Presence of norepinephrine and related enzymes in isolated brain microvessels

[t-3-monoxygenase/dopamine $\beta$-monoxygenase/amine oxidase (flavin-containing)]

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ABSTRACT  Norepinephrine and the enzymes involved in its synthesis and degradation were found to be associated with isolated brain microvessels. The significance of these results is discussed with respect to adrenergic innervation of the cerebral microvessels and thereby neural regulation of the cerebral microcirculation.

The cerebral vasculature consists of two systems of blood vessels: intracerebral and extracerebral vessels. Physiological and pharmacological studies of the brain blood vessels have generally been performed on isolated extracerebral arteries, such as pial arteries and basilar artery (1, 2), probably due to the difficulty in isolating the intracerebral vessels. Studies on the intracerebral vessels have usually been confined to histochemical and morphological procedures (1, 3).

We were able to isolate microvessels (arterioles, capillaries, and venules) from the rat brain with a modification of the method used by Brendel et al. (4). In the present study, we report on the presence of norepinephrine in these microvessels as well as the enzymes necessary for both its biosynthesis and catabolism.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Marland Farms, New Jersey) 7 to 8 weeks old were used in all studies.


Isolation of Cerebral Microvessels. Rat brain microvessels (arterioles, capillaries, and venules) were prepared by a modification of the method of Brendel et al. (4). Brains were removed immediately from decapitated rats, kept in ice-cold minimum essential medium (Joklik modified) (Grand Island Biological Co., Grand Island, N.Y.), and aerated with 95% O2 and 5% CO2. The surrounding pial membrane and arachnoidal plexuses in the ventricles were carefully and completely removed in ice-cold oxygenated medium. Pieces of brain tissue, freed from membrane, were collected in a test tube containing oxygenated medium. Mild homogenization of the tissue was performed with a hand-held Teflon pestle (Kontes Biological Products, Vineland, N.J.), which was loosely fitted to the tube (0.5 mm clearance). The homogenates were sieved over a 150-μm nylon fabric, and the filtrate was collected in a beaker immersed in an ice bath. This has been designated as brain filtrate. The brain tissues retained on the nylon cloth were transferred to another test tube and gently homogenized and sieved as before. A relatively homogeneous preparation of microvessels could be obtained by carrying out the homogenization and sieving procedure four times. Homogeneity of the microvessels was examined under a microscope during the course of isolation. The yield of cerebral microvessels obtained from one rat brain was approximately 2 mg wet weight. The combined brain filtrates were centrifuged at 150 × g for 10 min. The precipitate was then resuspended in suitable buffer for determination of enzyme activities.

Tyrosine hydroxylase [tyrosine 3-monoxygenase; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase(3-hydroxylating); EC 1.14.16.2] was determined by a modification of the method of Nagatsu et al. (5). All tissues were homogenized in phosphate buffer (67 mM; pH 6.2): whole brain in 20 volumes, mesenteric arteries in 10 volumes, and microvessels (from four to eight brains) in 80 μl. The reaction mixture consisted of 0.1 mM L-tyrosine containing about 2 × 106 cpm of L-[3,5-3H]tyrosine; 0.74 mM 2-amino-6-methyl-5,6,7,8-tetrahydropteridine; 140 mM 2-mercaptoethanol; 200 mM sodium acetate buffer (1 M; pH 6.0); 1000 units of catalase; and tissue homogenates in a total volume of 500 μl for brain tissue and 100 μl for blood vessels. The mixture was incubated for 15 min at 37°C, and the reaction was then terminated by adding 100 μl of 12.5% trichloroacetic acid for brain, and 500 μl of 2.5% trichloroacetic acid for vessels. After centrifugation of the sample, the supernatant was passed over a Dowex column (1H, 200–400 mesh). The effluent and 1.4 ml of water, used to wash the column, were collected and mixed with 15 ml of Aquasol in a counting vial. Radioactivity was measured in a Beckman Liquid Scintillation Counter.

