Regional Differences in Cerebrovascular Cholinergic Innervation in Goats

Domingo Triguero, DVM, Angel Luis López de Pablo, PhD, Bernardino Gómez, MD, and Carmen Estrada, MD

The presence and distribution of a cerebrovascular cholinergic system were studied in goats. Regional cerebral blood flow was measured in the parietal cerebral cortex, caudate nucleus, and white matter by the hydrogen clearance technique in unanesthetized goats. Intravenous low doses of physostigmine, but not of neostigmine, significantly increased regional blood flow without changing mean arterial blood pressure or behavior. Increases of blood flow were greater in cerebral cortex and caudate nucleus than in white matter although the vasodilation induced by hypercapnia was similar in the three regions. Intracerebral microvessels were isolated from cerebral cortex, caudate nucleus, and white matter to evaluate choline acetyltransferase activity as a marker for perivascular cholinergic nerves. The enzyme level was higher in vessels from cerebral cortex and caudate nucleus than in vessels from white matter, which is in accordance with the functional data. These results suggest the presence of a cholinergic perivascular innervation system in intracerebral microvessels. Such innervation has a nonhomogeneous distribution throughout the brain and might be implicated in the local regulation of cerebral blood flow. (Stroke 1988;19:736-740)

Cerebral blood flow (CBF) appears to be regulated in part by cholinergic mechanisms. Intravenous injections of cholinergic agonists increase total and local CBF. Perivascular application of these agonists also produces a dose-dependent vasodilation of pial vessels. In all these cases, the vasodilatory response was blocked by atropine administration, indicating that it was mediated by muscarinic receptors. The presence of such muscarinic receptor sites has been demonstrated also by radioiodinated techniques in pial and intracerebral blood vessels.

Choline acetyltransferase (ChAT) activity, the most reliable marker for cholinergic neurons, has been detected in pial arteries and in intracerebral microvessels from several species. Immunohistochemical studies with ChAT antibodies have shown ChAT-positive fibers and terminals in close contact with intracerebral blood vessels, the structure of the dendritic-vascular relation in these vessels is similar to that previously described for acetylcholinesterase (AChE) fibers in human substantia nigra.

Electrical stimulation of the rat cerebellar fastigial nucleus elicited an increase in cortical blood flow without changes in local metabolism. This CBF enhancement was inhibited by local application of atropine on the parietal cortex and by electrolytic lesions of the basal forebrain. These results suggest the existence of a functional intrinsic cholinergic pathway stimulated from the fastigial nucleus and whose cholinergic cell bodies might reside within either the cerebral cortex or the basal forebrain.

Part of the functional evidence favoring a cholinergic innervation of intracerebral blood vessels comes from the observation that intravenous administration of the AChE inhibitor physostigmine increases the regional cerebral blood flow (rCBF) in anesthetized animals without concomitant metabolic activation. However, in these experiments, the direct effect of physostigmine on cerebral blood vessels could be modified by either the use of anesthetics or blood pressure increases induced by the drug.

We studied the effect of physostigmine on rCBF in different brain regions of unanesthetized goats, at doses that do not produce systemic alterations. A biochemical correlate of the distribution of cholinergic innervation was obtained by the determination of ChAT activity in isolated intracerebral microvessels from the same brain regions.

Materials and Methods

rCBF was measured in seven adult female goats (25–30 kg) by the hydrogen clearance technique after chronic implantation of platinum electrodes in the parietal cerebral cortex, caudate nucleus, and subcortical white matter. Electrodes (0.35 mm thick) were insulated with epoxy resin except for the conical tip (0.25 mm long). The bare tip was subsequently cathodized in 5% hexachloroplatinic acid, providing a finely dispersed deposit of platinum on the electrode surface.

