Histamine H\textsubscript{2}-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 H\textsubscript{2}-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 H\textsubscript{2} 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—H\textsubscript{2}-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.\textsuperscript{1} The major cause of cellular injury is thought to be excess glutamate release\textsuperscript{2} mainly caused by the reverse function of its transporters\textsuperscript{3} 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.\textsuperscript{4,5}

Histamine (HA) plays an important role as a neurotransmitter and as a neuromodulator in the mammalian brain under normal and pathological conditions.\textsuperscript{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 H\textsubscript{2}-receptors leads to excitatory effects through a blockage of calcium-dependent potassium channels and the modulation of hyperpolarization-activated cation channels.\textsuperscript{7} Also, some reports have described that HA could positively modulate N-methyl-D-aspartate (NMDA) receptors,\textsuperscript{5,8} which are believed to mediate most of the ischemic-dependent neuronal injury.\textsuperscript{1}

HA receptor blockage in ischemia has provided contradictory results. Intraperitoneal administration of HA H\textsubscript{2}-receptor antagonists\textsuperscript{9} decreases brain edema formation after a common carotid artery occlusion. In contrast, there are some reports that describe adverse effects of these compounds. HA H\textsubscript{2}-receptor blockage in cerebral ischemia in gerbil has been reported to enhance neuronal damage.\textsuperscript{10,11} Moreover, Otsuka et al reported that this aggravating effect was associated with a facilitation of catecholamine metabolism in the rat.\textsuperscript{12} On the other hand, HA H\textsubscript{1}-receptor antagonists attenuated the ischemia-induced decrease in glucose metabolism in rat hippocampal slices.\textsuperscript{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 H\textsubscript{2}-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...
Antibodies
The antibody against procaspase 3 (H-277) was from Santa Cruz Biotechnology (Santa Cruz, Calif). The antibody against cleaved caspase 3 was from Cell Signaling (Beverly, Mass). Anti-microtubule–associated protein-2 (MAP-2) was from Chemicon (Temecula, Calif). The antihistidine decarboxylase was from AbCys. Secondary antibodies conjugated with fluorescein or Texas Red were from Jackson ImmunoResearch (West Grove, Pa). Horseradish peroxidase anti-rabbit secondary antibodies and anti-α-tubulin were from Transduction Biolabs (Bedford, Mass).

Cell Cultures
Primary cultures of mixed rat brain cortical cells containing neurons and glia were prepared as described, with modifications, from 40 fetal Wistar rats (Servei d’Estudis de la Universitat Autònoma de Barcelona, Barcelona, Spain) at 17 days of gestation. Dissociated cells were plated at a density of $3 \times 10^5$ cells/cm$^2$ in basal medium Eagle (BME) supplemented with 5% FCS, 5% horse serum, 2 mmol/L glutamine, and 50 μg/mL streptomycin, 50 μg/mL penicillin, 2 mmol/L glutamine, and 10 mmol/L glucose and plated onto poly-L-lysine precoated wells. Cultures were then placed in an airtight incubation chamber (CBS Scientific) equipped with inlet and outlet valves and were equilibrated for 15 minutes with a continuous flux of gas (5% CO$_2$/95% N$_2$). The chamber was then sealed and placed into a CO$_2$ 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 mmol/L of a mixture of radiolabeled and unlabeled HA. Cells were then rinsed to eliminate the extracellular $[^3]$H]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 $[^3]$H]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 (1:1000). 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. Cell 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 $\approx$50% of cell death (Figure 1A). Accordingly with previous reports, astrocyte viability was not affected by this period of OGD, as assessed in a pure astroglial culture (data not shown). OGD induced an increase in HA release ($\approx$30%
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 amphetamine, 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 μmol/L 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).
Effect of Ranitidine on OGD-Induced Caspase 3 Activation

Apoptotic cell death in cerebral ischemia has been associated with caspase 3 activation. 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 exposing cultures to normoxic (control) or ischemic (OGD) conditions with and without ranitidine demonstrates that MAP-2 (top) and cleaved caspase 3 primary antibodies (bottom) were used to analyze cell extracts. The same amount of protein (30 μg; quantified by Bradford assay) was loaded in each lane.

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. 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, 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, 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 H2-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 H1-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 also observed when it was added to cultures after OGD. Hence, addition of ranitidine ≥6 hours after OGD strongly reduced necrotic and apoptotic cell death in cortical cultures. To our knowledge, this is the first report to demonstrate the neuroprotective effects of H1-receptor antagonists on neural death associated with ischemic injury in the brain.

Some reports suggest that HA H1-receptor blockade aggravates ischemia-induced neuronal damage through an increase in released excitatory neurotransmitters. In contrast with these data, we have reported previously that in presynaptic terminals, blockade of HA H2-receptors reduces glutamate release, supporting a neuroprotective effect of blocking these HA receptors.

We do not know at present which molecular mechanism could be involved in the neuroprotective effects of HA H1-receptor antagonists. The possibility that ranitidine might act through other receptors besides HA H2 could not be excluded. It is also possible that HA released from microglia or astroglia during OGD could directly potentiate glutamate receptor-mediated cell death by interacting with the polyamine-binding site of the NMDA receptor complex. Ranitidine could then be able to antagonize HA interaction with NMDA receptors. However, no experimental data supporting this possibility has been described. Alternatively, ranitidine could act through its classical direct blockade of HA H2-receptor stimulation. Also, we could not rule out the possibility that the effect of ranitidine is mediated by inhibition of constitutively active H2-receptors. Up until now, there is no clear evidence about how HA receptors stimulation could promote cell death in the central nervous system. However, some reports have described an HA-mediated potentiation of NMDA receptors through H2-receptor stimulation and subsequent activation of potassium channels.

In summary, our present findings provide evidence that ranitidine, a commonly used antigastric ulcer drug, exerts neuroprotective actions on ischemic neural cell death even 6 hours after insult. All data together indicate that the HA H1-receptor blockers may be very interesting compounds to study novel and efficient treatments for cerebral ischemia.

References


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