For determination of aromatic-L-amino-acid decarboxylase (aromatic-L-amino-acid carboxyl-lyase, EC 4.1.1.28), whole brains were homogenized in 20 volumes, mesenteric arteries in 50 volumes, and microvessels (from eight brains) in 700 μl of phosphate buffer (5 mM; pH 7.2). The tissue homogenates (500 μl) were assayed for dopa decarboxylase (aromatic-L-amino-acid decarboxylase) activity using the method of Christenson et al. (6), which measures the 14CO2...
Table 1. Norepinephrine and related enzymes in brain and in blood vessels

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Norepinephrine (µg/wet tissue)</th>
<th>Tyrosine hydroxylase (tyrosine oxidized)</th>
<th>Dopa decarboxylase (CO₂ formed)</th>
<th>Dopamine β-hydroxylase (octopamine formed)</th>
<th>Monoamine oxidase (tyramine metabolized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>0.45 ± 0.06 (3)</td>
<td>0.49 ± 0.06 (3)c</td>
<td>31.0 ± 0.7 (3)</td>
<td>0.53 ± 0.07 (5)b</td>
<td>179.7 ± 8.4 (3)b</td>
</tr>
<tr>
<td>Brain filtrate</td>
<td>i</td>
<td>0.29 ± 0.02 (3)b,h</td>
<td>17.2 ± 0.8 (3)e</td>
<td>0.96 ± 0.17 (5)d</td>
<td>134.8 ± 4.7 (6)b</td>
</tr>
<tr>
<td>Brain microvessels</td>
<td>0.64 ± 0.09 (2)</td>
<td>0.07 ± 0.01 (3)</td>
<td>31.3 ± 4.6 (3)</td>
<td>1.75 ± 0.15 (5)</td>
<td>425.4 ± 23.1 (6)</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>2.76 ± 0.34 (3)c</td>
<td>1.17 ± 0.27 (3)d</td>
<td>9.2 ± 0.1 (3)</td>
<td>11.40 ± 1.3 (5)b</td>
<td>76.3 ± 8.4 (3)b</td>
</tr>
</tbody>
</table>

- Enzyme activities are expressed as nmol per mg of protein per hour with appropriate blanks subtracted. Figures represent mean ± SE.
- Numbers in parentheses refer to the number of experiments. Each experiment was carried out with four to eight rats.
- Significantly different from brain microvessels (P < 0.001).
- Significantly different from brain microvessels (P < 0.01).
- Significantly different from brain microvessels (P < 0.05).
- Significantly different from whole brain (P < 0.001).
- Significantly different from whole brain (P < 0.01).
- Significantly different from whole brain (P < 0.05).
- Not measured.

derived from carboxyl-labeled dopa. The preincubation period was 10 min, and the incubation in the presence of l-dopa (1 mM final concentration) containing about 1.3 × 10⁵ cpm of carboxyl-labeled DL-3,4-dopa was for 30 min. The second incubation period, after addition of trichloroacetic acid to trap the ¹⁴CO₂ on alkaline paper strips, was also 30 min. At the end of this incubation, the paper strips were moved and placed in a counting vial with 10 ml of Aquasol solution. Radioactivity was measured in a Beckman Liquid Scintillation Counter.

The dual-wavelength spectrophotometric method of Kato et al. (7) was used to estimate dopamine β-hydroxylase [dopamine β-monooxygenase;3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase(β-hydroxylation); EC 1.14.17.1]. In this assay, whole brains were homogenized in 10 volumes, mesenteric arteries in 50 volumes, and microvessels (from eight brains) were in 500 µl of phosphate buffer (100 mM; pH 7.4) which contained 0.1% Cutsicum. After extraction into 3 M NH₄OH, the difference between the absorbance of the generated p-hydroxybenzaldehyde at 333 and 360 nm was measured with a Cary 14 recording spectrophotometer.

Monoamine oxidase [amine oxidase(flavin-containing); aminooxygen oxidoreductase(deaminating)(flavin-containing); EC 1.4.3.4] activity was determined by the method of Goridis and Neff (8) with some modification. For assay, whole brains and mesenteric arteries were homogenized in 20 volumes, and microvessels (from four brains) were homogenized in 120 µl of phosphate buffer (67 mM; pH 7.2). The reaction mixture consisted of 2.5 mM tyramine that contained about 8.5 × 10⁴ cpm of [L-¹⁴C]tyramine and tissue homogenate in a total volume of 140 µl of buffer solution. The mixture was incubated for 30 min at 37°C, and the reaction stopped by the addition of 40 µl of 60% perchloric acid. After the protein was precipitated, the supernatant was pipetted into a Rexyn 101 column (+H, 200–400 mesh) and washed with 5 ml of distilled water. The effluent and washes were collected in a glass counting vial. The radioactivity of the samples was measured in a Beckman Liquid Scintillation Counter after 15 ml of Aquasol was added to the vial.

