Tryptophan Hydroxylase Activity in Rat Brain Base Arteries Related to Innervation Originating From the Dorsal Raphe Nucleus

María Jesús Moreno, PhD; Angel Luis López de Pablo, PhD; Emilio J. Marco, PhD

Background and Purpose Tryptophan hydroxylase activity was assayed in cell-free extracts of rat brain base arteries as marker of a serotonergic innervation.

Methods Estimation of the enzymatic activity was made in untreated male Sprague-Dawley rats (n = 53) and in those who underwent destruction of the dorsal and median raphe nuclei (n = 10).

Results Tryptophan hydroxylase activity was measured in rat cerebral arteries. The time-dependent 5-hydroxytryptophan production was undetectable in the absence of tryptophan or 6-methyltetrahydropterine and in the presence of 6-fluorotryptophan, and it was significantly reduced in the presence of p-chlorophenylalanine. Destruction of the dorsal raphe nucleus but not the median raphe nucleus brought about a significant reduction in enzyme activity.

Conclusions These results suggest that rat cerebral arteries receive a serotonergic innervation arising from the dorsal raphe nucleus. (Stroke. 1994;25:1046-1049.)

Key Words • cerebral arteries • serotonin • raphe nuclei • rats

Serotonin can be found in the cerebral blood vessels of several animal species, including man; its origin, however, remains a matter of controversy.

Biochemical and functional data indicate that the amine is stored in nerves arising from the raphe nuclei. Serotonin levels in cerebral blood vessels increase after the administration of tryptophan or a monoamine oxidase inhibitor and decrease after the animals are injected with p-chlorophenylalanine or the dorsal and medial raphe nuclei are destroyed. This serotonergic innervation seems functionally active, because the lesion of the dorsal raphe nucleus brings about an enhancement in the contractile response to serotonin of isolated cat middle cerebral artery and the electrical stimulation of the dorsal raphe nucleus reduces cerebral blood flow in several brain areas.

The morphological evidence, however, shows that serotonin is bound to sympathetic nerve endings, although there is not a true serotonergic innervation of the cerebral blood vessels. The serotonin-like immunofluorescence found in cerebral blood vessels disappears after cervical gangliectomy. It also disappears when the blood vessels are perfused with a saline solution or the animals are treated with amine uptake blockers. Thus, the serotonin revealed by histochemistry would be the result of its uptake by the sympathetic nerve terminals during the isolation procedure and not an actual neurotransmitter.

In vivo experiments indicate that tryptophan hydroxylase, the specific enzyme of the serotonin biosynthetic pathway, might be present in cerebral blood vessels. In animals the administration of an inhibitor of the aromatic amino acid decarboxylase brings about an increase in the 5-hydroxytryptophan (5-HTP) content in this kind of vessels. Such an increase is reduced when the main serotonergic pathway is destroyed with 5,7-dihydroxytryptamine.

However, other attempts to show the presence of tryptophan hydroxylase in the cerebral blood vessels have yielded conflicting results. Thus, Mathiau et al were unable to detect 5-HTP accumulation in isolated rat cerebral arteries after inhibiting the decarboxylase using an in vitro assay, whereas Cohen et al observed that the tryptophan hydroxylase–like immunoreactivity found in these vessels disappeared after cervical sympathectomy, although they could not demonstrate the same reactivity in the superior cervical ganglia.

The aim of the present work was to assay tryptophan hydroxylase activity in rat cerebral arteries by means of a classical in vitro biochemical method and determine whether this enzymatic activity might be linked to nerve fibers arising from the dorsal and median raphe nuclei.

Materials and Methods

Sixty-three male Sprague-Dawley rats, weighing 130 to 180 g, from the strain ICO:OFA SD (I.O.P.S. Caw) were used in the present study. The animals were housed in the proper facilities, complying with European Community directive 86/609/CEE and Spanish legislation (R.D. 223/1988) regarding the care of animals used in experimentation and other scientific purposes. The experiments reported here were approved by the Biosafety and Animal Care Unit Committee (Comisión de Bioseguridad y Gabinete Veterinario) of the Faculty of Medicine of the Autónoma University of Madrid.

