Research Report

Antihistamine terfenadine potentiates NMDA receptor-mediated calcium influx, oxygen radical formation, and neuronal death

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Abstract

We previously reported that the histamine H1 receptor antagonist terfenadine enhances the excitotoxic response to N-methyl-D-aspartate (NMDA) receptor agonists in cerebellar neurons. Here we investigated whether this unexpected action of terfenadine relates to its antihistamine activity, and which specific events in the signal cascade coupled to NMDA receptors are affected by terfenadine. Low concentrations of NMDA (100 μM) or glutamate (15 μM) that were only slightly (<20%) toxic when added alone, caused extensive cell death in cultures pre-exposed to terfenadine (5 μM) for 5 h. Terfenadine potentiation of NMDA receptor response was mimicked by other H1 antagonists, including chlorpheniramine (25 μM), oxatomide (20 μM), and triprolidine (50 μM), was prevented by histamine (1 mM), and did not require RNA synthesis. Terfenadine increased NMDA-mediated intracellular calcium and cGMP synthesis by approximately 2.4 and 4 fold respectively. NMDA receptor-induced cell death in terfenadine-treated neurons was associated with a massive production of hydrogen peroxides, and was significantly inhibited by the application of either (+)-alpha-tocopherol (200 μM) or the endogenous antioxidant melatonin (200 μM) 15 min before or up to 30 min after receptor stimulation. This operational time window suggests that an enduring production of reactive oxygen species is critical for terfenadine-induced NMDA receptor-mediated neurodegeneration, and strengthens the importance of antioxidants for the treatment of excitotoxic injury. Our results also provide direct evidence for antihistamine drugs enhancing the transduction signaling activated by NMDA receptors in cerebellar neurons. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: Interactions between neurotransmitters

Keywords: Terfenadine; excitatory aminoacid receptors; histamine; reactive oxygen species; intracellular calcium; cultured cerebellar neurons.

1. Introduction

Several lines of evidence indicate that histaminergic fibers could have a role in the modulation of the neurotransmission mediated by glutamate [3,38], and a role for histamine in NMDA-mediated excitotoxic death has been reported in a rat model of Wernicke’s encephalopathy [24]; however, the effects of antihistamine compounds on neuronal survival and function have been much less explored. Among the antihistamines that penetrate poorly into the central nervous system (CNS), terfenadine is widely used as a prototype of non-sedating histamine H1 receptor antagonist. Although terfenadine is generally devoid of CNS depressant activity, reported adverse effects include sedation, drowsiness, fatigue and weakness [30], suggesting that therapeutic concentrations of this drug may also act at the CNS. In addition to its antihistamine activity, terfenadine has been shown to possess a complex pharmacological profile that includes the ability to block voltage dependent ion channels [11,26,42], and to reverse drug resistance in a variety of cell types via its interaction with P glycoprotein [45]. Very limited data exist about the
effects of this drug on glutamate mediated neurotransmission and excitotoxicity.

Cerebellar granule neurons in culture represent a neuronal model that has proved very useful in the study of the biochemical events coupled to excitatory amino acid neurotransmission, and the conditions controlling excitotoxicity [29,32–35]. They express ionotropic NMDA glutamate receptors coupled with the synthesis of cGMP [32], as well as H\textsubscript{1} histamine receptors [20], which are associated with inositol phospholipid hydrolysis and calcium mobilization from intracellular stores [19]. We have recently reported [10] that terfenadine significantly potentiates the excitotoxic response elicited by NMDA in cultured cerebellar neurons. The aim of the present study was to extend these observations, specifically investigating (1) whether the potentiating effects of terfenadine relate to its antihistamine activity; for this purpose we tested other H\textsubscript{1} histamine antagonists and performed competition studies using different concentrations of histamine; (2) which specific events of the cascades triggered by NMDA receptor agonists in cultured cerebellar neurons are modulated by terfenadine and leading to excitotoxicity. In particular we focused on the NMDA-mediated influx of calcium, considered to be the key initial step in the biochemical processes following activation of NMDA receptors and evoking both NMDA receptor-mediated neuronal plasticity and neurotoxicity [5,8]. We also examined the formation of oxygen radicals, as glutamate exposure has been reported to be associated with an enhanced generation of reactive oxygen species [7,23]. The role of neosynthesized RNA in the potentiating effects of NMDA excitotoxicity induced by terfenadine in cultured cerebellar granule neurons has been also investigated.

