was monitored via the femoral artery, and the animals were transcardially perfusion-fixed at physiological pressure with 500 ml of 1% paraformaldehyde and 1.5% glutaraldehyde in Hanks’ balanced salts solution (H1387, Sigma Chemical Co., St. Louis, Mo.) at 37°C, pH=7.4. Perfusion was initiated with 100 ml of 37°C Hanks’ buffer prior to introducing the fixative. The mean±SEM systemic arterial blood pressure prior to perfusion-fixation was 85.5±2.5 mm Hg in the sham-operated group and 80.5±2.6 mm Hg in the ligated group (no significant difference). Arterial blood samples were withdrawn and blood gases were monitored at death and maintained within physiological limits. The brain was subsequently removed with the basilar artery in situ. Using a 24-gauge needle, the vertebral and basilar arteries were perfused with a small amount of 2% Evan’s blue dye for contrast with the brain tissue, and the ventral aspect of the brain was photographed on a copy stand. This photograph was used to determine the overall length of the basilar artery from its vertebral bifurcation to its junction with the circle of Willis. The lower third of the basilar artery was then dissected free and prepared for morphometric analysis by the following technique.

Sections of the basilar artery were immersed in the same fixative overnight, rinsed in Hanks’ buffer the next day, postfixed in 1% osmium tetroxide in this buffer (pH=7.4) for 1 hour, and rinsed again with buffer. The fixed tissue samples were then immersed in 0.05 M veronal acetate buffer and put in 3% uranyl acetate in 0.05 M veronal acetate overnight. They were then rinsed with buffer, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections (approximately 60–70 nm thick) were stained with 0.05 M lead citrate. Great care was taken to avoid geometric artifacts due to fixation procedures. Changes in vessel dimensions using this tissue processing protocol were <7% in thick sections (1 μm thick) and <1% in ultrathin sections of the arterial cross sections (G. Sleek and B. Duling, 1984, unpublished observations).

Transverse ultrathin sections from each of the four quadrants of the arterial cross section were cut with an ultramicrotome (Reichert Ultracut E, Cambridge Instruments, Deerfield, Ill.) and placed on 200-mesh grids. Five random, low-magnification transmission electron micrographs (Hitachi HU-12A, Mountain View, Calif.) were taken from each quadrant of the arterial cross section, for a total of 20 per artery. These micrographs included the entire width of the arterial wall (lumen to adventitia) at approximately ×3,000. The negatives were subsequently enlarged and, by applying standard point counting techniques with a 1 cm² matrix overlaying the photomicrographs, the percentage of the media occupied by smooth muscle cells was determined. The average of the 20 pictures yielded the value for each rabbit.

Thick sections (1 μm thick) of the arterial cross section were cut from the same blocks, mounted on glass slides, and stained with toluidine blue. For each artery, five photographs of the entire arterial cross...
section were taken with a Zeiss photomicroscope (Oberkochen, Germany). The luminal diameter, luminal circumference, and medial cross-sectional area were determined planimetrically using a Zeiss Kontron videoplan system. The average of the five measurements was calculated for each artery.

Smooth muscle cell numerical density was determined using the three-dimensional dissector technique and observing the "forbidden line rule." We applied the procedure as described elsewhere, except we stained the sections polychromatically with basic fuchsin and methylene blue by modification of the method of Huber et al. This technique was applied to a dissector volume that averaged 24,000 μm² for each artery, and the smooth muscle cell numerical density was then calculated from these figures. The number of smooth muscle cells per unit length of basilar artery was calculated by multiplying the cell numerical density by the medial cross-sectional area. The significance of differences between the means of the various parameters of the two experimental groups was tested by Student's two-tailed t test.

Reactivity of vessels from the normal control and ligated groups was assessed by isometric tension measurements on basilar artery ring segments. This procedure is described in detail elsewhere. Potassium chloride (KCl, 20–80 mM) was used to assess contractility of the vessels, and acetylcholine (ACh, 10⁻⁸ to 10⁻⁵ M) was applied after precontraction with 10⁻⁶ M serotonin (5-HT) to assess relaxation. Differences between the dose–response curves were tested using a general linear model for analysis of variance, and Scheffé's test was applied after one-way analysis of variance to compare points of maximal relaxation and contraction.

