Serotonin Release Into Plasma During Common Carotid Artery Thrombosis in Rats

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Background and Purpose: We have tested the hypothesis that platelet-derived serotonin is released into the bloodstream during cerebrovascular thrombosis.

Methods: Nonocclusive common carotid artery thrombosis was produced photochemically in 22 anesthetized adult male Wistar rats using the photosensitizing dye rose bengal and irradiation with an argon-pumped dye laser. Plasma serotonin levels were recorded before, during, and after photothrombosis by intra-arterial in vivo microdialysis with the probe placed distal to the site of thrombosis.

Results: During and immediately after the common carotid artery thrombosis, serotonin levels increased significantly to a peak value of 781 nmol/l (p<0.001 by analysis of variance), representing a 15-fold increase compared with baseline levels. The increased serotonin levels gradually decreased but remained significantly elevated for 90 minutes. Ultrastructural analysis of the carotid thrombi identified a dense mass of aggregated platelets at various stages of degranulation.

Conclusions: These results are the first to demonstrate directly that serotonin accumulation occurs in plasma during and after the acute phase of common carotid artery thrombosis. Increased plasma serotonin levels may play a major role in the cerebral blood flow and blood-brain barrier abnormalities previously documented in this model of large-vessel thrombotic stroke. (Stroke 1992;23:870-875)

Key Words • carotid arteries • cerebral ischemia, transient • serotonin • thrombosis • rats

It has been postulated that serotonin (5-hydroxytryptamine [5-HT]) is involved in the pathogenesis and the progression of stroke. In patients with cerebrovascular disorders, abnormal levels of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) have been documented in blood and cerebrospinal fluid samples. Reduced serotonin concentrations and increased levels of 5-HIAA in whole brain tissue extracts have also been observed.8 These alterations have been interpreted as a disturbed synthesis or release of serotonin from ischemic neurons. In experimentally induced cerebral ischemia, elevated levels of serotonin in the extracellular fluid have recently been documented using in vivo microdialysis techniques. Elevations in brain serotonin are important because 5-HT has been shown to potentiate the N-methyl-D-aspartate receptor site and thereby may potentially increase the vulnerability of neurons to excitotoxic processes. Further, serotonin is an important vasomodulator and has been shown to constrict or dilate cerebral vessels.

In addition to neuronally derived serotonin, platelets contain large amounts of serotonin that may be released during platelet aggregation and degranulation. Therefore, in the setting of cerebrovascular injury, endothelial damage and subsequent platelet activation may lead to increased levels of serotonin in the systemic circulation. Experimental studies determining whether elevated levels of serotonin can be demonstrated in the blood during cerebrovascular thrombosis have not been published previously.

To induce cerebrovascular thrombosis in the rat, a method of common carotid artery thrombosis has been developed. With nonocclusive common carotid artery (CCA) thrombosis, acute blood-brain barrier (BBB) leakage, abnormal patterns of local cerebral blood flow (ICBF), and embolic brain infarction have recently been documented. The microvascular consequences of CCA thrombosis have been shown to be mediated by blood-borne factors because similar hemodynamic and permeability abnormalities were induced in recipient rats receiving blood sampled downstream from a developing carotid thrombus. Various neuroactive and vasoactive substances including adenosine diphosphate and triphosphate, platelet activating factor, histamine,
vasopressin, thromboxane, and serotonin may be released in different proportions from aggregating platelets or from the blood coagulation system during thrombus formation.24 Because serotonin may potentially influence cerebrovascular permeability and ICBF, the purpose of this study was to characterize directly the temporal profile of serotonin concentration in downstream plasma in response to nonocclusive CCA thrombosis.

Materials and Methods
Experiments were performed on a total of 22 fasted adult male Wistar rats weighing between 225 and 325 g and heparinized with 50 units. Anesthesia was induced with 4% halothane for 5 minutes. Rats were then maintained on 1.5% halothane and a mixture of 70% nitrous oxide and 30% oxygen delivered by a closely fitting face mask. Femoral arterial and venous polyethylene catheters (PE-50) were inserted for measurements of arterial blood pressure and blood gases and for rose bengal administration. The rats were then intubated with PE-240 tubing and mechanically ventilated with 0.5% halothane, 70% nitrous oxide and 30% oxygen. Respiratory adjustments were made as needed to ensure normal arterial blood gases. Pancuronium bromide, 0.6 mg/kg, was injected intravenously, and additional doses of 0.2 mg/kg were administered to immobilize the animal. Rectal temperature was maintained at 36.7°C by means of a rectal thermistor probe and a thermostatically regulated heating pad (CMA/140, Carnegie Medicine, Stockholm, Sweden).