2. Materials and Methods

2.1. Cell cultures

Primary cultures of rat cerebellar neurons were prepared as previously described [34]. Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated and suspended in basal Eagle’s medium with 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly-L-Lysine coated (5 μg/ml) 35 mm dishes at 2.5×10⁵ cells/cm² and incubated at 37°C in a 5% CO\textsubscript{2}, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added after 20–24 h of culture to inhibit the replication of non-neuronal cells. After 8 days in vitro, morphologically identified granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation.

2.2. Neuronal treatment and survival

Neurons were used between 14 and 20 days in culture. As a general rule, drugs were dissolved in distilled water and pH was adjusted at approximately 7.4. Antihistamines were dissolved in ethanol and acidulated (5 mM HCl) water (1:1) and diluted at least 200 times in the culture medium. Vehicle controls were conducted containing the same amount of ethanol and HCl. All drugs were added to the growth medium for the indicated times, after which growth medium was removed and cultures were incubated for 5 min. with 1 ml incubation buffer containing 154 mM NaCl, 5.6 mM glucose, 8.6 mM HEPES, 1 mM MgCl\textsubscript{2}, 2.3 mM CaCl\textsubscript{2}, pH 7.4, to which the vital stain fluorescein diacetate (5 μg/ml) was added. The staining mixture was then aspirated, replaced with incubation buffer, and cultures were examined for neurotoxicity. Under fluorescent light, live neurons showed a bright green color in the cell body and neurites, while dead neurons did not retain any fluorescein diacetate, and their nuclei could be stained in red by 1 min exposure to 50 μg/ml ethidium bromide. Photographs of three randomly selected culture fields were taken, and live and dead neurons were counted. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the picture (~3000).

2.3. cGMP determination

Intracellular cGMP concentration was determined as previously reported [35] with minor modifications. Briefly, glutamate receptor agonists were added to the culture medium for 1 min. Terfenadine was added 5 h before agonists. MK-801 was added 1 min before agonists. Incubation was stopped by aspiration of the growth medium and addition of 1 ml HClO\textsubscript{4} (0.4 N). After neutralizing the perchlorate extract, cGMP content was determined by radioimmunoassay. Protein content was determined on the membrane pellet from the same sample.

2.4. Confocal microscopy

For intracellular calcium determination, neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester in an incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 MgCl\textsubscript{2}, 2.3 CaCl\textsubscript{2}, pH 7.4. At the moment of the record the dye was removed and the indicated drugs were added. Fluo-3 emission (>515 nm) was recorded in a Bio-Rad confocal microscope with a krypton–argon laser excitation source (488 nm). Signals were digitized using Bio-Rad interface and analyzed by NIH Image (1.61). Concentrations of intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) could be estimated as a
function of Fluo-3 intensity ($F$) using the calibration procedure described previously [40], and according to the following equation:

$$[\text{Ca}^{2+}]_i = K_d (F - F_{\text{min}})/(F_{\text{max}} - F)$$

where the dissociation constant $K_d$ has been estimated at 400 nM for Fluo-3 at vertebrate ionic strength [21], and the mean values obtained for the camera signal $F_{\text{min}}$ and for the maximum fluorescence $F_{\text{max}}$ were 6 and 230 respectively.

Oxygen radical formation was detected with carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA). Following uptake, the carboxy-H$_2$DCFDA is converted by endogenous esterases to carboxy-H$_2$DCF, which upon exposure to hydroperoxides is oxidized to the fluorescent probe carboxy-DCF. Neuronal cultures were treated with the indicated drugs and loaded with 20 μM carboxy-H$_2$DCFDA in the culture medium for 1 h, after which the dye was removed and cultures were washed twice with a buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 MgCl$_2$, 2.3 CaCl$_2$, pH 7.4. Carboxy-DCF fluorescence was recorded in a Bio-Rad fenadine (5 μM) containing 0.01% H$_2$O. Number of histamine-22 None TEF None TEF 2.5.