Results of the morphometric analysis and the in vitro study are reported as mean±SEM.

**Results**

The luminal diameter of the basilar arteries in the ligated group was 946±39 μm versus 761±40 μm in the sham-operated control group (p<0.01). The reported diameters were derived from measurements of the area of the arterial lumen, although diameters derived from measurement of the luminal circumference gave similar results. Eccentricity of the arterial cross sections (due to inexact cross sections or compression during sectioning) was measured and found to be minimal, with no significant difference between the groups. The length of the basilar artery, measured from the vertebral bifurcation to the basilar artery's junction with the circle of Willis, increased from 17.41±0.70 mm in the sham-operated group to 20.36±0.39 mm in the ligated group (p<0.005). The elongated vessels were visibly tortuous. The cross-sectional area of the media in the sham-operated group was 5.1±0.39x10⁴ μm² compared with 7.6±0.57x10⁴ μm² in the ligated group (p<0.005). The arterial media width:luminal radius ratio was not significantly different in the sham-operated and ligated groups (0.0557±0.0037 and 0.0542±0.0052, respectively). The percentage of the media occupied by smooth muscle cell was 73.2±1.4% in the sham-operated group and 74.6±1.2% in the ligated group (p<0.01). The reactivity of vessels from the normal control and ligated groups was assessed by isometric tension measurements on basilar artery ring segments. This procedure is described in detail elsewhere. Potassium chloride (KCl, 20–80 mM) was used to assess contractility of the vessels, and acetylcholine (ACh, 10⁻⁸ to 10⁻⁵ M) was applied after precontraction with 10⁻⁶ M serotonin (5-HT) to assess relaxation. Differences between the dose–response curves were tested using a general linear model for analysis of variance, and Scheffé's test was applied after one-way analysis of variance to compare points of maximal relaxation and contraction.

Results of the morphometric analysis and the in vitro study are reported as mean±SEM.

![Table 1. Percentage Change of Results of Morphometric Study](image_url)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-operated controls (n=8)</th>
<th>Ligated (n=8)</th>
<th>Change (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall basilar artery length (mm)</td>
<td>17.41±0.70</td>
<td>20.36±0.39</td>
<td>17</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Luminal diameter (μm)</td>
<td>761±40</td>
<td>946±39</td>
<td>24</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Medial cross-sectional area (10⁴ μm²)</td>
<td>5.1±0.3</td>
<td>7.6±0.57</td>
<td>48</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Smooth muscle cell volume (10⁴ μm³)</td>
<td>9.19±1.84</td>
<td>14.4±3.14</td>
<td>57</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Smooth muscle cell volume fraction (%)</td>
<td>73.2±1.4</td>
<td>74.6±1.2</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Media:radius ratio</td>
<td>0.0557±0.0037</td>
<td>0.0542±0.0052</td>
<td>-3</td>
<td>NS</td>
</tr>
<tr>
<td>Numerical cell density (10⁷ cells/μm³)</td>
<td>4.25±0.679</td>
<td>4.35±0.562</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Number of cells per unit length (cells/mm²)</td>
<td>21.5±3.6</td>
<td>31.0±2.1</td>
<td>44</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Significance determined by Student’s two-tailed t test. Values are mean±SEM; NS, not significant.
Stroke and related circulatory diseases are a major cause of mortality and morbidity. Patients whose collateral circulation could adjust to an increased load would be at a decided advantage over those whose collateral circulation could not. The mechanism that would confer such an advantage has not been investigated. In animal studies, chronically altering the blood flow in adult and juvenile arteries induces changes in arterial diameter.4-6,17-19 Furthermore, it is believed that there are also changes in medial mass, although definite evidence is lacking. This scenario may manifest itself in the large collateral circulation of patients with partial or total occlusion of vessels supplying the central nervous system.

