Serotonin as an Alternative Transmitter in Sympathetic Nerves of Large Cerebral Arteries of the Rabbit

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The distribution of serotonin (5-HT)-like immunoreactive (5-HT-LI) nerves and the potential role of 5-HT as a vasoconstrictor transmitter in large cerebral arteries of the rabbit were examined. 5-HT-LI fibers with weak immunofluorescence were observed in the anterior cerebral, middle cerebral, and basilar arteries when fixed by immersion after dissection from exsanguinated animals. The 5-HT-LI fibers, however, were not detected in these arteries when fixed either in vitro or in situ after first being perfused with Krebs solution in situ to flush the blood component from the lumen prior to dissection. In these arteries, 5-HT-LI nerve fibers with intense immunofluorescence, however, reappeared following incubation with 5-HT in vitro. The intensity of the 5-HT-LI fibers seemed to be proportional to the duration and 5-HT concentration during incubation. Following chronic surgical sympathectomy, 5-HT-LI fibers were not detected in arteries before or after incubation with 5-HT. Transmural nerve stimulation elicited constriction in 50% of the control arterial segments examined. The constriction was not affected by ketanserin but was prevented by guanethidine and chronic surgical sympathectomy. The remaining arterial segments that did not respond on transmural nerve stimulation, however, became constrictive on transmural nerve stimulation following incubation with 5-HT in vitro. The constriction was blocked by ketanserin and clonidine. These results demonstrate that the large cerebral artery of the rabbit brain has extremely sparse or no authentic 5-HT-LI nerves. The intense 5-HT-LI nerves observed following incubation with 5-HT are primarily due to uptake of 5-HT into sympathetic nerves, and on transmural nerve stimulation 5-HT can be released to induce vasoconstriction. It is suggested that 5-HT can act as an alternative sympathetic vasoconstrictor transmitter in cerebral arteries. (Circulation Research 1987;60:220-228)

Cerebral blood vessels from all species have been shown to receive dense sympathetic vasoconstrictor nerves. The exact functional role and transmitter mechanism of sympathetic nerves in controlling brain circulation, however, remains unsettled. It has been shown that sympathetic nerves may exert protective effects on cerebral circulation in acute and chronic hypertension. On the other hand, sympathetic nerves have been suggested to be involved in the pathogenesis of several cerebrovascular disorders. Furthermore, pharmacological studies using isolated preparations have shown that vasoconstriction elicited by sympathetic nerve stimulation is not blocked by α-adrenergic receptor antagonists. This finding suggests that a transmitter besides norepinephrine (NE) is involved in the sympathetic vasoconstriction of cerebral arteries. The nature of the second transmitter for vasoconstriction is not determined.

It has been demonstrated that perivascular nerves to cerebral arteries can take up serotonin (5-HT) or 5-hydroxytryptophan. The presence of 5-HT-like immunoreactive (5-HT-LI) nerves in major cerebral arteries of various species have also been demonstrated, although this finding was not supported by others. Furthermore, Griffith et al suggested that 5-HT was responsible for transmural nerve stimulation (TNS)-induced vasoconstriction in the rabbit vertebral artery. On the other hand, Edvinsson et al demonstrated that neurogenic vasoconstriction of the rabbit basilar artery was not affected by serotonin receptor antagonists such as ketanserin or methergoline.

Recently, several reports have shown that sympathetic nerves in peripheral and cerebral arteries can accumulate and release 5-HT. The presence of dense sympathetic innervation in cerebral arteries, therefore, prompted us to examine 1) if 5-HT is taken up into cerebral sympathetic nerves and is subsequently released on electrical stimulation of sympathetic nerves to induce vasoconstriction, and 2) if rabbit cerebral arteries receive an authentic serotoninergic innervation. We have found that, in contrast to some earlier reports, nerves to pial arteries of the rabbit normally are not serotoninergic. Sympathetic nerves to cerebral arteries, however, are able to take up and release 5-HT.