Surgery was carried out under 35 mg/kg pentobarbital anesthesia, and artificial respiration with room air was instituted with a Harvard respirator (South Natick, Massachusetts). Electrodes were implanted bilaterally under stereotactic control according to the atlas of Tindal et al, fixed to the bone with dental cement, and attached to an Amphenol connector. Two Ag/AgCl electrodes, placed subcutaneously, were used as reference and ground electrodes.
A recovery period of 7–10 days was allowed, during which the goats were acclimated to the experimental conditions. Hydrogen (3% in air) was administered by inhalation through a mask with a double-valvular system to provide a minimum dead space, and clearance curves were obtained and recorded with a Beckman polygraph (Fullerton, California). In all cases, gas inhalation and curve recording were held until a steady state of the generated potential was reached during both saturation and desaturation periods.

Arterial blood pressure (ABP) was continuously monitored with a Statham pressure transducer (Los Angeles, California) through a catheter (chronically implanted) in the temporal artery, which also allowed the periodic withdrawal of blood samples for arterial pH, PaO$_2$, and PaCO$_2$ determinations with a blood gas analyzer (model 165, Corning Scientific Instruments, Melffield, Massachusetts).

Physostigmine (eserine sulfate; Sigma Chemical, St. Louis, Missouri) and neostigmine (Prostigmine; Roche, Nutley, New Jersey) were injected intravenously slowly, and hydrogen clearance curves were obtained before (control curve) and 3 minutes after the intravenous administration of the drug.

The cerebrovascular response to hypercapnia was studied by inhalation of 5% CO$_2$-3% H$_2$ in air until a steady-state potential was reached, replacing the former gas mixture by 5% CO$_2$ in air during the clearance curve.

At the end of the experimental period, and under barbiturate anesthesia, electrolytic lesions were made by passing a high-intensity (50 mA) current through each electrode for 20 seconds. Goats were then killed by intravenous injection of saturated potassium chloride solution, and the head was perfused with saline and subsequently with 10% formaldehyde. The brain was removed and kept in fixative for histologic processing and staining by the Nissl method to confirm electrode placement (Figure 1).

Fractions of intracerebral microvessels were obtained from four goats according to described methods$^{4-22}$ with several modifications. In summary, goats were killed, and brains were removed and kept at 4°C. The arachnoid membranes were carefully peeled away, and pieces of tissue were removed with forceps from the cerebral cortex, caudate nucleus, and white matter and homogenized manually in 6 volumes of ice-cold isotonic saline buffered with 0.01 M sodium phosphate, pH 7.4 (PBS) with a glass-glass homogenizer. Homogenates were centrifuged at 1,000g for 10 minutes, washed with PBS, and recentrifuged. Each pellet was resuspended in PBS containing 15% dextran and centrifuged at 1,500g for 20 minutes in a swinging rotor. Supernatants were rehomogenized and centrifuged in the same conditions. The resulting pellets, which primarily contained blood vessels, were pooled, poured over a nylon mesh screen (149 µm), and washed with a strong stream of PBS. The fraction retained by the screen was used for ChAT measurements and will be referred to as "microvessels."

Freshly isolated microvessels and pieces of tissue from the three brain regions were homogenized in 0.05 M sodium phosphate buffer, pH 7.4, by sonication (Ultrasonics W 185 F, Farmingdale, New York) for 15 seconds. Homogenates were centrifuged at 27,000g for 45 minutes, and the supernatants were used for measuring ChAT activity following a modification of Fonnum's method.$^{23}$ The production of [14C]acetylcholine (ACh) from [14C]acetylcoenzyme A (acetyl-CoA) was determined in triplicate after a 50-minute incubation at 37°C. The [14C]ACh formed was extracted with sodium tetraphenylboron in a liquid scintillation fluid containing acetonitrile and measured with a Beckman LS 2800 counter.

Alkaline phosphatase and γ-glutamyl transpeptidase (γ-GTP) were measured according to described methods$^{24-25}$ in homogenates of gray matter and microvessels taken from the cerebral cortex. Protein was measured by the method of Lowry et al$^{26}$ with bovine serum albumin as standard.

