Histamine H₂-Receptor Antagonist Ranitidine Protects Against Neural Death Induced by Oxygen-Glucose Deprivation

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Background and Purpose—Administration of histamine receptor antagonists has been reported to produce contradictory results, either reducing or increasing neural damage induced by ischemia. In this study, we investigated the neuroprotective effects of histamine H₂-receptor antagonists in an “in vitro” model of ischemia.

Methods—Cultured rat brain cortical neurons were exposed to oxygen-glucose deprivation (OGD) in the presence or absence of different histaminergic drugs. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay. Necrosis and apoptosis were quantified by staining cells with propidium iodide and Hoechst 33258. Caspase 3 activation was determined by immunocytochemistry and Western blot.

Results—Pretreatment with H₂ antagonists effectively reduced neuronal cell death induced by OGD. Ranitidine decreased the number of necrotic and apoptotic cells. Caspase 3 activation and alteration of the neuronal cytoskeleton were also prevented by ranitidine pretreatment. The neuroprotective effect of ranitidine was still evident when added 6 hours after OGD.

Conclusions—H₂-receptor antagonists protected against OGD-induced neuronal death. Ranitidine attenuated cell death even when administered after OGD. These data suggest that this drug, which is currently used for the treatment of gastric ulcers, may be useful in promoting recovery after ischemia. (Stroke. 2004;35:2396-2401.)

Key Words: apoptosis □ cerebral ischemia □ neuronal death □ stroke

Cerebral ischemia triggers a sudden and severe necrotic cell death and a delayed neuronal degeneration with apoptotic features.¹ The major cause of cellular injury is thought to be excess glutamate release² mainly caused by the reverse function of its transporters³ and the consequent overactivation of glutamate receptors. In addition, there are other factors, such as cytokines or other released neurotransmitters, that may modulate the ischemic lesion.⁴,⁵

Histamine (HA) plays an important role as a neurotransmitter and as a neuromodulator in the mammalian brain under normal and pathological conditions.⁶ During the past decade, the histaminergic system has been implicated in the modulation of cell death in brain disorders. However, the exact mechanisms underlying HA action on ischemia-induced damage have not yet been fully defined. In this respect, it has been reported that activation of HA H₂-receptors leads to excitatory effects through a blockage of calcium-dependent potassium channels and the modulation of hyperpolarization-activated cation channels.⁷ Also, some reports have described that HA could positively modulate N-methyl-D-aspartate (NMDA) receptors,⁸,⁹ which are believed to mediate most of the ischemic-dependent neuronal injury.¹¹

HA receptor blockage in ischemia has provided contradictory results. Intraperitoneal administration of HA H₂-receptor antagonists⁹ decreases brain edema formation after a common carotid artery occlusion. In contrast, there are some reports that describe adverse effects of these compounds. HA H₂-receptor blockage in cerebral ischemia in gerbil has been reported to enhance neuronal damage.¹⁰,¹¹ Moreover, Otsuka et al reported that this aggravating effect was associated with a facilitation of catecholamine metabolism in the rat.¹² On the other hand, HA H₁-receptor antagonists attenuated the ischemia-induced decrease in glucose metabolism in rat hippocampal slices.¹³

In this context, the aim of this study was to explore the possible role of HA receptor antagonists on cell death in an in vitro model of cerebral ischemia on the basis of oxygen-glucose deprivation (OGD) exposure. We show here that pretreatment and post-treatment with HA H₂-receptor antagonists reduces cell death and also prevents caspase 3 activation.

Materials and Methods

Chemicals
Ranitidine, HA, propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were provided by...
Cultures were deprived of oxygen and glucose as described with modifications. The culture medium was replaced by a glucose-free Earle’s balanced salt solution (BSS) with the following composition (in mmol/L): NaCl 116, KCl 5.4, MgSO4·7H2O 1, NaH2PO4·2H2O 26.2, NaHCO3 0.01, glycine 1.8, CaCl2·2H2O and pH 7.4, which was previously saturated with 95% air/5% CO2 atmosphere for 7 days in vitro (DIV), when the plating medium was replaced by BME, supplemented as above without FCS and with 10% horse serum containing 10 μmol/L cytosine arabinoside. All experiments were performed with mature cultures (13 to 14 DIV). The procedures followed were in accordance with guidelines of the Comissió d’Ética en l’Experimentació Animal i Humana of the Universitat Autònoma de Barcelona.

Cell Viability
Cell viability was monitored by the colorimetric MTT assay as described. Results were expressed as the percentage of viable cells in OGD-exposed plates compared with control normoxic plates.

Fluorescent Analysis of Necrosis and Apoptosis
Cells were stained with PI and Hoechst 33258. PI (10 μmol/L) was added to cultures 12 to 16 hours after OGD. To perform the staining with Hoechst 33258, cells were fixed in ice-cold 4% paraformaldehyde and then incubated for 10 minutes at room temperature with 1 μg/mL Hoechst 33258. Cells were analyzed under a nonconfocal fluorescent Leica microscope. Because Hoechst 33258 stains all nuclei and PI stains the nuclei of cells with disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red round nuclei, and fragmented (or condensed) nuclei, respectively. Cells were counted by a blinded investigator from ≥3 independent experiments. In each experiment, >600 cells were examined in random fields from ≥3 culture wells for each condition. Our cultures showed ∼10% of apoptotic and 5% of necrotic cells under basal conditions (data not shown).