The right CCA and external carotid artery were exposed under a Zeiss operating microscope. The neck region containing the exposed CCA formed a cavity that was filled with saline. A microdialysis guide (CMA/50) was inserted for measurements of arterial blood pressure and blood gases and for rose bengal administration. The rats were then intubated with PE-240 tubing and mechanically ventilated with 0.5% halothane, 70% nitrous oxide and 30% oxygen. Respiratory adjustments were made as needed to ensure normal arterial blood gases. Pancuronium bromide, 0.6 mg/kg, was injected intravenously, and additional doses of 0.2 mg/kg were administered to immobilize the animal. Rectal temperature was maintained at 36.7°C by means of a rectal thermistor probe and a thermostatically regulated heating pad (CMA/140, Carnegie Medicine, Stockholm, Sweden).

The shaft of the microdialysis probe was thereby placed in the external carotid artery, and the active microdialysis probe tip was placed in the distal CCA. The microdialysis probe was perfused with Ringer's solution at a flow rate of 2.0 μl/min. After half an hour of equilibration, baseline recordings were collected over a 2-hour period before photothermal injury.

The photosensitizing dye rose bengal (15 mg/ml in 0.9% saline) was injected intravenously to a body dose of 20 mg/kg over 2 minutes simultaneously with the start of photothermal injury. The right CCA was irradiated for 10 minutes (n = 7) with the focused beam of a tuneable argon-pumped dye laser (Innova 70-4/CR599, Coherent, Calif.) operated at 562 nm and focused rectangularly (~1.5 x 1 mm) on the right CCA approximately 7 mm proximal to the CCA bifurcation. The laser was used at a power of 325 mW, corresponding to an average intensity of 22 W/cm² focused according to the efficiency criterion described previously.26 Control rats (n = 6) underwent the same surgical procedure and were infused with rose bengal, but no irradiation was conducted. To determine whether the photochemical injury produced significant elevations in blood temperature, direct temperature measurements were conducted in four additional rats using a temperature probe placed downstream from the irradiation site.

Microdialysis samples were collected at 30-minute intervals before and at 10 minutes during and after the first hour of CCA thrombosis, and thereafter at 30-minute intervals for an additional 2 hours. Samples were frozen and stored at −80°C until biochemical measurements were undertaken. Each collecting tube contained 5 or 30 μl (depending on the 10- or 30-minute sampling period) of 0.1 M perchloric acid and 8 mM L-cysteine. The mixture of perchloric acid and high concentrations of L-cysteine stabilized both catecholamines and indoleamines at room temperature for at least 12 hours. In addition, samples collected in this way resulted in over 90% stability of all major monoamines, their precursors, and metabolites when frozen for up to 2 months and then thawed. Concentrations of serotonin were determined by reversed-phase isocratic liquid chromatography with electrochemical detection slightly modified compared with previous methods described elsewhere.25-27 Briefly, the isocratic mobile phase, with a flow rate of 1.2 ml/min, consisted of a 100-mM citrate buffer including 0.3 mM Na₂EDTA, 0.334 mM octylsulfate, and 0.00940% (vol/vol) ascorbic acid at a pH of 2.35. The stationary phase consisted of a 150 x 4.6-mm stainless-steel column packed with 5 μm Nucleosil C-18 (Macherey-Nagel & Co. K.G., Düren, FRG). The substances were detected electrochemically with a BAS 3 electrochemical detector (Bioanalytical Systems, West Lafayette, Ind.) operated at a potential of +0.75 V versus Ag/AgCl reference electrode. The detector was coupled to an integrator (model SP4270, Spectraphysics, San Jose, Calif.). The recovery of 5-HT as measured in vitro at 37°C was found to be 18%. Transmitter concentrations presented are compensated for the in vitro recovery.

To document the acute morphological characteristics of the carotid thrombus, five rats were perfusion-fixed 10 minutes after thrombus formation for light and electron microscopic examination, as previously described.21 For scanning electron microscopy (n = 2), the CCA was first cut longitudinally to expose the luminal thrombus and microdialysis probe. Specimens were then dehydrated in graded ethanol, placed in Freon, and critical-point dried. After gold coating, arteries were examined and photographed with a JEOL JSM-35C scanning electron microscope. Specimens chosen for plastic embedding (n = 3) were dehydrated in ethanol and propylene oxide and embedded in EM bed 812. Thick plastic sections (2 μm) through the epicenter of the thrombus were cut and stained with toluidine blue. Thin sections were then sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10C TEM.

An analysis of variance (ANOVA) and post hoc analyses were performed with the system for statistics (SYSTAT).28 Because of multiple comparisons, the Bonferroni correction principle was used with the level of significance set at 0.005.