Data presentation and analysis

Positive cells, morphologically identified as mast cells

M, pH 7.5) containing 0.1% bovine serum albumin, 0.2% fetal calf serum, and 0.2% Triton-X. Samples were incubated (56%) in cultures pretreated with terfenadine for 4 h but not in cultures pretreated for shorter times (data not shown in Table 1, preincubation of neurons with terfenadine 2

Results

3.1. Potentiation by terfenadine of NMDA receptor-mediated neuronal death is prevented by histamine, does not require RNA synthesis, and is mimicked by other histamine H1-receptor antagonists.

We first investigated whether the effects of terfenadine on NMDA receptor mediated neurotoxicity were related to its antihistamine activity. As previously reported [10] and shown in Table 1, preincubation of neurons with terfenadine (5 μM, 5 h) significantly potentiated neurotoxicity by 100 μM NMDA, concentration which was not toxic when NMDA was added alone. Furthermore, terfenadine reduced by approximately 75% the number of surviving neurons after 12 h exposure to subtoxic concentrations of glutamate (15 μM) compared to cultures exposed to glutamate alone (Table 1). To test the time dependence of exposure to terfenadine, cultures were exposed to terfenadine for 15 min, 1 h, 3 h and 4 h prior to application of 100 μM NMDA or 15 μM glutamate. The number of surviving neurons was significantly reduced (56%) in cultures pretreated with terfenadine for 4 h but not in cultures pretreated for shorter times (data not shown in Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neuronal Survival (%)</th>
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<tbody>
<tr>
<td></td>
<td>-HIS</td>
</tr>
<tr>
<td>None</td>
<td>85±9</td>
</tr>
<tr>
<td>GLU</td>
<td>81±15</td>
</tr>
<tr>
<td>NMDA</td>
<td>80±12</td>
</tr>
<tr>
<td>MK-801 + GLU</td>
<td>84±7</td>
</tr>
<tr>
<td>MK-801 + NMDA</td>
<td>85±5</td>
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</tbody>
</table>

$^a$ Cerebellar neurons in primary culture were used at 14–18 days in culture and exposed to vehicle (None) or to the indicated drugs. Terfenadine (TEF, 5 μM) was added 5 h before glutamate (GLU, 15 μM) or N-methyl-D-aspartate (NMDA, 100 μM). Histamine (HIS, 5 μM) was added 15 min before TEF. NMDA receptor antagonist MK-801 (1 μM) was added 2 min before agonists. Neuronal viability was determined 12 h after application of GLU or NMDA by staining of cultures with fluorescein diacetate and ethidium bromide. Values are expressed as percentage of live neurons and are from three independent experiments. Statistical significance was determined by two-way analysis of variance (ANOVA) and two-tailed unpaired Student’s t-test.

$^b$ P<0.01 vs. None in the same line in the absence of HIS.

$^c$ P<0.01 vs. the same treatment in the absence of MK-801.

$^d$ P<0.01 vs. the same treatment in the absence of HIS.
shown). NMDA- and glutamate-induced excitotoxicity in terfenadine-treated cultures were confirmed to be specifically due to NMDA receptor stimulation by using the NMDA receptor antagonist (+)-10,11-dihydro-5-methyl-5H-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801), and in both cases neuronal death could be effectively prevented by the application of high concentrations of histamine (5 mM) (Table 1). To further check the nature of this antagonism we constructed dose-response experiments using concentrations of histamine from 500 μM to 10 mM. As shown in Fig. 1, the ability of terfenadine (5 μM) to potentiate excitotoxicity by subtoxic concentrations of glutamate (15 μM) was prevented by histamine in a concentration dependent manner. Although a significant increase in neuronal survival was already observed at 500 μM histamine, a concentration of at least 1 mM histamine was required to achieve maximum protection.