In 10 anesthetized rats (35 mg/kg IP sodium pentobarbital) the dorsal and median raphe nuclei were destroyed by electrocautery. The electrode was implanted according to the coordinates described by König and Klippel, and the rats

Received November 1, 1993; accepted January 19, 1994.
were killed 15 days later. The control group rats were subjected to the same procedure, but no current was passed through the electrode. The accuracy of the lesion placement was tested by measurement of tryptophan hydroxylase activity in the hippocampus and striatum.

After the rats were decapitated, the brain was quickly removed. The circle of Willis with some of its branches was dissected out, with the left hippocampus and striatum as well as the lesioned animals. The dissected tissues, and in some cases the whole brain, were frozen on dry ice and stored at −8°C.

The frozen brains were placed on a metal block cooled to −8°C, and frontal slices 0.5 to 1 mm thick were cut freehand with a razor blade. The slices were kept frozen on the block, and the dorsal raphe nucleus was punched out. The nucleus was identified following the description by Königs and Klippel.

The assay of tryptophan hydroxylase was similar to that described by Meek and Neeckers. The tissues were homogenized by sonication in 0.05 mol/L Tris buffer (pH 7.4) containing 10−3 mol/L mercaptoethanol and 0.05% Triton 100-X. The volume used for the samples of cerebral arteries and dorsal raphe nucleus was 300 μL; for the hippocampi and striata, 600 μL. The homogenates were centrifuged at 12 000 rpm for 10 minutes in a Beckman microfuge. One hundred μL of the supernatant was added to an Eppendorf microcentrifuge tube with 20 μL of a standard reaction mixture containing, in 0.05 mol/L Tris buffer (pH 7.4), tryptophan (2 mmol/L), 6-methyltetrahydropterine (16 mmol/L), mercaptoethanol (1 mmol/L), and catalase (2.5 mg/mL). The tubes were incubated in a Suppelco Blok Heater at 37°C for 1 hour. Some cell-free extracts from cerebral blood vessels were incubated under the same conditions for 2 hours. The reaction was stopped by adding 20 μL of 11.64 mol/L HClO4. Blank or 0-hour tubes were made by adding the perchloric acid to the supernatants and shaking them before adding the reaction mixture. The samples were centrifuged at 12 000 rpm for 5 minutes, and 5-HTP was assayed in 20 μL of the supernatant by high-pressure liquid chromatography (HPLC) with fluorometric detection. Proteins were determined in the precipitates of the first homogenates by the method of Lowry et al, with 5-HTP enzymatic activity that was unchanged in hippocampus and striatum and cerebral arteries showed a decreased enzymatic activity that was unchanged in hippocampus (Fig 3).

Tryptophan hydroxylase activity measured in samples of dorsal raphe nucleus was 858±160 pmol/mg protein per hour.

Discussion

The present results support the existence of a serotonergic innervation impinging on the rat brain base arteries.

Tryptophan hydroxylase can be assayed in these blood vessels by means of a biochemical method. When a standard reaction mixture is in contact with cell-free extracts of rat cerebral arteries, there is a time-dependent production of 5-HTP. This 5-HTP is synthesized from tryptophan by the action of tryptophan hydroxy-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5-HTP (pmol/mg protein/hr)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.25±4.28</td>
<td>8</td>
</tr>
<tr>
<td>+p-Chlorophenylalanine (1 mmol/L)</td>
<td>5.83±6.00</td>
<td>5</td>
</tr>
<tr>
<td>+6-Fluorotryptophan (1 mmol/L)</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>−Tryptophan</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>−6-Methyltetrahydropterine</td>
<td>ND</td>
<td>5</td>
</tr>
</tbody>
</table>

n indicates number of samples; ND, not detectable. *P<.05 compared with control.
lase, since it is absent after removal of the amino acid or the cofactor from the medium or diminished in the presence of enzyme inhibitors. On the other hand, destruction of the dorsal but not the median raphe nucleus brings about a significant decrease in the enzymatic activity, indicating that the enzyme is bound at least in part to nerve fibers whose cell bodies are located in that brain stem nucleus.