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This work was supported by grants from NIH (HL 27763, 24683), AHA/IHA (83-1040, 83-1048), Southern Illinois University School of Medicine, and the University of Tsukuba Project Research.

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Received May 5, 1986; accepted October 13, 1986.
**Immunohistochemistry**

In Vitro Tissue Bath Studies

All rabbits were perfused in situ with Krebs solution through the left ventricle under sodium pentobarbital anesthesia (50 mg/kg, i.v.) and exsanguinated. The brain was removed and the basilar artery dissected. Ring segments (4 mm long) of the arteries were carefully cannulated with a stainless steel rod (30-gauge hemispherical section) and a short piece of platinum wire, and mounted in an isolated tissue bath containing 30 ml Krebs solution at room temperature. The platinum wire was bent into an inverted U shape and anchored to a plastic gate, which could be moved up and down by a fine control micrometer (unislide series A1500). The steel rod was connected to a Gould UC-2 strain gauge for isometric recording of changes in force. After 10 minutes, the temperature of the Krebs solution was gradually increased to and maintained at 37°C. Resting tension was then adjusted to 0.5 g and a period of 1 hour was allowed for equilibration. A pair of stimulating electrodes, one on either side of the vessel, was arranged for TNS, which was delivered across the electrodes via a stimulator and a modified coupling device, which provided a low source impedance. Stimulation parameters (trains of 100 biphasic square wave pulses of 0.3 milliseconds, 160 mA across the electrodes) were selected to achieve supramaximal nerve stimulation. Actual stimulation parameters were monitored with an oscilloscope (Tektronix).

Since the response obtained in this stimulating condition was completely blocked by tetrodotoxin (9 x 10^-7 M), this stimulation was referred to as TNS. After the first response of the tissue on TNS was recorded, the tissue was then incubated with 5-HT of different concentrations for 30 minutes. The tissue was then washed with Krebs solution and the TNS repeated.

**Sympathetic Denervation**

In three rabbits, unilateral superior cervical ganglion was extirpated under anesthesia with ketamine (35 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) 1 week before sacrifice. The completeness of sympathetic denervation was confirmed by the total disappearance of the catecholamine (CA)-fluorescence, using the glyoxylic acid method.

**Statistical Methods and Drugs**

Statistical analyses were made using paired and unpaired t tests. Drugs used were serotonin creatinine sulfate (Calbiochem-Behring Corp., La Jolla, Calif.), guanethidine sulfate (Sigma Chemical Co., St. Louis, Mo.), ketanserin (Janssen), tetrodotoxin (Sigma) and clonidine HCl (Sigma).

**Results**

**Immunohistochemistry**

When cerebral arteries were dissected from anesthetized animals that were killed by exsanguination, and were fixed by immersion, weak 5-HT-LI nerves were frequently observed in these arteries (Figure 1a). In 5 of 5 animals, blood was observed in the lumen of these arteries at the time of dissection under a dissecting microscope. The intensity of the 5-HT-LI, however, varied among the tissues; in some, immunofluorescence was almost absent.

When the brain arteries were fixed by perfusion in situ before exsanguination, in 4 of 4 animals 5-HT-LI nerves were not observed in the arteries examined (anterior communicating, middle cerebral, internal carotid, basilar, and vertebral arteries) (Figure 1b). In these tissues, no trace of blood remained in the lumen at the time of dissection.

In the third series of experiments, brains of 4 rabbits were perfused in situ with Krebs solution. The cerebral and ear arteries were then dissected and incubated in the presence or absence of 5-HT (10−7−10−4 M) for 30 minutes at 37° C. The tissues fixed immediately after dissection without incubation, or after incubation in Krebs solution in the absence of 5-HT, exhibited no 5-HT-LI nerves (Figure 2a). On the other hand, cerebral arteries (anterior communicating, middle cerebral, and basilar arteries) and central ear arteries incubated with 5-HT for 30 minutes at 37° C exhibited dense distribution of 5-HT-LI nerves (Figure 2b-d). Although the immunofluorescence was weak in tissues incubated with 10−8 M 5-HT for 30 minutes, the intensity was enhanced by incubation with a higher concentration of 5-HT or longer (1 hour) incubation time (not shown). Following chronic superior cervical ganglionectomy, 5-HT-LI nerves were not observed in cerebral arteries (3 of 3 sympathectomized animals) before or after incubation with 5-HT (Figure 3). Successful sympathetic denervation was confirmed by the absence of CA-fluorescent nerves (Figure 3) in adjacent segments of the artery.