The long exposures necessary to potentiate the excitotoxic response to NMDA led us to consider the possibility that newly synthesized proteins were involved in the effects of terfenadine and for that purpose, we tested the effect of terfenadine on NMDA receptor-mediated excitotoxicity in the presence of actinomycin D. We found no significant differences due to the presence of actinomycin D. Thus, exposure of cultures to terfenadine (5 μM, 4 h) and then to NMDA (100 μM) induced after 24 h a dramatic reduction in the number of surviving neurons from 97±1% to 10±5% and to 11±7% live neurons in the absence and in the presence of actinomycin D respectively. Exposure of neurons to terfenadine alone had no effect on cell survival, and the concentration of NMDA (100 μM) used in these experiments induced very little toxicity (from 97±1% to 71±5% live neurons) when added alone.

To exclude the possibility that potentiation of excitotoxicity by NMDA receptor agonists could be just a terfenadine-related effect, we tested the ability of other histamine H1 receptor antagonists to increase the excitotoxic response to glutamate in cerebellar granule neurons. Pretreatment of cultures for 5 h with (±)-chlorpheniramine (25 μM) or oxatomide (20 μM), significantly increased cell death in cultures exposed to subtoxic concentrations (15 μM) of glutamate (Fig. 2). A smaller but still significant potentiation of the excitotoxic response to glutamate was also observed for trans-triprolidine (50 μM) using the same exposure conditions.

3.2. Terfenadine increases the intracellular calcium response induced by NMDA

Calcium imaging experiments showed that in cultured cerebellar neurons stimulation of NMDA receptors elicits

![Fig. 1](image1.png)

Fig. 1. Potentiation by terfenadine of NMDA receptor-mediated excitotoxicity is prevented by histamine in a concentration-dependent manner. Neurons were exposed to 5 μM terfenadine (TEF) for 5 h and then treated with a subtoxic concentration (15 μM) of glutamate (GLU) in the absence (inset figure and first point of the curve) or in the presence of the indicated concentrations of histamine. Histamine was added 10 min before TEF. Neuronal survival was determined 24 h after the addition of GLU by staining live neurons with fluorescein diacetate (5 min) and dead neurons nuclei with ethidium bromide (1 min), and compared with survival of neurons in sister cultures treated with vehicle (None) or with TEF or GLU alone (see inset figure). Data represent the mean ± S.D. of triplicate values from three separate experiments. *P<0.01 vs. 0 mM histamine; **P<0.001 vs. TEF or GLU.

![Fig. 2](image2.png)

Fig. 2. Histamine H1 receptor antagonists potentiate NMDA receptor-mediated excitotoxicity. Neuronal survival after 24 h exposure to 15 μM glutamate (GLU) was determined in vehicle-pretreated neurons (None) and in neurons pretreated for 5 h with 20 μM oxatomide (OXA), 25 μM chlorpheniramine (CHP) or 50 μM trans-triprolidine (TRP). Represented values are the mean ± S.D. (n=6). *P<0.05 vs. None within the same treatment.
an intracellular calcium signal that can be significantly increased by terfenadine (Fig. 3). Thus, application of 25 μM NMDA induced a rather small increase in cellular fluorescence intensity (Figs. 3A and B), consistent with a limited influx of calcium into neurons, while stimulation of terfenadine-treated neurons with this low concentration of NMDA resulted in a rapid and a strong rise in fluorescence intensity in most cell bodies (Figs. 3C and D), indicating a large influx of extracellular calcium into cells. Quantification of the fluorescence intensity in about 20–30 neurons per field revealed an NMDA-induced mean increase from 55±18 to 127±16 in untreated and from 62±16 to