Values are expressed as mean ± SEM (n, number of goats). The significance was assessed either by analysis of variance and Student's t test corrected with
Bonferroni’s method or by Student’s $t$ test for paired samples when required. Differences between values were considered significant when $p<0.05$.

**Results**

**In Vivo Experiments**

Low doses of physostigmine (20–30 µg/kg i.v.) in all cases consistently increased rCBF in the cerebral cortex, caudate nucleus, and white matter (Table 1). The increases of rCBF, expressed as percentage of control values, were similar in the caudate nucleus and cerebral cortex, but significantly lower in the white matter (Figure 2). These effects were not accompanied by significant changes in mean ABP (MABP) or behavior. No changes in arterial pH, PaCO$_2$, or PaO$_2$ were observed.

The administration of the same doses of neostigmine did not change rCBF in any region studied (Table 1, Figure 2). MABP remained constant, and behavioral alterations were absent during neostigmine administration.

Carbon dioxide inhalation significantly increased rCBF in the three regions without changing MABP (Table 1). This vasodilatory response was similar in the cerebral cortex and caudate nucleus, as well as in the white matter, when expressed as a percentage of change (Figure 2). Breathing 5% CO$_2$ significantly changed ($p<0.01$) arterial pH (from 7.43 ± 0.03 to 7.36 ± 0.03, $n=4$), PaCO$_2$ (from 35 ± 0.8 to 39 ± 0.8 mm Hg, $n=4$), and PaO$_2$ (from 77 ± 2.3 to 94 ± 3.1 mm Hg, $n=4$).

**In Vitro Experiments**

The composition and purity of the microvessel fractions were assessed by light microscopy. As shown in Figure 3, this fraction consisted of small arteries and veins (15–80 µm in diameter) containing smooth muscle. Often, capillary segments were seen branching off the larger vessels. Contamination by nonvascular elements and cellular debris appeared negligible in our preparations.

Specific activities of alkaline phosphatase and γ-GTP, marker enzymes for cerebral endothelium, were 11 and four times higher, respectively, in the microvesSEL fractions than in the cortical gray matter.

ChAT activity was detected in microvessels isolated from the cerebral cortex and caudate nucleus, whereas in white matter microvessels, the enzyme activity was below our detection limits (Figure 4). In all cases, enzyme levels in vascular fractions were significantly lower than in brain tissue. ChAT activities in cerebral cortex and caudate nucleus microvessels were similar, despite the significant difference observed in brain tissue samples from both regions ($p<0.005$) (Figure 4).

**Discussion**

A significant increase in rCBF was observed in goats after the administration of low doses of physostigmine. The fact that no changes were produced in MABP indicates that the effect of the drug on CBF is not secondary to an autonomic excitation or other general cardiovascular changes. Signs of discomfort or behavioral alterations, which are associated with increases in cerebral metabolic rates and CBF, were absent during the hydrogen clearance measurements. The few experiments in which discomfort was apparent after physostigmine administration were discarded.

Cerebral metabolism was not measured in our experiments. However, doses of physostigmine from five to 10 times higher than those we used did not pro-

![Figure 2. Bar graph of percent change in regional cerebral blood flow (rCBF) in cerebral cortex (CX), caudate nucleus (CN), and white matter (WM) after 30 µg/kg physostigmine i.v. (open bars), 30 µg/kg neostigmine i.v. (black bars), or 5% CO$_2$ inhalation (gray bars). Values are mean ± SEM. Number of goats are in parentheses. Significance was assessed by analysis of variance and Student’s $t$ test for unpaired data. **$p<0.01$.](http://stroke.ahajournals.org/)

