Bifemelane Protects Cultured Cortical Neurons Against N-Methyl-D-aspartate Receptor-Mediated Glutamate Cytotoxicity

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ABSTRACT—This study was performed to examine the neuroprotective action of bifemelane against glutamate cytotoxicity in cultured cortical neurons obtained from fetal rats. Cell viability was markedly reduced by a brief exposure to glutamate. Glutamate cytotoxicity was reduced by exposing cultures to bifemelane at concentrations of 1–10 μM for 24 hr prior to glutamate exposure. In contrast, glutamate cytotoxicity was not affected by adding bifemelane to the glutamate-containing-medium without pretreatment. Bifemelane did not affect N-methyl-D-aspartate (NMDA)-induced currents, although the higher concentration (100 μM) of the drug reduced them. These findings suggest that bifemelane protects against glutamate neurotoxicity without affecting NMDA receptors.

Keywords: Cerebral cortex, Glutamate, Bifemelane

Bifemelane is used clinically as a cerebral metabolic activator. This drug is effective for the treatment of cognitive and emotional disturbances related to cerebrovascular disease (1). Pharmacological studies have demonstrated that bifemelane suppresses ischemic cerebral edema (2) and has a protective effect against ischemic injury of hippocampal CA1 neurons in gerbils (3). Recent studies suggest possible mechanisms of the protective action of bifemelane against ischemia-induced neurodegeneration. Gu et al. (4) showed evidence suggesting that bifemelane inhibits an ischemia-induced increase in c-Fos-like immunoreactivity in the cerebral cortex. Bifemelane enhances ischemic tolerance in gerbil hippocampal CA1 neurons (5). Moreover, bifemelane has an inhibitory effect on superoxide generation by neutrophils though the drug does not have a radical-scavenging effect (6). These findings suggest that bifemelane possesses neuroprotective actions against stress caused by brain ischemia.

Accumulating evidence has indicated that certain excitatory amino acids such as glutamate play an important role in the neurodegeneration observed in hypoxic-ischemic brain injury (7). Moreover, brief glutamate-exposure induces delayed death in cultured neurons of the cerebral cortex (8). Because N-methyl-D-aspartate (NMDA)-receptor antagonists protect cells from glutamate cytotoxicity in cortical cultures, it is generally accepted that NMDA receptors play crucial roles in glutamate-induced cytotoxicity in cortical neurons. Glutamate neurotoxicity mediated by NMDA receptors involves Ca2+ influx and subsequent formation of nitric oxide (9). Therefore, this study was performed to examine whether or not bifemelane prevents glutamate neurotoxicity in cortical cultures.

Primary cultures were obtained from the cerebral cortex of fetal rats (16–18 days gestation). The procedures have been described previously (10). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were plated on plastic coverslips which were placed in 60-mm Falcon dishes. Cultures were incubated in Eagle's minimal essential salt medium (Eagle's MEM) supplemented with 10% heat-inactivated fetal bovine serum (1–8 days after plating) or 10% heat-inactivated horse serum (9–12 days after plating), glutamine (2 mM), glucose (total of 11 mM), NaHCO3 (24 mM) and HEPES (10 mM). Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. After 8 days of plating, non-neuronal cells were removed by adding 10 μM cytosine arabinoside. Only mature (10–14 days in vitro) cultures were studied.

Neurotoxicity induced by EAAs was quantified by examining cultures under Hoffman modulation microscopy. All experiments were performed in Eagle's MEM at 37°C. Cell viability was assessed by means of trypan blue
exclusion. Cells stained with trypan blue were regarded as non-viable. The viability of the cultures was calculated as the percentage of the ratio of the number of unstained cells (viable cells) against the total number of cells counted (viable cells plus non-viable cells). Over 200 cells per coverslip were counted to determine the viability. In each experiment, cells on 5 coverslips were counted to obtain means ± S.E.M. of cell viability.

Recordings of whole cell currents were performed as described previously (11, 12). Cultured cortical neurons 10–12 days after seeding were maintained in the bath solution containing 124 mM NaCl, 2 mM KCl, 1.24 mM KH₂PO₄, 26 mM NaHCO₃, 3 mM CaCl₂, 10 mM glucose and 1 μM tetrodotoxin (pH 7.3). The micropipette solution was composed of 129 mM K-gluconate, 7 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 1 mM EGTA and 2 mM HEPES (pH 7.3). The patch microelectrodes had a resistance of 2–4 MΩ. Whole cell currents were recorded at room temperature (20–25°C) with a patch clamp amplifier (CES-2200; Nihon Kohden, Tokyo). A glass pipette with a tip diameter of 5 μm was placed near the cell. NMDA was ejected from the pipette by gas pressure (10–30 psi) with a duration of 40–60 msec (puff application, IM-200J; Narishige, Tokyo). Glycine (10 μM) was added to the NMDA-containing solution.

Bifemelane hydrochloride was synthesized at Mitsubishi Chemical Co., (Tokyo). The other chemicals were purchased commercially: (+)-MK-801 hydrogen maleate (MK-801; Research Biochemicals Co., Natick, MA, USA), NMDA (Sigma Chemical, St. Louis, MO, USA), monosodium L-glutamate (Nacalai Tesque, Tokyo). The drugs were dissolved in Eagle’s MEM immediately before the experiments.

Data were expressed as means ± S.E.M. The statistical significance of the data was determined by the Dunnett’s two-tailed test for glutamate neurotoxicity and the paired Student’s t-test for whole cell currents.