![Fig. 3. Terfenadine increases intracellular calcium upon stimulation of NMDA receptors. Neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester and then examined under a laser confocal microscope. (A–D) Representative images taken before (A,C) or 30 s after (B,D) the addition of 100 μM NMDA in the absence (A,B) or in the presence (C,D) of 5 μM terfenadine (TEF) for 5 h. Scale color bar spans from black (minimum) to red (maximum). (E) Images as the ones represented in A–D were used to estimate intracellular calcium concentrations before (None) or after application of NMDA (100 μM) to cultures treated with vehicle (–TEF) or with terfenadine (5 μM) for 5 h (+TEF). Concentrations of intracellular calcium were calculated from the fluorescence intensity averaged for 20–30 neurons per field as previously described [40]. Intracellular calcium after application of NMDA in the presence of the NMDA receptor antagonist MK-801 is also represented. Results represent the mean±S.D. of two independent experiments (n = 50–60). *P < 0.01 vs. NMDA in the absence of TEF.](image-url)
171 ± 18 fluorescence units (f.u.) in terfenadine-treated neurons respectively within 30 s, and in both cases it could be abolished by MK-801 (Fig. 3 E). The increase in the intracellular concentration of calcium induced by NMDA was estimated [40] (see Materials and Methods) to be from approximately 112 ± 34 to 470 ± 60 nM and from approximately 133 ± 30 to 1120 ± 125 nM in the absence and in the presence of terfenadine respectively.

To investigate the effect of terfenadine on calcium-dependent signal transduction we measured the intracellular formation of cGMP stimulated by NMDA. As shown in Table 2, preincubation of neurons with terfenadine (5 μM, 4 h) resulted in an approximately 4 fold increase in cGMP formation induced by the selective stimulation of NMDA receptors with 1 mM NMDA, and also increased by approximately 2 fold the cGMP formation induced by 100 μM NMDA. The effect was specific for NMDA receptors that it was specifically blocked by the NMDA receptor antagonist MK-801, and that terfenadine had no effect on cGMP formation stimulated by the non-NMDA receptor agonists AMPA or domoic acid (data not shown).

3.3. Terfenadine potentiates the formation of oxygen radicals following stimulation of NMDA receptors

To test for the participation of oxidative stress in the potentiation by terfenadine of NMDA receptor-mediated response we used the specific probe dichlorodihydrofluorescein diacetate (H2DCFDA) to measure the generation of reactive oxygen species, and in particular hydrogen peroxides, in cultures exposed to subtoxic concentrations (15 μM) of glutamate both in the presence and in the absence of terfenadine. As shown in Fig. 4, stimulation of neurons with this low concentration of glutamate did not result in a significant generation of hydrogen peroxides (Fig. 4C) compared to unstimulated cultures (Fig. 4A). In contrast, a massive formation of oxygen radicals occurred in cultures exposed to this low concentration of glutamate in the presence of terfenadine (Fig. 4D). Radical formation could be specifically abolished by the NMDA receptor antagonist MK-801 (Fig. 4E), and was not observed in neurons exposed to terfenadine alone (Fig. 4B), indicating that activation of NMDA receptors was necessary. Similar results were obtained in experiments in which NMDA was used as NMDA-receptor agonist (not shown). These findings suggested a key role for hydrogen peroxides as mediators of the neurodegeneration coupled to overactivation of NMDA receptors in cultured cerebellar neurons. Indeed, stimulation of neurons with 50 μM glutamate or 500 μM NMDA induced extensive formation of hydrogen peroxides and resulted in over 80% neurotoxicity, due to excessive stimulation of NMDA receptors as it could be completely abolished by MK-801 (data not shown).

3.4. (+)-alpha-tocopherol and melatonin protect from terfenadine potentiation of NMDA toxicity

The role of oxygen radical formation in the pathway mediating toxicity by NMDA receptors in the presence of terfenadine was further evaluated by testing the protective effects of two different antioxidant compounds: the pineal secretory product melatonin, and (+)-alpha-tocopherol (vitamin E). In particular, we investigated whether melatonin and (+)-alpha-tocopherol were able to prevent cell death produced by low concentrations of NMDA in the presence of terfenadine, and whether addition of these antioxidant drugs after the excitotoxic insult could rescue neurons. Both melatonin (200 μM) and (+)-alpha-tocopherol (200 μM) markedly inhibited NMDA-mediated cell death in terfenadine treated neurons when added 15 min prior to NMDA application (Fig. 5A), reducing neuronal death by approximately 70%. To determine whether addition of these drugs after initiation of the excitotoxic insult exerted neuroprotective effects, we performed experiments in which melatonin or (+)-alpha-tocopherol were added 0, 5, 15, and 30 min following stimulation with NMDA. As shown in Fig. 5B, addition of melatonin 0, 10 or 15 min after exposure to 100 μM NMDA reduced neuronal death significantly, while no protection was observed in cultures to which melatonin had been added 30 min after NMDA. As for (+)-alpha-tocopherol, this compound showed a greater effectiveness, and significant protection from NMDA in terfenadine treated neurons was observed in cultures to which (+)-alpha-tocopherol was added up to 30 min after NMDA (Fig. 5B).