In Vitro Tissue Bath Studies

TNS induced vasoconstriction in 16 of 33 basilar arterial rings. The vasoconstriction was not affected by ketanserin (Figure 4a), a result consistent with our previous reports.11 In the remaining arteries that did not respond to TNS, exogenous 5-HT (10−6 M) caused constriction (110.4 ± 19.5 mg/mg wt). 5-HT was then kept in the tissue bath for another 30 minutes. After 5-HT was washed out from the bath, TNS was repeated and all arteries responded with constriction (Figures 4b and 5). This TNS-induced vasoconstriction after 5-HT incubation was blocked by ketanserin (10−7 M) (Figure 4b) and abolished by guanethidine (Figure 5). The inhibition of the TNS-induced vasoconstriction at 8 and 16 Hz by ketanserin (10−7 M) was 40.7 ± 11.0% and 45.6 ± 6.7%, respectively (Figure 6). The remaining vasoconstrictor responses were abolished by tetrodotoxin (TTX, 9 × 10−7 M) or guanethidine (10−6 − 3 × 10−6 M) (Figures 4, 5, and 7).

The effects of clonidine, an α2-adrenergic agonist, on the TNS-induced constriction in basilar arteries following incubation with 5-HT were also examined. Clonidine (10−8 − 10−6 M), which did not affect the resting tone of the basilar artery (Figure 7), decreased the TNS-induced vasoconstriction in this artery (Figures 7 and 8). The residual vasoconstriction was blocked by ketanserin (10−7 M). On the other hand, in arterial segments that failed to respond to TNS, pretreatment with NE (10−6 M) for 30 minutes did not render the tissue constrictive on TNS (Figure 5).

Discussion

Reports on the presence of 5-HT-containing nerves in large cerebral arteries have been inconsistent. Using immunohistochemical methods, 5-HT-LI nerves have been demonstrated to be present in large cerebral arteries of the rat and rabbit1−15,23 but Itakura et al14 did not find them in the cerebral arteries of the rat. In the present study, large cerebral arteries of the rabbit exhibited 5-HT-LI nerves with weak immunofluorescence when arteries contained blood in the lumen at the time of dissection and immersion fixation. However, no 5-HT-LI nerves were detected in arteries that were
perfused with physiological solution in situ to remove blood from the lumen before dissection and fixation. Similarly, 5-HT-LI nerves were not observed in the arteries fixed in situ by perfusion with fixative. One explanation for the latter finding may be that perfusion with the physiological solution or fixative may result in depletion of 5-HT from serotonergic nerves. This is unlikely, however, since similar perfusion procedures did not appreciably affect the CA-fluorescence nerves in cerebral arteries. These results indicate that authentic 5-HT-LI nerves are extremely sparse or not present in large cerebral arteries of the rabbit. The presence of dense 5-HT-containing nerves in the isolated cerebral blood vessel wall may therefore be related to procedures used to remove the artery prior to the fixation. It is interesting to note that in the reports by Griffith et al., Edvinsson et al., and Alafaci et al., the cerebral arteries were dissected and fixed by immersion in vitro, while in the work of Itakura et al., cerebral arteries were initially fixed by in situ perfusion.

