Ischemia-Induced Extracellular Release of Serotonin Plays a Role in CA1 Neuronal Cell Death in Rats

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Background and Purpose: Serotonin, via 5-HT₂ receptors, exerts an excitatory effect on CA1 neurons and may play a role in ischemia-induced excitotoxic damage. To evaluate the role of serotonin in ischemia, both neurochemical and histopathological studies were performed.

Methods: Neurochemical studies included rats that were subjected to 12.5 or 20 minutes of normothermic ischemia by two-vessel occlusion plus hypotension, and extracellular serotonin levels were measured in the hippocampus (12.5 minutes' ischemia, n=5) or striatum (20 minutes' ischemia, n=13) by microdialysis. In the histopathological study the effect of 8 mg/kg ritanserin, a 5-HT₂ antagonist, administered continuously from 30 minutes prior to ischemia until 1 hour of recirculation was evaluated in five rats subjected to 10 minutes of ischemia. After 3 days, the numbers of normal-appearing neurons in the CA1 subregions were counted.

Results: Ischemia of 12.5 minutes' duration induced a fourfold increase in serotonin in the hippocampus (mean±SEM baseline, 1.86±0.25 pmol/ml perfusate; during ischemia, 8.14±0.89 pmol/ml; p<0.05 by analysis of variance). Twenty minutes of ischemia induced a 25-fold increase in serotonin in the dorsolateral striatum (baseline, 0.98±0.15 pmol/ml; ischemia, 24.4±5.93 pmol/ml; p<0.001). The histopathological study demonstrated severe ischemic damage in all CA1 subregions of nontreated animals (medial, 34±16 normal-appearing neurons, middle, 52.2±22.9 neurons; lateral, 56.6±21.8 neurons). Treatment with ritanserin significantly attenuated ischemic damage (medial, 117.6±6.5 neurons; middle, 131.4±4.9 neurons; lateral, 130±7.5 neurons; p<0.01 different from nontreated).

Conclusions: Taken together, these results suggest that serotonin plays a detrimental role, mediated by 5-HT₂ receptors, in the development of ischemic damage. (Stroke 1992;23:1595–1601)

KEY WORDS • cerebral ischemia • ritanserin • serotonin • rats
ischemic outcome. In support of this hypothesis, several studies have shown the beneficial effect of blocking serotonin function in various models of brain injury. Pretreatment with the serotonin neurotoxin p-chlorophenylalanine attenuates the behavioral consequences of unilateral common carotid artery occlusion in gerbils. Using a spinal cord ischemia model in rabbits, early posts ischemic treatment with a serotoninergic antagonist was shown to prevent or reduce spinal cord damage assessed behaviorally. Taken together, these results suggest that serotonin may play an important role in the pathogenesis of ischemia, although the mechanism of action is not clear. In the present study, we evaluated the effect of transient global ischemia on the extracellular levels of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) in two vulnerable brain regions, the dorsal hippocampus and the dorsolateral striatum. To evaluate the importance of these neurochemical changes we determined whether specific 5-HT2 blockers can attenuate ischemic neuronal damage in the dorsal hippocampus.

Materials and Methods

Male Wistar rats weighing 280–300 g were subjected to 12.5 or 20 minutes of global cerebral ischemia by two-vessel occlusion combined with systemic hypotension. Femoral vessels were first cannulated under halothane anesthesia, and polyethylene ligatures were placed around each common carotid artery. The rats were then intubated and mechanically ventilated to maintain PaCO2 and PaO2 within the normal ranges. The animals were then mounted on a stereotoxic frame (David Kopf Instruments, Tujunga, Calif.), and microdialysis probes (CMA/10, 3 mm dialysis membrane, Carnegie Medicin, Sweden) were stereotaxically implanted in either the dorsal hippocampus or the dorsolateral striatum. The probes were subsequently perfused with Ringer's solution at a flow rate of 2 μl/min by means of a microinfusion pump (Carnegie Medicin). The animals were maintained on 1% halothane combined with nitrous oxide and oxygen and were immobilized with 0.6 mg/kg i.v. pancuronium bromide; additional doses of 0.3 mg/kg were administered at half-hour intervals.