HA Release Determination
Cultures were incubated for 1 hour at 37°C with BSS containing 5 μmol/L of a mixture of radiolabeled and unlabeled HA. Cells were then rinsed to eliminate the extracellular [3H]HA, and BSS was added for 20 minutes (pre-OGD). OGD was then performed as indicated above. After OGD, BSS was added to cultures for 20 minutes (post-OGD). Two 10-minute samples of BSS were collected from the pre-OGD period together with the BSS from normoxic and OGD-treated cells. Radioactivity was measured, and released HA was calculated taking into account the specific activity in the incubation mixture. The amount of [3H]HA released during OGD was expressed as a percentage over pre-OGD samples.

Immunohistochemistry
Cells were plated on BIOCOAT 8-well culture slides (Becton and Dickinson) and fixed in 4% paraformaldehyde in Tris-buffered saline (TBS; 100 mmol/L Tris, 0.9% NaCl, pH 7.6) for 1 hour at 4°C. After washing, cells were blocked for 1 hour in TBS-Tween 20 0.1% containing 5% BSA and then incubated overnight at 4°C with the primary antibodies against the active form of caspase 3, (diluted 1:50 in blocking buffer) and the cell-specific marker for neurons, MAP-2 (BME). Cells were washed with TBS-Tween 0.1% and then incubated with the appropriate secondary antibodies conjugated with fluorescein isothiocyanate or tetramethylrhodamine B isothiocyanate (1:500) in blocking buffer. Culture slides were then mounted and the cells observed under epifluorescence.

Immunoblotting
Cell culture extracts were prepared by lysis in Mammalian Protein Extraction Reagent (M-PER; Pierce). Protein content was determined by the Bradford method. A total of 25 μg of protein was resolved on a 15% SDS-PAGE gel and transferred onto Hybond-P (Amersham Biosciences) polyvinylidene difluoride membranes. Blots were blocked with 5% BSA in TBS containing 0.1% Tween 20 and incubated overnight at 4°C in a blocking buffer containing primary antibodies against caspase 3 (1:1000), MAP-2 (1:1000), histidine decarboxylase (1:5000), or α-tubulin (1:10000). Blots were then incubated with horseradish peroxidase–conjugated secondary antibodies (1:10 000) in the blocking buffer and developed using the Super Signal West Pico Chemiluminescent Substrate method (Pierce).

Statistical Analysis
Statistical significance was determined by 1-way ANOVA followed by Tukey multiple comparison test. A value of P<0.05 was considered statistically significant.

Results
OGD-Induced Cell Death and HA Release in Cortical Cell Cultures
We initially established that 75 minutes of OGD exposure induced ∼50% of cell death (Figure 1A). Accordingly, with previous reports, astrocyte viability was not affected by this period of OGD, as assessed in a pure astroglial culture (data not shown). OGD induced an increase in HA release (≈30%
over normoxic cultures; Figure 1B), whereas protein levels of histidine decarboxylase were not altered (Figure 1B, inset).

Effect of HA H₂-Receptor Drugs on OGD-Induced Neuronal Cell Death

When cultures were exposed to OGD in the presence of HA or the H₂-agonist amthamine, cell death was increased up to 75% and 35% over control, respectively (Figure 2). Pretreatment with the H₂ antagonists ranitidine, cimetidine, and tiotidine reduced OGD-induced neuronal death (Figure 2).

Identical results were obtained when cell viability was assessed 48 hours after OGD exposure (data not shown). In preliminary experiments, we found that 100 μM ranitidine was the minimal concentration needed to have the maximal reduction in OGD-mediated cell death. Similar results were obtained with other H₂-antagonists tested (data not shown). None of the tested antagonists altered the basal rate of cell death.

Effect of Ranitidine on OGD-Induced Necrosis and Apoptosis

To better characterize the effects of ranitidine on OGD-induced neuronal cell death, PI and Hoechst 33258 were added to cultured neurons exposed to OGD in the presence or absence of ranitidine. Twenty-four hours after OGD exposure, we observed necrotic cells with round red nuclei but also pyknotic cells that exhibited bright blue nuclei (Figure 3A). Ranitidine significantly (P<0.05) reduced pyknosis in cells exposed to OGD (Figure 3B), whereas it had no effect on chromatin condensation in sham control cultures (data not shown). Ranitidine also reduced the number of necrotic PI-positive cells (Figure 3B).