Several studies have demonstrated that sympathetic adrenergic nerves in peripheral arteries can take up and store 5-HT. Perivascular nerves in cerebral arteries have also been shown to be capable of taking up radio-labelled 5-HT. In the present study, no 5-HT-LI nerve was found in the cerebral arteries dissected from
brains preperfused with Krebs solution. These cerebral arteries, however, after incubation with 5-HT in vitro exhibited dense mesh-like 5-HT-LI nerves similar to those of sympathetic adrenergic nerves. It is possible that 5-HT is taken up and stored in sympathetic nerves in cerebral arteries. This possibility was confirmed by the observation that both 5-HT-LI and noradrenergic nerves disappeared completely in cerebral arteries after chronic superior cervical ganglionectomy. Furthermore, the rabbit central ear artery, like the basilar artery, also exclusively receives sympathetic nerves. In these arteries, 5-HT-LI nerves appeared only after incubation with 5-HT. In the sympathetically denervated ear arteries, the 5-HT-LI nerves were not detected even after incubation with 5-HT. These results indicate that sympathetic adrenergic nerves in cerebral and ear arteries indeed can take up and store 5-HT. The exact source of 5-HT is not known. 5-HT may originate primarily from the circulation. During exsanguination or dissection, 5-HT may be released into and/or from the blood. It is known that platelets and mast cells contain 5-HT that can be released during various conditions.

It has been demonstrated that TNS-induced vasoconstriction of the rabbit basilar artery is blocked by guanethidine but is partially resistant to $\alpha$-adrenoceptor antagonists. Accordingly, TNS-induced vasoconstriction was suggested to be due to release of a...
second transmitter in addition to NE from the sympathetic nerve. It has been suggested that neuropeptide Y (NPY) may be the transmitter mediating vasoconstriction in cerebral arteries. Positive identification of this conclusion, however, has not been presented. Griffith et al further suggested that 5-HT was the transmitter substance responsible for neurogenic vasoconstriction in the rabbit vertebral artery since the TNS-induced vasoconstriction in this artery was blocked by ketanserin. This is in conflict with our previous report that the TNS-induced vasoconstriction in the rabbit basilar artery was resistant to ketanserin and methergoline. This latter finding further supports the presence of a nonserotonergic, nonadrenergic vasoconstrictor transmitter in the rabbit basilar artery. In the present study, TNS elicited constriction in 50% of the basilar arteries examined. The neurogenic vasoconstriction, as previously described, was resistant to ketanserin (Figure 4A). On the other hand, the arteries, which failed to constrict on TNS, became responsive.

**FIGURE 4.** The effect of ketanserin on the vasoconstriction induced by transmural nerve stimulation (TNS) at 8 and 16 Hz in the basilar artery before and after incubation with 5-HT (10^{-6} M). Panel A: TNS-induced constriction of the artery without 5-HT preincubation. The constriction was not blocked by ketanserin. Panel B: The basilar artery failed to respond on TNS. Following incubation with 5-HT (10^{-6} M) for 30 minutes, the artery became responsive with constriction on TNS at 8 and 16 Hz. The constrictions on TNS at both 8 and 16 Hz were reduced by ketanserin (10^{-7} M).

**FIGURE 5.** TNS-induced vasoconstriction in arteries pretreated with 5-HT (10^{-6} M) and NE (10^{-6} M). The basilar artery was divided into 3 segments. Each arterial segment is indicated by the heavy bar (arrowhead). All 3 segments failed to respond to TNS prior to incubation with 5-HT. 5-HT constricted the artery while NE at the same concentration did not cause a response. 5-HT and NE remained in the bath for 30 minutes before wash (W). TNS-induced constriction in arteries preincubated with 5-HT but not in those with NE. The TNS-induced constriction was abolished by guanethidine.
with constriction on TNS only after incubation with 5-HT. This neurogenic vasoconstriction was then blocked by ketanserin (Figure 4B), which suggests that vasoconstriction was due to release of stored 5-HT from the sympathetic nerves in the cerebral arterial wall. Taken together, these results demonstrate that 5-HT not only can be taken up by sympathetic nerves, but also can be released from sympathetic nerves to induce vasoconstriction. According to Baldessarini and Fischer, a substance can act as an alternative transmitter if the substance is not normally present but can be accumulated by nerve terminals and released on nerve impulse. 5-HT therefore may be considered as an alternative vasoconstrictor transmitter in the rabbit basilar artery. The nature of the transmitter(s) mediating the ketanserin-resistant vasoconstriction remains to be identified.