4. Discussion

In the present study we have investigated the potentiation by terfenadine of the excitotoxic response to NMDA receptor agonists in cultured cerebellar neurons. We found that the effects of terfenadine on NMDA receptor-mediated

<table>
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<tr>
<th>Treatment</th>
<th>cGMP (pmol/mg protein)</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>None</td>
</tr>
<tr>
<td>NMDA (100 μM)</td>
<td>3.3 ± 0.3*</td>
</tr>
<tr>
<td>NMDA (1 mM)</td>
<td>17 ± 2*</td>
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*Cerebellar neurons in primary culture were used at 12–14 days in culture and vehicle (None) or the indicated drugs were added to the growth medium. Terfenadine (TEF, 5 μM) was added 5 h before NMDA. After 1 min exposure to NMDA culture medium was aspirated and 1 ml HClO4 (0.4 N) was added. The perchlorate extract was neutralized with NaOH and the cGMP content was determined by radioimmunoassay. Data are from triplicate values from two independent experiments. Statistical significance was determined by two-way analysis of variance (ANOVA) and two-tailed Student’s t-test. P < 0.01 vs. unstimulated in the same column. P < 0.01 vs. the same treatment in the absence of TEF.
Fig. 4. Terfenadine enhances the formation of hydrogen peroxides induced by stimulation of NMDA receptors. Neuronal cultures untreated (A) or exposed to 5 μM terfenadine (B), glutamate (C), and to terfenadine plus glutamate in the absence (D) or in the presence of MK-801 (E), were loaded with 20 μM carboxy-H$_2$DCFDA in the culture medium for 1 h. Cultures were washed twice with a buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 MgCl₂, 2.3 CaCl₂, pH 7.4, and the carboxy-DCF fluorescence was recorded in a Bio-Rad confocal microscope with a krypton–argon laser excitation source (488 nm). Signals were digitized using Bio-Rad interface and analyzed by NIH Image (1.61). Scale color spans from white (minimum) to black (maximum). Terfenadine was added 5 h before glutamate and carboxy-H$_2$DCFDA was added 30 min after glutamate.
Fig. 5. Antioxidants melatonin and (+)-alpha-tocopherol prevent potentiation by terfenadine of NMDA excitotoxicity. Neurons at 14–17 DIC were either untreated (None) or exposed to the indicated drugs in the growth medium, and neuronal survival was determined 24 h later by staining of live neurons with fluorescein diacetate and dead neurons nuclei with ethidium bromide, and observation of cultures under a fluorescence light. Terfenadine (TEF) was added 5 h before NMDA. Melatonin (MEL) and (+)-alpha-tocopherol (α-TCF) were added 10 min before TEF (Panel A) or at the indicated times after application of NMDA (Panel B). Concentrations were: TEF, 5 μM; NMDA, 100 μM; MEL, 200 μM; α-TCF, 200 μM. Data represent the mean±S.D. of duplicate values from four independent experiments. *P<0.001 vs. NMDA in panel A and *P<0.01 vs. TEF+NMDA in panel B.