Results
Physiological findings recorded just before rose bengal injection (controls) and photochemically-induced CCA thrombosis were as follows (mean±SD): PaO₂
FIGURE 1. Light and electron micrographs of common carotid artery 10 minutes after photochemically induced thrombosis. Panel a: Scanning electron micrograph showing relation between carotid thrombus (T) and microdialysis probe (DP). Magnification, ×350. Panel b: Higher magnification of carotid thrombus showing aggregated platelets with pseudopodia. Magnification, ×9,000. Panel c: Thick plastic section of thrombus occupying approximately 70% of luminal (L) area. Toluidine blue stain. Magnification, ×400. Panel d: Transmission electron micrograph of degranulated platelets with clear cytoplasmic vacuoles. Magnification, ×12,300.

154±22.8 and 149±7.1 mm Hg; PaCO₂ 37.9±3.89 and 39.2±1.54 mm Hg; and pH 7.39±0.05 and 7.40±0.02 in the control and thrombus groups, respectively. Continuous mean arterial blood pressure monitoring before, during, and after rose bengal infusion with (93.3±7.2 mm Hg) or without (95.0±4.5 mm Hg) laser irradiation revealed no significant changes during the experiment. In animals in which the blood temperature was measured before, during, and after thrombus formation, a very modest temperature increase from a preirradiation
value of $37.2\pm0.1$ to $37.7\pm0.1 ^\circ C$ was observed during the 10-minute irradiation period. Immediately after irradiation, temperature returned to preirradiation levels. During CCA thrombosis, the blood flow was confirmed to move in an anterograde direction over the thrombosed CCA segment by visual inspection under the operating microscope.

Scanning electron microscopic examination of thrombosed carotid arteries identified a large platelet thrombus at the site of carotid irradiation (Figures 1A and 1B). Remote from the thrombus, the microdialysis probe appeared smooth and relatively free of adhering platelets. Examination of thick plastic sections revealed a fairly homogeneous aggregate of platelets occupying 50–75% of the luminal area (Figure 1C). Transmission electron microscopy of the thrombus identified aggregated platelets with well-developed pseudopodia at different stages of degranulation (Figure 1D).

Stable baseline concentrations of plasma 5-HT were detected in four consecutive samples collected before CCA thrombosis (mean $51.4\pm7.8$ nmol/l of perfusate). During and immediately after the nonocclusive CCA thrombosis, 5-HT levels in plasma increased significantly to a maximum of $781\pm159$ nmol/l ($p<0.001$ by ANOVA), representing a 15-fold increase (Figure 2). The increased 5-HT levels gradually decreased but remained significantly elevated for 90 minutes ($p<0.005$). However, as shown in Figure 2, the serotonin concentrations did not reach baseline levels during the 3-hour postirradiation period. In control rats, plasma levels of 5-HT were stable during baseline recordings as well as during and after intravenous administration of rose bengal (Figure 2) ($p>0.1$ by ANOVA).

Discussion

These results show a substantial release of serotonin into the downstream plasma during experimentally induced CCA thrombosis. The microdialysis technique, originally developed by Ungerstedt, was used in the present study for intra-arterial recordings. This new approach for measuring arterial blood substances has several advantages. First, temporal profiles of serotonin release over several hours can be documented by obtaining multiple samples from the same animal without altering blood volume. Second, direct liquid chromatographic plasma-5HT measurements can be performed without any further handling of samples. This is important because additional plasma extraction procedures are time consuming and may result in variable serotonin concentrations. In sham-operated control animals, plasma 5-HT concentrations were stable during the 5-hour sampling period and agreed with earlier published values. Scanning electron microscopy of the microdialysis probe revealed a relatively smooth membrane surface devoid of thrombotic material. Thus, local thrombosis at the site of the microdialysis probe tip was not responsible for the elevated plasma serotonin levels seen after CCA thrombosis.

The photochemical insult used in this study is a consequence of light-induced excitation of the endothelium-bound rose bengal dye. This light–dye reaction is considered to result in singlet molecular oxygen production leading to direct peroxidation of endothelial fatty acids and proteins. The endothelial damage leads to massive platelet aggregation at the luminal surface of the vessel wall. In this study, a very modest increase in blood temperature ($0.5 ^\circ C$) was demonstrated during CCA thrombosis immediately distal to the developing thrombus. This small increase in blood temperature strongly suggests that thermal effects are not primarily involved in thrombus formation using rose bengal and laser irradiation at its 562-nm absorbance peak in tissue.