On the other hand, dense CA- or NE-fluorescence is always observed in the rabbit basilar arteries, and its appearance in the present study does not seem to be affected by the fixation procedure. The rabbit basilar artery, however, is very insensitive to NE. The arteries that failed to constrict on TNS still did not elicit constriction on TNS following incubation with NE, a result different from that of 5-HT-incubation. This result is consistent with the previous reports that NE is probably not the primary vasoconstrictor in cerebral sympathetic nerves. The transmitter role of NE in the cerebral sympathetic nerve remains unanswered.

The functional significance of 5-HT uptake into, and subsequent release from, sympathetic nerves in vivo is not known. Cerebrospinal fluid (CSF) in normal subjects has been shown to contain less than $10^{-9}$ M 5-HT. This concentration of 5-HT can cause a rise in CSF of patients with ruptured intracranial aneurysm. The enhanced 5-HT concentration in CSF may be the genesis of the initial vasospasm. Therefore, one important role of sympathetic nerves based on the results of the present study may be that these nerves serve as a "sink" for taking up vasoactive substance such as 5-HT and protect cerebral arteries from massive constriction.

On the other hand, the neuronally accumulated 5-HT may be released to act like a vasoconstrictor transmitter, also shown in the present study. It is known that 5-HT is involved in the pathogenesis of several cerebral vascular diseases. For example, it has been suggested that 5-HT is involved in the etiology of vascular headaches associated with the intracranial disorder headaches. Furthermore, vascular headache such as migraine, which is characterized by an initial intracranial arterial constriction, is sometimes prevented by 5-HT receptor antagonists such as methysergide and cyproheptadine, and adrenergic agents such as clonidine and propranolol. It should be pointed out that clonidine and propranolol have been shown to impair adrenergic neurotransmission by activating the inhibitory presynaptic $\alpha_2$-adrenoceptors and inhibiting the facilitatory presynaptic $\beta_2$-adrenoceptors, respectively. Although the presynaptic $\alpha$-adrenoceptors in rabbit cerebral arteries have not been positively clarified, the presynaptic $\alpha$-adrenoceptors in cerebral arteries of the cat have been shown to be of the $\alpha_2$ subtype. Our results have also demonstrated that neurogenic vasoconstriction in rabbit basilar arteries is potentiated by phentolamine,
Thus, the pathophysiologic role of 5-HT as an alternative vasoconstrictor transmitter in cerebral arteries, especially in the predominant presynaptic α2-adrenoceptors in the rabbit cerebral arteries. It has, however, been reported that clonidine induces vasodilatation of the rabbit middle cerebral arteries by stimulating postsynaptic histamine H2-receptors.9 The specific receptors in mediating the neurogenic effect of clonidine in cerebral blood vessels in the present study remain to be identified. However, in concentrations that did not affect the vessel tone, clonidine blocked the neurogenic vasoconstriction in arteries pretreated with 5-HT. Preliminary results obtained with 10−6 M propranolol were similar. These results emphasize that clonidine and propranolol block neurogenic vasoconstriction, most likely by acting presynaptically to interrupt 5-HT transmission or decrease release of 5-HT from the sympathetic nerves. Thus, the pathophysiologic role of 5-HT as an alternative vasoconstrictor transmitter in cerebral arteries, especially in those regions receiving dense sympathetic innervation, becomes evident and deserves further detailed examination.

Acknowledgments

We thank Susan Sarwinski for excellent technical assistance and Salie Fluckiger for preparing the manuscript.

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Key Words • serotonin • sympathetic nerves • cerebral artery • alternative transmitter
Serotonin as an alternative transmitter in sympathetic nerves of large cerebral arteries of the rabbit.
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Circ Res. 1987;60:220-228
doi: 10.1161/01.RES.60.2.220

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