Previous studies have shown that the serotonergic innervation of rat cerebral arteries originates from dorsal and median raphe nuclei. Our present data suggest that only dorsal raphe nucleus innervates rat brain base arteries. The reason for this difference might be that in the present study only the major cerebral arteries were used, whereas previous studies have also included other cerebral microvessels. A similar result is obtained in cats, in which lesion of the dorsal but not the median raphe nucleus induces supersensitivity to serotonin in the middle cerebral artery.

The results of the present study agree with experiments demonstrating tryptophan hydroxylase activity in cerebral blood vessels in vivo. The discrepancy of the findings by Mathiau et al with this evidence might be due to methodological differences. These authors incubated the cerebral blood vessels in a medium devoid of enzyme cofactor that was in addition thoroughly bubbled with oxygen. Because cofactor concentration is critical for the correct activity of tryptophan hydroxylase, it is possible that their assay did not reach subsaturating conditions, as they assumed. Another explanation might be that they were under the limit of detection in their cerebral blood vessel preparation because according to the findings shown here, tryptophan hydroxylase activity in the nerve terminals of the vessels is 50 times less than that in dorsal raphe nucleus. Their own data also support this explanation: when these authors measure the enzyme activity by giving labeled tryptophan and estimating the formation of \[^{14}C\]5-HTP, some incorporation of radioactivity into the reaction product is found.

Regarding the differences between the morphological evidence and the results published here, no explanation can be given. The tryptophan hydroxylase-like immunoreactivity found in cerebral blood vessels seems to belong to the peripheral sympathetic innervation, for cervical gangliectomy dramatically reduces it, whereas lesions of the central serotonergic pathways have no effect. The same technique could not, however, unveil the presence of the enzyme in the cerebral ganglia. This is remarkably similar to the results obtained when a similar technique was used to detect serotonin in the cerebral blood vessels. The serotonin-like immunohistochemical fluorescence disappears after cervical gangliectomy or treatment of the animals with an amine uptake inhibitor or when the blood vessels are perfused with a saline solution before dissection of the tissue. However, biochemical analyses of the serotonin content of the cerebral arteries give the opposite results, with no change in the amine levels after cervical sympathectomy or superfusion of the blood vessels with saline solution. The present findings, then, support the biochemical evidence and stress once more the divergence between the morphological techniques and the rest of the methodologies used to approach the problem exposed here.

In any case, the morphological evidence is not as homogeneous as it seems. For instance, when horseradish peroxidase (HRP) is applied in the cat middle cerebral artery, HRP-labeled neurons are found in the dorsal raphe nucleus, and when the serotonin-like immunoreactivity is compared with the immunoreactivity to noradrenaline in rabbit cerebral arteries, they do not superimpose.

**Acknowledgment**

Supported by Fondo de Investigaciones Sanitarias grant 93/0316.
References


Editorial Comment

The preceding article describes biochemical evidence to support the existence of tryptophan hydroxylase within nerve fibers surrounding the rat circle of Willis. Because this enzyme is specific for the biosynthesis of serotonin and because destruction of the dorsal raphe nucleus significantly reduces enzyme activity within large cerebral arteries, the authors conclude that serotonin is contained within nerve fibers projecting from parenchymal brain stem neurons located within the dorsal raphe. This study of Moreno et al is important because it adds data to the controversy over whether or not serotonin is contained within perivascular sympathetic fibers by an uptake-dependent mechanism and/or whether it is contained within ascending projections from dorsal raphe nucleus. Although the data presented support the latter possibility, the existence of an uptake-dependent mechanism within sympathetics has not been excluded by this study.

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*Stroke*. 1994;25:1046-1049
doi: 10.1161/01.STR.25.5.1046

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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http://stroke.ahajournals.org/content/25/5/1046

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