neurotoxicity appear to be related to its anti-histamine activity for they can be effectively antagonized by high concentrations of histamine in the growth medium (see Table 1) and mimicked by other histamine H1 antagonists (Fig. 2). These observations support a specific relationship between terfenadine activity as an histamine receptor antagonist and the effects of this drug on excitotoxicity. An aggravation of ischemic neuronal damage in the rat hippocampus has been described in rats in which histaminergic neurotransmission was impaired with the histidine decarboxylase inhibitor alpha-fluoromethylhistidine [1]. Our results would be consistent with the hypothesis that terfenadine inhibited a tonic stimulation of H1 receptors. Approximately 50% of the histamine content of rat brain may derive from mast cells [43], and the presence in the cerebellum of meninges, difficult to remove completely, may allow for a certain amount of these cells in the cultures. By immunohistochemistry experiments using a polyclonal antihistamine antibody we have estimated the extent of mast cell contamination in our cultures to be about 1–2 mast cell/2500 neurons (see section 2.5 of Materials and Methods), while cerebellar neurons were histamine-negative (not shown). So, it cannot be excluded that the presence of this reduced number of mast cells may allow for a low amount of histamine in the culture medium. Alternatively, it is also possible that by acting as an inverse agonist terfenadine may be blocking spontaneous agonist-independent activity of histamine receptors. Interestingly, constitutive activity of wild-type human H1 receptors and inverse agonistic activity of several histamine H1 antagonists has been recently reported [2]. Although taken together our findings suggest the specific involvement of histamine H1 receptors, non-selective cross-reactivity with other histamine receptor subtypes cannot be excluded completely given the relatively high concentrations of terfenadine, as well as of other histamine H1 receptor antagonists, needed to achieve potentiation of NMDA excitotoxicity.

Our results show that the increase in toxicity induced by terfenadine in neurons stimulated with subtoxic concentrations of NMDA or glutamate is associated with a greater (about 2.5 fold increase) influx of extracellular calcium into neurons (Fig. 3 and data not shown). The inhibitory effect of MK-801 indicates that calcium entry occurs via NMDA receptor channels (Fig. 3). Although the mechanisms underlying terfenadine potentiation of calcium influx upon stimulation of NMDA receptors remain to be established, the observation that it required at least 4 h exposure of neurons to terfenadine before NMDA application (data not shown) suggests an intracellular regulation of NMDA channels permeability rather than an extracellular interaction of terfenadine with NMDA receptors. The simplest mechanistic explanation may be related to a progressive depolarization consequent to terfenadine blockade of several types of K+ currents [4], and leading to a reduction of Mg2+ block of the NMDA channel and the facilitation of
excitotoxicity. Against this possibility however, it should be considered that the concomitant block of voltage sensitive sodium channels by terfenadine [11,27] may slow down such depolarization process, and allow for progressive use-dependent inactivation of the NMDA channel by released endogenous glutamate [13,28], resulting in neuroprotection. Furthermore, a reversible inactivation of NMDA channels by intracellular calcium has been shown in hippocampal neurons [25,31,44] as well as in recombinant NMDA receptors [22]. Neither ligand binding nor channel opening was required to elicit this calcium-dependent inactivation [25], that could be prevented by blocking voltage sensitive calcium channels (VSCC) [31]. Histamine H1 receptors are coupled to a guanylnucleotide-sensitive G protein and the phosphoinositide hydrolysis. Thus, by reducing tonic stimulation or spontaneous activity of H1-receptors terfenadine may decrease cytosolic calcium levels due to both the impairment of the release from intracellular stores and the inhibition of the inositol-1,4,5-triphosphate (IP3)-induced calcium influx via VSCC [9].

A direct action of terfenadine on VSCC may be also considered. Terfenadine is structurally related to diphenylalkylamine L-type Ca<sup>2+</sup> channel blockers, and in Ca<sup>2+</sup> channel affinity studies using rat cortical membranes terfenadine showed a pK<sub>d</sub> of about 6.4 [46]. An inhibition of calcium currents possibly via L-type channels by concentrations of terfenadine in the low micromolar range has been reported in ventricular myocytes [26]. Moreover, we have shown that terfenadine is also effective in reducing the depolarization-induced influx of calcium via VSCC in cerebellar neurons [12]. Therefore, an inhibition of VSCC currents by terfenadine could result in partial recovering of NMDA receptors from inactivation as cytosolic calcium levels decrease, and lead to a higher number of ‘normal’ (not inactivated) NMDA receptors on the neuronal membrane. According to this hypothesis, the ability of histamine to antagonize the effects of terfenadine (Fig. 1) could be associated to a compensatory increase in the release of calcium from intracellular stores due to the stimulation of histamine H1 receptors. Experiments are currently in progress to investigate this possibility.