<p>| Table 1. Regional Cerebral Blood Flow and Mean Arterial Blood Pressure Before and After Acetylcholinesterase Inhibitors and CO$_2$ Administration |
|-----------------|-----------------|-------------|-------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Phystostigmine (30 µg/kg)</th>
<th>Control</th>
<th>Neostigmine (30 µg/kg)</th>
<th>Control</th>
<th>5% CO$_2$</th>
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<tbody>
<tr>
<td>rCBF (ml/min/100 g)</td>
<td></td>
<td></td>
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<tr>
<td>Caudate nucleus</td>
<td>35 ± 3.7 (4)</td>
<td>44 ± 4.5 (4)*</td>
<td>33 ± 5.2 (4)</td>
<td>31 ± 5.9 (4)</td>
<td>35.3 ± 2.1 (5)</td>
<td>52 ± 3.5 (5)†</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>43 ± 3.9 (4)</td>
<td>56 ± 5.5 (4)*</td>
<td>46 ± 8.5 (4)</td>
<td>46 ± 7.5 (4)</td>
<td>44 ± 3.1 (6)</td>
<td>65 ± 4.2 (6)†</td>
</tr>
<tr>
<td>White matter</td>
<td>15.3 ± 0.8 (4)</td>
<td>17.5 ± 1.1 (4)*</td>
<td>15.6 ± 1 (4)</td>
<td>14.9 ± 1.3 (4)</td>
<td>15.4 ± 0.7 (4)</td>
<td>22 ± 1.2 (4)†</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>102 ± 9.2 (4)</td>
<td>106 ± 10.2 (4)</td>
<td>106 ± 8.4 (4)</td>
<td>109 ± 7.6 (4)</td>
<td>101 ± 6.1 (6)</td>
<td>103 ± 6.5 (6)</td>
</tr>
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</table>

Values are mean ± SEM. Number of goats given in parentheses. Significance levels were assessed by Student’s $t$ test for paired data. rCBF, regional cerebral blood flow; MABP, mean arterial blood pressure.

* $p<0.05$; † $p<0.01$. 

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duce metabolic activation in anesthetized rabbits despite significant changes in rCBF. Awake animals seem to be more sensitive to the cerebral vasodilatory effect of the drug. Recent autoradiographic studies in unanesthetized rats have shown that 50 μg/kg physostigmine produced a maximum increase of cortical CBF versus the 150–300 μg/kg physostigmine that was necessary in anesthetized rats to obtain the same effect. No change in cerebral cortex glucose consumption was observed in awake rats during the cortical vasodilation induced by AChE inhibition. According to these previous studies, it is very unlikely that the rCBF enhancement we found in our experiments was related to a metabolic activation but rather to a direct effect of physostigmine on cerebral blood vessels.

The effect of physostigmine is due to its AChE inhibitory action and is indirectly mediated by muscarinic receptors since it is completely blocked by local atropine administration. The absence of vasodilatory action of neostigmine allows us to locate the effect of physostigmine beyond the blood–brain barrier since neostigmine and physostigmine have very similar potency for inhibiting AChE in vitro.

Local vasodilatory responses to carbon dioxide inhalation were of similar magnitude in the cerebral cortex, caudate nucleus, and white matter, indicating that there is no difference in the intrinsic vascular reactivity among these three regions. However, upon physostigmine administration, a lower response was found in the white matter than in the cerebral cortex or caudate nucleus, probably because of a poorer cholinergic innervation of blood vessels within the former region. Biochemical data support this suggestion since the cholinergic-marker enzyme ChAT was undetectable in white matter microvessels. No significant differences were found between ChAT activity in cerebral cortex and caudate nucleus microvessels, which is in contrast to the great difference detected in the corresponding tissue extracts. These results argue against the possibility of vascular ChAT being the result of contamination from brain tissue.

According to our data, physostigmine might increase the endogenous availability of ACh released from cholinergic synapses of neighboring cerebral blood vessels. This cerebrovascular cholinergic system might originate in the nearby cholinergic neuronal elements and is probably involved in the control of rCBF with a vasodilatory action. There are regional differences in the density of this cholinergic innervation. Such anisotropic distribution would allow local blood flow (and probably transport through the blood–brain barrier) to be adapted to the requirements of every brain region in a fine-tuned way, according to its precise functional state.

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References


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