In our previous studies (10, 13), the neurotoxic effects of glutamate on the rat cultured cortical neurons were studied in detail. A 10-min exposure to 1 mM glutamate followed by a 1-hr incubation with glutamate-free medium was established as the appropriate conditions under which to examine drug-induced protection against glutamate neurotoxicity. Therefore, glutamate-induced cytotoxicity was examined using this procedure in the following experiments. Figure 1 summarizes the effects of bifemelane on glutamate-induced cytotoxicity. Protective effects of bifemelane were assessed by chronic and acute drug application. To study chronic bifemelane application, the drug was added to the incubation medium for 24 hr prior to glutamate exposure. The drug was removed from the incubation medium immediately before glutamate application. Chronic application of bifemelane at 1–10 μM reduced glutamate cytotoxicity in a concentration-dependent manner. A 24-hr exposure to 10 μM bifemelane alone did not affect the viability in comparison with that of non-treated cultures (data not shown). To examine whether acute application of bifemelane is effective against glutamate cytotoxicity, the cultures were maintained with the standard medium without bifemelane prior to glutamate exposure. Bifemelane was added to both the glutamate-containing and the following in-

![Fig. 1. Effect of bifemelane on neurotoxicity induced by glutamate (Glu). Cultures were exposed to 1 mM glutamate for 10 min and then incubated with glutamate-free medium for 1 hr. In chronic application, the drug was added to the incubation medium for 24 hr prior to glutamate exposure. In acute application, the drug was added to both the glutamate-containing and following glutamate-free media. *P < 0.05, **P < 0.01, compared with glutamate alone.](image)
cubating media. Concentrations of bifemelane was set to those used in the chronic application. Acute application of bifemelane at 1 μM slightly but significantly reduced glutamate cytotoxicity. However, the higher concentration (10 μM) of the drug did not affect glutamate cytotoxicity. In contrast, acute administration of MK-801 at 10 μM markedly reduced glutamate cytotoxicity.

Figure 2 shows an example of the effects of bifemelane on NMDA-induced whole-cell currents. Considerable inward currents were induced by the puff application of NMDA (500 μM) at a holding potential of −60 mV. When bifemelane at 10 μM was added to the bath, no alteration of the NMDA-induced currents was observed. Marked reduction of NMDA-induced currents was observed by increasing the concentration of the drug to 100 μM. The results obtained from 5 neurons are summarized in Table 1. Bifemelane at a concentration of 10 μM did not affect NMDA-induced currents, although there was a significant reduction of the currents when 100 μM of the drug was applied.

This study demonstrated that glutamate cytotoxicity was reduced by the chronic application of bifemelane, a well-known cerebral metabolic activator. The results indicate that bifemelane possesses neuroprotective effects against glutamate cytotoxicity in cultured cortical neu-

![Bifemelane effect on NMDA-induced currents](image)

**Fig. 2.** Effect of bifemelane on NMDA-induced currents. An upward trend indicates an inward current in panel A and an outward current in panel B. A: a continuous recording showing that puff application of NMDA to the cultured cells induced inward whole-cell currents in the absence (a and b) and in the presence (b, c and d) of bifemelane. Bifemelane (10–100 μM) was added to the perusing solution during the period of time indicated by the bar above the trace. B: part of the NMDA-induced currents shown in panel A on an expanded time scale.

**Table 1.** Effect of bifemelane on NMDA-induced currents in cultured cortical neurons

<table>
<thead>
<tr>
<th>Concentration of bifemelane</th>
<th>n</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM</td>
<td>5</td>
<td>96.2 ± 5.8</td>
</tr>
<tr>
<td>30 μM</td>
<td>5</td>
<td>75.5 ± 9.1</td>
</tr>
<tr>
<td>100 μM</td>
<td>5</td>
<td>31.2 ± 9.5**</td>
</tr>
</tbody>
</table>

n, number of neurons; *, mean percentage of the ratio of the amplitude of NMDA-induced currents in the presence of bifemelane to that in the absence of bifemelane (control). **P < 0.01, compared with the control.
rons. Though chronically applied bifemelane induced an obvious protective effect against glutamate cytotoxicity, the effect of acute application of the drug was weak and independent of concentration. It is unlikely that bifemelane at 10 μM induced cytotoxic action since this concentration of the drug did not affect the viability of cultures. Therefore, we concluded that chronic application of bifemelane is necessary to induce consistent protection against glutamate neurotoxicity. Bifemelane was removed from the glutamate-containing medium between applications in the experiments with chronic drug application. Moreover, NMDA-induced currents were not affected by bifemelane at 10 μM, although this concentration of the drug significantly reduced glutamate cytotoxicity. Therefore, it is unlikely that direct interaction of bifemelane with NMDA receptors plays a role in its neuroprotective effect.

Although the exact mechanism of the protective effect of bifemelane has not yet been fully elucidated, bifemelane is incorporated into the cell membrane and may alter membrane function (14). Recent evidence suggest the effectiveness of long-term bifemelane treatment on post-ischemic brain injury (15). Moreover, it has been reported that bifemelane inhibited superoxide production by neutrophils stimulated with a phorbol ester (6). Bifemelane did not show radical scavenging effects, but had an inhibitory effect on superoxide generation by neutrophils. We have previously suggested the involvement of superoxide as well as nitric oxide in the process of glutamate neurotoxicity according to the finding that both superoxide dismutase and an inhibitor of nitric oxide synthase prevented glutamate-induced cytotoxicity in cultured cortical neurons (9). Therefore, the neuroprotective action of bifemelane may be due to its inhibitory effect on superoxide generation.

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