Following a 2-hour stabilization period, 12.5 (n = 5) or 20 (n = 13) minutes of ischemia were produced by tightening the carotid ligatures bilaterally and maintaining the mean arterial blood pressure at 45–50 mm Hg by gradual withdrawal of blood. Brain temperature was carefully regulated at 36–37°C throughout the experiment as we have previously shown brain temperature to be a critical determinant of ischemic outcome. To permit posts ischemic reperfusion, the carotid ligatures were removed and the blood, kept at 36–37°C, was reinfused to restore mean arterial blood pressure to normal values. In the hippocampus, samples of the microdialysis perfusate, representing extracellular fluid, were collected at 10-minute intervals during the 30 minutes before ischemia, during the 12.5 minutes of ischemia, and during the first 30 minutes and at the end of 1–4 hours of reperfusion. In the striatum microdialysis samples were collected at 10-minute intervals during the 30 minutes prior to ischemia, during the 20 minutes of ischemia, and during the first 30 minutes and at the end of every 30-minute period for up to 3 hours of reperfusion. All samples were immediately frozen and stored at −80°C until analysis. The location of the microdialysis probe was verified histologically in each animal at the end of the experiment.

Concentrations of serotonin and 5-HIAA were determined by reverse-phase isocratic liquid chromatography with electrochemical detection, slightly modified compared with previous methods described elsewhere. 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 ethylenediaminetetraacetic acid, 0.334 mM octylsulfate, and 6%/94% (vol/vol) acetonitrile/water at a pH of 2.35. The stationary phase consisted of a 150×4.6 mm stainless steel column packed with 5 μM NuSorb C-18 (Macherey-Nagel and Co., Duren, FRG). The substances were detected electrochemically with a BAS 3 electrochemical detector (Bioanalytical Systems, Inc., West Lafayette, Ind.) operated at a potential of 0.75 V versus an Ag/AgCl reference electrode. The detector was coupled to a model SP 4270 integrator (Spectrophysics, San Jose, Calif.).

For the histopathological studies, the rats were subjected to 10 minutes of ischemia and allowed to survive for 72 hours. Two groups of animals were included, a nontreated ischemic group (n = 5) and a group (n = 5) treated with 8 mg/kg ritanserin administered intravenously continuously from 30 minutes prior to ischemia until 1 hour of recirculation. After 3 days of reperfusion the animals were transcardially perfused with a mixture of 40% formaldehyde, glacial acetic acid, and absolute methanol (1:1:8 by volume). Brain sections were then stained with hematoxylin and eosin and examined for ischemic cell changes by an observer blinded to the experimental conditions. For quantitative histopathological analysis of the CA1 sector of the hippocampus, this structure was divided into three equal segments (medial, middle, and lateral). In each segment, the number of normal-appearing CA1 neurons per 1,000 μm length of stratum pyramidale was counted.

The effects of ischemia on serotonin and 5-HIAA levels were assessed by comparison of the microdialysis data during and after ischemia with baseline levels using analysis of variance (ANOVA), followed by Scheffé and Dunn tests. The effect of ritanserin on the histopathological outcome was evaluated by intergroup comparison of the number of normal-appearing neurons by ANOVA. The level of significance was set at 0.05. Results are given as mean±SEM.

Results

Microdialysis sampling was carried out before, during, and after ischemia. The time courses of changes in the extracellular levels of serotonin and 5-HIAA in the hippocampus and striatum during 12.5- and 20-minute ischemic insults, respectively, are shown in Figures 1 and 2. In both regions, stable levels of serotonin and 5-HIAA were detected during the 30 minutes prior to the ischemic insult. In the hippocampus, 12.5 minutes of ischemia induced a fourfold increase in serotonin levels (baseline, 1.86±0.25; ischemia, 8.14±0.89 pmol/ml perfusate). Serotonin levels gradually returned to baseline by 20–30 minutes of reperfusion and stayed within normal limits for up to 4 hours of recirculation. In parallel to the increase in serotonin levels during ischemia, a significant 54% decrease in extracellular...
5-HIAA levels was observed during the first 10 minutes of recirculation. In the striatum, 20 minutes of ischemia induced an even greater increase in extracellular serotonin levels. Serotonin levels rose from a baseline of 0.98±0.15 to 24.4±5.93 pmol/ml perfusate during ischemia, representing a 25-fold increase. The temporal profiles were similar in the striatum and hippocampus; serotonin levels normalized by 30 minutes of recirculation and stayed within the normal limits for up to 3 hours of reperfusion. In the striatum, the ischemia-induced increases in serotonin levels were accompanied by a 64% decrease in 5-HIAA levels. Levels of 5-HIAA normalized during early reperfusion.

The effects of 10 minutes of ischemia on the magnitude of histopathological damage in the three subregions of the hippocampus are depicted in Figures 3 and 4. Ten minutes of ischemia produced severe damage in the CA1 sector of the hippocampus in all animals. As shown in Figures 3 and 4, few normal-appearing neurons were detected in any CA1 subregion, and the number of normal neurons was decreased significantly compared with nonischemic controls. In rats treated with ritanserin prior to ischemia, normal-appearing neurons were detected throughout the CA1 subregions (Figures 3 and 4). Significant differences in the number of preserved neurons were documented between the treated and nontreated groups.