Endo et al.20 using angiography, reported an enlarged and tortuous basilar artery following bilateral carotid artery occlusion in rabbits. The mortality from our procedure is higher than that reported by Endo et al., who experienced a 30% mortality with a similar procedure. This discrepancy is likely due to our addition of external carotid artery ligation to the common carotid artery ligation. Our study indicates that collateral arteries subjected to increased blood flow increase their luminal diameters. However, this enlargement is not due to simple dilatation, but rather to an increase in wall volume or mass, evidenced by the fact that the media:radius ratio was unchanged between groups despite an increase in luminal diameter in the ligated group. This finding is corroborated by the direct measurement of increased medial volume. In an artery with no remodeling, a continual decrease in the media:radius ratio as the vessel dilates would be expected.

This increase in the total volume of smooth muscle was shown to be due to cell hyperplasia rather than hypertrophy. This distinction will be important in attempts to identify the regulatory signals involved in remodeling since there is good evidence that the signals for smooth muscle cell hypertrophy and hyperplasia are quite different.21 The results of our in vitro study corroborated our morphometric findings. Arteries that possess more smooth muscle cells per unit length of artery would be expected to exhibit an increased capacity for contraction and vasodilation to such agonists as KCl and ACh. Thus, the arteries have been reconstructed but have maintained the same relative proportions (media:radius ratio) and are perfectly capable of normal vasoregulatory activity in the face of a heavier load.

Morphometric studies on arteries from hypertensive patients and in models of hypertension have usually revealed the presence of restructured arteries characterized by a smaller luminal diameter and a greater medial mass. The relation between the findings of smooth muscle cell hyperplasia in the flow-induced enlargement of arteries in the present model with the findings of increased medial mass in models of hypertension is unclear. Mulvany et al.22 are among those who clearly demonstrated an increase in arterial medial mass in models of hypertension. Efforts to provide a mechanism for the increase appear inconclusive. Experiments using various models of hypertension and human pathological specimens including those by Furuyama,23 Olivetti et al.,24 and Owens et al25-27 indicate that hypertrophy of the smooth muscle cells was responsible; other workers, such as Bevan et al28,29 and Owens and Reidy,30 have concluded that hypertension is associated with vascular muscle cell hyperplasia. The growth response of vascular smooth muscle (hypertrophy versus hyperplasia) is reported to vary between vascular beds and between hypertensive models.21

Overall consensus on a mechanism for increased medial mass associated with hypertension appears to be greatly hampered by the diversity of animal mod-

---

**FIGURE 1.** Mean±SEM contractile response of basilar artery ring segments to potassium chloride (KCl) is more potent in ligated rabbits (○, n=10) than in normal controls (◆, n=10). Contractile force is expressed in milligrams (mg).

**FIGURE 2.** Relaxation of basilar artery ring segments to acetylcholine (ACh) expressed as mean±SEM percent relaxation after precontraction by 10^-6M serotonin is more sensitive in ligated rabbits (○, n=10) than in normal controls (◆, n=10). Maximal relaxation is the same in both groups.
Drag or wall shear stress is probably detected by the endothelium and communicated to the smooth muscle cells via endothelial filaments penetrating the internal elastic lamina.\(^2\) Recent reports\(^3\)–\(^6\) have presented evidence that flow-induced vessel dilatation is a local, endothelium-dependent phenomenon. Endothelial cells are the source of a variety of smooth muscle cell growth factors and vasoactive substances.\(^33\) It remains to be determined if the release or production of these substances is modulated by hemodynamic factors.

**Acknowledgments**

The authors would like to acknowledge the expert technical assistance of Bonnie L. Sheppard and Jan A. Redick of the Central Electron Microscope Facility at the University of Virginia and the skillful assistance of Helen Tuan.

**References**

8. Saino K: Ischemic cerebral damage and EEG in the rabbit (dissertation). University of Helsinki, Finland, 1972
22. Mulvany MJ, Hansen PK, Aalkjaer C: Direct evidence that the greater contractility of resistance vessels in spontaneously hypertensive rats is associated with a narrowed lumen, thickened media, and an increased number of smooth muscle cell layers. *Circ Res* 1978;43:854–863

**KEY WORDS** • cerebral arteries • hyperplasia • rabbits
Mechanism of enlargement of major cerebral collateral arteries in rabbits.
R M Lehman, G K Owens, N F Kassell and K Hongo

doi: 10.1161/01.STR.22.4.499

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/22/4/499