Our observations that terfenadine significantly potentiate the NMDA-dependent synthesis of cGMP (Table 2), which depends upon extracellular calcium influx [32,35], provide for the first time evidence for the modulation by an antihistamine drug of the transduction signaling activated by NMDA receptors in cerebellar neurons. The increase in NMDA receptor-mediated formation of oxygen radicals observed in terfenadine-treated cultures (Fig. 4) further support this idea. Reactive oxygen species have been demonstrated to play a pivotal role in the pathogenesis of excitotoxic death [7,36,41], and in cultured cerebellar granule cells, NMDA receptor stimulation has been shown to produce marked elevations in superoxide anion and hydroxy radicals due to the activation by calcium of phospholipase A2 and the metabolism of arachidonic acid [23]. Our results further confirm the generation of oxygen radicals, and in particular hydrogen peroxides, as a key event in the occurrence of NMDA receptor-mediated neurotoxicity. Indeed, formation of radicals preceded neuronal death of cultures exposed to terfenadine plus glutamate (Fig. 4D) but it was not detectable in neurons exposed to terfenadine alone (Fig. 4B), and only a slight formation of hydrogen peroxides was observed in cultures exposed to subtoxic concentrations of glutamate (Fig. 4C). It should be noted that terfenadine and glutamate alone had no significant effect on neuronal viability (see Table 1). Moreover, the ability of free radical scavengers such as the pineal hormone melatonin and (+)-alpha-tocopherol in protecting neurons from death by NMDA in terfenadine-treated cultures (Fig. 5) further supports a prominent role for oxygen free radicals in excitotoxicity. Melatonin has been reported to be neuroprotective ‘in vivo’ against kainic acid-induced lesions [6,18], and ‘in vitro’ from cell death of cultured cerebellar neurons following exposure to kainic acid [17]. In contrast, melatonin treatment was suggested to be ineffective in protecting neurons from toxicity induced by exposure of neurons to NMDA [17]. A possible explanation for the discrepancy between the latter observation and the protective effect of melatonin we describe here may be that our experimental paradigm relies on neurotoxicity precipitated by enhancing the excitatory signal induced by a subtoxic concentration of NMDA. Indeed, melatonin has been shown to prevent death of hippocampal neurons induced by enhanced excitatory neurotransmission via NMDA receptors [41]. Melatonin is believed to work via electron donation to directly detoxify free radicals including the highly toxic hydroxyl radical and peroxynitrite [16], and it has been also shown to inhibit cerebellar NO synthase [37] and to maintain the cellular homeostasis of reduced glutathione, a key component of the cellular defense cascade against injury caused by reactive oxygen species [15]. Moreover, melatonin and vitamin E inhibited NO-induced lipid peroxidation in rat brain homogenates [14]. Further studies are necessary to establish the specific contribution of these pathways to the neuroprotective action of melatonin and alpha-tocopherol we report here.

Cerebellar granule cells have been frequently used to study the signaling pathways triggered by excitatory amino acids [29,32–35]. In the present study we have used this
primary cell culture system to investigate the molecular mechanism by which the antihistamine terfenadine potentiates the excitotoxic response elicited by NMDA receptor agonists. We have shown that this novel action of terfenadine involves an increase in the influx of calcium via NMDA receptors and the NMDA receptor mediated intracellular synthesis of cGMP, and does not depend upon RNA synthesis. Terfenadine potentiation of NMDA receptor-induced cell death was histamine sensitive, associated with a massive production of hydrogen peroxides, and was prevented by the antioxidants vitamin E and melatonin. Our findings provide for the first time direct evidence for antihistamine drugs enhancing the transduction signaling activated by NMDA receptors, and strengthen the importance of antioxidants for the treatment of excitotoxic injury.

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