Norepinephrine was assayed by the method of Schlumpf et al. (9). Fluorescence (activation 385 and emission 485 nm) was measured in the Amino-Bowman Spectrophotofluorometer.

Tissue protein was determined according to the method of Lowry et al. (10), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The preparations of brain microvessels were quite homogeneous, as shown in Fig. 1. A portion of a brain microvessel is shown at higher amplification in Fig. 2. It should be noted (Table 1) that these microvessels contain more decarboxylase, dopamine β-hydroxylase, and monoamine oxidase than...
do the brain filtrates. The enzyme activities in the vessels are as high as or higher than those of intact brain. This shows a high degree of localization of sympathetic neurons in the isolated microvessels. Only tyrosine hydroxylase activity is lower in microvessels than in the brain as a whole. Nevertheless it is present in appreciable amount. It is also possible that some tyrosine hydroxylase may have been lost during the repeated washings and sievings of the microvessels. Comparison with mesenteric artery also reveals that some of the enzymes are present in even higher concentration in the brain microvessels. Preliminary studies indicate that cerebral microvessels contain large amounts of catechol-o-methyl transferase, also more than in mesenteric artery.

Dopamine \( \beta \)-hydroxylase, which converts dopamine to norepinephrine, has been used as a marker of noradrenergic neurons both in the central and peripheral sympathetic systems (11–13). The fact that dopamine \( \beta \)-hydroxylase activity is present in the cerebral microvessels would indicate that the latter are innervated by noradrenergic nerve fibers. It is commonly recognized that extracerebral vessels are innervated by nerve fibers (3, 14). Whether the intracerebral vessels are also accompanied by nerve fibers has been contro-

versial (15, 16). The present findings are consistent with those of Hartman and Udenfriend (17) and Hartman et al. (12), who contend that the intracerebral blood vessels are innervated by central noradrenergic neurons whose cell bodies might be located in the locus coeruleus. Those studies were based on immunofluorescence. More recent evidence, using electron microscopy, also indicates that intracerebral arteri-oles in the cat are innervated by noradrenergic nerve fibers (18).

It has been reported that brain slices taken from animals pretreated with L-dopa show a large accumulation of dop-amine in the endothelium of brain capillaries, but not in the brain tissue (19, 20). It was suggested, therefore, that brain capillaries function as a barrier that prevents dopamine, which is formed there from dopa, from entering the brain. It is equally possible that the high dopamine concentration observed in the capillaries after administration of dopa is due to the intimate association of the microvessels with noradrenergic neurons. As shown in Table 1 the latter are richer in the decarboxylase than they are in dopamine \( \beta \)-hydroxylase and might, therefore, form and accumulate dopamine and even norepinephrine.

**FIG. 2.** A portion of a microvessel fragment is shown in phase contrast photograph. Scale marker, 20 \( \mu m \).
Although studies on the responsiveness of the extracerebral blood vessels to administered norepinephrine have demonstrated that the amine causes cerebral arteries to constrict both in vivo (21, 22) and in vitro (23, 24), its effect on cerebral blood flow is controversial. In a recent review (25) it has been reported that norepinephrine administration can increase, decrease, or elicit no effect on cerebral blood flow. Also, changes in hypothalamic blood flow have been shown to depend on the dose of norepinephrine administered (26). The immunofluorescence studies of Hartman and his colleagues (12, 17) and the electron microscopic evidence of Cervos-Navarro and Matakas (18) have, thus far, been the most conclusive evidence for adrenergic innervation of cerebral microvessels. Demonstration of the presence in isolated brain microvessels of norepinephrine and the enzymatic machinery necessary for its biosynthesis and catabolism provides additional evidence for neuronal regulation of the cerebral microcirculation.

We thank Dr. J. Lambert for determining dopa decarboxylase activity in the tissues.