Increased plasma serotonin concentrations after carotid thrombosis are consistent with previous reports using other methods of thrombus formation in canine coronary arteries. In one study, a 40-fold increase...
of downstream plasma 5-HT was observed as a consequence of complete coronary occlusion induced by electrical stimulation of the vessel wall. Interestingly, that study reported a significant elevation of plasma 5-HT when a nonocclusive thrombosis with 25–30% obstruction of the coronary artery lumen was produced. In the present study, a 15-fold increase in plasma serotonin levels was observed after a 50–75% occlusion of the CCA. It is therefore possible that the degree of stenosis during thrombus formation may critically determine whether elevated levels of plasma serotonin can be detected. A nonlinear relation between the degree of stenosis, platelet aggregation, and elevated plasma serotonin may therefore exist. Because increased 5-HT enhances platelet aggregation through platelet 5-HT receptor activation,18 a vicious cycle of platelet degranulation and subsequent serotonin release. This suggestion is supported by the fact that the half-life of blood serotonin is relatively short (1–2 minutes).55

Elevated levels of plasma serotonin after vascular injury may contribute to various pathophysiological processes. For example, in some studies serotonin has been shown to affect BBB function.36–38 In one investigation, serotonin infusion (10–20 μg/kg/min during 10 minutes) into the jugular vein caused a transient leakage of the BBB to iodine-131-labeled sodium that was blocked by the 5-HT receptor antagonist cyproheptadine.39 Interestingly, the serotonin concentration required to induce BBB leakage in that study is similar to the peak plasma 5-HT values measured in the present study during CCA thrombosis. Thus, if one assumes that 5-HT is evenly distributed in the blood and that a 300-g rat contains approximately 17.1 ml blood,19 a 10–20 μg/kg/min intravenous infusion of 5-HT would be equivalent to a plasma level of 453–906 nmol/l/min. However, because not all studies have demonstrated serotonin-induced BBB abnormalities,40,41 further investigation is required to establish the conditions under which plasma 5-HT, perhaps in concert with other vasoactive substances, may induce BBB dysfunction.

In addition to permeability changes, serotonin is an important vasomodulator and has been shown to have complex effects on various vascular beds.16–18 In many studies, the topical application of serotonin onto pial vessels or in vitro vessel preparations has been used to demonstrate a vascular response to 5-HT. In contrast, intravascular infusion of serotonin has been shown to produce inconsistent alterations in ICBF depending on animal species, 5-HT concentrations, routes of 5-HT administration, and the integrity of the BBB.38,42–45 For example, when the BBB is artificially perturbed, widespread reductions in ICBF have been documented with serotonin infusion.42–44 The elevated levels of plasma serotonin reported in the present study might therefore be expected to produce alterations in ICBF if BBB dysfunction occurs after CCA thrombosis. In this regard, studies have demonstrated both BBB breakdown and alterations in ICBF immediately after photochemically induced CCA thrombosis.21,23

In conclusion, increased serotonin concentrations in downstream plasma in response to CCA thrombosis have been demonstrated. The serotonin increase may be of importance as a mediator of BBB disruption, edema formation and abnormal cerebral blood flow after vascular injury. In the clinical setting, blood-borne factors released from the site of vascular thrombosis might be expected to play a major role in the pathophysiology of acute stroke and transient ischemic attacks. Based on experimental findings obtained using the present model of carotid thrombosis, it is anticipated that therapeutic strategies for acute stroke should involve thrombolysis and specific pharmacological agents that antagonize the effects of vasoactive substances.

Acknowledgments

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References

18. Garrison JC: Histamine, bradykinin, 5-hydroxytryptamine, and their antagonists, in Gilman AG, Rall TW, Nies AS, Taylor P
vigorously to serotonin than do normal vessels. For example, it was shown by Faraci et al. 1 that systemic atherosclerotic cerebral vessels respond much more to cause modest vasoconstriction. It is known that concentration of serotonin in the blood that would be expected to thrombus. There was a resultant increase in the concentration of serotonin in the blood of sera to serotonin in monkeys. 1


Editorial Comment

The established mechanisms by which thrombosis of large cerebral arteries can affect the cerebral circulation include the mechanical obstruction caused by the thrombus as well as embolization of downstream vessels from fragments of the thrombus. The study by Wester and colleagues points out another mechanism by which arterial thrombosis can affect the cerebral circulation. These authors demonstrated the release of serotonin into the bloodstream from platelets incorporated in the thrombus. There was a resultant increase in the concentration of serotonin in the blood that would be expected to cause modest vasoconstriction. It is known that atherosclerotic cerebral vessels respond much more vigorously to serotonin than do normal vessels. For example, it was shown by Faraci et al. that systemic administration of serotonin in doses that were not vasoactive in normal animals caused an 81% reduction in retinal blood flow in atherosclerotic monkeys, a change that would be expected to have functional consequences. It is likely that further investigation will show that additional chemical constituents of the platelets may have similar adverse effects on the cerebral circulation.

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Reference