**Discussion**

We have previously demonstrated that during transient forebrain ischemia the extracellular concentrations of various neurotransmitters are markedly increased.4,7,9 These neurotransmitters include glutamate, \( \gamma \)-aminobutyric acid, glycine, dopamine, and norepinephrine. In the present study, we describe the effects of moderate-to-severe transient global ischemia on extracellular serotonin and 5-HIAA. Basal levels of serotonin and 5-HIAA were within the ranges of earlier published data.34 Ischemia induced a significant increase in the levels of serotonin, in both the dorsal hippocampus and the dorsolateral striatum. The rise in serotonin concentration was observed during the ischemic period, and the magnitude of the increase was dependent on the duration of ischemia; changes induced by 20 minutes of ischemia were significantly greater than those following 12.5 minutes of ischemia. Similar results have been described in other ischemia models.25-28

The extracellular levels of serotonin are determined by the balance between release and uptake. Under
normal conditions, serotonin release from nerve endings is triggered by calcium influx via voltage-operated calcium channels in the presynaptic plasma membrane. Both glial cells and neuronal presynaptic and postsynaptic elements have a high-affinity serotonin uptake system. Thus, during ischemia energy failure and membrane depolarization probably trigger the surge in extracellular serotonin, via both increased presynaptic influx and a perturbed uptake process. An additional source, which may contribute to the elevated serotonin levels, includes leakage of plasma serotonin through a disrupted blood–brain barrier. Finally, platelets activated during ischemia may provide an additional source of serotonin. The two latter sources of increased serotonin may be of particular importance in the setting of acute thrombus formation by activated platelets, which has been recently demonstrated to be associated with release of serotonin into the plasma. The rapid decrease of dialysate serotonin levels during reperfusion is consistent with reinstatement of active...
reuptake mechanisms on termination of the ischemic insult and with clearance of serotonin into the cerebrospinal fluid and systemic circulation. The mechanisms underlying the marked decreases in extracellular 5-HIAA during ischemia are also not clearly understood. These decreases likely reflect ischemia-induced attenuation of monoamine oxidase activity, leading to a reduction of oxidative deamination. During reperfusion, deamination is restored and levels of 5-HIAA return to baseline.

The importance of ischemia-induced increases in extracellular serotonin is underscored by the results of the histopathological study, demonstrating that blocking the 5-HT₂ receptor has a significant beneficial effect on preserving the integrity of the CA1 neurons after 10 minutes of global ischemia in rats. Ritalserin, at the dose we used, acts predominantly at the 5-HT₁ receptors; only at higher doses does the drug affect the dopaminergic or serotonergic systems. These studies also demonstrate that systemic administration of ritalserin has central effects evaluated by ex vivo studies, suggesting that the drug preferentially affects the blood-brain barrier. Previous studies have demonstrated that administration of a 5-HT₁ agonist can confer protection during both global and focal ischemia. Since the 5-HT₁ receptors are presynaptic and control by a feedback mechanism the amount of serotonin released, the protective effect of an agonist may involve attenuation of ischemia-induced serotonin release. Our results demonstrate that in addition to the 5-HT₁ agonists, the 5-HT₂ antagonists are also protective against ischemic neuronal damage. The neuroprotective effect of ritalserin is in accordance with the effect of other mixed- and nonselective 5-HT₂ receptor antagonists, such as nafidrofuryl and emopamil, investigated in a variety of ischemia models. While the exact mechanism by which serotonin plays a detrimental role in the setting of ischemia remains unclear, several mechanisms can be contemplated.

Imbalance between excitation and inhibition induced by the interaction of several neurotransmitters has been suggested to play a major role in the development of ischemic neuronal damage in vulnerable brain regions such as the CA1 sector of the hippocampus. Serotonin has been described as having a potentiating effect on neuronal excitation by glutamate in different brain regions. High concentrations of serotonin have been shown to depolarize motor neurons for activation of 5-HT₂ receptors. In the neocortex, serotonin potentiates the release of excitatory amino acids and has also been demonstrated. N-Methyl-D-aspartate (NMDA), quisqualate, and glutamate depolarizations were all enhanced by iontophoretically or topically applied serotonin in neocortical slices from cats. A similar facilitatory effect of serotonin on the electrophysiological response to NMDA was demonstrated in neocortical neurons. One can postulate, therefore, that the protective effect of a 5-HT₂ blocker may involve reduction of the excitotoxic process induced by glutamate. However, the distribution of 5-HT₂ receptors in the hippocampus as revealed by autoradiographic studies in rat and human brains indicates that the density of 5-HT₂ receptors in the CA1 sector is relatively low compared with the cortex. This diminishes the probability that the beneficial effect of the 5-HT₂ antagonist involves a direct effect on CA1 neurons. Interestingly, the entorhinal cortex contains a high concentration of 5-HT₁ receptors. Since the major excitatory input to the hippocampus is from the entorhinal cortex, it can be assumed that ritalserin exerts its neuroprotective effects by depression of the entorhinal output. This hypothesis is supported by studies demonstrating that both lesions of the entorhinal afferents and destruction of the entorhinal cortex protect CA1 pyramidal neurons from ischemic degeneration. Therefore, 5-HT₂ antagonists may protect by depressing CA1 neuronal hyperexcitation by inhibiting the action of neurons in the dentate gyrus and/or entorhinal cortex.