Figure 1. OGD-induced cell death and HA release in cortical cell cultures. A, Neuronal death triggered by OGD. Cells were exposed for 75 minutes to OGD. Degree of viability was quantified 24 hours later using the MTT reduction assay. Results are expressed as a percentage of the value obtained in normoxic cultures. Data shown are mean±SEM of 5 experiments run in quadruplicate. *P<0.05 compared with normoxia. B, OGD-induced HA release. Cultures were loaded with [³H]HA and exposed for 75 minutes to OGD after 20 minutes of stabilization, and HA released over basal was determined as indicated in Materials and Methods. Basal release was 7.56±0.54 ng HA per hour per well. Results are mean±SEM (n=6) of a representative experiment performed in triplicate. Inset, Detection of histidine decarboxylase (HDC; 44 kDa) by Western blot in normoxic and OGD-treated cultures.

Figure 2. Effects of histaminergic drugs on OGD-induced cell death. Cell cultures were pretreated with HA (100 μM/L), the H₂-receptor agonist amthamine (Am; 10 μM/L), or the H₂-receptor antagonists ranitidine (Ran; 100 μM/L), cimetidine (Cim; 100 μM/L), and tiotidine (Tiot; 100 μM/L) for 20 minutes, and then cultures were exposed for 75 minutes to OGD. The histaminergic drugs were present during the OGD period and afterward until the determination of cell viability. Cell viability was assessed 24 hours later using the MTT reduction assay. Results are expressed as a percentage of cell death induced by 75 minutes of OGD. Data are mean±SEM of 5 experiments run in quadruplicate. Asterisks denote significant differences from vehicle (P<0.05).

Figure 3. Ranitidine protects against necrosis and apoptosis induced by OGD. Cells exposed to 75 minutes of OGD were treated with 100 μM/L of ranitidine as indicated in the Figure 2 legend. Degree of necrosis and apoptosis was quantified 24 hours later using PI or Hoechst 33258, respectively. A, Representative microphotographies showing necrotic (n) and pyknotic (a) nuclei in the same field. B, Quantification of necrotic and apoptotic nuclei in each condition. Results are expressed as percentage of total nuclei. Data are mean±SEM of 3 experiments run in quadruplicate. Asterisks denote significant differences from vehicle (P<0.05).
Effect of Ranitidine on OGD-Induced Caspase 3 Activation

Apoptotic cell death in cerebral ischemia has been associated with caspase 3 activation.16 Accordingly, we decided to study the effect of ranitidine on OGD-induced caspase 3 activation. Six hours after OGD, there is a substantial activation of caspase 3 in neurons visualized with a specific antibody against the cleaved enzyme (Figure 4A, green label). A clearly disorganized MAP-2 labeled network (in red) is also noticeable. Preincubation with ranitidine before OGD diminished caspase 3 cleavage and prevented alteration of MAP-2 staining (Figure 4). Western blot analysis of cell extracts collected 6 hours after OGD (Figure 4B) demonstrates that MAP-2 degradation, which results from OGD (lane 2), was effectively prevented by pretreatment with ranitidine (lane 4). A reduction of caspase 3 cleavage in cell extracts from ranitidine-treated OGD cultures is clearly evident compared with untreated OGD cells (Figure 4B).

Effect of Ranitidine Treatment After OGD

To examine whether ranitidine exerts a neuroprotective action after induction of OGD, cultures were treated with ranitidine 3 or 6 hours after OGD. Twenty-four hours later, cell viability was assessed by MTT reduction assay, and parallel cultures were stained with PI and Hoechst 33258 to quantify the necrotic and apoptotic nuclei. Ranitidine significantly decreased OGD-induced cell death (Figure 5A). A significant reduction of apoptotic (67%) and necrotic (65%) cells versus untreated OGD-exposed cultures was also observed (Figure 5B).

Discussion

It is well established that during brain ischemia, a release of glutamate and cytokines takes place.2 HA is also suggested to be released during ischemia. We have found an increase in HA release in cortical cultures deprived of oxygen and glucose. We have also detected the presence of L-histidine decarboxylase, the enzyme responsible for HA synthesis in brain. Because no histaminergic neurons are present in the cerebral cortex,6 it is likely that glial cells present in our cultures are the source of released HA. Because in vitro studies have shown that HA potentiates glutamate-mediated excitotoxicity,8,17 it is tempting to suggest that inhibition of HA action during ischemia could exert a beneficial effect. In this respect, and supporting the neuroprotective effect of blocking HA action, we observed that several HA H1-receptor antagonists decreased OGD-mediated cell death, as measured by MTT reduction.

Although ischemic neuronal death was traditionally described as necrosis, in recent years evidence of ischemic-
induced apoptosis is arising. Thus, it is now believed that a good neuroprotective therapy for cerebral ischemia should be able to reduce apoptotic death. By monitoring the effect of H2-receptor antagonists on the necrotic and apoptotic component of OGD-mediated cell death, we have observed that ranitidine reduced OGD-mediated necrosis and apoptosis. The antiapoptotic effect of ranitidine was confirmed by a decrease in OGD-mediated activation of caspase 3 and MAP-2 degradation. The neuroprotective effect of ranitidine was observed when it was added to cultures after OGD. Hence, addition of ranitidine to culture medium from 20 minutes before OGD until 24 hours after insult. All data together indicate that the HA H2-receptor blockers may be very interesting compounds to study novel and efficient treatments for cerebral ischemia.

References


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