Cerebral blood vessels are innervated by serotonergic neurons and there is evidence that this innervation arises, at least in part, from neuronal processes originating at mesencephalic sites. In addition to its activity as a neuromodulator, serotonin is a potent vasoconstrictor and its release during ischemia may have a significant vasoconstrictor effect, leading to decreased blood flow and aggravating ischemic neuronal damage. Furthermore, the vasoconstrictor effect of serotonin can be augmented during ischemia and recirculation because of endothelial damage. The vasoconstrictor effect of serotonin is known to be mediated by 5-HT₂ receptors. This suggests that a possible mechanism underlying the protective effect of serotonin involves blocking the 5-HT₂ receptor located in blood vessels and attenuating the blood flow reduction induced by the excessive release of serotonin during ischemia. In support of this hypothesis, it has been demonstrated that ketanserin prevents decreases in cerebral blood flow that are observed in cortical areas remote from thrombotic infarction.

We and others have demonstrated that mild decreases in brain temperature can have a protective effect on ischemic neuronal damage. It is well known that serotonin is involved in thermoregulation. The possible role of serotonin in thermoregulation has also been investigated with relation to 5-HT receptor subtypes. Activation of the central 5-HT₁ receptor elicits hypothermia, while stimulation of the central 5-HT₂ receptor leads to hyperthermia in rats. This may suggest that the protective effect of a 5-HT₂ antagonist may also involve reducing hyperthermia in animals. However, in our experimental protocols, brain temperature was carefully maintained at normothermic levels, which excludes possible hypothalamic effects underlying the protective effect of the drug.

Recently it has been demonstrated that serotonin stimulates the release of arachidonic acid in hippocampal neurons. This pharmacological study suggests that the release of arachidonic acid and inositol phosphate was mediated by 5-HT₂ receptors. Arachidonic acid and its eicosanoid metabolites have also been implicated in a number of pathophysiological processes, including hypoxia/ischemia, and are released in the brain during stroke and seizures. Therefore, the detrimental effects of serotonin may potentially be mediated through receptor-generated arachidonic acid or eicosanoids. The protective effect of 5-HT₂ antagonists may involve this route.

In summary, our results demonstrate that moderate-to-severe ischemia induces a significant increase in extracellular serotonin levels and that blocking the
5-HT_{2} receptor confers protection from ischemic neuronal damage in the CA1 sector of the hippocampus. The mechanism underlying the detrimental effect of serotonin may involve both neuronal and vascular actions, and further studies are necessary to clarify the contribution of each component.

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This paper provides neurochemical evidence that cerebral ischemia and reperfusion are accompanied by the release of serotonin and histopathological evidence that a 5-HT2 receptor antagonist reduces ischemic damage of CA1 neurons. In relation to pathophysiology, the findings are important because they suggest that serotonin may contribute to ischemic damage. In relation to therapy, the findings are important because they imply that a 5-HT2 receptor antagonist may be useful in attenuation of ischemic damage.

Several mechanisms may contribute to the detrimental effect of serotonin during ischemia and reperfusion. First, serotonin may produce neuronal excitation, either directly or by release of excitatory neurotransmitters. Second, ischemia/reperfusion impairs cerebral vasodilator mechanisms (especially endothelium-dependent vasodilatation) so that vasoconstrictor effects of serotonin may not be opposed by vasodilator responses to other stimuli. Third, sympathetic nerves to cerebral blood vessels take up serotonin. It is possible that serotonin that is released during cerebral ischemia/reperfusion may be taken up by nerves, released, and produce pronounced cerebral vasoconstriction.

This study has exciting implications for therapeutic approaches, but before therapeutic uses are considered, it probably will be necessary to clarify mechanisms by which 5-HT2 receptor antagonists are protective after ischemia/reperfusion. It seems likely that therapeutic approaches will differ if the detrimental effect of serotonin is mediated by neuronal excitation, impaired cerebral vasodilator responses, uptake and release of serotonin by sympathetic nerve endings, or release of nitric oxide. Clarification of these mechanisms may lead to useful therapy after cerebral ischemia/reperfusion.

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